# Improving heterologous cytochrome P450 function in *S. cerevisiae*

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# Abstract of Dissertation

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Membrane-attached cytochrome P450 monooxygenases (CYPs) are versatile and industrially important enzymes, playing major roles in drug metabolism as well as production of fine chemicals, pharmaceutical compounds or flavors and fragrances. However, a number of limitations have restricted their use in industrial processes including narrow substrate specificity, the association of the enzymes with membranous structures, the co-expression of an appropriate cytochrome P450 reductase, the need for a complex system of cofactor regeneration and low turnover numbers. Therefore, approaches for improving overall CYP activity are versatile and highly complex. The aim of this study was the establishment of a genetically modified *S. cerevisiae* strain serving as expression platform for overally improved CYP activity. Displaying many cellular features and metabolic pathways similar or identical to those of higher eukaryotes, yeast cells are highly amenable to express recombinant CYPs. Furthermore, yeasts have the ability to synthesize diverse substrates like plant terpenes intracellularly upon expression of appropriate terpene synthases. This option helps to circumvent phase transfer issues of hydrophobic substances into yeast cells and allows the direct CYP-mediated conversion to produce terpenoids. Using both, resting and self-sufficient, terpene-producing cells, multiple approaches were evaluated for their potential to improve CYP activity like comparing different expression hosts, supporting enzyme stabilities and increasing intracellular cofactor and substrate supply.

# Zusammenfassung der Dissertation

Improving heterologous cytochrome P450 function in *S. cerevisiae*

Membrangebundene Cytochrom P450 Enzyme (CYPs) sind vielseitige und industriell bedeutsame Enzyme, die eine wichtige Rolle in der Verstoffwechslung von Medikamenten und bei der Produktion von Feinchemikalien, pharmazeutischen Wirkstoffen, aber auch diversen Duft- und Geschmackstoffen spielen. Der Einsatz von CYPs in industriellen Anwendungen wird jedoch von einer Anzahl an Faktoren, wie zum Beispiel enger Substratspezifitäten, Membranständigkeit der Enzyme, der Co-Expression einer passenden Cytochrom P450-Reduktase, der Notwendigkeit eines komplexen Cofaktor-Regenerationssystems und geringen spezifischen Aktivitäten beeinträchtigt. Daher sind auch die Ansätze zur Beeinflussung und Steigerung von CYP-Aktivitäten vielfältig und komplex. Für die heterologe Expression von CYPs höherer Eukaryoten eignen sich vor allem Hefezellen ausgezeichnet, da sie einen ähnlichen zelluläre Aufbau und fast idente Stoffwechselwege wie Pflanzenzellen und tierische Zellen aufweisen. In dieser Arbeit wurden in der Hefe *S. cerevisiae* unterschiedliche, membrangebundene P450 Enzyme exprimiert, wobei das Hauptziel darin bestand, Substratumsetzungen zu steigern. Hydrophobe Substrate, wie zum Bespiel diverse Terpene, können in Hefen intrazellulär hergestellt werden, indem man die jeweilige Synthase co-exprimiert. Somit verhindert man Limitationen im Import von hydrophoben Verbindungen in die Hefezellen. Diese Terpene werden von ausgewählten CYPs stereospezifisch hydroxyliert. Sowohl „resting cells“, als auch Terpene intrazellulär produzierende und umsetzende Zellen wurden verwendet um diverse Ansätze zur Optimierung von CYP-Enzymaktivitäten zu testen. Es wurden unterschiedliche Expressionsstämme eingesetzt, um die Steigerung der Stabilität heterologer Enzyme und die verbesserte intrazelluläre Cofaktor- und Substratverfügbarkeit zu bearbeiten.

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# Aim and outline of this thesis

During the last two decades, heterologous expression of membrane-linked cytochrome P450 monooxygenases (CYPs) has proven to be as popular as demanding. In nature, CYPs are involved in the detoxification of hydrophobic substances but also act as important drug metabolizers in the human liver (Crespi and Miller, 1999; Danielson, 2002). Their ability to hydroxylate complex, hydrophobic substrates stereospecifically renders them highly interesting for synthetic chemistry. To date, CYPs are already used in a significant number of industrial applications like the production of fine chemicals, pharmaceutical compounds, flavors and fragrances (Chang and Keasling, 2006; Kirby and Keasling, 2009; Paddon et al., 2013; Wriessnegger and Pichler, 2013). However, membrane-bound CYPs are challenging enzymes because they are difficult to express, unstable and complex regarding their need for correct membranous surroundings and a corresponding CYP reductase responsible for regeneration and cofactor supply (Bernhardt, 2006; Gu et al., 2003; Henderson et al., 2003; Omura, 2010). Therefore, most CYP-mediated bioconversions are performed with whole-cell systems. High-level expression is not only a burning issue for membrane protein crystallography but also for enhanced production of high-value products.

Finding the ideal expression host is a crucial and important step when working with membrane-bound cytochrome P450 enzymes (Geier et al., 2012). Success stories employing *E. coli* are spread rarely, because of its rather simple cellular machinery and the lack of ER membranes normally serving as anchoring site for CYPs and their reductases (Chang et al., 2007; Kim et al., 2011; Yun et al., 2006). In different studies it has proven beneficial to cut off or modify the N-terminal transmembrane domain of CYPs to increase their expression levels (McDougle et al., 2013; Zelasko et al., 2013). However, this modification can also lead to reduced or even total loss of activity. The membrane-anchor is believed to be linked to substrate availability and/or interaction with the reductase for electron transfer (Denisov et al., 2012; Isin and Guengerich, 2008; Otyepka et al., 2012). The application of yeasts for recombinant protein expression is an attractive alternative as they comprise low-cost cultivation and high-quantity production platforms meeting the demand for criteria of safety and authentically processed proteins (Gellissen, 2005; Gellisson, 2004). Additionally, yeasts display many tools desired for correct folding, cellular translocation and posttranslational modifications of higher eukaryotic proteins (extensively reviewed by Freigassner et al. (2009).

In Chapter I an overview of challenges and pitfalls of heterologous membrane protein expression is given with a focus on yeasts. The review delineates in detail the state of the art considering advantages of different yeast expression systems, but also gives a summary of membrane protein classes successfully expressed in yeasts so far. General strategies to improve expression using optimized cultivation protocols or helper factors are explained. The importance and challenge of choosing the

ideal expression host for cytochrome P450 enzymes is shown in Chapter II (Geier et al., 2012). Four different expression hosts, *E. coli* and the three yeasts *S. cerevisiae*, *P. pastoris* and *Y. lipolytica* were genetically modified for co-expression of human cytochrome P450 enzyme 2D6 and human cytochrome P450 reductase. Although only poorly expressed, human hepatic CYP2D6 naturally acts as main detoxifier in the human liver (Zhuge et al., 2004). The strains were compared regarding their ability to express heterologous proteins and further convert the model substrate bufuralol to 6-hydroxy-bufuralol. Strengths and weaknesses of the single host systems are discussed. Baker's yeast started to qualify as excellent expression host for human and plant CYP enzymes back in the early 1990's (Pompon et al., 1996; Truan et al., 1993; Urban et al., 1994). One enormous success in this field was the production of the antimalarial drug artemisinin-precursor artemisinic acid using self-sufficient cells of *S. cerevisiae* yielding 25 g/L of yeast culture (Paddon et al., 2013).

In chapters III and IV, the overall aim and the results of this PhD thesis are presented, i.e. improving heterologous expression and activity of cytochrome P450 enzymes in *S. cerevisiae*. One huge advantage when working with *S. cerevisiae* is the availability of knockout collections, e.g. the EUROSCARF collection, consisting of approximately 5000 non-essential single gene knockout mutants (Winzeler et al., 1999). This knockout library was used to screen for gene disruptions improving overall CYP activity. Possible cellular targets ranged from changing membranous surroundings, stabilization of heterologous enzymes, increase of intracellular NADPH pool and better transport of hydrophobic substrates across the cell wall. *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO) (Takahashi et al., 2007) co-expressed with a cytochrome P450 reductase from *Arabidopsis thaliana* (CPR) was chosen as model enzyme. HPO and CPR stereospecifically hydroxylate (+)-valencene, a main fragrance of oranges, to give trans-nootkatol. The screening of the knockout library is described in detail in chapter III. During this screening the Δ*ice2* mutant was found to show reduced stability of CPR. This observation led to the construction of a strain over-expressing the *ICE2* gene in yeast.

In chapter IV, the effect of *ICE2* overexpression on trans-nootkatol production is presented. Similar to biosynthesis in plants, (+)-valencene can directly be synthesized in *S. cerevisiae* cells upon conversion of farnesyl-diphosphate (FPP) by a co-expressed (+)-valencene synthase (ValS) (Beekwilder et al., 2014). Additionally, the whole terpenoid biosynthesis pathway can be engineered by over-expression of t*HMG1* (Polakowski et al., 1998). Over-expression of *ICE2* improved overall trans-nootkatol production 1.4-fold in resting cells upon external addition of (+)-valencene as well as in self-sufficient *S. cerevisiae* cells. In addition to HPO/CPR, other cytochrome P450 enzyme and reductase combinations were tested for alterations in substrate conversion and reductase stability, i.e. CYP2D6 with human reductase (see chapter II) and a limonene-3-hydroxylase (L3H) derived from *Mentha piperita* co-expressed with CPR from *Arabidopsis thaliana*, which converts (-)-limonene to trans-isopiperitenol. As an alternative expression host, *Pichia pastoris* was employed for expression of the

same cytochrome P450 enzymes in our group (Wriessnegger et al., manuscript in revision). Often, these two yeasts mistakenly are believed to be similar, but our and diverse other studies have shown that there are clear cellular differences (see Chapters I and II). Direct comparison of *S. cerevisiae* and *P. pastoris* expressing the same cytochrome P450 enzymes always proved the latter to be the better expression and converting host (Geier et al., 2012)(Emmerstorfer et al., manuscript in preparation)(Wriessnegger et al., manuscript in revision). Possible reasons might be a combination of better tolerance to hydrophobic compounds, growth to extremely high cell densities and the use of the extraordinarily strong *AOX1* promoter for high-level protein expression (Chapter I).

# References


Beekwilder, J., Van Houwelingen, A., Cankar, K., Van Dijk, A.D.J., De Jong, R.M., Stoopen, G., Bouwmeester, H., Achkar, J., Sonke, T., Bosch, D., 2014. Valencene synthase from the heartwood of Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene. Plant biotechnology journal 12, 174–182.

Bernhardt, R., 2006. Cytochromes P450 as versatile biocatalysts. Journal of biotechnology 124, 128–45.

Chang, M.C.Y., Eachus, R.A., Trieu, W., Ro, D.-K., Keasling, Jay D, 2007. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. Nature chemical biology 3, 274–277.

Chang, M.C.Y., Keasling, Jay D, 2006. Production of isoprenoid pharmaceuticals by engineered microbes. Nature chemical biology 2, 674–681.

Crespi, C.L., Miller, V.P., 1999. The use of heterologously expressed drug metabolizing enzymes--state of the art and prospects for the future. Pharmacology & therapeutics 84, 121–31.

Danielson, P.B., 2002. The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. Current drug metabolism 3, 561–97.

Denisov, I G, Shih, A.Y., Sligar, S G, 2012. Structural differences between soluble and membrane bound cytochrome P450s. Journal of inorganic biochemistry 108, 150–8.

Freigassner, M., Pichler, H., Glieder, A., 2009. Tuning microbial hosts for membrane protein production. Microbial Cell Factories 8, 69.

Geier, M., Braun, A., Emmerstorfer, A., Pichler, H., Glieder, A., 2012. Production of human cytochrome P450 2D6 drug metabolites with recombinant microbes - a comparative study. Biotechnology journal 1–13.

Gellissen, G., 2005. Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems, Wiley- VCH, Weinheim.

Gellisson, G., 2004. Key and Criteria to the Selection of An Expression Platform. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Gu, J., Weng, Yan, Zhang, Q.-Y., Cui, H., Behr, M., Wu, L., Yang, W., Zhang, Li, Ding, X., 2003. Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. The Journal of Biological Chemistry 278, 25895–25901.

Henderson, C.J., Otto, D.M.E., Carrie, D., Magnuson, M.A., McLaren, A.W., Rosewell, I., Wolf, C.R., 2003. Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. The Journal of Biological Chemistry 278, 13480–13486.

Isin, E.M., Guengerich, F.P., 2008. Substrate binding to cytochromes P450. Analytical and bioanalytical chemistry 392, 1019–30.

Kim, Y.H., Kwon, T., Yang, H.J., Kim, W., Youn, H., Lee, J.Y., Youn, B., 2011. Gene engineering, purification, crystallization and preliminary X-ray diffraction of cytochrome P450 p-coumarate-3-hydroxylase (C3H), the Arabidopsis membrane protein. Protein expression and purification 79, 149–55.

Kirby, J., Keasling, Jay D, 2009. Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annual review of plant biology 60, 335–355.

McDougle, D.R., Palaria, A., Magnetta, E., Meling, D.D., Das, A., 2013. Functional studies of N-terminally modified CYP2J2 epoxygenase in model lipid bilayers. Protein science: a publication of the Protein Society 22, 964–79.

Omura, T., 2010. Structural diversity of cytochrome P450 enzyme system. Journal of Biochemistry 147, 297–306.

Otyepka, M., Berka, K., Anzenbacher, P., 2012. Is there a relationship between the substrate preferences and structural flexibility of cytochromes P450? Current drug metabolism 13, 130–42.

Paddon, C.J., Westfall, P.J., Pitera, D.J., Benjamin, K., Fisher, K., McPhee, D., Leavell, M.D., Tai, A., Main, A., Eng, D., Polichuk, D.R., Teoh, K.H., Reed, D W, Treynor, T., Lenihan, J., Fleck, M., Bajad, S., Dang, G., Dengrove, D., Diola, D., Dorin, G., Ellens, K.W., Fickes, S., Galazzo, J., Gaucher, S.P., Geistlinger, T., Henry, R., Hepp, M., Horning, T., Iqbal, T., Jiang, H., Kizer, L., Lieu, B., Melis, D., Moss, N., Regentin, R., Secrest, S., Tsuruta, H., Vazquez, R., Westblade, L.F., Xu, L., Yu, M., Zhang, Y., Zhao, L., Lievense, J., Covello, P S, Keasling, J D, Reiling, K.K., Renninger, N.S., Newman, J.D., 2013. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496, 528–532.

Polakowski, T., Stahl, U., Lang, C., 1998. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Applied Microbiology and Biotechnology 49, 66–71.

Pompon, D., Louerat, B., Bronine, A., Urban, P., 1996. Yeast expression of animal and plant P450s in optimized redox environments. Methods in enzymology 272, 51–64.

Takahashi, S., Yeo, Y.-S., Zhao, Yuxin, O´Maille, P.E., Greenhagen, B.T., Noel, J.P., Coates, R.M., Chappell, J., 2007. Functional characterization of premnaspirodiene oxygenase, a cytochrome P450 catalyzing regio- and stereo-specific hydroxylations of diverse sesquiterpene substrates. The Journal of Biological Chemistry 282, 31744–31754.

Truan, G., Cullin, C., Reisdorf, P., Urban, P., Pompon, D., 1993. Enhanced in vivo monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b5. Gene 125, 49–55.

Urban, P., Werck-Reichhart, D, Teutsch, H.G., Durst, F., Regnier, S., Kazmaier, M., Pompon, D., 1994. Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway. European journal of biochemistry / FEBS 222, 843–50.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., et al., 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science (New York, N.Y.) 285, 901–6.

Wriessnegger, T., Pichler, H., 2013. Yeast metabolic engineering--targeting sterol metabolism and terpenoid formation. Progress in lipid research 52, 277–93.

Yun, C.-H., Yim, S.-K., Kim, D.-H., Ahn, T., 2006. Functional expression of human cytochrome P450 enzymes in Escherichia coli. Current drug metabolism 7, 411–29.

Zelasko, S., Palaria, A., Das, A., 2013. Optimizations to achieve high-level expression of cytochrome P450 proteins using *Escherichia coli* expression systems. Protein expression and purification 92, 77–87.

Zhuge, J., Yu, Y.-N., Wu, X.-D., 2004. Stable expression of human cytochrome P450 2D6*10 in HepG2 cells. World Journal of Gastroenterology 10, 234–237.

# Chapter I

# Overexpression of membrane proteins from higher eukaryotes in yeasts

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***Invited review for***

***'Applied Microbiology and Biotechnology'***

## Abstract

Heterologous expression and characterization of the membrane proteins of higher eukaryotes is of paramount interest in basic research as well as in the development of pharmaceuticals. Due to the rather simple and well-established methods for their genetic modification and cultivation, yeast cells are attractive host systems for recombinant protein production. In contrast to the cell lines of higher eukaryotes, yeasts permit efficient library screening methods. This review provides an overview on the remarkable progress and pitfalls in applying various yeast host strains for high-level expression of eukaryotic membrane proteins. Genetically modified yeasts are used as high-throughput screening tools for heterologous membrane protein functions or as benchmark for analyzing drug-target relationships, e.g. by using yeasts as sensors. Furthermore, yeasts have proven to be powerful hosts for revealing (membrane) protein interactions. We also discuss the stress responses of yeasts upon heterologous expression of membrane proteins, and highlight how these observations enable target-oriented intervention into metabolic pathways. Through co-expression of chaperones and/or optimizing yeast cultivation and expression strategies, yield-optimized hosts were created for membrane protein crystallography or efficient whole-cell production of fine chemicals.

# 1. Introduction

Approximately one third of all genes encode integral membrane proteins in every kingdom of life (Krogh et al. 2001). More than 50 % of current medication are targeting membrane proteins (Petschnigg et al. 2011). Therefore, it is not surprising that heterologous expression of membrane proteins for biochemical characterization, structural analysis or industrial applications are attracting ever-increasing attention. Although elucidation of membrane protein structures is heavily worked on in dedicated research centres, success rates are mediocre due to low protein yields, poor solubility in aqueous milieu and tedious, time-consuming purification methods employing detergents. Many eukaryotic membrane protein structures are still obtained either via direct purification from rich native sources (Kiser et al. 2009; Nyblom et al. 2013; Toyoshima et al. 2013), over-expression in mammalian cells (reviewed by Andréll and Tate 2013), or upon heterologous expression in *E. coli* (Bernaudat et al. 2011). In contrast to the latter, yeasts provide endogenously many of the factors required for correct folding, posttranslational modification and intracellular transport of eukaryotic proteins (extensively reviewed by Freigassner et al. 2009). Furthermore, yeasts offer low-cost screening and high-level production platforms meeting the demands of safety and authentically processed proteins (Gellissen 2005; Gellisson 2004). Since the first groups published expression of human uncoupling proteins (Murdza-Inglis et al. 1991), human $D_{25}$ dopamine receptor (Sander et al. 1994) or rabbit SERCA1a (Centeno et al. 1994) in yeast, lots of effort were put into the field of recombinant membrane protein production in these hosts. In many cases, yeasts were employed to confirm the modes of action expected for selected membrane proteins of higher eukaryotes. Often, membrane proteins of interest were expressed from high copy number plasmids and strong promoters to prove protein functionality as described for plant aquaporins (reviewed by Kaldenhoff et al. 2007), G-protein coupled receptors (reviewed by Sarramegna et al. 2003) or uncoupling proteins (reviewed by Klingenberg 2001). Though, not in every case strong expression yielded the best result. Based on progress in understanding cellular functions, fine-tuning of induction conditions increased the applicability of yeasts in membrane protein production. In this review, we point out the vast potential of yeasts in screening for diverse interaction processes, for example in drug-target protein relationships, protein-lipid and protein-protein interactions. As an update and extension of Freigassner et al. (2009), we provide a deeper insight into recent achievements focusing on yeast host engineering strategies, highlighting specific applications and listing membrane protein families successfully expressed for means of fundamental or applied research.

## 2. Yeast expression systems for membrane protein expression

S*accharomyces cerevisiae* is still the host of choice for straightforward heterologous protein expression, especially in fundamental research. Baker's yeast exhibits incontestable advantages compared to other yeasts, i.e. several whole genome sequences of laboratory strains, well-characterized cell biology and metabolism, and many different strain collections, e.g. single-gene knockout collections (Entian et al. 1999; Giaever et al. 2002; Winzeler et al. 1999), GFP-tag collections (Huh et al. 2003) and GST-tag collections (Sopko et al. 2006; Zhu et al. 2001). Nevertheless, the use of *S. cerevisiae* in heterologous protein expression may hold some noteworthy drawbacks. *S. cerevisiae* has a tendency to hyperglycosylate proteins by attaching numerous mannose residues to N-linked carbohydrate chains, which can severely influence protein activity and translocation, and can also channel the recombinant protein to the endoplasmic reticulum associated degradation (ERAD) pathway. The option of using episomal plasmids for protein expression allows high copy number expression and flexibility of transforming one and the same expression vector easily into different strains, but entails instability of recombinant strains due to vector loss or vector incompatibility (reviewed by Gellissen et al. 2005).

However, another single-celled fungus, the methylotrophic yeast *Pichia pastoris*, is gaining popularity in heterologous protein expression (reviewed by Gonçalves et al. 2013; Ramón and Marín 2011). Comprising many advantages such as high cell density cultivation, strong and regulable promoter systems for expression and genetic manipulation techniques similar to *S. cerevisiae* make it a powerful expression host (Cereghino et al. 2002; Cregg et al. 2009; Macauley-Patrick et al. 2005). Although similarly organized in cell structure, *S. cerevisiae* and *P. pastoris* yield in substantial differences in expression success (reviewed by Darby et al. 2012; Mattanovich et al. 2012). Many heterologously expressed membrane protein classes can be produced in both yeast hosts. For example, human monoamine oxidase was first expressed successfully in *S. cerevisiae*, but researchers very quickly changed to the *P. pastoris* expression system as yields were much higher (Table 2). Moreover, *P. pastoris* plays a prominent role as recombinant membrane protein producer among all yeast hosts in protein crystallography (Table 1). Though, baker's yeast is favored over *P. pastoris* when it comes to recombinant expression of uncoupling proteins or oleosins. A slight disadvantage in using the strong *AOX1* promoter for heterologous protein expression in *P. pastoris* still is the need for methanol induction.

Although thoroughly promising, other non-conventional yeasts are only in the starting blocks to be extensively employed for membrane protein expression. For example, *Yarrowia lipolytica* naturally offers interesting properties in conversion of hydrophobic substrates and,

therefore, holds potential in expressing membrane-associated proteins especially for metabolism of hydrophobic substrates (Thevenieau et al. 2009; Nicaud 2012). *Y. lipolytica* was already successfully used as production host of several green notes used as aroma components and diverse substances such as gamma-decalactone for food chemistry (Fickers et al. 2005; Schrader et al. 2004). *Y. lipolytica* has shown to be a powerful strain when it comes to studies on membrane-anchored cytochrome P450 enzymes (CYPs) like the human hepatical CYP2D6 (Braun et al. 2012; Geier et al. 2012), CYP1A1 (Nthangeni et al. 2004) or plant CYP53B1 (Shiningavamwe et al. 2006).

*Schizosaccharomyces pombe*, distantly related to *S. cerevisiae* (Sipiczki 2000), is called fission yeast because it reproduces by means of fission, technically similar to the proliferation of higher eukaryotic cells. By sharing many molecular, genetic and biochemical features with multicellular organisms, *S. pombe* is a particularly useful model for studying the function and regulation of genes from higher eukaryotes. Many cellular processes of more complex organisms, such as mRNA splicing, post-translational modification and cell-cycle control resemble those of S*. pombe* more closely than those of *S. cerevisiae* (Takegawa et al. 2009; Zhao and Lieberman 1995). Therefore, *S. pombe* was successfully used for expression of leukotriene LTC4 synthase (Ago et al. 2007), cytochrome P450 enzymes (Drăgan et al. 2005; Ewen et al. 2008; Hakki et al. 2008; Peters et al. 2009) and human $D_{25}$ dopamine receptor (Sander et al. 1994). In general, *S. pombe* has a potential as producer of receptors, especially G-protein coupled receptors (GPCRs), based on its more suitable $G_a$-subunit than *S. cerevisiae*, a more highly developed intracellular membrane system and better ligand accessibility (Ladds et al. 2003). *S. pombe* is a very interesting host for analysis of olfactory receptors (Davey and Ladds 2011). Proteins comprising difficult glycosylation patterns can be produced in *S. pombe* (De Pourcq et al. 2010).

Like *P. pastoris*, the thermotolerant, methylotrophic yeast *Hansenula polymorpha* is able to grow to high cell densities. Its optimal growth temperature of 37 to 43°C reduces the risk of contaminations in large-scale bioreactor cultivations. Additionally, growth at higher temperatures may be favorable for expression of human proteins (van Dijk R et al. 2000; Gellissen et al. 2005). Furthermore, *H. polymorpha* efficiently produces recombinant N-glycosylated proteins, which are much less hypermannosylated due to sophisticated glyco-engineering (Kim et al. 2006; Kim et al. 2004). This yeast was successfully used for expression of complex human membrane-bound β-1,2-N-acetylglucosaminyltransferase I (GnTI) (Cheon et al. 2012).

# 3. Heterologous membrane protein expression in different fields of research and applications

## 3.1. Determination of high resolution structures

In 2002, human monoamine oxidase B, a mitochondrial outer membrane protein, was the first higher eukaryotic membrane protein to be expressed in *P. pastoris* for crystallisation (Binda et al. 2002). Table 1 gives an overview of all membrane protein structures elucidated at high resolution including their used yeast host system for overexpression. A noteworthy milestone was set by Long et al. (2007) crystallizing a Kv channel expressed in *P. pastoris* in a defined lipid environment. For the very first time, the structure of a G-protein-gated potassium-selective channel was clarified by Whorthon and Mackinnon (2011). The more transmembrane domains (TMDs) a protein contains, the more difficult it is to be expressed heterologously. The protein structure with the highest number of transmembrane domains solved so far, a $H^+$-translocating pyrophosphatase from *Vigna radiata* with 16 TMDs, was achieved upon expression in *S. cerevisiae* (Lin et al. 2012). It is quite obvious that the structures of membrane bound oxidases, were mainly obtained upon expression in yeast (Table 1). Interestingly, beta-barrel transmembrane proteins were hardly expressed in yeasts for crystallization attempts, which can be explained by the achievements in expressing this membrane protein group in *E. coli*.

**Table 1: Membrane proteins of higher eukaryotes heterologously expressed in yeast for high resolution structure determination. (data obtained from http://blanco.biomol.uci.edu/mpstruc/)**

| PROTEIN | ORIGINAL HOST | EXPRESSION HOST | PDB COORDINATES | RESOLUTION | REFERENCE |
|---|---|---|---|---|---|
| **MONOTOPIC MEMBRANE PROTEINS** | | | | | |
| **Oxidases** | | | | | |
| **Monoamine Oxidase B, 1OJA: bound with Isatin** | ***H. sapiens*** | ***P. pastoris*** | **1GOS, 1OJA** | **3.0 Å/1.70 Å** | (Binda et al. 2003, 2002) |
| **Monoamine Oxidase A** | ***R. norvegicus*** | ***S. cerevisiae*** | **1O5W** | **3.20 Å** | (Ma et al. 2004) |
| **Monoamine Oxidase A with bound Clorglycine** | ***H. sapiens*** | ***P. pastoris*** | **2BXR** | **3.00 Å** | (De Colibus et al. 2005) |
| **Monoamine Oxidase A with bound Harmine:,** 2Z5Y: G110A mutant with bound Harmine | ***H. sapiens*** | ***S. cerevisiae*** | **2Z5X, 2Z5Y** | **2.20 Å/ 2.17 Å** | (Son et al. 2008) |
| **TRANSMEMBRANE PROTEINS: ALPHA HELICAL** | | | | | |
| **GPCRs** | | | | | |
| **$A_{2A}$ adenosine receptor in complex inverse-agonist antibody,** 3VGA: with bound mouse Fab2838 in the presence of the antagonist ZM241385 | ***H. sapiens*** | ***P. pastoris*** | **3VG9, 3VGA** | **2.70 Å/ 3.10 Å** | (Hino et al. 2012) |
| **Histamine $H_1$ receptor, complexed with doxepin** | ***H. sapiens*** | ***P. pastoris*** | **3RZE** | **3.10 Å** | (Shimamura et al. 2011) |

| | | | | | |
|---|---|---|---|---|---|
| **Channels: potassium and sodium ion- selectivity** | | | | | |
| **Two-Pore Domain Potassium Channel $K_{2P}$1.1 (TWIK-1)** | ***H. sapiens*** | ***P. pastoris*** | **3UKM** | **3.40 Å** | (Miller and Long 2012) |
| **Two-Pore Domain Potassium Channel $K_{2P}$4.1 (TRAAK)** | ***H. sapiens*** | ***P. pastoris*** | **3UM7** | **3.80 Å** | (Brohawn et al. 2012) |
| **Two-Pore Domain Potassium Channel $K_{2P}$4.1 (TRAAK)** | ***H. sapiens*** | ***P. pastoris*** | **4I9W** | **2.75 Å** | (Brohawn et al., 2013) |
| **Kv1.2 Voltage-gated potassium Channel,** 3LUT**:** Re-refinement of 2A79 above using normal-mode x-ray crystallographic refinement | ***R. norvegicus*** | ***P. pastoris*** | **2A79, 3LUT** | **2.9 Å/ 2.9 Å** | (Chen et al. 2010; Long et al. 2005a, 2005b) |
| **Kv1.2/Kv2.1 Voltage-gated potassium channel chimera, 3LNM:** F233W Mutant | ***R. norvegicus*** | ***P. pastoris*** | **2R9R, 3LNM** | **2.4 Å** | (Long et al. 2007; Tao et al. 2010) |
| **Kir2.2 Inward-Rectifier Potassium Channel, 3SPI,** 3SPC**:** In complex with dioctanoylglycerol pyrophosphate (DGPP), 3SPH: I223L mutant in complex with $PIP_2$, 3SPJ:I223L mutant, apo form, 3SPG: R186A mutant in complex with $PIP_2$ | ***G. gallus*** | ***P. pastoris*** | **3JYC, 3SPI, 3SPC, 3SPH, 3SPJ, 3SPG** | **3.1 Å/ 3.31 Å/ 2.45 Å/ 3.00 Å/ 3.31 Å/ 2.61 Å** | (Hansen et al. 2011; Tao et al. 2009) |
| **GIRK2 (Kir3.2) G-protein-gated $K^+$ channel:** 3SYA: Wild-type protein + $PIP_2$, 3SYC: D228N mutant, 3SYP: R201A mutant, 3SYQ: R201A mutant + $PIP_2$, 4KFM***:** The β and γ subunits of *Homo sapiens* expressed in *S. frugiperda* | ***M. musculus*** | ***P. pastoris*** | **3SYO, 3SYA, 3SYC, 3SYP, 3SYQ, 4KFM*** | **3.60 Å/3.00 Å/ 3.4 Å / 3.1 Å / 3.45 Å / 3.45 Å** | (Whorton and MacKinnon 2013, 2011) |
| **Channels: Ca-ion selective** | | | | | |
| **Orai Calcium release-activated calcium (CRAC) channel,** 4HKS: K163W mutant | ***D. melanogaster*** | ***P. pastoris*** | **4HKR, 4HKS** | **3.35 Å/ 3.35 Å** | (Hou et al. 2012) |
| **Channels: aquaporins and glyceroporins** | | | | | |
| **AQP4 aquaporin water channel** | ***H. sapiens*** | ***P. pastoris*** | **3GD8** | **1.8 Å** | (Ho et al. 2009) |
| **AQP5 aquaporin water channel (HsAQP5)** | ***H. sapiens*** | ***P. pastoris*** | **3D9S** | **2.0 Å** | (Horsefield et al. 2008) |
| **SoPIP2;1 plant aquaporin (closed conformation),** 2B5F: Open conformation, **3CLL: S115E mutant,** 3CN5: S115E:S274E mutant, 3CN6: S274E mutant | ***S. oleracea*** | ***P. pastoris*** | **1Z98,2B5F, 3CLL, 3CN5, 3CN6** | **2.10 Å/ 3.90 Å/ 2.30 Å/ 2.05 Å/ 2.95 Å** | (Nyblom et al. 2009; Törnroth-Horsefield et al. 2006) |
| **Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG)** | | | | | |
| **Leukotriene $LTC_4$ Synthase in complex with glutathione** | ***H. sapiens*** | ***S. pombe*** | **2PNO** | **3.3 Å** | (Ago et al. 2007) |
| **Leukotriene $LTC_4$ Synthase in complex with glutathione,** 2UUI: apo form | ***H. sapiens*** | ***P. pastoris*** | **2UUH, 2UUI** | **2.15 Å/ 2.00 Å** | (Martinez Molina et al. 2007) |
| **Major facilitator superfamily (MFS) transporters** | | | | | |
| **PiPT high-affinity phosphate transporter** | ***P. indica*** | ***S. cerevisiae*** | **4J05** | **2.90 Å** | (Pedersen et al. 2013) |
| **ATP binding cassette transporter** | | | | | |
| **P-Glycoprotein,** 3G60: With bound QZ59-RRR, 3G61: With bound QZ59-SSS | ***M. musculus*** | ***P. pastoris*** | **3G5U, 3G60, 3G61** | **3.8 Å/ 4.40 Å/ 4.35 Å** | (Aller et al. 2009) |
| **P-Glycoprotein** | ***C. elegans*** | ***P. pastoris*** | **4F4C** | **3.40 Å** | (Jin et al. 2012) |
| **Membrane integral pyrophosphatases (M-PPases)** | | | | | |
| **$H^+$-translocating M-PPase** in complex with the non-hydrolysable substrate analog imidodiphosphate (IDP) | ***V. radiata*** | ***S. cerevisiae*** | **4A01** | **2.35 Å** | (Lin et al. 2012) |
| **$Na^+$-translocating M-PPase with metal ions in active site,** 4AV6: In complex with phosphate and magnesium | ***T. maritima*** | ***S. cerevisiae*** | **4AV3, 4AV6** | **2.60 Å/ 4.00 Å** | (Kellosalo et al. 2012) |

## 3.2. Yeast recombinant screening systems (drugs and protein functionality)

The analysis of membrane protein structure-function relationships is a major focus in basic and applied research. A popular strategy for correlating protein function and structure is to perform site-directed mutagenesis and to analyse by simple assays the effects on protein activity. Revealing and understanding drug-target relationships is of great interest, because understanding the bioactivities of drugs is crucial for the early-stages of drug discovery, toxicology studies and clinical trials. Focused on medical indications, several databases list drug-membrane protein relationships with clinical relevance (Hecker et al. 2012; Sun et al. 2012).

### 3.2.1. Receptors and olfactory signaling pathways: yeasts as sensors

The most extensively studied group of membrane proteins commonly targeted by drugs are by far GPCRs and their effectors. GPCR research in yeasts mainly deals with two approaches characterizing either the specificity of docking a defined ligand to a given receptor or screening 'orphan' GPCRs for their unknown effector ligand in recombinant sensor cells (reviewed by Ladds et al. 2005; Ladds et al. 2003; Minic et al. 2005b; Suga and Haga 2007). Yeasts constitute great recombinant hosts providing a low endogenous background for mammalian GPCRs and G-proteins. GPCRs comprise one of the largest human protein families and, in fact, are the most popular drug-targets. Yeast is an interesting host for screening of (olfactory) receptors and their corresponding stimulants (Pausch 1997). There are remarkable similarities between the signal transduction cascades of GPCRs in mammalian cells and the pheromone response pathway in yeast. Upon cellular engineering, an effector docking to a heterologous GPCR actuates a MAP kinase pathway leading to expression of the yeast Ste12p transcription factor. Ste12p in turn specifically activates the $P_{FUS1}$ promoter cloned in front of a signal molecule like β-galactosidase, fluorophores or auxotrophy markers allowing yeast to grow on defined media (Dowell and Brown 2009; Ladds et al. 2005). Also termed the 'smelling yeast', *S. cerevisiae* strains expressing mammalian, e.g. human, olfactory receptors were established as biosensors producing signal molecules like luciferase or GFP as soon as odor ligands bind (Crowe et al. 2000; Dhanasekaran et al. 2009; Minic et al. 2005a). For example, a *S. cerevisiae* strain aiding in the detection of environmental toxines such as dinitrotoluene (DNT) was created (Radhika et al. 2007). On average, only half of all heterologously expressed GPCRs have been shown to couple to the pheromone signaling pathway of *S. cerevisiae* (Dowell and Brown 2009; Ladds

et al. 2005). Beside baker's yeast *S. pombe* was successfully established as heterologous expression host for olfactory receptors (reviewed by Davey and Ladds 2011).

### 3.2.2. Mitochondrial flavin containing oxidoreductases

Monoamine oxidases (MAOs) are situated in the outer mitochondrial membrane of higher eukaryotic cells, mainly in neurons and the intestinal tract. They catabolize monoamines such as adrenalin and serotonin, which may accumulate due to endogenous signaling processes or due to uptake from foods. MAO dysfunction is linked to a number of psychiatric and neurological disorders. Thus, inhibitors of MAOs are applied as anti-depressive drugs (Meyer et al. 2006; reviewed in Tipton et al. 2004). In 1990, human liver MAO-A expression in *S. cerevisiae* yielded 15 mg of purified enzyme per liter of cell culture (Weyler et al. 1990). Significantly higher levels were obtained in *P. pastoris* at 329 mg of MAO-A per liter of cell culture later on (Li et al. 2002). Several further MAOs were successfully expressed to high yields in *P. pastoris* (Table 2), paving the way for further drug screening and enzyme crystallization attempts (Arslan and Edmondson 2010; Binda et al. 2002; Newton-Vinson et al. 2000; Wang and Edmondson 2010).

### 3.2.3. Transport Proteins

Transport proteins have been investigated extensively as they may specifically enable drug entry into cells as described for peptidomimetic drugs such as β-lactam antibiotics, angiotensin-converting enzyme inhibitors, selected peptidase inhibitors and prodrugs (Rubio-Aliaga and Daniel 2002). Targeted import of small peptides was studied by generating *P. pastoris* strains heterologously expressing mammalian peptidase transporters PEPT1 and PEPT2 (Döring et al. 1998a; Döring et al. 1997; Foltz et al. 2004). PEPT1 and PEPT2 are highly expressed in epithelial cells located in the human small intestine and kidney, which makes them a favored target for drug delivery (reviewed in Brandsch 2009). It was also shown, that delta-aminolevulinic acid (δ-ALA) is transported into *P. pastoris* cells by the same peptidase transporters and, therefore, explains the observed effect of δ-ALA accumulation in patients' epithelial cells after oral administration (Döring et al. 1998b). *S. cerevisiae* producing phytochelatin was used as screening system for functionality of plant ABC-type transporters abrogating growth deficiencies caused by high heavy metal concentrations (Park et al. 2012; Song et al. 2010).

### 3.2.4. Investigating cellular 'plumbing' systems: Aquaporins

Aquaporins are important in all kingdoms of life, because it is vital that water entry and exit is very accurately organized in cells (King et al. 2004). Aquaporins play a role in the onset of many diseases such as dry skin, obesity and even cancer (reviewed in Verkman 2012). Modulators and stimulators of aquaporins may help to alleviate symptoms like brain swelling, epidermal dehydration and pain. Heterologous expression of aquaporins in *P. pastoris* has been described by several groups (Daniels and Yeager 2005; Karlsson et al. 2003; Nyblom et al. 2007). To screen for potential inhibitors/effectors of aquaporin function an assay was established based on isolating spheroplasts of recombinant *P. pastoris* strains followed by spectroscopic measurement of their swelling degree (Azad et al. 2009; Azad et al. 2008). Crystal structures of aquaporin water channels were resolved upon heterologous expression in *P. pastoris* (Table 1). Taking advantage of known protein structures and homology modeling, structure-function relationships were elucidated by mutational analysis of aquaporins concomitantly measuring water channel activities of the spheroplasts of recombinant *P. pastoris* expression strains (Azad et al. 2012; Murata et al. 2000). Functional heterologous expression of plant aquaporins has also been described in *S. cerevisiae* (Kaldenhoff et al. 2007; Murozuka et al. 2013; Otto et al. 2010).

### 3.2.5. Transmembrane ATPases: cell-membrane counter traders

Transmembrane ATPases actively transport small molecules across cellular membranes, which is driven by ATP hydrolysis. The most prominent family are P-type ATPases (reviewed in Bublitz et al. 2010). The first study describing the heterologous expression of a transmembrane ATPase, i.e. rabbit SERCA1a, in yeast was published by Centeno et al. (1994). A very effective enzyme purification method based on the BAD fusion strategy was applied to analyze altered ATPase activity of interesting muteins created on the basis of homology modeling. BAD stands for biotin acceptor domain, which is cloned C-terminally to the protein of interest provoking the expression host to autonomously biotinylate the recombinant protein *in vivo* (Cardi et al. 2010a; Cardi et al. 2010b; Jidenko et al. 2006).

# 4. Overexpression of membrane proteins for fundamental research

Besides applying higher eukaryotic membrane proteins heterologously expressed in yeasts for investigation of drug-target relationships or for production of industrial chemicals, research is also focused on elucidating the basic membrane protein functions.

## *4.1. Ion channels: Guards of the gates*

Analysis, screening and characterization of ion channels from higher eukaryotes can elegantly be achieved with yeast strains engineered to lack endogenous ion channels. For example, potassium channels were studied in different smartly constructed yeast strains. First, *S. cerevisiae* was engineered to be deficient for $K^+$ uptake - by deletion of *trk1* and *trk2* - to function as library screening host for identification of new heterologous Kir family channels that compensate growth deficiency under low $K^+$ conditions (Tang et al. 1995). The strain was used to test mammalian cDNA libraries for $K^+$-channel activity (Grishin et al. 2006) or peptide sequences fused to channel libraries and different GPCRs for correct surface expression (Okamoto and Shikano 2011; Shikano et al. 2005). Furthermore, such a strain can be used to determine structural elements relevant for channel functionality as described by Yi et al. (2001). Elucidating channel functionality in the opposite direction, a *S. cerevisiae* strain deficient in $K^+$ export has been constructed rendering it unable to grow under high $K^+$ influence (Kolacna et al. 2005). This strain was used for identification and structural investigation of members of the $K^+$ channel family by mutational analysis and simple growth complementation assays under high $K^+$ conditions (Bernstein et al. 2013; Schwarzer et al. 2008). Different cation/$H^+$ exchangers from *A. thaliana* were expressed in a cation-handling deficient *S. cerevisiae* strain and cell growth as well as protein transport was investigated under different alkaline pH values (Chanroj et al. 2011; Hernández et al. 2009; Maresova and Sychrova 2006).

## *4.2. Pump transporting or transporter pumping*

An overview of different plant $H^+$-pyrophosphatases ($H^+$-PPases) and their characterization in engineered yeast strains is given in Serrano et al. (2007). Over-expression of $H^+$-PPases chimera may lead to altered intracellular localization in *S. cerevisiae* (Drake et al. 2010), may alleviate the phenotype of a *S. cerevisiae* strains with vacuolar ATPase deficiency (Pérez-

Castiñeira et al. 2011) or may alter resistance to high salinity and metal stressors (Yoon et al. 2013). A new site-directed method called DREAM (Directed ReCombination-Assisted Mutagenesis) was established and used for analyzing the human bile salt export pump (BSEP) in *S. cerevisiae* (Stindt et al. 2013; Stindt et al. 2011). *P. pastoris* was also chosen as expression host for human liver BSEP (ABCB11) and MDR3 (ABCB4). More than 100 detergents were tested for BSEP extraction and ATPase activity was assessed via ATP agarose binding and malachite green assays, respectively (Ellinger et al. 2013). Human hepatical thiazide-sensitive NaCl-cotransporter (NCC, SLC12A3), containing 12 TMDs, was heterologously expressed in *S. cerevisiae*. Site-specific mutations causing Gitleman syndrome were shown to target the cotransporter for ERAD (Needham et al. 2011).

Metal deficiency is one of the most common nutritional disorders in plants, thus transporters causative for or capable of relieving metal deficiency are of highest interest. Iron transporters from *A. thaliana* (Korshunova et al. 1999; Vert et al. 2001), apple (Xiao et al. 2008; Zhang et al. 2013a), tomato (Bereczky et al. 2003; Eckhardt et al. 2001) and peas (Cohen et al. 2004) were expressed in an iron-uptake deficient *S. cerevisae* mutant. Most of these transporters were found not only to restore growth deficiencies under low $Fe^{2+}$ conditions, but also to complement uptake of other metals such as Mn, Cu and Zn in the corresponding mutant strains. Moreover, zinc transporters from barley and rice (Ishimaru et al. 2005; Pedas et al. 2009) or a manganese transporter from barley (Pedas et al. 2008) were functionally characterized in zinc and manganese deficient S*. cerevisiae* strains, respectively.

### *4.3. Uncoupling proteins: 'Heating' up the cells*

Altogether, five distinct isoforms of uncoupling proteins (UCP1 to UCP5) have been found in mammals based on sequence homologies (Jezek et al. 2004; Krauss et al. 2005). Already in 1991 the first heterologous expression of an uncouplin protein, rat UCP, was successfully performed in *S. cerevisiae* (Murdza-Inglis et al. 1991). A considerable number of human and mammalian UCPs have since been expressed in *S. cerevisiae* (reviewed by Klingenberg et al. 2001). It is not surprising that *S. cerevisiae* still is the model organism of choice for expression and analysis of uncoupling proteins, because of the availability of detailed mitoproteome maps providing comprehensive "gene-to-protein" datasets (Sickmann et al. 2003). The first insect uncoupling protein derived from *Drosophila melanogaster* was expressed in *S. cerevisiae* by Fridell et al. (2004). Douette et al. (2006) heterologously expressed human UCP1 and showed that it has a dual influence in free radical generation. Furthermore, they documented regulation of the mitochondrial proteome by hUCP1. Two enzymes of skung cabbage were shown to exhibit uncoupling activity in baker's yeast, while

one enzyme was found to possess a unique structure lacking transmembrane domain 5 of the usual 6 (Ito et al. 2006). The first UCP from an invertebrate, the amphioxus *Branchiostoma belcheri*, was successfully expressed in *S. cerevisiae* (Chen et al. 2010).

### 4.4. GPCRs: the receptor in general

Besides using yeasts expressing GPCRs as whole-cell sensors, fundamental research is pursuing biochemical characterization of GPCRs expressed in yeasts (Table 2). Marsango et al. (2011) investigated dimerization of human prokineticin binding receptor PKR2 in *S. cerevisiae* by Western blot analyses. High-quantity production and purification of beta2-adrenergic receptor in *S. cerevisiae* was realized at the mg/L scale (Gerasimov et al. 2012; Niebauer and Robinson 2006; O'Malley et al. 2009). *P. pastoris* was used for production and characterization of neuromedin U type 1 and 2 receptor ($NmU_1R$/ $NmU_2R$). The addition of 2% DMSO during expression improved protein yield from 1 to 5 pmol per mg total membrane protein (Shukla et al. 2007a). Cherouati et al. (2006) compared expression levels of 20 GPCRs in *P. pastoris* under different cultivation conditions. The need for large amounts of purified membrane proteins for crystallization experiments considerably pushed the efforts in obtaining high-yielding yeast expression strains (Table 2).

**Table 2: Some examples of recombinant protein levels of different families heterologously expressed in yeast. For each class of protein, obtained yields are listed chronologically.**

| PROTEIN | INITIAL HOST | EXPRESSION HOST | AMOUNTS | REFERENCE |
|---|---|---|---|---|
| **GPCRs** | | | | |
| OR17 and OR17-40 | *R. norvegicus, H. sapiens* | *S. cerevisiae* | **327 pmol** per mg of membrane protein, 1.44 *$10^5$ receptor entities per cell | (Minic et al. 2005) |
| Adenosine (A2a) receptor | *H. sapiens* | *S. cerevisiae* | **4 mg** active protein per L of yeast culture | (Niebauer and Robinson 2006) |
| Neuromedin U type 1 and 2 receptor ($NmU_1R$/ $NmU_2R$) | *H. sapiens* | *P. pastoris* | **6-9 pmol** receptor per mg of total membrane protein | (Shukla et al. 2007a) |
| 12 different GPCRs (e.g. hFSHR, hA2aR, CB2R, NK1R…) | *H. sapiens* | *S. cerevisiae* | Ranking from **0.7 ± 0.1 mg** to 10.0 ± 1.0 mg per L of yeast culture | (O'Malley et al. 2009) |
| Muscarinic acetylcholine receptor subtype 2 (CHRM2) | *H. sapiens* | *P. pastoris* | **51.2 pmol** per mg of membrane protein; 1.9 mg per L of yeast culture | (Yurugi-Kobayashi et al. 2009) |
| β2-adrenergic receptor | *H. sapiens* | *P. pastoris* | **20 mg per L** of yeast culture | (Gerasimov et al. 2012) |
| **Channels: Aquaporins** | | | | |
| Aquaporin 1 (hAQP1) | *H. sapiens* | *S. cerevisiae* | **0.5 mg** purified protein per L of culture | (Saparov et al. 2001) |
| Aquaporin PM28A | *S. oleracea* | *P. pastoris* | **25 mg** of purified protein per L of culture | (Karlsson et al. 2003) |

| | | | | |
|---|---|---|---|---|
| PvTIP3;1 | *P. vulgaris* | *P. pastoris* | **~1 mg** of purified protein from 50 g of wet cells | (Daniels and Yeager 2005) |
| Aquaporin 1 (hAQP1) | *H. sapiens* | *P. pastoris* | **90 mg** purified hAQP1 per L of culture | (Nyblom et al. 2007) |
| Aquaporin 4 (hAQP4) | *H. sapiens* | *P. pastoris* | **~15 mg** purified protein per L of culture | (Ho et al. 2009) |
| Aquaporin 1 | *H. sapiens* | *S. cerevisiae* | **1,500 pmol** per mg of total membrane protein; 8.5 % of total membrane protein | (Bomholt et al. 2013) |
| **Uncoupling proteins** | | | | |
| UCP | *R. norvegicus* | *S. cerevisiae* | **70-100 pg** per mg of mitochondrial protein | (Murdza-Inglis et al. 1991) |
| UPC1 | *M. musculus* | *S. cerevisiae* | **11 µg** per mg of mitochondrial protein | (Stuart et al. 2001) |
| UCP1 | *H. sapiens* | *S. cerevisiae* | **~10 µg** per mg of total mitochondrial protein | (Douette et al. 2006) |
| **Transporter** | | | | |
| ABC transporter BSEP and MDR3 | *H. sapiens* | *P. pastoris* | **~1 mg** BSEP and **~6 mg** MDR3 per 100 g of wet cell weight | (Ellinger et al. 2013) |
| **ATPases** | | | | |
| SERCA1a | *H. sapiens* | *S. cerevisiae* | **100 pmol** per mg of membrane protein | (Jidenko et al. 2005) |
| SERCA1a | *H. sapiens* | *S. cerevisiae* | **200–500 µg** of a 50% pure SERCA1 per L of yeast culture | (Cardi et al. 2010a) |
| **Membrane bound oxidases** | | | | |
| Monoamine oxidase A | *H. sapiens* | *S. cerevisiae* | **15 mg** per L of culture | (Weyler et al. 1990) |
| Monoamine oxidase B | *H. sapiens* | *P. pastoris* | 1700 U or **200 mg** of purified protein per 2 L of culture | (Newton-Vinson et al. 2000) |
| Monoamine oxidase A | *H. sapiens* | *P. pastoris* | 1170 units or **660 mg** of purified protein per 2 L of culture | (Li et al. 2002) |
| Monoamine oxidase A | *R. norvegicus* | *S. cerevisiae* | **10 mg** highly pure protein per L of culture | (Ma et al. 2004) |
| Monoamine oxidase A | *R. norvegicus* | *P. pastoris* | 700 U or **200 mg** of purified protein per L of culture | (Wang and Edmondson 2010) |
| Monoamine oxidase A | *D. rerio* | *P. pastoris* | 300 U or **200 mg** of purified protein per L of culture | (Arslan and Edmondson 2010) |
| Cytochrome $b_{561}$ | *Bos primigenius taurus* | *P. pastoris* | **0.7 mg** detergent-solubilized cyt b561 per L of culture | (Liu et al. 2005) |
| Cytochrome $b_{561}$ | *M. musculus* | *S. cerevisiae* | **15 nmol** per mg of total protein | (Bérczi et al. 2005) |
| Cytochrome $b_{561}$ | *A. thaliana* | *P. pastoris* | **7.7 mg** per 2 L of culture | (Cenacchi et al. 2012) |

# 5. Interaction analyses

## 5.1. Membrane protein expression in yeast for interaction studies

Membrane proteins frequently associate in complexes and, therefore, the identification of subunits and interaction partners is of great importance for understanding membrane protein mode of action. Heterologous expression in yeast can be a valuable tool to study the interaction of membrane proteins with other proteins or lipids. Several methods have been established and applied to identify interaction partners of membrane proteins.

## 5.2. Protein-protein interaction

Yeast two-hybrid (Y2H) systems are standard strategies to investigate protein-protein interactions *in vivo*. However, Y2H is not applicable to membrane proteins due to the requirement for nuclear localization of the proteins to be tested. To overcome this problem, a split-ubiquitin assay was established (Johnsson and Varshavsky 1994) and further optimized for the screening of membrane protein interaction partners (Stagljar et al. 1998). In the so-called membrane yeast two-hybrid (MYTH) assay, the carboxy-terminal part of ubiquitin (Cub) is fused to a membrane protein together with the artificial transcription factor protein A-LexA-VP16 (PLV). Upon interaction with another membrane protein, which itself is fused to the amino-terminal part of ubiquitin (NubG), the reconstituted ubiquitin is recognized by a specific protease, which mediates cleavage of the transcription factor. The transcription factor, in turn, enters the nucleus and induces the transcription of reporter genes *lacZ* and *HIS3*, respectively. Recently, this method was used to identify ten novel interacting proteins of the μ-opioid receptor by screening human brain cDNA libraries(Petko et al. 2013). This GPCR is an important target for clinically relevant opioid agonist drugs with analgesic properties. Whereas homooligomerization of GPCRs has been proven important for GPCR activity (Wade et al. 2011), heterooligomerization is believed to have a huge impact as well, but still is rather hard to prove and analyze. However, the MYTH method is a powerful method in screening for oligomer- or dimer-partners and their favoured ligands (Nakamura et al. 2013), e.g. searching for novel ATP13A2 interactors (Usenovic et al. 2012), finding new partners of CLN8 (Passantino et al. 2013) or investigating physical interaction of $K^+$-channel TASK-2 with human heterotrimeric G-protein subunits (Añazco et al. 2013). A modified version is the mating based split-ubiquitin assay (mbSUS) established by Obrdlik et al. (2004). mbSUS allows the detection of interactions of full-length membrane proteins and is optimized for systematic large-scale analyses of membrane interactions by employing *in vivo* cloning strategies. For example, this assay was used for interactome mapping of *Arabidopsis* membrane proteins (Chen et al. 2012).

Another well-established protein complementation method for the investigation of membrane protein interaction in yeast is the bimolecular fluorescence complementation assay, shortly BiFC (reviewed by Kerppola 2010). In this assay, a fluorophor such as GFP is split and each half is fused to the proteins believed to interact. As soon as the two supposed interaction partners indeed interact, a fluorescence signal can be detected. The major advantage of this method is that the site of interaction can be concomitantly determined through microscopy in living cells. The interaction between different major intrinsic membrane proteins, mainly responsible for the transport of water and small neutral solutes in *A. thaliana,* were studied

lately by using this technique in *S. cerevisiae* (Murozuka et al. 2013). The same assay had been used earlier to study the heterotetrameric assembly of Tobacco Aquaporins NtAQP1 and NtPIP2;1 in *S. cerevisiae* by Otto et al. (2010). Further methods for detecting membrane protein interactions and their successful applications are reviewed in detail by Petschnigg et al. (2011) describing additionally the Ras recruitment systems, G-protein fusion technology or FRET and BRET based systems. *S. cerevisiae* and bioluminescence resonance energy transfer (BRET) methods have been employed to analyze the protein interactions of GPCRs (Sanz and Pajot-Augy 2013). Library screening of randomly mutagenized GPCRs, for example human UDP-glucose receptor or muscarinic acetylcholine receptor, was preferentially performed in baker's yeast (Ault and Broach 2006; Stewart et al. 2010; and reviewed by Beukers and Ijzerman 2005; Celić et al. 2004).

## 5.3. Protein-lipid interaction

The impact of certain lipid molecular species on the activity and stability of membrane proteins has been reviewed extensively (Adamian et al. 2011; Hunte 2005; Lee 2004; Opekarová and Tanner 2003). Membrane proteins may be surrounded by an annular lipid layer, or undergo specific protein-lipid binding interactions. These interactions are of crucial importance for proper membrane targeting and protein folding, as well as membrane protein stability and activity. Several studies underscore that cholesterol, for instance, is a strong interaction partner of certain mammalian membrane proteins as evidenced by defined cholesterol binding sites in crystal structures (Cherezov et al. 2007; Hanson et al. 2008; Liu et al. 2012; Manglik et al. 2012; Shinoda et al. 2009). Although membrane protein-lipid interaction studies are often performed using model membranes (reviewed by Zhao and Lappalainen 2012), also heterologous expression in engineered yeast cells can give insights into lipid requirements of these proteins. Especially membrane-engineered yeast can be a valuable tool to study the influence of certain lipid composition on membrane protein activity and stability *in vivo*. These engineered strains can be, furthermore, a tool for expression of membrane proteins that require specific lipids that do not naturally occur in yeast. When membrane proteins from higher eukaryotes are expressed in yeast, one might encounter the situation that the different lipid composition negatively influences the yield of stable and active recombinant protein. Usually, yeasts contain ergosterol as major sterol compound, and through metabolic engineering, *S. cerevisiae* and *P. pastoris* cells were re-programmed to produce cholesterol, the major mammalian sterol (Souza et al. 2011; Hirz et al., 2013). The effect of this change in the sterol composition was studied with regard to effects on yeast

growth as well as homologous and heterologous membrane protein expression. A set of *S. cerevisiae* sterol-engineered strains was produced in order to characterize sterol structure requirements of membrane proteins, in particular of the yeast ABC transporter Pdr12p as well as tryptophan and arginine transporters. Pdr12p function required ergosterol and, thus, cells with modified sterol composition were not resistant to weak organic acids. Speculating that GPCRs require specific sterol interaction, sterol-engineered *S. cerevisiae* strains producing cholesterol-like sterols were employed for expression of β3-adrenergic and μ-opioid receptors and did show 2-3 fold higher yield than wild type strains (Kitson et al. 2011). Morioka et al. (2013) furthermore investigated the influence of sterol composition on the activity of Ste2p, an endogenous yeast GPCR. Specifically, cholesterol exerted a negative effect on the signalling activity of Ste2p. A cholesterol-producing *P. pastoris* strain was superior in functional expression of human Na,K-ATPase α3β1 isoform to the wild type strain background. The modification in membrane sterol composition resulted in enhanced stability and proper plasma membrane localization of the human Na,K-ATPase α3β1 isoform leading to a significantly higher receptor-ligand binding sites on the cell surface (Hirz et al. 2013). A detailed list of membrane proteins that require specific sterols for their activity was recently published in a comprehensive review about sterol engineered yeast (Wriessnegger and Pichler 2013).

Bocer et al. (2012) demonstrated that membrane lipid composition, especially the availability of phosphatidylserine, influenced the activity of the murine A class ABC transporter (ABCA1), which was heterologously expressed in a protease-deficient *S. cerevisiae* strain using the *MET25* promoter. Functional expression of this lipid transporter in yeast furthermore led to increased susceptibility to membrane-directed compounds such as amphotericin B and papuamide B. Moreover, phosphatidylserine stabilizes the α2β1 isoform of human Na,K-ATPase (Kapri-Pardes et al. 2011). In a follow-up study, it was reported that neutral phospholipids such as phosphatidylcholine and phosphatidylethanolamine stimulate Na,K-ATPase α1β1 activity (Haviv et al. 2013). These examples demonstrate that heterologous membrane protein expression in yeast can give insights into lipid-membrane protein interaction influencing protein localization, activity and stability.

## 6. Industrial applications: Fine chemical production

Heterologous expression of membrane proteins may play a key role in the production of hydrophobic fine chemicals, representing an environmentally friendly alternative to commonly used chemical synthesis routes. In this respect, oxidoreductases are a most exploited enzyme class and especially cytochrome P450 (CYP450) are industrially interesting enzymes. CYP450 enzymes functionalize hydrophobic substances by stereo- and regioselectively introducing a hydroxyl group and, hence, synthesize highly demanded compounds (reviewed by Bernhardt 2006). CYP450 activity is irrevocably linked to a finely balanced system of cofactor recycling, oxygen supply, correct integration of iron into the active site and, of course, a perfect teamwork with their corresponding reductase that functions as electron donor (Gu et al. 2003; Henderson et al. 2003; Omura 2010). A prominent example for the application of CYP450 proteins is the production of high-value terpenoids, e.g. the antimalarial drug artemisinin (reviewed by Arsenault et al. 2008; Brown 2010). Numerous engineering strategies employing yeast in producing terpenoids, carotenoids or steroidal hormones such as cortisone have been reviewed recently (Wriessnegger and Pichler, 2013).

Membrane protein expression can also be used for production of (poly)-unsaturated fatty acids (PUFAs). PUFAs are produced by expression of the corresponding desaturases, which almost exclusively originate from plant and animal cells (Pereira et al., 2003). An endoplasmic reticulum (ER)-associated oleate desaturase (FAD2-like fatty acid desaturase) from *Crepis alpina* was analyzed for structure-function relationship by mutation of conserved regions elicited by ClustalW alignment (Gagné et al. 2009). FAD2 desaturases from different plants, amongst others soybean, olive or moss, were cloned and expressed in *S. cerevisiae* for the accumulation of fatty acids normally not present in yeast such as linoleic acid (18:3) (Chodok et al. 2013; Hernández et al. 2005; Li et al. 2007). Aiming for functional analysis, a sterol desaturase from *Tetrahymena thermophila* was expressed in a *S. cerevisiae erg3* knockout mutant to show desaturation of the yeast sterol episterol thus alleviating hypersensitivity to cycloheximide (Poklepovich et al. 2012). Plants differ from other higher eukaryotes by harboring two different types of desaturases, ER-associated as well as plastidial ones (Ohlrogge and Browse 1995). Evolving from the same ancestor though, they display different substrate specificities arising from their distinct cellular localization (Sperling et al. 2003). Due to the lack of chloroplast membranes in yeast, expression of heterologous plastidial Ω3-desaturases led to unsatisfying quantities and activities in yeast (Domergue et al. 2003a, Domergue et al. 2003b). Venegas-Calerón et al. (2009) showed that co-expressing sunflower plastidial *Ha*FAD7 in *S. cerevisiae* with photosynthetic ferredoxin

originating from the same species resulted in a 10-fold increase in desaturase activity (over 4.5% conversion of linoleic acid) compared to the values measured without the additional electron donor. The same authors could also shed some light on the question, whether the low activity was an effect of cofactor depletion, protein degradation or cellular mistargeting. Through deleting the putative plastidial leading sequences desaturation activity was improved ten-fold and, therefore, suggested the reduced activity to be caused by mistargeting (Pelham 2000; Venegas-Calerón et al. 2010).

A similar strategy was used for studying oleosins, which are structural proteins found in plant oil bodies protecting maturating parts from desiccation (Hsieh and Huang 2007; reviewed by Capuano et al. 2007). Several groups studied intracellular translocation of oleosins for example in *S. cerevisiae sec* mutant strains defective in aspects of endomembrane protein trafficking (Beaudoin et al. 2000). C- and N-terminal truncations of wild type oleosins did not seem to have a detrimental effect on microsomal localization in yeast (Beaudoin and Napier 2002). Very recent studies have shown that over-expressed oleosins mainly accumulate in lipid droplet (LDs) and only accumulate in ER membranes if lipid droplet formation is impaired (Jacquier et al. 2013; Jamme et al. 2013; Vindigni et al. 2013). Interestingly, oleosins are not only structural proteins, but also comprise enzyme activities as monoacylglycerol acyltransferases and phospholipases (Parthibane et al. 2012). Fusion of recombinant proteins to oleosins for over-expression in yeast has become a highly interesting possibility for biotechnological applications (reviewed by Bhatla et al. 2010). Oleosin-fusion proteins accumulating in oil bodies or targeted to oleosomes can easily be separated via floating centrifugation. Subsequently, oleosins are removed by site specific peptidases as shown for expression of mCherry and cohesin linked to sesame oleosins in *Y. lipolytica* (Han et al. 2013). This strategy is a smart and easy to handle protein purification approach with growing potential for the future.

## 7. Strategies for improving membrane protein expression in yeast

Despite many recent breakthroughs in the field of membrane protein expression, progress in structural analysis of membrane proteins lags behind studies of soluble proteins. Membrane protein structures are still dramatically under-represented in the structural databases. The substantial medical importance of many membrane proteins, e.g. GPCRs, is the driving force for developing high-throughput heterologous expression strategies and the investigation of these challenging proteins at the molecular level. The major bottleneck in many of the membrane protein studies is obtaining sufficient amount of stable functional proteins for structural studies. Usually, membrane proteins perform poorly in over-expression systems

and tend to be instabile in detergent solutions required for the membrane extraction and purification steps. Consequently, large efforts have been undertaken in optimizing target proteins, diverse expression systems or purification strategies to yield sufficient protein for structural determination (Bannwarth and Schulz 2003; Vinothkumar et al. 2013).

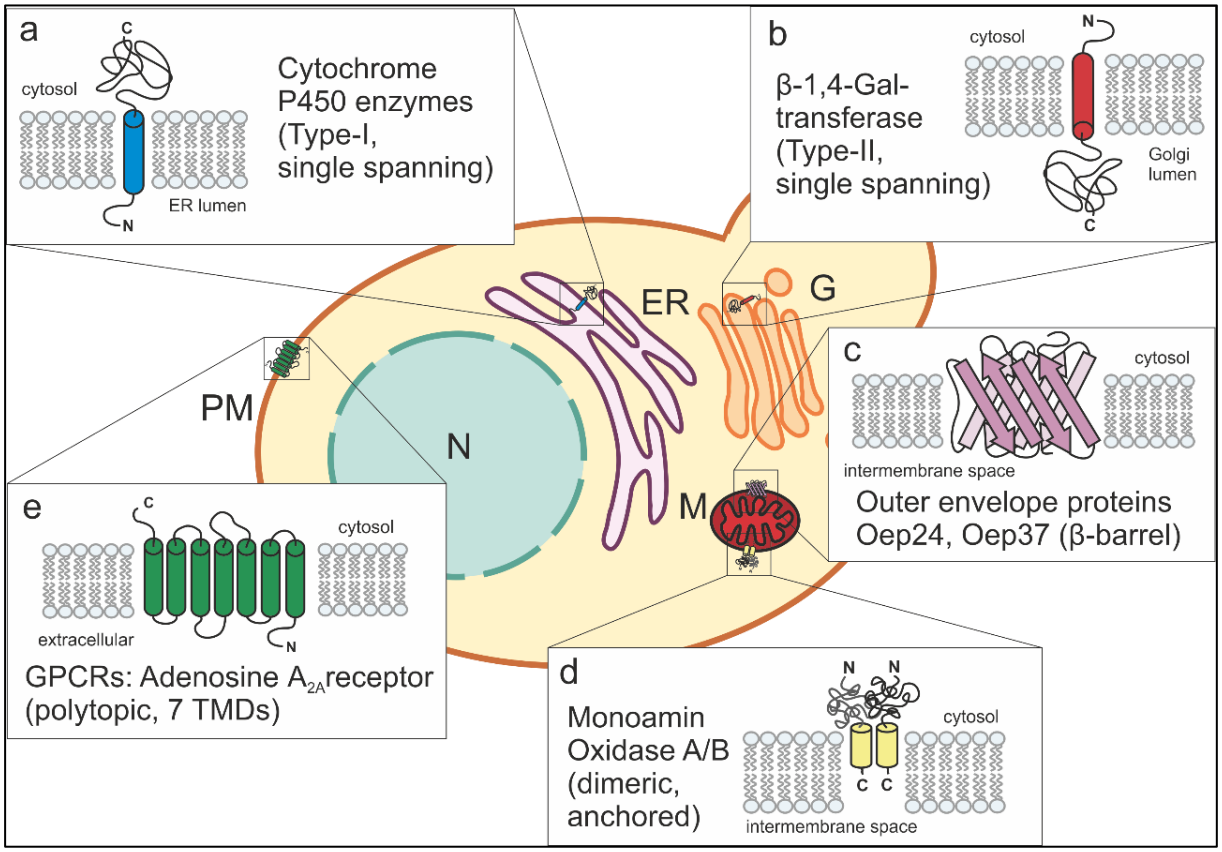


**Figure 1: Prominent membrane proteins expressed in yeast: topology and localization. This schematic drawing demonstrates the basic topologies membrane proteins can adopt, highlighting examples that have been successfully expressed in yeast. Upon heterologous expression, membrane proteins can be furthermore localized to different compartments, which has to be taken into account for functional studies as well as for cell engineering purposes. Cytochrome P450 enzymes are typical Type-I single spanning membrane proteins (a), localized to the endoplasmic reticulum (ER), with their largest, C-terminal part facing the cytosolic side. They have been heterologously expressed in different yeasts, which are used as versatile whole-cell biocatalysts** (Bernhardt 2006)**. Using a yeast membrane anchor, namely the N-terminal part of Golgi (G) localized Kre2p, a human β-1,4-Gal-transferase was functionally expressed in S. cerevisiae, enabling also proper localization of this Type-II membrane protein (b) to the yeast Golgi apparatus** (Schwientek et al. 1995)**. This strategy was further successfully employed to engineer the protein glycosylation pathway of *P. pastoris*** (Vervecken et al., 2004)**. Beta-barrel membrane proteins (c) are typically found in the outer membranes of Gram-negative bacteria, mitochondria and chloroplasts. The plant chloroplast outer envelope membrane proteins Oep24 and Oep37 were exclusively localized to the mitochondria (M) when heterologously expressed in yeast, indicating a conserved membrane import mechanism. Monoamine oxidases A and B were found to be α-helically anchored to mitochondrial outer membranes and to form a dimeric complex in the active state (d). Polytopic membrane proteins (e) consisting of multiple membrane spanning alpha helices frequently localize to the plasma membrane of recombinant yeast, e.g. GPCRs such as the A2A adenosine receptor comprising 7 TMDs. N = Nucleus**

### 7.1. Characteristics of overexpressed membrane proteins in yeast

Membrane proteins are very diverse in structure and physico-chemical properties, hence, they behave in an unpredictable way upon overexpression. Therefore, it is often necessary to test various constructs in diverse expression hosts. Frequently, an appropriate combination and optimization of target gene, vector and expression host maximizes the amount and quality of protein produced (Bernaudat et al. 2011). Several studies using *E. coli* and/or *S. cerevisiae* investigated the correlation between protein expression level and protein characteristics. Overexpression of 300 membrane proteins in *E. coli* led to the assumption that there is no link between protein features and protein expression level. In contrast, White et al. (2008) investigated the correlation of yeast membrane protein expression and protein specific parameters, i.e. size, number of transmembrane domains or hydrophobicity, by analyzing the expression levels of 1092 predicted membrane proteins in *S. cerevisiae*. They showed that similar to soluble proteins, the size of the proteins is one decisive factor for high-level membrane protein expression. More than 40% of homologous membrane proteins smaller than 60 kDa were expressed well in *S. cerevisiae* compared to less than 20% of proteins larger than 80 kDa. The inverse correlation between the number of transmembrane segments in a protein and level of expression had been reported previously (Gelperin et al. 2005) and could be confirmed in the study of White et al. (2008). Interestingly, high-level membrane protein expression was positively correlated with the hydrophobicity of predicted transmembrane segments. Hence, it was postulate that increasing the proportion of hydrophobic amino acids in transmembrane segments of membrane proteins or decreasing the overall content of aromatic residues could be favorable for membrane protein yields.

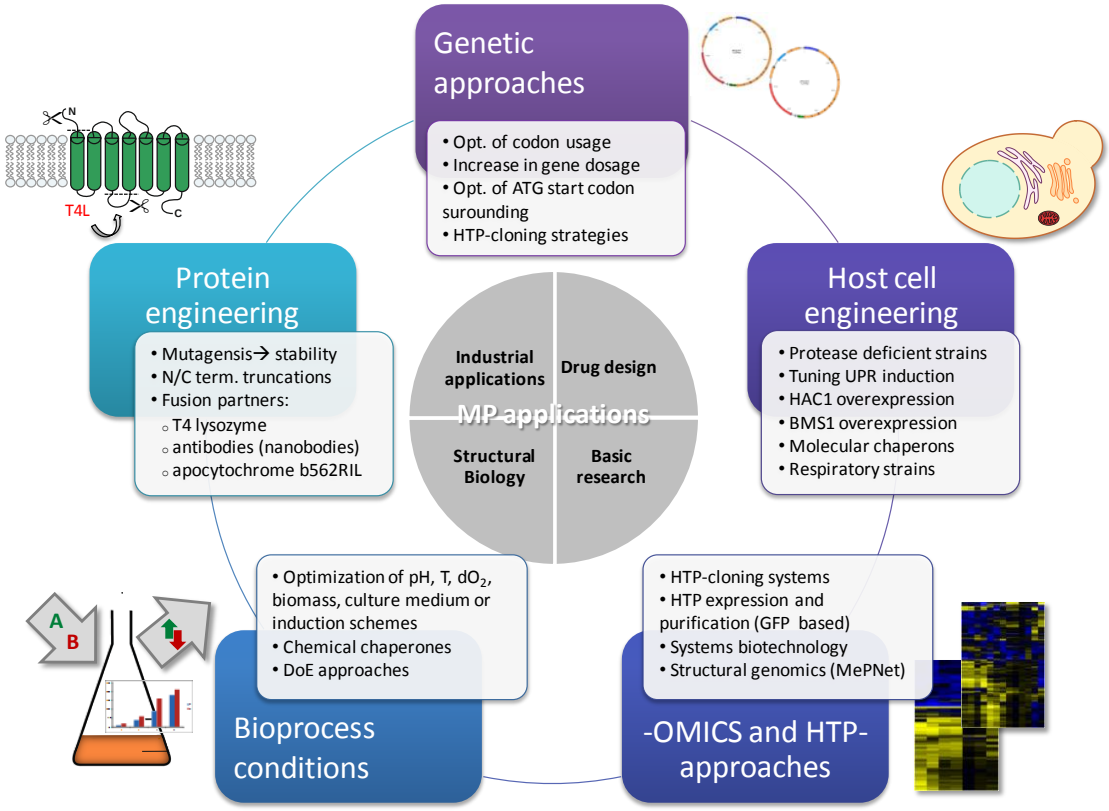


**Figure 2: Strategies for improvements in recombinant membrane protein expression in yeast.**

## *7.2. Improvements on the genetic level: adaptation of sequences*

Recently, the yeast *P. pastoris* has turned out as a most attractive host for high-level production of GPCRs (Krettler et al. 2013; Oberg et al. 2009; Singh et al. 2012a, Singh et al. 2012b). GPCR yield could mainly be enhanced by (1) optimization of the nucleotide sequence of the respective genes, (2) co-production of accessory proteins and (3) optimization of the growth conditions (Ramón and Marín 2011b; Hedfalk 2013). Heterologous expression of aquaporins in *P. pastoris* strongly responded to an increase in recombinant gene dosage, whereupon protein folding and membrane localisation seemed to be unaffected by increased expression levels (Nordén et al. 2011). Another expression study showed that a combination of codon optimisation, high gene dosage and clone selection was important for production of aquaporins in *P. pastoris* (Öberg et al. 2011). Similarly, the human μ-opioid receptor could by expressed at extended level in *P. pastoris* after optimizing gene copy number, strain background, temperature, pH, and methanol induction

(Sarramegna et al., 2002a). For the HT5A 5-hydroxytryptamin and the human β2-adrenergic receptors, the yield of functional protein was increased up to 2-fold when the number of gene copies was increased from one to two or six (Weiss et al. 1998a). Beside codon optimization of the target gene, another important aspect is the improvement of translation initiation efficiency by optimization of the surrounding of the start codon (Oberg and Hedfalk, 2013). Oberg et al. (2009) showed that small changes in the nucleotide or protein sequence of aquaporins could strongly influence expression level, maturation, folding and stability of the nascent peptide chain when produced in *P. pastoris*. Optimization of the start ATG codon context by insertion of adenosine residues upstream of the CYP2D6 gene improved the yields of the recombinant protein produced in yeast (Krynetski et al. 1995).

### *7.3. Protein engineering for enhanced protein stability and/or activity*

Several studies showed that the expression levels of membrane proteins, e.g. GPCRs, can be improved by changing protein features. Introduction of specific mutations into the coding sequences may lead to improved stability and, therefore, higher yields of functional recombinant proteins as demonstrated for human aquaporin produced in *P. pastoris* (Oberg and Hedfalk 2013; Oberg et al. 2009) or for a eukaryotic nucleobase-ascorbate transporter expressed in *S. cerevisiae* (Leung et al. 2013). Mutagenesis techniques are also frequently and successfully applied to study the interaction of GPCRs with ligands, their mechanism of activation and their interaction with G proteins (Beukers and Ijzerman 2005; Lundstrom et al. 2006). High-resolution structures of GPRCs represent a good starting point for drug design (Congreve et al. 2011). For several examples of GPCRs, protein engineering was required prior to crystallization studies. This involved a combination of 1) truncation of N- and/or C-terminal domains, ( 2) implementation of point mutations in order to stabilize the receptor in a specific conformation (Lebon et al. 2011; Serrano-Vega and Tate 2009; Serrano-Vega et al. 2008; Standfuss et al. 2007), and 3) insertion of T4 Lysozyme (T4L) into the third intracellular loop (Rosenbaum et al. 2007) or, more recently, at the N-terminus of the receptor (Rasmussen et al. 2011). The strategy of truncating the N- and/or C-terminal domains of GPCRs was usually accompanied by further protein engineering or the addition of stabilizing antibodies/nanobodies (Day et al. 2007; Rasmussen et al. 2007). To date, the most successful methodology has been the T4L engineering method, involving the insertion of T4 lysozyme into the third intracellular loop of the proteins, which enabled the crystallization of many membrane proteins, e.g. β2-adrenergic receptor (β2AR) (Rasmussen et al. 2011; Rosenbaum et al. 2007), A2A adenosine receptor (A2AAR) (Ashok et al. 2013; Jaakola et al.

2008; Xu et al. 2011), chemokine CXCR4 receptor (Wu et al. 2010), or the sphingosine 1-phosphate receptor 1. Another example is the human histamine H1 receptor, which was stably expressed in *P. pastoris* at large scale after deleting 19 amino acid residues at the N-terminus and a replacement of the third cytoplasmic loop of the protein with T4-lysozyme (Shiroishi et al. 2011). In another study, the same authors established an easy-to-handle platform using *S cerevisiae* for the rapid construction and evaluation of functional GPCR variants for structural studies. This platform can be used as fast pre-screening method to identify a suitable GPCR variant improved in expression level and stability for application in crystallization studies (Shiroishi et al. 2012).

Beside T4 lysozyme also thermostabilized apocytochrome b562RIL (Liu et al. 2012) was successfully employed to stabilize not only the inactive state of GPCRs but also their extremely unstable active state in complex with trimeric G protein. Further developments and improvements in membrane protein structure determination have been recently reviewed (Bill et al. 2011; Lieberman et al. 2011; Sonoda et al. 2010).

### *7.4. Yeast host cell engineering strategies/metabolic engineering*

Membrane proteins can be expressed in various systems, but the choice of the right host system is an important factor for the efficiency of membrane protein expression (Midgett and Madden, 2007; Bernaudat et al. 2011). Comparison of membrane protein yield in several expression hosts revealed that the best host was the evolutionarily most closely related one to the source of the target protein (Grisshammer and Tate 1995). Lately, major focus was placed on strategies to improve the productivity of host systems and especially to understand the molecular mechanisms and bottlenecks for achieving high membrane protein yields (Ashe and Bill 2011). Using the *P. pastoris* expression system, protease-deficient expression strains, such as the SMD1163 strain, and the use of the *S. cerevisiae* α-factor leader sequence led to improvement in receptor expression levels sufficient for subsequent biophysical and structural studies (Weiss et al. 1998a, Weiss et al. 1995). A clear relationship between Fps1 (eukaryotic glycerol facilitator) yield and an increase in *BMS1* transcript number was observed when comparing high-yielding host strains to wild-type (Bonander et al. 2009). Engineering of host cells by tuning *BMS1* transcript levels in a doxycycline-dependent manner resulted in optimized yields of functional membrane and soluble protein targets. Polysome profiling revealed that the reason for this high-yielding phenotype is a changed ribosomal subunit stoichiometry, hence, a change in translational properties. This finding is consistent with the role of the gene product of *BMS1* in ribosome biogenesis (Ashe and Bill 2011).

In a recent study by Ferndahl et al. (2010), a respiratory *S. cerevisiae* strain TM6* showed at least a doubling in productivity over wild-type strains for three recombinant membrane proteins (Ferndahl et al. 2010). This strain mediates low sugar uptake rates and, for that reason, the strain does not produce ethanol even in the presence of high external sugar levels (Elbing et al. 2004). Hence, this respiratory *S. cerevisiae* strain seems to represent a valuable host system as its improved biomass properties do lead to increased volumetric yields without the need of special cultivation schemes. The expression level of rat $Na^+/H^+$ antiporters in yeast plasma membrane was improved by using a strain harboring a *npi1* mutation, which significantly lowered the amount of Rsp5 ubiquitin ligase in the cell leading to enhanced stability of the protein in the plasma membrane (Flegelova et al. 2006). A physiological response at the translational level towards recombinant protein production in *S. cerevisiae* was described by Steffensen and Pedersen (2006). The heterologous expression of soluble or integral membrane proteins derepressed translation of *GCN4* mRNA. The transcription factor Gcn4p is a master regulator of gene expression in yeast and is particularly involved in responses to various kinds of stress (Hinnebusch and Natarajan 2002). The expression of the Na,K-ATPase integral membrane protein induced *GCN4* translation up to 70-fold compared to the control, depending on the strain background and the expression system. These findings suggest that production of heterologous membrane proteins represses global cap-dependent translation initiation.

## *7.5. Yeast stress response and membrane protein expression*

### 7.5.1. Environmental stress

Environmental stress response (ESR) of the cell may be imposed by the cultivation conditions, and will largely depend on the design of the fermentation strategy. Genome-wide transcriptional changes after exposure of yeast cells to environmental stresses like heat shock, acid, high osmolarity and different chemical substances were intensively investigated (Causton et al. 2001; Gasch et al. 2000; Lelandais and Devaux 2010). However, little information is available on yeast stress response in context with recombinant protein expression or industrial fermentations. Generally, it was observed that the ESR genes are up- or down-regulated transiently as a reaction to the stressful conditions, and are back to the near-normal expression level after adaptation to the new environmental conditions. During this transient phase the cell reprograms the metabolism in order to reach a new homeostasis (Mattanovich et al. 2004).The ESR triggered by elevated temperature or heat

shock has been investigated, but adaptation to cold shock or reduced temperatures are not well described, although lower temperatures are often applied for the production of heterologous proteins in yeast. Several expression studies, especially with soluble target proteins, described the advantage of lowering the cultivation temperature (Jahic et al. 2003b) and/or pH (Curvers et al. 2001; Jahic et al. 2003a; Cregg et al. 2000) on protein production due to reduced cell lysis or lower host cell protease activity.

### 7.5.2. Metabolic stress

As transcript abundance does apparently not limit membrane protein production in many cases (Sarramegna et al. 2002b), assembly of membrane proteins often works better when synthesis rates are slowed down to better match the rates of insertion and assembly. Frequently, a high gene copy number entails an increase in total protein yield, but not in amounts of functional protein as a consequence of improper balance of protein synthesis and folding (Shukla et al. 2007b; Weiss et al. 1998b; Weiss et al. 1995). In contrast to secretory proteins, however, the most prominent bottleneck in overexpression of membrane proteins is the physical space within or at a lipid bilayer. Difficulties arise from limitations in membrane capacity when accommodating additional proteins. Each membrane has an optimal ratio between lipids and membrane proteins. Thus, variations caused by massive protein insertion affect membrane integrity and cell functionality. Upon disturbance of this balance, expression systems often react with stress responses including protein degradation. Accumulation of unfolded and misfolded proteins in the ER triggers the activation of the unfolded protein response (UPR).The UPR senses the increase in unfolded protein within the ER and regulates the transcription of UPR target genes encoding chaperones, foldases, and proteins involved in glycosylation or lipid metabolism. UPR in yeast is activated by non-conventional splicing of the *HAC1* mRNA. Mature Hac1p binds to UPR-responsive elements in the promoter of UPR target genes (Patil and Walter 2001; Walter and Ron 2011; Drew and Kim 2012a). UPR has been identified as a target for improvements in heterologous protein yield in order to produce recombinant membrane proteins (Mattanovich et al. 2004; Griffith et al. 2003). Overexpression of *P. pastoris HAC1* increased the yields of some heterologous proteins in *P. pastoris*, including the doubling of functional yields of adenosine A2A receptor. However, there seems to be no general effect and, therefore, *HAC1* overexpression needs to be evaluated on a case to case basis (Guerfal et al. 2010).

Investigation of the expression levels of 12 GPCRs from the rhodopsin family of receptors in the yeast *S. cerevisiae* indicated that problems with GPCR folding and trafficking start with protein translocation through the ER membrane, which leads to the activation of downstream cellular stress responses (O'Malley et al. 2009). Previously, it has been shown that *S.*

*cerevisiae* activates the UPR pathway when unfolded or partially misfolded proteins accumulate within the ER due to expression of soluble (Mori et al. 1992) or membrane-bound (Griffith et al. 2003) proteins. The expressed GPRCs were analysed for their association with the ER-resident chaperone, Kar2p, which is known to bind to exposed hydrophobic regions of misfolded proteins. Kar2p associated with most of the receptors except for hA2aR, which is consistent with other analysis showing proper folding and trafficking out of the ER for hA2aR. A study on $hCB_2R$ has also suggested that signal sequence processing may be an issue in GPCR production in *P. pastoris* (Zhang et al. 2007).

### *7.6. Optimization of yeast cultivation conditions*

Beside genetic engineering, optimization of culture parameters for yeast host systems may increase membrane protein yield. In contrast to *S. cerevisiae*, the *P. pastoris* system can be cultured to very high cell densities, potentially producing large quantities of the protein of interest (Cereghino and Cregg 2000; Macauley-Patrick et al. 2005). The use of bioreactors, which allow straightforward culturing of large volumes together with optimal control of growth parameters to maximise cell densities and expression of the target receptors, are an attractive alternative to shake flask cultivation. *P. pastoris* has been described to adapt easily to large-scale cultivation in bioreactors (Cereghino et al. 2002). Hence, Singh et al. (2008) developed a large-scale fermentation protocol for the production of significantly higher levels of functional $A_{2A}R$ compared to traditional shake flask cultures. The described protocol was also suitable for large-scale production of the human dopamine D2 and the human serotonin 5HT1D receptors. A beneficial effect of increased pre-induction biomass acccumulation on protein yield was described for several soluble proteins (Holmes et al. 2009), whereas for membrane proteins this correlation was not necessarily observed (Bonander et al. 2005). It was shown that higher cell densities can cause cellular stresses leading to modifications in yeast membrane composition (Mattanovich et al. 2004) and, thus, affecting the activity of recombinant proteins. Consequently, medium cell density fermentation procedures, e.g. for GPCR production, have been suggested to be more advantageous compared to high biomass cultivations (Singh et al. 2008). Bonander et al. (2005) investigated the host cell response to high-yielding and low-yielding production conditions by analyzing the protein production yield as function of culture conditions for the eukaryotic glycerol facilitator Fps1p. They showed that the most rapid growth conditions are not optimal for protein production. Moreover, it was described that it is crucial to grow cells under tightly-controlled conditions and to harvest them prior to glucose exhaustion, just before the diauxic shift. Further analysis

revealed that low-membrane protein yields obtained under different culture conditions are not reflected in corresponding changes in mRNA levels of *FPS1*, but rather can be related to defects in yeast's secretory pathway and its cellular physiology (Bonander et al. 2005).

A recent study showed that an optimized induction scheme using non-selective rich medium yielded higher biomass and improved protein production by a factor >3 in *S. cerevisiae* (Drew and Kim 2012b). Bonander and Bill (2012) investigated the influence of YNB-based media and CBS media on membrane protein production in *S. cerevisiae*. Growth in the simple YNB-based medium typically yielded low biomass and less recombinant protein compared to CBS medium. The latter was much more labour intensive to prepare, though. Medium optimisation studies showed that supplementing YNB-based medium with myo-inositol to levels similar to the CBS medium yielded improved growth rates and protein levels. A similar effect was seen after supplementation with biotin. The positive effect of myo-inosotol was attributed to its essential role in the relief of cellular stresses during membrane protein expression (Gaspar et al. 2006). It has been shown previously that addition of soy peptides improve yeast tolerance to freeze-thaw stress or changes in lipid metabolism (Ikeda et al. 2011). Recently, the production of six out of eight selected GPCRs in *S. cerevisiae* was enhanced ~ 2.3-fold when using soy peptide containing medium in contrast to free amino acid-based medium (Ito et al. 2012).

## *7.7. Improving membrane protein expression by chaperones and auxiliary proteins*

### 7.7.1. Molecular chaperones

Prerequisite for the expression of functionally active and correctly targeted integral membrane proteins are proper folding, maturation and transport processes in the host cells. Typically, membrane proteins - excluding proteins destined for peroxisomal or mitochondrial membranes - enter the secretory pathway by translocating into the ER membrane where folding and maturation of the proteins take place (Alder and Johnson 2004; Hebert and Molinari 2007). One strategy to improve the functional yield of membrane proteins has therefore been to co-express molecular chaperones that are known to facilitate folding. Co-expression of ER-resident chaperones and foldases have been show to alleviate ER-folding processes yielding improved levels of heterologous (soluble) proteins produced in *E. coli* (Schlapschy and Skerra 2011; Hoffmann and Rinas 2004) or insect cells (Tate et al. 1999; Kato et al. 2005). The successful assistance of molecular chaperones and auxiliary proteins

in heterologous membrane protein synthesis in yeast is hardly documented. Butz et al. (2003) investigated whether the co-overexpression of ER-resident proteins like PDI, calnexin or Kar2p would improve total and/or active GPCR yields. They showed that the expression of human $A_2a$ adenosine receptor ($A_2a$) and mouse substance P receptor (SPR) in yeast was not limited by an ER-bottleneck, due to (1) unchanged receptor yield by co-expression of ER-chaperones; (2) no ER-retention of GPCRs observed by confocal-microscopy; (3) insensitivity of both GPCR yields to gene copy number.

Another strategy to improve GPCR expression for structural elucidation in yeast is the addition of ligands that specifically bind the receptors (Grünewald et al. 2004; Weiss et al. 1998a; Fraser 2006). There is evidence that a specific ligand can serve as a molecular chaperone during protein folding (Bernier et al. 2004; Petäjä-Repo et al. 2002; Grünewald et al. 2004). GPCR expression was further optimized in *P. pastoris* by combining low temperature induction with with addition of ligands to the *P. pastoris* culture media at a concentration close to 100-fold $K_d$ (Lundstrom et al. 2006; André et al. 2006). Following this strategy, the yield of functional GPCR was increased more than eightfold over standard expression conditions.

### 7.7.2. Chemical chaperones

Supplementation of yeast culture media with chemical chaperones, such as DMSO, histidine or glycerol has been beneficial for membrane protein yield (André et al. 2006; Drew and Kim 2012b; Figler et al. 2000; Fraser 2006; Shukla et al. 2007c; Weiss et al. 1998a). Addition of DMSO to the growth medium dramatically altered the expression pattern of yeast (Zhang et al. 2003) and altered the membrane properties of several organisms by up-regulating genes involved in lipid synthesis (Murata et al. 2003). Recent studies using genome-wide screens in *S. cerevisiae* identified major cellular processes which are sensitive for DMSO including Golgi/ER transport, chromatin remodeling, DNA repair and cell wall integrity (Gaytán et al. 2013; Zhang et al. 2013). Furthermore, DMSO also increased the permeability of membranes, thus enhancing the access of externally added ligands to membrane proteins (Yu and Quinn 1994). The addition of 2.5 % DMSO to *Pichia* cultivation medium resulted in six-fold increased protein production compared to standard conditions for 16 out of 20 tested receptors (André et al. 2006). Like for many chaperones, DMSO supplementation was not key to high membrane protein yields, as for several proteins no or a negative effect was observed (Shiroishi et al. 2012).

The addition of histidine to the culture medium was ascribed to have a positive effect on the production yield of membrane proteins. 12 out of 20 tested GPCRs showed enhanced production yields upon supplementation of *P. pastoris* cultivation medium with histidine, but with a moderate improvement factor compared to other tested optimization parameters (André et al. 2006). It is assumed that histidine, rather than other amino acids, can act as a physiological “antioxidant” in yeast cells (Murakami et al. 1997), but actually there is no data available to support this assumption. Glycerol was used as chemical chaperone to obtain high yields of active human P-glycoprotein in *S. cerevisiae* (Figler et al. 2000). Cells cultured in media supplemented with 10% glycerol showed a 3.3-fold increase in membrane-localized P-gp relative to controls. The positive effect of the glycerol was ascribed to the ability of glycerol to stabilize the conformation of the proteins and to enhance folding and maturation.

## 8. Conclusion and Outlook

Yeasts are a versatile and extremely powerful system for expression and investigation of higher eukaryotic membrane proteins, despite the many obstacles exacerbating the applicability of recombinant membrane proteins in biochemical and structural studies. They are tricky to handle in solution, need correct membrane targeting, appropriate membrane environment for optimal activity and, of course, large amounts of membrane proteins are needed for crystallization. Nevertheless, engineering strategies help to overcome many of these difficulties. Fine-tuning of expression protocols by adapting induction and cultivation conditions like decreasing growth temperatures and, thus, slowing down protein biosynthesis enabled increased yields in regard of protein quantity. Strategies paving the way for expression success on a molecular level are codon-optimization, altering yeast membrane composition and co-expression of helper proteins, e.g. chaperones supporting correct folding or membrane targeting. Eliciting strengths and weaknesses of different yeast systems applied so far did ease the decision on to which host to choose from the start. Interestingly, yeasts differ in their applicability for expression of different membrane protein types more than one might believe. *S. cerevisiae* was caught up recently by other yeasts, primarily *P. pastoris*. The potential of their relatives *S. pombe*, *Y. lipolytica* and *H. polymorpha* is not even close to be fully exploited. Although eukaryotic expression systems are applied roughly as often as yeast systems, they are inferior concerning growth rates, applicability as hosts for mutant library screenings and lacking standard protein interaction analysis tools like Y2H and MYTH. Based on many achievements in tuning and, hence, pushing high-level protein production, the majority of all membrane protein crystal structures originate from homologous overexpression. During the last five years, eukaryotic protein structures resolved with the help of yeast were increased almost three-fold, suggesting yeast to be a promising host for further crystallization studies. Structural resolution enables the elucidation of structure-function relationships, which is indispensable for state-of-the-art scientific work. Yeasts engineered to serve as screening tool for drug-target interactions is one of the most encouraging research fields currently. Summing up all smaller and bigger recent success stories reached so far offers an overall nice perspective for yeast continuing to be a valuable system for studies on higher eukaryotic membrane proteins.

# References


Adamian, L., Naveed, H., Liang, J., 2011. Lipid-binding surfaces of membrane proteins: evidence from evolutionary and structural analysis. Biochimica et biophysica acta 1808, 1092–1102.

Ago, H., Kanaoka, Y., Irikura, D., Lam, B.K., Shimamura, T., Austen, K.F., Miyano, M., 2007. Crystal structure of a human membrane protein involved in cysteinyl leukotriene biosynthesis. Nature 448, 609–12.

Alder, N.N., Johnson, A.E., 2004. Cotranslational membrane protein biogenesis at the endoplasmic reticulum. The Journal of biological chemistry 279, 22787–90.

Aller, S.G., Yu, J., Ward, A., Weng, Yue, Chittaboina, S., Zhuo, R., Harrell, P.M., Trinh, Y.T., Zhang, Qinghai, Urbatsch, I.L., Chang, G., 2009. Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science (New York, N.Y.) 323, 1718–22.

Añazco, C., Peña-Münzenmayer, G., Araya, C., Cid, L.P., Sepúlveda, F. V, Niemeyer, M.I., 2013. G protein modulation of $K_{2P}$ potassium channel TASK-2: A role of basic residues in the C terminus domain. Pflugers Archiv: European Journal of physiology 465, 1715–1726

André, N., Cherouati, N., Prual, C., Steffan, T., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R., Reinhart, C., 2006. Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. Protein science: a publication of the Protein Society 15, 1115–26.

Andréll, J., Tate, C.G., 2013. Overexpression of membrane proteins in mammalian cells for structural studies. Molecular membrane biology 30, 52–63.

Arsenault, P.R., Wobbe, K.K., Weathers, P.J., 2008. Recent advances in artemisinin production through heterologous expression. Current medicinal chemistry 15, 2886–96.

Arslan, B.K., Edmondson, D.E., 2010. Expression of zebrafish (Danio rerio) monoamine oxidase (MAO) in *Pichia pastoris*: purification and comparison with human MAO A and MAO B. Protein expression and purification 70, 290–7.

Ashe, M.P., Bill, R.M., 2011. Mapping the yeast host cell response to recombinant membrane protein production: relieving the biological bottlenecks. Biotechnology Journal 6, 707–714.

Ashok, Y., Nanekar, R.T., Jaakola, V.-P., 2013. Crystallogenesis of adenosine A(2A) receptor-T4 lysozyme fusion protein: a practical route for the structure. Methods in enzymology 520, 175–98.

Ault, A.D., Broach, J.R., 2006. Creation of GPCR-based chemical sensors by directed evolution in yeast. Protein engineering, design & selection: PEDS 19, 1–8.

Azad, A.K., Katsuhara, M., Sawa, Y., Ishikawa, T., Shibata, H., 2008. Characterization of four plasma membrane aquaporins in tulip petals: a putative homolog is regulated by phosphorylation. Plant & cell physiology 49, 1196–208.

Azad, A.K., Sawa, Y., Ishikawa, T., Shibata, H., 2009. Heterologous expression of tulip petal plasma membrane aquaporins in *Pichia pastoris* for water channel analysis. Applied and Environmental Microbiology 75, 2792–2797.

Azad, A.K., Yoshikawa, N., Ishikawa, T., Sawa, Y., Shibata, H., 2012. Substitution of a single amino acid residue in the aromatic/arginine selectivity filter alters the transport profiles of tonoplast aquaporin homologs. Biochimica et biophysica acta 1818, 1–11.

Bannwarth, M., Schulz, G.E., 2003. The expression of outer membrane proteins for crystallization. Biochimica et biophysica acta 1610, 37–45.

Beaudoin, F, Wilkinson, B.M., Stirling, C.J., Napier, J A, 2000. In vivo targeting of a sunflower oil body protein in yeast secretory (sec) mutants. The Plant journal: for cell and molecular biology 23, 159–70.

Beaudoin, F., Napier, J.A., 2002. Targeting and membrane-insertion of a sunflower oleosin in vitro and in *Saccharomyces cerevisiae*: the central hydrophobic domain contains more than one signal sequence, and directs oleosin insertion into the endoplasmic reticulum membrane using a signal a. Planta 215, 293–303.

Bérczi, A., Su, D., Lakshminarasimhan, M., Vargas, A., Asard, H., 2005. Heterologous expression and site-directed mutagenesis of an ascorbate-reducible cytochrome b561. Archives of biochemistry and biophysics 443, 82–92.

Bereczky, Z., Wang, H.-Y., Schubert, V., Ganal, M., Bauer, P., 2003. Differential regulation of nramp and irt metal transporter genes in wild type and iron uptake mutants of tomato. The Journal of biological chemistry 278, 24697–704.

Bernaudat, F., Frelet-Barrand, A., Pochon, N., Dementin, S., Hivin, P., Boutigny, S., Rioux, J.-B., Salvi, D., Seigneurin-Berny, D., Richaud, P., Joyard, J., Pignol, D., Sabaty, M., Desnos, T., Pebay-Peyroula, E., Darrouzet, E., Vernet, T., Rolland, N., 2011. Heterologous expression of membrane proteins: choosing the appropriate host. PloS one 6, e29191.

Bernhardt, R., 2006. Cytochromes P450 as versatile biocatalysts. Journal of biotechnology 124, 128–45.

Bernier, V., Bichet, D.G., Bouvier, M., 2004. Pharmacological chaperone action on G-protein-coupled receptors. Current opinion in pharmacology 4, 528–33.

Bernstein, J.D., Okamoto, Y., Kim, M., Shikano, S., 2013. Potential use of potassium efflux-deficient yeast for studying trafficking signals and potassium channel functions. FEBS open bio 3, 196–203.

Beukers, M.W., Ijzerman, A.P., 2005. Techniques: how to boost GPCR mutagenesis studies using yeast. Trends in pharmacological sciences 26, 533–9.

Bhatla, S.C., Kaushik, V., Yadav, M.K., 2010. Use of oil bodies and oleosins in recombinant protein production and other biotechnological applications. Biotechnology advances 28, 293–300.

Bill, R.M., Henderson, P.J.F., Iwata, S., Kunji, E.R.S., Michel, Hartmut, Neutze, R., Newstead, S., Poolman, B., Tate, Christopher G, Vogel, H., 2011. Overcoming barriers to membrane protein structure determination. Nature biotechnology 29, 335–40.

Binda, C., Li, M., Hubalek, Frantisek, Restelli, N., Edmondson, Dale E, Mattevi, A., 2003. Insights into the mode of inhibition of human mitochondrial monoamine oxidase B from high-resolution crystal structures. Proceedings of the National Academy of Sciences of the United States of America 100, 9750–9755.

Binda, C., Newton-Vinson, Paige, Hubálek, Frantisek, Edmondson, Dale E, Mattevi, A., 2002. Structure of human monoamine oxidase B, a drug target for the treatment of neurological disorders. Nature Structural Biology 9, 22–26.

Bocer, T., Zarubica, A., Roussel, A., Flis, K., Trombik, T., Goffeau, A., Ulaszewski, S., Chimini, G., 2011. The mammalian ABC transporter ABCA1 induces lipid-dependent drug sensitivity in yeast. Biochimica et Biophysica Acta 3–10.

Bomholt, J., Hélix-Nielsen, C., Scharff-Poulsen, P., Pedersen, P.A., 2013. Recombinant Production of Human Aquaporin-1 to an Exceptional High Membrane Density in *Saccharomyces cerevisiae*. PloS one 8, e56431.

Bonander, N., Bill, R.M., 2012. Optimising yeast as a host for recombinant protein production (review). Methods in molecular biology (Clifton, N.J.) 866, 1–9.

Bonander, N., Darby, R.A., Grgic, L., Bora, N., Wen, J., Brogna, S., Poyner, D.R., O'Neill, M.A., Bill, R.M., 2009. Altering the ribosomal subunit ratio in yeast maximizes recombinant protein yield. Microbial cell factories 8, 10.

Bonander, N., Hedfalk, K., Larsson, C., Mostad, P., Chang, C., Gustafsson, L., Bill, R.M., 2005. Design of improved membrane protein production experiments: quantitation of the host response. Protein science: a publication of the Protein Society 14, 1729–40.

Brandsch, M., 2009. Transport of drugs by proton-coupled peptide transporters: pearls and pitfalls. Expert opinion on drug metabolism & toxicology 5, 887–905.

Braun, A., Geier, M., Bühler, B., Schmid, A., Mauersberger, Stephan, Glieder, A., 2012. Steroid biotransformations in biphasic systems with Yarrowia lipolytica expressing human liver cytochrome P450 genes. Microbial cell factories 11, 106.

Brohawn, S.G., Campbell, E.B., MacKinnon, R., 2013. Domain-swapped chain connectivity and gated membrane access in a Fab-mediated crystal of the human TRAAK K+ channel. Proceedings of the National Academy of Sciences of the United States of America 110, 2129–34.

Brohawn, S.G., Del Mármol, J., MacKinnon, R., 2012. Crystal structure of the human K2P TRAAK, a lipid- and mechano-sensitive K+ ion channel. Science (New York, N.Y.) 335, 436–41.

Brown, G.D., 2010. The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua* L. (Qinghao). Molecules (Basel, Switzerland) 15, 7603–98.

Bublitz, M., Poulsen, H., Morth, J.P., Nissen, P., 2010. In and out of the cation pumps: P-type ATPase structure revisited. Current opinion in structural biology 20, 431–9.

Butz, J.A., Niebauer, R.T., Robinson, Anne Skaja, 2003. Co-expression of molecular chaperones does not improve the heterologous expression of mammalian G-protein coupled receptor expression in yeast. Biotechnology and bioengineering 84, 292–304.

Capuano, F., Beaudoin, Frédéric, Napier, Johnathan A., Shewry, P.R., 2007. Properties and exploitation of oleosins. Biotechnology Advances 25, 203–206.

Cardi, D., Montigny, C., Arnou, B., Jidenko, M., Marchal, E., Le Maire, M., Jaxel, C., 2010a. Heterologous expression and affinity purification of eukaryotic membrane proteins in view of functional and structural studies: The example of the sarcoplasmic reticulum Ca(2+)-ATPase. Methods in molecular biology (Clifton, N.J.) 601, 247–67.

Cardi, D., Pozza, A., Arnou, B., Marchal, E., Clausen, J.D., Andersen, J.P., Krishna, S., Møller, J. V, Le Maire, M., Jaxel, C., 2010b. Purified E255L mutant SERCA1a and purified *Pf*ATP6 are sensitive to SERCA-type inhibitors but insensitive to artemisinins. The Journal of biological chemistry 285, 26406–16.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S., Young, R.A., 2001. Remodeling of yeast genome expression in response to environmental changes. Molecular biology of the cell 12, 323–37.

Celić, A., Connelly, S.M., Martin, N.P., Dumont, M.E., 2004. Intensive mutational analysis of G protein-coupled receptors in yeast. Methods in molecular biology (Clifton, N.J.) 237, 105–20.

Cenacchi, L., Busch, M., Schleidt, P.G., Müller, F.G., Stumpp, T.V.M., Mäntele, W., Trost, P., Lancaster, C.R.D., 2012. Heterologous production and characterisation of two distinct dihaem-containing membrane integral cytochrome b(561) enzymes from *Arabidopsis thaliana* in *Pichia pastoris* and *Escherichia coli* cells. Biochimica et biophysica acta 1818, 679–88.

Centeno, F., Deschamps, S., Lompré, A.-M., Anger, M., Moutin, M.-J., Dupont, Y., Palmgren, M.G., Villalba, J.M., Møller, J.V., Falson, P., Le Maire, M., 1994. Expression of the sarcoplasmic reticulum $Ca^{2+}$-ATPase in yeast. FEBS Letters 354, 117–122.

Cereghino, G.P.L., Cereghino, J.L., Ilgen, C., Cregg, J.M., 2002. Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Current opinion in biotechnology 13, 329–32.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*, FEMS Microbiology Reviews.

Chanroj, S., Lu, Y., Padmanaban, S., Nanatani, K., Uozumi, N., Rao, R., Sze, H., 2011. Plant-specific cation/H+ exchanger 17 and its homologs are endomembrane K+ transporters with roles in protein sorting. The Journal of biological chemistry 286, 33931–41.

Chen, J., Lalonde, S., Obrdlik, P., Noorani Vatani, A., Parsa, S.A., Vilarino, C., Revuelta, Jose Luis, Frommer, W.B., Rhee, S.Y., 2012. Uncovering Arabidopsis membrane protein interactome enriched in transporters using mating-based split ubiquitin assays and classification models. Frontiers in plant science 3, 124.

Chen, K., Sun, G., Lv, Z., Wang, C., Jiang, Xueyuan, Li, D., Zhang, C., 2010. Molecular cloning of amphioxus uncoupling protein and assessment of its uncoupling activity using a yeast heterologous expression system. Biochemical and biophysical research communications 400, 701–6.

Chen, X., Wang, Q., Ni, F., Ma, J., 2010. Structure of the full-length Shaker potassium channel Kv1.2 by normal-mode-based X-ray crystallographic refinement. Proceedings of the National Academy of Sciences of the United States of America 107, 11352–7.

Cheon, S.A., Kim, H., Oh, D.-B., Kwon, O., Kang, H.A., 2012. Remodeling of the glycosylation pathway in the methylotrophic yeast *Hansenula polymorpha* to produce human hybrid-type N-glycans. Journal of microbiology (Seoul, Korea) 50, 341–8.

Cherezov, V., Rosenbaum, D.M., Hanson, M.A., Rasmussen, S.G.F., Thian, F.S., Kobilka, T.S., Choi, H.-J., Kuhn, P., Weis, W.I., Kobilka, B.K., Stevens, R.C., 2007. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science (New York, N.Y.) 318, 1258–65.

Cherouati, N., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R., Reinhart, C., 2006. Enhancing functional production of G protein-coupled receptors in *Pichia pastoris* to levels required for structural studies via a single expression screen. Protein Science 1115–1126.

Chodok, P., Eiamsa-ard, P., Cove, D.J., Quatrano, R.S., Kaewsuwan, S., 2013. Identification and functional characterization of two Δ12-fatty acid desaturases associated with essential linoleic acid biosynthesis in *Physcomitrella patens*. Journal of industrial microbiology & biotechnology 40, 901–13.

Cohen, C.K., Garvin, D.F., Kochian, L. V, 2004. Kinetic properties of a micronutrient transporter from *Pisum sativum* indicate a primary function in Fe uptake from the soil. Planta 218, 784–92.

Congreve, M., Langmead, C., Marshall, F.H., 2011. The use of GPCR structures in drug design. Advances in pharmacology (San Diego, Calif.) 62, 1–36.

Cregg, J., L Cereghino, J., Shi, J., R Higgins, D., 2000. Recombinant protein expression in *Pichia pastoris*. Molecular Biotechnology 16, 23–52.

Cregg, J.M., Tolstorukov, I., Kusari, A., Sunga, J., Madden, K., Chappell, T., 2009. Expression in the yeast *Pichia pastoris*., Methods in Enzymology. Elsevier Inc.

Crowe, M.L., Perry, B.N., Connerton, I.F., 2000. Golf complements a GPA1 null mutation in *Saccharomyces cerevisiae* and functionally couples to the STE2 pheromone receptor. Journal of receptor and signal transduction research 20, 61–73.

Curvers, S., Brixius, P., Klauser, T., Thömmes, J., Weuster-Botz, D., Takors, R., Wandrey, C., 2001. Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing. Part 1. Fermentation. Biotechnology progress 17, 495–502.

Daniels, M.J., Yeager, M., 2005. Phosphorylation of aquaporin *Pv*TIP3;1 defined by mass spectrometry and molecular modeling. Biochemistry 44, 14443–54.

Darby, R.A.J., Cartwright, S.P., Dilworth, M. V, Bill, R.M., 2012. Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris*. Methods in molecular biology (Clifton, N.J.) 866, 11–23.

Davey, J., Ladds, G., 2011. Heterologous expression of GPCRs in fission yeast. Methods in molecular biology (Clifton, N.J.) 746, 113–31.

Day, P.W., Rasmussen, S.G.F., Parnot, C., Fung, J.J., Masood, A., Kobilka, T.S., Yao, X.-J., Choi, H.-J., Weis, W.I., Rohrer, D.K., Kobilka, B.K., 2007. A monoclonal antibody for G protein-coupled receptor crystallography. Nature methods 4, 927–9.

De Colibus, L., Li, M., Binda, C., Lustig, A., Edmondson, Dale E, Mattevi, A., 2005. Three-dimensional structure of human monoamine oxidase A (MAO A): relation to the structures of rat MAO A and human MAO B. Proceedings of the National Academy of Sciences of the United States of America 102, 12684–9.

De Pourcq, K., De Schutter, K., Callewaert, N., 2010. Engineering of glycosylation in yeast and other fungi: current state and perspectives. Applied microbiology and biotechnology 87, 1617–31.

Dhanasekaran, D.N., Radhika, V., Proikas-Cezanne, T., Jayaraman, M., Ha, J., 2009. Heterologous expression of olfactory receptors for targeted chemosensing. Annals of the New York Academy of Sciences 1170, 157–60.

Domergue, F., Abbadi, A., Ott, C., Zank, T.K., Zähringer, U., Heinz, E., 2003a. Acyl carriers used as substrates by the desaturases and elongases involved in very long-chain polyunsaturated fatty acids biosynthesis reconstituted in yeast. The Journal of biological chemistry 278, 35115–26.

Domergue, F., Spiekermann, P., Lerchl, J., Beckmann, C., Kilian, O., Kroth, P.G., Boland, W., Zähringer, U., Heinz, E., 2003b. New insight into Phaeodactylum tricornutum fatty acid metabolism. Cloning and functional characterization of plastidial and microsomal delta12-fatty acid desaturases. Plant physiology 131, 1648–60.

Döring, F., Michel, T., Rösel, A., Nickolaus, M., Daniel, H, 1998a. Expression of the mammalian renal peptide transporter PEPT2 in the yeast *Pichia pastoris* and applications of the yeast system for functional analysis. Molecular membrane biology 15, 79–88.

Döring, F., Theis, S, Daniel, H, 1997. Expression and functional characterization of the mammalian intestinal peptide transporter PepT1 in the methylotropic yeast *Pichia pastoris*. Biochemical and biophysical research communications 232, 656–62.

Döring, F., Walter, J., Will, J., Föcking, M., Boll, M., Amasheh, S., Clauss, W., Daniel, H, 1998b. Delta-aminolevulinic acid transport by intestinal and renal peptide transporters and its physiological and clinical implications. The Journal of clinical investigation 101, 2761–7.

Douette, P., Gerkens, P., Navet, R., Leprince, P., De Pauw, E., Sluse, F.E., 2006. Uncoupling protein 1 affects the yeast mitoproteome and oxygen free radical production. Free radical biology & medicine 40, 303–15.

Dowell, S.J., Brown, A.J., 2009. Yeast assays for G protein-coupled receptors. Methods in molecular biology (Clifton, N.J.) 552, 213–29.

Drăgan, C.-A., Zearo, S., Hannemann, F., Bernhardt, R., Bureik, M., 2005. Efficient conversion of 11-deoxycortisol to cortisol (hydrocortisone) by recombinant fission yeast *Schizosaccharomyces pombe*. FEMS yeast research 5, 621–5.

Drake, R., Serrano, A., Pérez-Castiñeira, J.R., 2010. N-terminal chimaeras with signal sequences enhance the functional expression and alter the subcellular localization of heterologous membrane-bound inorganic pyrophosphatases in yeast. The Biochemical journal 426, 147–157.

Drew, D., Kim, H., 2012a. Large-scale production of membrane proteins in *Saccharomyces cerevisiae*: using a green fluorescent protein fusion strategy in the production of membrane proteins. Methods in molecular biology (Clifton, N.J.) 866, 209–16.

Drew, D., Kim, H., 2012b. Optimizing *Saccharomyces cerevisiae* induction regimes. Methods in molecular biology (Clifton, N.J.) 866, 191–5.

Eckhardt, U., Mas Marques, A., Buckhout, T.J., 2001. Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. Plant molecular biology 45, 437–48.

Elbing, K., Ståhlberg, A., Hohmann, S., Gustafsson, L., 2004. Transcriptional responses to glucose at different glycolytic rates in *Saccharomyces cerevisiae*. European journal of biochemistry / FEBS 271, 4855–64.

Ellinger, P., Kluth, M., Stindt, J., Smits, S.H.J., Schmitt, L., 2013. Detergent screening and purification of the human liver ABC transporters BSEP (ABCB11) and MDR3 (ABCB4) expressed in the yeast *Pichia pastoris*. PloS one 8, e60620.

Entian, K.D., Schuster, T., Hegemann, J.H., Becher, D., Feldmann, H., Güldener, U., Götz, R., Hansen, M., Hollenberg, C.P., Jansen, G., Kramer, W., Klein, S., Kötter, P., Kricke, J., Launhardt, H., Mannhaupt, G., Maierl, A., Meyer, P., Mewes, W., Munder, T., Niedenthal, R.K., Ramezani Rad, M., Röhmer, A., Römer, A., Hinnen, A., 1999. Functional analysis of 150 deletion mutants in Saccharomyces cerevisiae by a systematic approach. Molecular & general genetics: MGG 262, 683–702.

Ewen, K.M., Schiffler, B., Uhlmann-Schiffler, H., Bernhardt, R., Hannemann, F., 2008. The endogenous adrenodoxin reductase-like flavoprotein arh1 supports heterologous cytochrome P450-dependent substrate conversions in *Schizosaccharomyces pombe*. FEMS yeast research 8, 432–41.

Ferndahl, C., Bonander, N., Logez, C., Wagner, R., Gustafsson, L., Larsson, C., Hedfalk, K., Darby, R.A.J., Bill, R.M., 2010. Increasing cell biomass in *Saccharomyces cerevisiae* increases recombinant protein yield: the use of a respiratory strain as a microbial cell factory. Microbial cell factories 9, 47.

Fickers, P., Benetti, P.-H., Waché, Y., Marty, A., Mauersberger, S, Smit, M.S., Nicaud, J-M, 2005. Hydrophobic substrate utilisation by the yeast Yarrowia lipolytica, and its potential applications. FEMS yeast research 5, 527–43.

Figler, R.A., Omote, H, Nakamoto, R.K., Al-Shawi, M.K., 2000. Use of chemical chaperones in the yeast *Saccharomyces cerevisiae* to enhance heterologous membrane protein expression: high-yield expression and purification of human P-glycoprotein. Archives of biochemistry and biophysics 376, 34–46.

Flegelova, H., Haguenauer-Tsapis, R., Sychrova, H., 2006. Heterologous expression of mammalian Na/H antiporters in *Saccharomyces cerevisiae*. Biochimica et biophysica acta 1760, 504–16.

Foltz, M., Meyer, A., Theis, S., Demuth, H.-U., Daniel, H., 2004. A rapid in vitro screening for delivery of peptide-derived peptidase inhibitors as potential drug candidates via epithelial peptide transporters. The Journal of pharmacology and experimental therapeutics 310, 695–702.

Fraser, N.J., 2006. Expression and functional purification of a glycosylation deficient version of the human adenosine 2a receptor for structural studies. Protein expression and purification 49, 129–37.

Freigassner, M., Pichler, H., Glieder, A., 2009. Tuning microbial hosts for membrane protein production. Microbial Cell Factories 8, 69.

Fridell, Y.-W.C., Sánchez-Blanco, A., Silvia, B.A., Helfand, S.L., 2004. Functional characterization of a *Drosophila* mitochondrial uncoupling protein. Journal of bioenergetics and biomembranes 36, 219–28.

Gagné, S.J., Reed, Darwin W, Gray, G.R., Covello, Patrick S, 2009. Structural control of chemoselectivity, stereoselectivity, and substrate specificity in membrane-bound fatty acid acetylenases and desaturases. Biochemistry 48, 12298–304.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., Brown, P.O., 2000. Genomic expression programs in the response of yeast cells to environmental changes. Molecular biology of the cell 11, 4241–57.

Gaspar, M.L., Aregullin, M.A., Jesch, S.A., Henry, S.A., 2006. Inositol induces a profound alteration in the pattern and rate of synthesis and turnover of membrane lipids in *Saccharomyces cerevisiae*. The Journal of biological chemistry 281, 22773–85.

Gaytán, B.D., Loguinov, A. V, De La Rosa, V.Y., Lerot, J.-M., Vulpe, C.D., 2013. Functional genomics indicates yeast requires Golgi/ER transport, chromatin remodeling, and DNA repair for low dose DMSO tolerance. Frontiers in genetics 4, 154.

Geier, M., Braun, A., Emmerstorfer, A., Pichler, H., Glieder, A., 2012. Production of human cytochrome P450 2D6 drug metabolites with recombinant microbes--a comparative study. Biotechnology journal 1–13.

Gellissen, G., 2005. Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems, Wiley-VCH, Weinheim.

Gellissen, G., Kunze, G., Gaillardin, C., Cregg, J.M., Berardi, E., Veenhuis, Marten, Van der Klei, I., 2005. New yeast expression platforms based on methylotrophic *Hansenula polymorpha* and *Pichia pastoris* and on dimorphic *Arxula adeninivorans* and *Yarrowia lipolytica*--a comparison. FEMS yeast research 5, 1079–96.

Gellisson, G., 2004. Key and Criteria to the Selection of An Expression Platform. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

Gelperin, D.M., White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, Lixia, Piccirillo, S., Yu, H., Gerstein, Mark, Dumont, M.E., Phizicky, E.M., Snyder, Michael, Grayhack, E.J., 2005. Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. Genes & development 19, 2816–26.

Gerasimov, A.S., Zeĭnalov, O.A., Él'darov, M.A., Shul'ga, A.A., 2012. Biosynthesis and purification of human beta2-adrenergic receptor expressed in methylotrophic yeast *Pichia pastoris*. Molekuliarnaia biologiia 46, 308–16.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., et al., 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 418, 387–91.

Gonçalves, A.M., Pedro, A.Q., Maia, C., Sousa, F., Queiroz, J.A., Passarinha, L.A., 2013. *Pichia pastoris*: a recombinant microfactory for antibodies and human membrane proteins. Journal of microbiology and biotechnology 23, 587–601.

Griffith, D.A., Delipala, C., Leadsham, J., Jarvis, S.M., Oesterhelt, D., 2003. A novel yeast expression system for the overproduction of quality-controlled membrane proteins. FEBS letters 553, 45–50.

Grishin, A., Li, H., Levitan, E.S., Zaks-Makhina, E., 2006. Identification of gamma-aminobutyric acid receptor-interacting factor 1 (TRAK2) as a trafficking factor for the K+ channel Kir2.1. The Journal of Biological Chemistry 281, 30104–30111.

Grisshammer, R., Tate, C G, 1995. Overexpression of integral membrane proteins for structural studies. Quarterly reviews of biophysics 28, 315–422.

Grünewald, S., Haase, W., Molsberger, E., Michel, H., Reiländer, H., 2004. Production of the human D2S receptor in the methylotrophic yeast *P. pastoris*. Receptors & channels 10, 37–50.

Gu, J., Weng, Yan, Zhang, Q.-Y., Cui, H., Behr, M., Wu, L., Yang, W., Zhang, Li, Ding, X., 2003. Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. The Journal of Biological Chemistry 278, 25895–25901.

Guerfal, M., Ryckaert, S., Jacobs, P.P., Ameloot, P., Van Craenenbroeck, K., Derycke, R., Callewaert, N., 2010. The HAC1 gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. Microbial cell factories 9, 49.

Hakki, T., Zearo, S., Drăgan, C.-A., Bureik, M., Bernhardt, R., 2008. Coexpression of redox partners increases the hydrocortisone (cortisol) production efficiency in CYP11B1 expressing fission yeast *Schizosaccharomyces pombe*. Journal of biotechnology 133, 351–9.

Han, Z., Madzak, C., Su, W.W., 2013. Tunable nano-oleosomes derived from engineered *Yarrowia lipolytica*. Biotechnology and bioengineering 110, 702–10.

Hansen, S.B., Tao, X., MacKinnon, R., 2011. Structural basis of PIP2 activation of the classical inward rectifier K+ channel Kir2.2. Nature 477, 495–8.

Hanson, M.A., Cherezov, V., Griffith, M.T., Roth, C.B., Jaakola, V.-P., Chien, E.Y.T., Velasquez, J., Kuhn, P., Stevens, R.C., 2008. A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor. Structure (London, England: 1993) 16, 897–905.

Haviv, H., Habeck, M., Kanai, R., Toyoshima, C., Karlish, S.J.D., 2013. Neutral phospholipids stimulate Na,K-ATPase activity: a specific lipid-protein interaction. The Journal of biological chemistry 288, 10073–81.

Hebert, D.N., Molinari, M., 2007. In and out of the ER: protein folding, quality control, degradation, and related human diseases. Physiological reviews 87, 1377–408.

Hecker, N., Ahmed, J., Von Eichborn, J., Dunkel, M., Macha, K., Eckert, A., Gilson, M.K., Bourne, P.E., Preissner, R., 2012. SuperTarget goes quantitative: update on drug-target interactions. Nucleic acids research 40, D1113–7.

Hedfalk, K., 2013. Further advances in the production of membrane proteins in *Pichia pastoris*. Bioengineered 4.

Henderson, C.J., Otto, D.M.E., Carrie, D., Magnuson, M.A., McLaren, A.W., Rosewell, I., Wolf, C.R., 2003. Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. The Journal of Biological Chemistry 278, 13480–13486.

Hernández, A., Jiang, X., Cubero, B., Nieto, P.M., Bressan, R.A., Hasegawa, P.M., Pardo, J.M., 2009. Mutants of the *Arabidopsis thaliana* cation/$H^+$ antiporter *At*NHX1 conferring increased salt tolerance in yeast: the endosome/prevacuolar compartment is a target for salt toxicity. The Journal of biological chemistry 284, 14276–85.

Hernández, M.L., Mancha, M., Martínez-Rivas, J.M., 2005. Molecular cloning and characterization of genes encoding two microsomal oleate desaturases (FAD2) from olive. Phytochemistry 66, 1417–26.

Hinnebusch, A.G., Natarajan, K., 2002. Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. Eukaryotic cell 1, 22–32.

Hino, T., Arakawa, T., Iwanari, H., Yurugi-Kobayashi, T., Ikeda-Suno, C., Nakada-Nakura, Y., Kusano-Arai, O., Weyand, S., Shimamura, T., Nomura, N., Cameron, A.D., Kobayashi, Takuya, Hamakubo, T., Iwata, S., Murata, Takeshi, 2012. G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. Nature 482, 237–40.

Hirz, M., Richter, G., Leitner, E., Wriessnegger, T., Pichler, H., 2013. A novel cholesterol-producing *Pichia pastoris* strain is an ideal host for functional expression of human Na,K-ATPase α3β1 isoform. Applied microbiology and biotechnology.

Ho, J.D., Yeh, R., Sandstrom, A., Chorny, I., Harries, W.E.C., Robbins, R.A., Miercke, L.J.W., Stroud, R.M., 2009. Crystal structure of human aquaporin 4 at 1.8 A and its mechanism of conductance. Proceedings of the National Academy of Sciences of the United States of America 106, 7437–42.

Hoffmann, F., Rinas, U., 2004. Roles of heat-shock chaperones in the production of recombinant proteins in *Escherichia coli*. Advances in biochemical engineering/biotechnology 89, 143–61.

Holmes, W.J., Darby, R.A., Wilks, M.D., Smith, R., Bill, R.M., 2009. Developing a scalable model of recombinant protein yield from *Pichia pastoris*: the influence of culture conditions, biomass and induction regime. Microbial cell factories 8, 35.

Horsefield, R., Nordén, K., Fellert, M., Backmark, A., Törnroth-Horsefield, S., Terwisscha van Scheltinga, A.C., Kvassman, J., Kjellbom, P., Johanson, U., Neutze, R., 2008. High-resolution x-ray structure of human aquaporin 5. Proceedings of the National Academy of Sciences of the United States of America 105, 13327–32.

Hou, X., Pedi, L., Diver, M.M., Long, S.B., 2012. Crystal structure of the calcium release-activated calcium channel Orai. Science (New York, N.Y.) 338, 1308–13.

Hsieh, K., Huang, A.H.C., 2007. Tapetosomes in *Brassica tapetum* accumulate endoplasmic reticulum-derived flavonoids and alkanes for delivery to the pollen surface. The Plant cell 19, 582–96.

Huh, W.-K., Falvo, J. V, Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., O'Shea, E.K., 2003. Global analysis of protein localization in budding yeast. Nature 425, 686–91.

Hunte, C., 2005. Specific protein-lipid interactions in membrane proteins. Biochemical Society transactions 33, 938–42.

Ikeda, K., Kitagawa, S., Tada, T., Iefuji, H., Inoue, Y., Izawa, S., 2011. Modification of yeast characteristics by soy peptides: cultivation with soy peptides represses the formation of lipid bodies. Applied Microbiology and Biotechnology 89, 1971–7.

Ishimaru, Y., Suzuki, M., Kobayashi, Takanori, Takahashi, M., Nakanishi, H., Mori, S., Nishizawa, N.K., 2005. OsZIP4, a novel zinc-regulated zinc transporter in rice. Journal of experimental botany 56, 3207–14.

Ito, K., Hikida, A., Kitagawa, S., Misaka, T., Abe, K., Kawarasaki, Y., 2012. Soy peptides enhance heterologous membrane protein productivity during the exponential growth phase of *Saccharomyces cerevisiae*. Bioscience, biotechnology, and biochemistry 76, 628–31.

Ito, K., Sugawara, T., Shiroishi, M., Tokuda, N., Kurokawa, A., Misaka, T., Makyio, H., Yurugi-Kobayashi, T., Shimamura, T., Nomura, N., Murata, Takeshi, Abe, K., Iwata, S., Kobayashi, Takuya, 2008. Advanced method for high-throughput expression of mutated eukaryotic membrane proteins in *Saccharomyces cerevisiae*. Biochemical and biophysical research communications 371, 841–5.

Ito, K., Matsukawa, K., Kato, Y., 2006. Functional analysis of skunk cabbage SfUCPB, a unique uncoupling protein lacking the fifth transmembrane domain, in yeast cells. Biochemical and biophysical research communications 349, 383–90.

Jaakola, V.-P., Griffith, M.T., Hanson, M.A., Cherezov, V., Chien, E.Y.T., Lane, J.R., Ijzerman, A.P., Stevens, R.C., 2008. The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science (New York, N.Y.) 322, 1211–7.

Jacquier, N., Mishra, S., Choudhary, V., Schneiter, R., 2013. Expression of oleosin and perilipins in yeast promote formation of lipid droplets from the endoplasmatic reticulum. Journal of cell science.

Jahic, M., Gustavsson, M., Jansen, A.-K., Martinelle, M., Enfors, S.-O., 2003a. Analysis and control of proteolysis of a fusion protein in Pichia pastoris fed-batch processes. Journal of biotechnology 102, 45–53.

Jahic, M., Wallberg, F., Bollok, M., Garcia, P., Enfors, S.-O., 2003b. Temperature limited fed-batch technique for control of proteolysis in Pichia pastoris bioreactor cultures. Microbial cell factories 2, 6.

Jamme, F., Vindigni, J.-D., Méchin, V., Cherifi, T., Chardot, T., Froissard, M., 2013. Single Cell Synchrotron FT-IR Microspectroscopy Reveals a Link between Neutral Lipid and Storage Carbohydrate Fluxes in S. cerevisiae. PloS one 8, e74421.

Jezek, P., Zácková, M., Růzicka, M., Skobisová, E., Jabůrek, M., 2004. Mitochondrial uncoupling proteins-facts and fantasies. Physiological research Academia Scientiarum Bohemoslovaca 53 Suppl 1, S199–S211.

Jidenko, M., Lenoir, G., Fuentes, J.M., Le Maire, M., Jaxel, C., 2006. Expression in yeast and purification of a membrane protein, SERCA1a, using a biotinylated acceptor domain. Protein expression and purification 48, 32–42.

Jidenko, M., Nielsen, R.C., Sørensen, T.L.-M., Møller, J. V, Le Maire, M., Nissen, P., Jaxel, C., 2005. Crystallization of a mammalian membrane protein overexpressed in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America 102, 11687–91.

Jin, M.S., Oldham, M.L., Zhang, Q., Chen, J., 2012. Crystal structure of the multidrug transporter P-glycoprotein from *Caenorhabditis elegans*. Nature 490, 566–9.

Johnsson, N., Varshavsky, A., 1994. Split ubiquitin as a sensor of protein interactions *in vivo*. Proceedings of the National Academy of Sciences of the United States of America 91, 10340–4.

Kaldenhoff, R., Bertl, A., Otto, B., Moshelion, M., Uehlein, N., 2007. Characterization of plant aquaporins. Methods in enzymology 428, 505–31.

Kapri-Pardes, E., Katz, A., Haviv, H., Mahmmoud, Y., Ilan, M., Khalfin-Penigel, I., Carmeli, S., Yarden, O., Karlish, S.J.D., 2011. Stabilization of the alpha2 isoform of Na,K-ATPase by mutations in a phospholipid binding pocket. The Journal of biological chemistry 286, 42888 –42899.

Karlsson, M., Fotiadis, D., Sjvall, S., Johansson, I., Hedfalk, K., Engel, A., Kjellbom, P., 2003. Reconstitution of water channel function of an aquaporin overexpressed and purified from *Pichia pastoris*. FEBS Letters 537, 68–72.

Kato, T., Murata, Takeomi, Usui, T., Park, E.Y., 2005. Improvement of the production of GFPuv-beta1,3-N-acetylglucosaminyltransferase 2 fusion protein using a molecular chaperone-assisted insect-cell-based expression system. Biotechnology and bioengineering 89, 424–33.

Kellosalo, J., Kajander, T., Kogan, K., Pokharel, K., Goldman, A., 2012. The structure and catalytic cycle of a sodium-pumping pyrophosphatase. Science (New York, N.Y.) 337, 473–6.

Kerppola, T. K., 2008. Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. *Annual review of biophysics* 37, 465–7

Kim, M.W., Kim, E.J., Kim, J.-Y., Park, J.-S., Oh, D.-B., Shimma, Y., Chiba, Y., Jigami, Y., Rhee, S.K., Kang, H.A., 2006. Functional characterization of the *Hansenula polymorpha* HOC1, OCH1, and OCR1 genes as members of the yeast OCH1 mannosyltransferase family involved in protein glycosylation. The Journal of biological chemistry 281, 6261–72.

Kim, M.W., Rhee, S.K., Kim, J.-Y., Shimma, Y., Chiba, Y., Jigami, Y., Kang, H.A., 2004. Characterization of N-linked oligosaccharides assembled on secretory recombinant glucose oxidase and cell wall mannoproteins from the methylotrophic yeast *Hansenula polymorpha*. Glycobiology 14, 243–51.

King, L.S., Kozono, D., Agre, P., 2004. From structure to disease: the evolving tale of aquaporin biology. Nature Reviews Molecular Cell Biology 5, 687–698.

Kiser, P.D., Golczak, M., Lodowski, D.T., Chance, M.R., Palczewski, K., 2009. Crystal structure of native RPE65, the retinoid isomerase of the visual cycle. Proceedings of the National Academy of Sciences of the United States of America 106, 17325–30.

Kitson, S.M., Mullen, W., Cogdell, R.J., Bill, R.M., Fraser, N.J., 2011. GPCR production in a novel yeast strain that makes cholesterol-like sterols. Methods (San Diego, Calif.) 55, 287–92.

Klingenberg, M., 2001. Uncoupling proteins--how do they work and how are they regulated. IUBMB life 52, 175–9.

Klingenberg, M., Winkler, E., Echtay, K., 2001. Uncoupling protein, $H^+$ transport and regulation. Biochemical Society transactions 29, 806–11.

Kolacna, L., Zimmermannova, O., Hasenbrink, G., Schwarzer, S., Ludwig, J., Lichtenberg-Fraté, H., Sychrova, H., 2005. New phenotypes of functional expression of the mKir2.1 channel in potassium efflux-deficient *Saccharomyces cerevisiae* strains. Yeast Chichester England 22, 1315–1323.

Korshunova, Y.O., Eide, D., Clark, W.G., Guerinot, M.L., Pakrasi, H.B., 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant molecular biology 40, 37–44.

Krauss, S., Zhang, C.-Y., Lowell, B.B., 2005. The mitochondrial uncoupling-protein homologues. Nature Reviews Molecular Cell Biology 6, 248–261.

Krettler, C., Reinhart, C., Bevans, C.G., 2013. Expression of GPCRs in *Pichia pastoris* for structural studies. Methods in enzymology 520, 1–29.

Krogh, A., Larsson, B., Von Heijne, G., Sonnhammer, E.L., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. Journal of molecular biology 305, 567–80.

Krynetski, E.Y., Drutsa, V.L., Kovaleva, I.E., Luzikov, V.N., 1995. High yield expression of functionally active human liver CYP2D6 in yeast cells. Pharmacogenetics 5, 103–9.

Ladds, G., Davis, K., Hillhouse, E.W., Davey, J., 2003. Modified yeast cells to investigate the coupling of G protein-coupled receptors to specific G proteins. Molecular microbiology 47, 781–92.

Ladds, G., Goddard, A., Davey, J., 2005. Functional analysis of heterologous GPCR signalling pathways in yeast. Trends in biotechnology 23, 367–73.

Lebon, G., Bennett, K., Jazayeri, A., Tate, Christopher G, 2011. Thermostabilisation of an agonist-bound conformation of the human adenosine A(2A) receptor. Journal of molecular biology 409, 298–310.

Lee, A.G., 2004. How lipids affect the activities of integral membrane proteins. Biochimica et biophysica acta 1666, 62–87.

Lelandais, G., Devaux, F., 2010. Comparative functional genomics of stress responses in yeasts. Omics: a journal of integrative biology 14, 501–15.

Leung, J., Cameron, A.D., Diallinas, G., Byrne, B., 2013. Stabilizing the heterologously expressed uric acid-xanthine transporter UapA from the lower eukaryote *Aspergillus nidulans*. Molecular membrane biology 30, 32–42.

Li, L., Wang, X., Gai, J., Yu, D., 2007. Molecular cloning and characterization of a novel microsomal oleate desaturase gene from soybean. Journal of plant physiology 164, 1516–26.

Li, M., Hubálek, F., Newton-Vinson, P., Edmondson, D.E., 2002. High-level expression of human liver monoamine oxidase A in *Pichia pastoris*: comparison with the enzyme expressed in *Saccharomyces cerevisiae*. Protein expression and purification 24, 152–62.

Lieberman, R.L., Culver, J.A., Entzminger, K.C., Pai, J.C., Maynard, J.A., 2011. Crystallization chaperone strategies for membrane proteins. Methods (San Diego, Calif.) 55, 293–302.

Lin, S.-M., Tsai, J.-Y., Hsiao, C.-D., Huang, Y.-T., Chiu, C.-L., Liu, M.-H., Tung, J.-Y., Liu, T.-H., Pan, R.-L., Sun, Y.-J., 2012. Crystal structure of a membrane-embedded $H^+$-translocating pyrophosphatase. Nature 484, 399–403.

Liu, J., Zhu, Y., Du, G., Zhou, J., Chen, J., 2013. Response of *Saccharomyces cerevisiae* to D-limonene-induced oxidative stress. Applied microbiology and biotechnology 97, 6467–75.

Liu, W., Chun, E., Thompson, A.A., Chubukov, P., Xu, F., Katritch, V., Han, G.W., Roth, C.B., Heitman, L.H., IJzerman, A.P., Cherezov, V., Stevens, R.C., 2012. Structural basis for allosteric regulation of GPCRs by sodium ions. Science (New York, N.Y.) 337, 232–6.

Liu, W., Kamensky, Y., Kakkar, R., Foley, E., Kulmacz, R.J., Palmer, G., 2005. Purification and characterization of bovine adrenal cytochrome b561 expressed in insect and yeast cell systems. Protein expression and purification 40, 429–39.

Long, S.B., Campbell, E.B., Mackinnon, R., 2005a. Voltage sensor of Kv1.2: structural basis of electromechanical coupling. Science (New York, N.Y.) 309, 903–8.

Long, S.B., Campbell, E.B., Mackinnon, R., 2005b. Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. Science (New York, N.Y.) 309, 897–903.

Long, S.B., Tao, X., Campbell, E.B., MacKinnon, R., 2007. Atomic structure of a voltage-dependent K+ channel in a lipid membrane-like environment. Nature 450, 376–82.

Lundstrom, K., Wagner, R., Reinhart, C., Desmyter, A., Cherouati, N., Magnin, T., Zeder-Lutz, G., Courtot, M., Prual, C., André, N., Hassaine, G., Michel, H., Cambillau, C., Pattus, F., 2006. Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems. Journal of structural and functional genomics 7, 77–91.

Ma, J., Yoshimura, M., Yamashita, E., Nakagawa, A., Ito, A., Tsukihara, T., 2004. Structure of rat monoamine oxidase A and its specific recognitions for substrates and inhibitors. Journal of molecular biology 338, 103–14.

Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M., 2005. Heterologous protein production using the *Pichia pastoris* expression system. Yeast Chichester England 22, 249–270.

Manglik, A., Kruse, A.C., Kobilka, T.S., Thian, F.S., Mathiesen, J.M., Sunahara, R.K., Pardo, L., Weis, W.I., Kobilka, B.K., Granier, S., 2012. Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. Nature 485, 321–6.

Maresova, L., Sychrova, H., 2006. Arabidopsis thaliana CHX17 gene complements the kha1 deletion phenotypes in *Saccharomyces cerevisiae*. Yeast (Chichester, England) 23, 1167–71.

Marsango, S., Bonaccorsi di Patti, M.C., Barra, D., Miele, R., 2011. Evidence that prokineticin receptor 2 exists as a dimer in vivo. Cellular and molecular life sciences : CMLS 68, 2919–29.

Martinez Molina, D., Wetterholm, A., Kohl, A., McCarthy, A.A., Niegowski, D., Ohlson, E., Hammarberg, T., Eshaghi, S., Haeggström, J.Z., Nordlund, P., 2007. Structural basis for synthesis of inflammatory mediators by human leukotriene C4 synthase. Nature 448, 613–6.

Mattanovich, D., Branduardi, P., Dato, L., Gasser, B., Sauer, M., Porro, D., 2012. Recombinant protein production in yeasts. Methods in molecular biology (Clifton, N.J.) 824, 329–58.

Mattanovich, D., Gasser, B., Hohenblum, H., Sauer, M., 2004. Stress in recombinant protein producing yeasts. Journal of biotechnology 113, 121–35.

Meyer, J.H., Ginovart, N., Boovariwala, A., Sagrati, S., Hussey, D., Garcia, A., Young, T., Praschak-Rieder, N., Wilson, A.A., Houle, S., 2006. Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. Archives of general psychiatry 63, 1209–16.

Midgett, C.R., Madden, D.R., 2007. Breaking the bottleneck: eukaryotic membrane protein expression for high-resolution structural studies. Journal of structural biology 160, 265–74.

Miller, A.N., Long, S.B., 2012. Crystal structure of the human two-pore domain potassium channel K2P1. Science (New York, N.Y.) 335, 432–6.

Minic, J, Sautel, M., Salesse, R, Pajot-Augy, E, 2005a. Yeast system as a screening tool for pharmacological assessment of g protein coupled receptors. Current medicinal chemistry 12, 961–9.

Minic, J., Persuy, M.-A., Godel, E., Aioun, J., Connerton, I., Salesse, Roland, Pajot-Augy, E., 2005. Functional expression of olfactory receptors in yeast and development of a bioassay for odorant screening. The FEBS journal 272, 524–37.

Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M.J., Sambrook, J.F., 1992. A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast KAR2 (BiP) gene by unfolded proteins. The EMBO journal 11, 2583–93.

Morioka, S., Shigemori, T., Hara, K., 2013. Effect of sterol composition on the activity of the yeast G-protein-coupled receptor Ste2. Applied Microbiology and Biotechnology 97, 4013–20.

Murakami, K., Onoda, Y., Kimura, J., Yoshino, M., 1997. Protection by histidine against oxidative inactivation of AMP deaminase in yeast. Biochemistry and molecular biology international 42, 1063–9.

Murata, K., Mitsuoka, K., Hirai, T., Walz, T., Agre, P, Heymann, J.B., Engel, A, Fujiyoshi, Y., 2000. Structural determinants of water permeation through aquaporin-1. Nature 407, 599–605.

Murata, Y., Watanabe, T., Sato, M., Momose, Y., Nakahara, T., Oka, S., Iwahashi, H., 2003. Dimethyl sulfoxide exposure facilitates phospholipid biosynthesis and cellular membrane proliferation in yeast cells. The Journal of Biological Chemistry 278, 33185–33193.

Murdza-Inglis, D.L., Patel, H. V, Freeman, K.B., Jezek, P., Orosz, D.E., Garlid, K.D., 1991. Functional reconstitution of rat uncoupling protein following its high level expression in yeast. The Journal of biological chemistry 266, 11871–5.

Murozuka, E., Hanisch, S., Pomorski, T.G., Jahn, Thomas Paul, Schjoerring, J.K., 2013. Bimolecular fluorescence complementation and interaction of various Arabidopsis major intrinsic proteins expressed in yeast. Physiologia plantarum 148, 422–31.

Nakamura, Y., Ishii, J., Kondo, A., 2013. Rapid, Facile Detection of Heterodimer Partners for Target Human G-Protein-Coupled Receptors Using a Modified Split-Ubiquitin Membrane Yeast Two-Hybrid System. PloS one 8, e66793.

Needham, P.G., Mikoluk, K., Dhakarwal, P., Khadem, S., Snyder, A.C., Subramanya, A.R., Brodsky, J.L., 2011. The thiazide-sensitive NaCl cotransporter is targeted for chaperone-dependent endoplasmic reticulum-associated degradation. The Journal of biological chemistry 286, 43611–21.

Newton-Vinson, P, Hubalek, F, Edmondson, D E, 2000. High-level expression of human liver monoamine oxidase B in *Pichia pastoris*. Protein expression and purification 20, 334–45.

Nicaud, Jean-Marc, 2012. *Yarrowia lipolytica*. Yeast (Chichester, England) 29, 409–18.

Niebauer, R.T., Robinson, A.S., 2006. Exceptional total and functional yields of the human adenosine (A2a) receptor expressed in the yeast *Saccharomyces cerevisiae*. Protein expression and purification 46, 204–11.

Nordén, K., Agemark, M., Danielson, J.Å.H., Alexandersson, E., Kjellbom, P., Johanson, U., 2011. Increasing gene dosage greatly enhances recombinant expression of aquaporins in *Pichia pastoris*. BMC biotechnology 11, 47.

Nthangeni, M.B., Urban, P., Pompon, D., Smit, M.S., Nicaud, J-M, 2004. The use of *Yarrowia lipolytica* for the expression of human cytochrome P450 CYP1A1. Yeast (Chichester, England) 21, 583–92.

Nyblom, M., Frick, A., Wang, Yi, Ekvall, M., Hallgren, K., Hedfalk, K., Neutze, R., Tajkhorshid, E., Törnroth-Horsefield, S., 2009. Structural and functional analysis of *So*PIP2;1 mutants adds insight into plant aquaporin gating. Journal of molecular biology 387, 653–68.

Nyblom, M., Oberg, F., Lindkvist-Petersson, K., Hallgren, K., Findlay, H., Wikström, J., Karlsson, A., Hansson, O., Booth, P.J., Bill, R.M., Neutze, R., Hedfalk, K., 2007. Exceptional overproduction of a functional human membrane protein. Protein expression and purification 56, 110–20.

Nyblom, M., Poulsen, H., Gourdon, P., Reinhard, L., Andersson, M., Lindahl, E., Fedosova, N., Nissen, P., 2013. Crystal Structure of Na+, K+-ATPase in the Na+-Bound State. Science (New York, N.Y.) 342, 123–127.

O'Malley, M.A., Mancini, J.D., Young, C.L., McCusker, E.C., Raden, D., Robinson, A.S., 2009. Progress toward heterologous expression of active G-protein-coupled receptors in *Saccharomyces cerevisiae*: Linking cellular stress response with translocation and trafficking. Protein science a publication of the Protein Society 18, 2356–70.

Oberg, F., Ekvall, M., Nyblom, M., Backmark, A., Neutze, R., Hedfalk, K., 2009. Insight into factors directing high production of eukaryotic membrane proteins; production of 13 human AQPs in *Pichia pastoris*. Molecular membrane biology 26, 215–27.

Oberg, F., Hedfalk, K., 2013. Recombinant production of the human aquaporins in the yeast *Pichia pastoris* (Invited Review). Molecular membrane biology 30, 15–31.

Öberg, F., Sjöhamn, J., Conner, M.T., Bill, R.M., Hedfalk, K., 2011. Improving recombinant eukaryotic membrane protein yields in *Pichia pastoris*: the importance of codon optimization and clone selection. Molecular membrane biology 28, 398–411.

Obrdlik, P., El-Bakkoury, M., Hamacher, T., Cappellaro, C., Vilarino, C., Fleischer, C., Blaudez, D., Sanders, D., Revuelta, Jose L, Ellerbrok, H., Kamuzinzi, R., Boles, E., Andre, B., 2004. K+-channel interactions detected by a genetic system optimized for systematic studies of membrane protein interactions. PNAS 101, 12242–12247

Ohlrogge, J., Browse, J., 1995. Lipid biosynthesis. The Plant cell 7, 957–70.

Okamoto, Y., Shikano, S., 2011. Phosphorylation-dependent C-terminal binding of 14-3-3 proteins promotes cell surface expression of HIV co-receptor GPR15. The Journal of biological chemistry 286, 7171–81.

Omura, T., 2010. Structural diversity of cytochrome P450 enzyme system. Journal of Biochemistry 147, 297–306.

Opekarová, M., Tanner, W., 2003. Specific lipid requirements of membrane proteins - a putative bottleneck in heterologous expression. Biochimica et Biophysica Acta (BBA) - Biomembranes 1610, 11–22.

Otto, B., Uehlein, N., Sdorra, S., Fischer, M., Ayaz, M., Belastegui-Macadam, X., Heckwolf, M., Lachnit, M., Pede, N., Priem, N., Reinhard, A., Siegfart, S., Urban, M., Kaldenhoff, R., 2010. Aquaporin tetramer composition modifies the function of tobacco aquaporins. The Journal of biological chemistry 285, 31253–60.

Park, J., Song, W.-Y., Ko, D., Eom, Y., Hansen, T.H., Schiller, M., Lee, T.G., Martinoia, E., Lee, Y., 2012. The phytochelatin transporters *At*ABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. The Plant journal: for cell and molecular biology 69, 278–88.

Parthibane, V., Rajakumari, S., Venkateshwari, V., Iyappan, R., Rajasekharan, R., 2012. Oleosin is bifunctional enzyme that has both monoacylglycerol acyltransferase and phospholipase activities. The Journal of biological chemistry 287, 1946–54.

Passantino, R., Cascio, C., Deidda, I., Galizzi, G., Russo, D., Spedale, G., Guarneri, P., 2013. Identifying protein partners of CLN8, an ER-resident protein involved in neuronal ceroid lipofuscinosis. Biochimica et biophysica acta 1833, 529–40.

Patil, C., Walter, P, 2001. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Current opinion in cell biology 13, 349–55.

Pausch, M.H., 1997. G-protein-coupled receptors in high-throughput screening assays for drug discovery. Trends in biotechnology 15, 487–494.

Pedas, P., Schjoerring, J.K., Husted, S., 2009. Identification and characterization of zinc-starvation-induced ZIP transporters from barley roots. Plant physiology and biochemistry: PPB / Société française de physiologie végétale 47, 377–83.

Pedas, P., Ytting, C.K., Fuglsang, A.T., Jahn, T.P., Schjoerring, J.K., Husted, S., 2008. Manganese efficiency in barley: identification and characterization of the metal ion transporter *Hv*IRT1. Plant physiology 148, 455–66.

Pedersen, B.P., Kumar, H., Waight, A.B., Risenmay, A.J., Roe-Zurz, Z., Chau, B.H., Schlessinger, A., Bonomi, M., Harries, W., Sali, A., Johri, A.K., Stroud, R.M., 2013. Crystal structure of a eukaryotic phosphate transporter. Nature 496, 533–6.

Pelham, H.R., 2000. Using sorting signals to retain proteins in endoplasmic reticulum. Methods in enzymology 327, 279–83.

Pereira, S.L., Leonard, A.E., Mukerji, P., 2003. Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. Prostaglandins, leukotrienes, and essential fatty acids 68, 97–106.

Pérez-Castiñeira, J.R., Hernández, A., Drake, R., Serrano, A., 2011. A plant proton-pumping inorganic pyrophosphatase functionally complements the vacuolar ATPase transport activity and confers bafilomycin resistance in yeast. The Biochemical journal 437, 269–78.

Petäjä-Repo, U.E., Hogue, M., Bhalla, S., Laperrière, A., Morello, J.-P., Bouvier, M., 2002. Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. The EMBO journal 21, 1628–37.

Peters, F.T., Dragan, C.-A., Schwaninger, A.E., Sauer, C., Zapp, J., Bureik, M., Maurer, H.H., 2009. Use of fission yeast heterologously expressing human cytochrome P450 2B6 in biotechnological synthesis of the designer drug metabolite N-(1-phenylcyclohexyl)-2-hydroxyethanamine. Forensic science international 184, 69–73.

Petko, J., Justice-Bitner, S., Jin, J., Wong, V., Kittanakom, S., Ferraro, T.N., Stagljar, I., Levenson, R., 2013. MOR Is Not Enough: Identification of Novel mu-Opioid Receptor Interacting Proteins Using Traditional and Modified Membrane Yeast Two-Hybrid Screens. PloS one 8, e67608.

Petschnigg, J., Moe, O.W., Stagljar, I., 2011. Using yeast as a model to study membrane proteins. Current Opinion in Nephrology and Hypertension 20, 425–432.

Poklepovich, T.J., Rinaldi, M.A., Tomazic, M.L., Favale, N.O., Turkewitz, A.P., Nudel, C.B., Nusblat, A.D., 2012. The cytochrome b5 dependent C-5(6) sterol desaturase DES5A from the endoplasmic reticulum of Tetrahymena thermophila complements ergosterol biosynthesis mutants in *Saccharomyces cerevisiae*. Steroids 77, 1313–20.

Radhika, V., Proikas-Cezanne, T., Jayaraman, M., Onesime, D., Ha, J.H., Dhanasekaran, D.N., 2007. Chemical sensing of DNT by engineered olfactory yeast strain. Nature chemical biology 3, 325–30.

Ramón, A., Marín, M., 2011. Advances in the production of membrane proteins in *Pichia pastoris*. Biotechnology journal 6, 700–6.

Rasmussen, S.G.F., Choi, H.-J., Rosenbaum, D.M., Kobilka, T.S., Thian, F.S., Edwards, P.C., Burghammer, M., Ratnala, V.R.P., Sanishvili, R., Fischetti, R.F., Schertler, G.F.X., Weis, W.I., Kobilka, B.K., 2007. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. Nature 450, 383–7.

Rasmussen, S.G.F., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., Chae, P.S., Pardon, E., Calinski, D., Mathiesen, J.M., Shah, S.T.A., Lyons, J.A., Caffrey, M., Gellman, S.H., Steyaert, J., Skiniotis, G., Weis, W.I., Sunahara, R.K., Kobilka, B.K., 2011. Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature 477, 549–55.

Rosenbaum, D.M., Cherezov, V., Hanson, M.A., Rasmussen, S.G.F., Thian, F.S., Kobilka, T.S., Choi, H.-J., Yao, X.-J., Weis, W.I., Stevens, R.C., Kobilka, B.K., 2007. GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. Science (New York, N.Y.) 318, 1266–73.

Rubio-Aliaga, I., Daniel, H., 2002. Mammalian peptide transporters as targets for drug delivery. Trends in pharmacological sciences 23, 434–40.

Sander, P., Grünewald, S, Reiländer, H, Michel, H., 1994. Expression of the human D2S dopamine receptor in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*: a comparative study. FEBS letters 344, 41–6.

Sanz, G., Pajot-Augy, e., 2013. Deciphering activation of olfactory receptors using heterologous expression in *Saccharomyces cerevisiae* and bioluminescence resonance energy transfer. Methods in molecular biology (Clifton, N.J.) 1003, 149–60.

Saparov, S.M., Kozono, D., Rothe, U., Agre, P., Pohl, P., 2001. Water and ion permeation of aquaporin-1 in planar lipid bilayers. Major differences in structural determinants and stoichiometry. The Journal of biological chemistry 276, 31515–20.

Sarramegna, V., Talmont, F, Demange, P, Milon, A, 2003. Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification. Cellular and molecular life sciences: CMLS 60, 1529–46.

Sarramegna, V., Demange, P., Milon, A., Talmont, F., 2002a. Optimizing functional versus total expression of the human mu-opioid receptor in *Pichia pastoris*. Protein expression and purification 24, 212–20.

Sarramegna, V., Talmont, F., Seree de Roch, M., Milon, A., Demange, P., 2002b. Green fluorescent protein as a reporter of human mu-opioid receptor overexpression and localization in the methylotrophic yeast *Pichia pastoris*. Journal of biotechnology 99, 23–39.

Schlapschy, M., Skerra, A., 2011. Periplasmic chaperones used to enhance functional secretion of proteins in *E. coli*. Methods in molecular biology (Clifton, N.J.) 705, 211–24.

Schrader, J., Etschmann, M.M.W., Sell, D., Hilmer, J.M., Rabenhorst, J., 2004. Applied biocatalysis for the synthesis of natural flavour compounds-current industrial processes and future prospects. Biotechnology letters 26, 463–72.

Schwarzer, S., Kolacna, L., Lichtenberg-Fraté, H., Sychrova, H., Ludwig, J., 2008. Functional expression of the voltage-gated neuronal mammalian potassium channel rat ether a go-go1 in yeast. FEMS Yeast Research 8, 405–413.

Schwientek, T., Lorenz, C., Ernst, J.F., 1995. Golgi localization in yeast is mediated by the membrane anchor region of rat liver sialyltransferase. The Journal of biological chemistry 270, 5483–5489.

Serrano, A., Pérez-Castiñeira, J.R., Baltscheffsky, M., Baltscheffsky, H., 2007. H+-PPases: yesterday, today and tomorrow. IUBMB life 59, 76–83.

Serrano-Vega, M.J., Magnani, F., Shibata, Y., Tate, C.G., 2008. Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. Proceedings of the National Academy of Sciences of the United States of America 105, 877–82.

Serrano-Vega, M.J., Tate, Christopher G, 2009. Transferability of thermostabilizing mutations between beta-adrenergic receptors. Molecular membrane biology 26, 385–96.

Shikano, S., Coblitz, B., Sun, H., Li, M., 2005. Genetic isolation of transport signals directing cell surface expression. Nature Cell Biology 7, 985–92.

Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, Wei, Han, G.W., Kobayashi, Takuya, Stevens, R.C., Iwata, S., 2011. Structure of the human histamine H1 receptor complex with doxepin. Nature 475, 65–70.

Shiningavamwe, A., Obiero, G., Albertyn, J., Nicaud, Jean-Marc, Smit, M., 2006. Heterologous expression of the benzoate para-hydroxylase encoding gene (CYP53B1) from *Rhodotorula minuta* by *Yarrowia lipolytica*. Applied microbiology and biotechnology 72, 323–9.

Shinoda, T., Ogawa, H., Cornelius, F., Toyoshima, C., 2009. Crystal structure of the sodium-potassium pump at 2.4 A resolution. Nature 459, 446–50.

Shiroishi, M., Kobayashi, Takuya, Ogasawara, S., Tsujimoto, H., Ikeda-Suno, C., Iwata, S., Shimamura, T., 2011. Production of the stable human histamine $H_1$ receptor in *Pichia pastoris* for structural determination. Methods (San Diego, Calif.) 55, 281–6.

Shiroishi, M., Tsujimoto, H., Makyio, H., Asada, H., Yurugi-Kobayashi, T., Shimamura, T., Murata, Takeshi, Nomura, N., Haga, T., Iwata, S., Kobayashi, Takuya, 2012. Platform for the rapid construction and evaluation of GPCRs for crystallography in *Saccharomyces cerevisiae*. Microbial cell factories 11, 78.

Shukla, A.K., Haase, W., Reinhart, C., Michel, H., 2007a. Heterologous expression and comparative characterization of the human neuromedin U subtype II receptor using the methylotrophic yeast *Pichia pastoris* and mammalian cells. The international journal of biochemistry & cell biology 39, 931–42.

Shukla, A.K., Haase, W., Reinhart, C., Michel, H., 2007b. Heterologous expression and characterization of the recombinant bradykinin B2 receptor using the methylotrophic yeast *Pichia pastoris*. Protein expression and purification 55, 1–8.

Shukla, A.K., Haase, W., Reinhart, C., Michel, H., 2007c. Heterologous expression and characterization of the recombinant bradykinin B2 receptor using the methylotrophic yeast *Pichia pastoris*. Protein expression and purification 55, 1–8.

Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H.E., Schönfisch, B., Perschil, I., Chacinska, A., Guiard, B., Rehling, P., Pfanner, N., Meisinger, C., 2003. The proteome of *Saccharomyces cerevisiae* mitochondria. Proceedings of the National Academy of Sciences of the United States of America 100, 13207–12.

Singh, S., Gras, A., Fiez-Vandal, Cedric, Martinez, M., Wagner, R., Byrne, B., 2012a. Screening for high-yielding *Pichia pastoris* clones: the production of G protein-coupled receptors as a case study. Methods in molecular biology (Clifton, N.J.) 866, 65–73.

Singh, S., Gras, A., Fiez-Vandal, Cedric, Martinez, M., Wagner, R., Byrne, B., 2012b. Large-scale production of membrane proteins in *Pichia pastoris*: the production of G protein-coupled receptors as a case study. Methods in molecular biology (Clifton, N.J.) 866, 197–207.

Singh, S., Gras, A., Fiez-Vandal, Cédric, Ruprecht, J., Rana, R., Martinez, M., Strange, P.G., Wagner, R., Byrne, B., 2008. Large-scale functional expression of WT and truncated human adenosine A2A receptor in *Pichia pastoris* bioreactor cultures. Microbial cell factories 7, 28.

Sipiczki, M., 2000. Where does fission yeast sit on the tree of life? Genome Biology 1, reviews1011.1– reviews1011.4

Son, S.-Y., Ma, Jichun, Kondou, Y., Yoshimura, M., Yamashita, E., Tsukihara, T., 2008. Structure of human monoamine oxidase A at 2.2-A resolution: the control of opening the entry for substrates/inhibitors. Proceedings of the National Academy of Sciences of the United States of America 105, 5739–44.

Song, W.-Y., Park, J., Mendoza-Cózatl, D.G., Suter-Grotemeyer, M., Shim, D., Hörtensteiner, S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J.I., Lee, Y., Martinoia, E., 2010. Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. Proceedings of the National Academy of Sciences of the United States of America 107, 21187–92.

Sonoda, Y., Cameron, Alex, Newstead, S., Omote, Hiroshi, Moriyama, Y., Kasahara, M., Iwata, S., Drew, D., 2010. Tricks of the trade used to accelerate high-resolution structure determination of membrane proteins. FEBS letters 584, 2539–47.

Sopko, R., Huang, D., Preston, N., Chua, G., Papp, B., Kafadar, K., Snyder, Mike, Oliver, S.G., Cyert, M., Hughes, T.R., Boone, C., Andrews, B., 2006. Mapping pathways and phenotypes by systematic gene overexpression. Molecular cell 21, 319–30.

Souza, C.M., Schwabe, T.M.E., Pichler, H., Ploier, B., Leitner, E., Guan, X.L., Wenk, M.R., Riezman, I., Riezman, Howard, 2011. A stable yeast strain efficiently producing cholesterol instead of ergosterol is functional for tryptophan uptake, but not weak organic acid resistance. Metabolic Engineering 13, 555–569.

Sperling, P., Ternes, P., Zank, T K, Heinz, E, 2003. The evolution of desaturases. Prostaglandins, leukotrienes, and essential fatty acids 68, 73–95.

Stagljar, I, Korostensky, C., Johnsson, N., Te Heesen, S., 1998. A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. Proceedings of the National Academy of Sciences of the United States of America 95, 5187–92.

Standfuss, J., Xie, G., Edwards, P.C., Burghammer, M., Oprian, D.D., Schertler, G.F.X., 2007. Crystal structure of a thermally stable rhodopsin mutant. Journal of molecular biology 372, 1179–88.

Steffensen, L., Pedersen, P.A., 2006. Heterologous expression of membrane and soluble proteins derepresses GCN4 mRNA translation in the yeast *Saccharomyces cerevisiae*. Eukaryotic cell 5, 248–61.

Stewart, G.D., Sexton, P.M., Christopoulos, A., 2010. Prediction of functionally selective allosteric interactions at an M3 muscarinic acetylcholine receptor mutant using Saccharomyces cerevisiae. Molecular pharmacology 78, 205–14.

Stindt, J., Ellinger, P., Stross, C., Keitel, V., Häussinger, D., Smits, S.H.J., Kubitz, R., Schmitt, L., 2011. Heterologous overexpression and mutagenesis of the human bile salt export pump (ABCB11) using DREAM (Directed REcombination-Assisted Mutagenesis). PloS one 6, e20562.

Stindt, J., Ellinger, P., Weissenberger, K., Dröge, C., Herebian, D., Mayatepek, E., Homey, B., Braun, S., Schulte Am Esch, J., Horacek, M., Canbay, A., Schmitt, L., Häussinger, D., Kubitz, R., 2013. A novel mutation within a transmembrane helix of the bile salt export pump (BSEP, ABCB11) with delayed development of cirrhosis. Liver international 33. 1527–35

Stuart, J.A., Harper, J.A., Brindle, K.M., Jekabsons, M.B., Brand, M.D., 2001. A mitochondrial uncoupling artifact can be caused by expression of uncoupling protein 1 in yeast. The Biochemical journal 356, 779–789.

Suga, H., Haga, T., 2007. Ligand screening system using fusion proteins of G protein-coupled receptors with G protein alpha subunits. Neurochemistry international 51, 140–64.

Sun, J., Wu, Y., Xu, H., Zhao, Z., 2012. DTome: a web-based tool for drug-target interactome construction. BMC bioinformatics 13 Suppl 9, S7.

Takegawa, K., Tohda, H., Sasaki, M., Idiris, A., Ohashi, T., Mukaiyama, H., Giga-Hama, Y., Kumagai, H., 2009. Production of heterologous proteins using the fission-yeast (*Schizosaccharomyces pombe*) expression system. Biotechnology and applied biochemistry 53, 227–35.

Tang, W., Ruknudin, A., Yang, W.P., Shaw, S.Y., Knickerbocker, A., Kurtz, S., 1995. Functional expression of a vertebrate inwardly rectifying K+ channel in yeast. Molecular Biology of the Cell 6, 1231–1240.

Tao, X., Avalos, J.L., Chen, J., MacKinnon, R., 2009. Crystal structure of the eukaryotic strong inward-rectifier K+ channel Kir2.2 at 3.1 A resolution. Science (New York, N.Y.) 326, 1668–74.

Tao, X., Lee, A., Limapichat, W., Dougherty, D.A., MacKinnon, R., 2010. A gating charge transfer center in voltage sensors. Science (New York, N.Y.) 328, 67–73.

Tate, C G, Whiteley, E., Betenbaugh, M.J., 1999. Molecular chaperones improve functional expression of the serotonin (5-hydroxytryptamine) transporter in insect cells. Biochemical Society transactions 27, 932–6.

Thevenieau, F., Nicaud, J.-M., Gaillardin, C., 2009. Yeast Biotechnology: Diversity and Applications. Springer Netherlands, Dordrecht.

Tipton, K.F., Boyce, S., O'Sullivan, J., Davey, G.P., Healy, J., 2004. Monoamine oxidases: certainties and uncertainties. Current medicinal chemistry 11, 1965–82.

Törnroth-Horsefield, S., Wang, Yi, Hedfalk, K., Johanson, U., Karlsson, M., Tajkhorshid, E., Neutze, R., Kjellbom, P., 2006. Structural mechanism of plant aquaporin gating. Nature 439, 688–94.

Toyoshima, C., Iwasawa, S., Ogawa, H., Hirata, A., Tsueda, J., Inesi, G., 2013. Crystal structures of the calcium pump and sarcolipin in the Mg2+-bound E1 state. Nature 495, 260–4.

Usenovic, M., Knight, A.L., Ray, A., Wong, V., Brown, K.R., Caldwell, G.A., Caldwell, K.A., Stagljar, I., Krainc, D., 2012. Identification of novel ATP13A2 interactors and their role in α-synuclein misfolding and toxicity. Human molecular genetics 21, 3785–94.

Van Dijk R, Faber, K., Kiel, J., Veenhuis, M, Van der Klei I, 2000. The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory. Enzyme and microbial technology 26, 793–800.

Venegas-Calerón, M., Beaudoin, F., Garcés, R., Napier, J.A., Martínez-Force, E., 2010. The sunflower plastidial omega3-fatty acid desaturase (*Ha*FAD7) contains the signalling determinants required for targeting to, and retention in, the endoplasmic reticulum membrane in yeast but requires co-expressed ferredoxin for activity. Phytochemistry 71, 1050–8.

Verkman, A.S., 2012. Aquaporins in clinical medicine. Annual review of medicine 63, 303–16.

Vert, G., Briat, J.F., Curie, C., 2001. Arabidopsis IRT2 gene encodes a root-periphery iron transporter. The Plant journal: for cell and molecular biology 26, 181–9.

Vervecken, W., Kaigorodov, V., Callewaert, N., Geysens, S., Vusser, K. De, Contreras, R., 2004. *In vivo* synthesis of mammalian-like , hybrid-type N-glycans in *Pichia pastoris*. Applied and Environmental Biotechnology 70, 2639–2646.

Vindigni, J.-D., Wien, F., Giuliani, A., Erpapazoglou, Z., Tache, R., Jagic, F., Chardot, T., Gohon, Y., Froissard, M., 2013. Fold of an oleosin targeted to cellular oil bodies. Biochimica et biophysica acta 1828, 1881–8.

Vinothkumar, K.R., Edwards, P.C., Standfuss, J., 2013. Practical aspects in expression and purification of membrane proteins for structural analysis. Methods in molecular biology (Clifton, N.J.) 955, 17–30.

Walter, P., Ron, D., 2011. The unfolded protein response: from stress pathway to homeostatic regulation. Science (New York, N.Y.) 334, 1081–6.

Wang, J., Edmondson, D.E., 2010. High-level expression and purification of rat monoamine oxidase A (MAO A) in *Pichia pastoris*: comparison with human MAO A. Protein expression and purification 70, 211–7.

Weiss, H.M., Haase, W, Michel, H, Reiländer, H, 1995. Expression of functional mouse 5-HT5A serotonin receptor in the methylotrophic yeast *Pichia pastoris*: pharmacological characterization and localization. FEBS letters 377, 451–6.

Weiss, H.M., Haase, W, Michel, H, Reiländer, H, 1998a. Comparative biochemical and pharmacological characterization of the mouse 5HT5A 5-hydroxytryptamine receptor and the human beta2-adrenergic receptor produced in the methylotrophic yeast *Pichia pastoris*. The Biochemical journal 330, 1137–47.

Weiss, H.M., Haase, W, Reiländer, H, 1998b. Expression of an integral membrane protein, the 5HT5A receptor. Methods in molecular biology (Clifton, N.J.) 103, 227–39.

Weyler, W., Titlow, C.C., Salach, J.I., 1990. Catalytically active monoamine oxidase type A from human liver expressed in *Saccharomyces cerevisiae* contains covalent FAD. Biochemical and biophysical research communications 173, 1205–11.

White, M.A., Clark, K.M., Grayhack, E.J., Dumont, M.E., 2008. Characteristics affecting expression and solubilization of yeast membrane proteins 365, 621–636.

Whorton, M.R., MacKinnon, R., 2011. Crystal structure of the mammalian GIRK2 K+ channel and gating regulation by G proteins, PIP2, and sodium. Cell 147, 199–208.

Whorton, M.R., MacKinnon, R., 2013. X-ray structure of the mammalian GIRK2-βγ G-protein complex. Nature 498, 190–7.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., et al., 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science (New York, N.Y.) 285, 901–6.

Wriessnegger, T., Pichler, H., 2013. Yeast metabolic engineering--targeting sterol metabolism and terpenoid formation. Progress in lipid research 52, 277–93.

Wu, B., Chien, E.Y.T., Mol, C.D., Fenalti, G., Liu, Wei, Katritch, V., Abagyan, R., Brooun, A., Wells, P., Bi, F.C., Hamel, D.J., Kuhn, P., Handel, T.M., Cherezov, V., Stevens, R.C., 2010. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. Science (New York, N.Y.) 330, 1066–71.

Xiao, H., Yin, L., Xu, X., Li, T., Han, Z., 2008. The iron-regulated transporter, MbNRAMP1, isolated from *Malus baccata* is involved in Fe, Mn and Cd trafficking. Annals of botany 102, 881–9.

Xu, F., Wu, H., Katritch, V., Han, G.W., Jacobson, K.A., Gao, Z.-G., Cherezov, V., Stevens, R.C., 2011. Structure of an agonist-bound human A2A adenosine receptor. Science (New York, N.Y.) 332, 322–7.

Yi, B.A., Lin, Y.F., Jan, Y.N., Jan, L.Y., 2001. Yeast screen for constitutively active mutant G protein-activated potassium channels. Neuron 29, 657–667.

Yoon, H.-S., Kim, S.-Y., Kim, I.-S., 2013. Stress response of plant H+-PPase-expressing transgenic *Escherichia coli* and *Saccharomyces cerevisiae*: a potentially useful mechanism for the development of stress-tolerant organisms. Journal of applied genetics 54, 129–33.

Yu, Z.W., Quinn, P.J., 1994. Dimethyl sulphoxide: a review of its applications in cell biology. Bioscience reports 14, 259–81.

Yurugi-Kobayashi, T., Asada, H., Shiroishi, M., Shimamura, T., Funamoto, S., Katsuta, N., Ito, Keisuke, Sugawara, T., Tokuda, N., Tsujimoto, H., Murata, Takeshi, Nomura, N., Haga, K., Haga, T., Iwata, S., Kobayashi, Takuya, 2009. Comparison of functional non-glycosylated GPCRs expression in *Pichia pastoris*. Biochemical and biophysical research communications 380, 271–6.

Zhang, L., Liu, N., Ma, X., Jiang, L., 2013. The transcriptional control machinery as well as the cell wall integrity and its regulation are involved in the detoxification of the organic solvent dimethyl sulfoxide in *Saccharomyces cerevisiae*. FEMS yeast research 13, 200–18.

Zhang, R., Kim, T.-K., Qiao, Z.-H., Cai, J., Pierce, W.M., Song, Z.-H., 2007. Biochemical and mass spectrometric characterization of the human CB2 cannabinoid receptor expressed in *Pichia pastoris*-importance of correct processing of the N-terminus. Protein expression and purification 55, 225–35.

Zhang, W., Needham, D.L., Coffin, M., Rooker, A., Hurban, P., Tanzer, M.M., Shuster, J.R., 2003. Microarray analyses of the metabolic responses of *Saccharomyces cerevisiae* to organic solvent dimethyl sulfoxide. Journal of industrial microbiology & biotechnology 30, 57–69.

Zhang, X.-N., Han, Z.-H., Yin, L.-L., Kong, J., Xu, X.-F., Zhang, X.-Z., Wang, Yi, 2013a. Heterologous functional analysis of the *Malus xiaojinensis Mx*IRT1 gene and the His-box motif by expression in yeast. Molecular biology reports 40, 1499–504.

Zhao, H., Lappalainen, P., 2012. A simple guide to biochemical approaches for analyzing protein-lipid interactions. Molecular biology of the cell 23, 2823–30.

Zhao, Y, Lieberman, H.B., 1995. *Schizosaccharomyces pombe*: a model for molecular studies of eukaryotic genes. DNA and cell biology 14, 359–71.

Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Jansen, R., Bidlingmaier, S., Houfek, T., Mitchell, T., Miller, P., Dean, R.A., Gerstein, M, Snyder, M, 2001. Global analysis of protein activities using proteome chips. Science (New York, N.Y.) 293, 2101–5.

# Chapter II

# Production of human cytochrome P450 2D6 drug metabolites with recombinant microbes – a comparative study

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My work included cloning of different co-expression constructs harboring CYP2D6 and human reductase for expression in *S. cerevisiae*. The constructs were transformed into the strain W303 *MAT*a to be cultivated according to an optimized expression protocol established by Anita Emmerstorfer (Emmerstorfer et al., publication in progress).

## Abstract

The processes of drug development require efficient strategies to produce the respective drug metabolites, which are often difficult to obtain. Biotransformations employing recombinant microorganisms as whole-cell biocatalysts have become an attractive alternative to the chemical syntheses of such metabolites. For the first time the potential of four different microbial systems expressing the human cytochrome P450 2D6 (CYP2D6), which is one of the most important drug metabolizing enzymes, were compared and evaluated for such applications. The microbial host *Pichia pastoris* showed to be the most efficient for expressing CYP2D6. Without additional over-expression of chaperons, the achieved yield of CYP2D6 was the highest compared to microbial hosts reported so far. Therefore, the system described in this study outperformed the previously reported expression of the N-terminally modified enzyme. It was also shown that the activities of the whole-cell conversions of bufuralol in recombinant *P. pastoris* were significantly higher than the *Escherichia coli* catalyst, which expressed the same unmodified gene.

# 1 Introduction

Cytochromes P450 (CYPs) constitute a super-familiy of heme-containing monooxygenases with pivotal roles in all kingdoms of life. In humans, CYPs are the principal enzyme system for the clearance of drugs and xenobiotics [1]. CYPs are predominantly expressed in hepatocytes, where they take part in the phase 1 metabolism by introducing an oxygen atom into the substrate molecule. Several CYP isoforms with different substrate spectra are involved in this process, CYP2D6 being among the most important. Although CYP2D6 is expressed only weakly in the human liver, i.e. <2% of total CYP liver enzyme content, it is involved in the metabolism of ~ 25% of drugs currently in use [2]. Furthermore, CYP2D6 is of pharmacological interest as it displays a very high degree of inter-individual variability. This variability is mainly caused by an extensive genetic polymorphism; over 80 alleles and allele variants have been described [3]. Consequently, a variety of phenotypes arises displaying - in extreme cases - no or even greater than normal CYP2D6 activity, which are referred to as “poor metabolizer” and “ultrarapid metabolizer”, respectively. The consequences for a patient might therefore be the suffering from severe side effects or the complete lack of a drug response. To study such phenotypic implications, the availability of drug metabolites is a prerequisite. Furthermore, safety testings of drug metabolites have become an important issue in the drug discovery and development process lately. In 2008, the U.S. Food and Drug Administration defined that metabolites present at >10% of the parent compound in the human metabolism have to be subjected to toxicity studies [4].

Chemical synthesis of drug metabolites is often not feasible, since the stereo- and regioselective hydroxylation of a non-activated carbon atom is hardly achieved by standard chemical means. Whole-cell biotransformations employing recombinant microorganism are an elegant and scalable possibility to employ the native enzymes in metabolite syntheses. Using whole cells as biocatalysts is advantageous in many aspects [5]. First of all, enzyme isolation and purification steps can be circumvented which present significant time- and cost-factors. Whole cells can also be regarded as cover protecting the enzymes from shear forces or organic solvents and increase, thus, their stability. Mammalian CYPs almost always rely on the presence of cytochrome P450 reductase (CPR), the enzyme required for the electron transfer from the co-factor NAD(P)H. Co-expressing CPR or taking advantage of an endogenous redox partner in whole cells yields fully functional monooxygenase systems. Intact cells provide in addition a membrane environment which is often required for CYP functionality and favors the conversion of hydrophobic substrates. Furthermore, the cellular metabolism can be exploited for the regeneration of NAD(P)H required in CYP reactions. Thus, the addition of costly co-factors can be avoided. One of the main challenges in the context of whole-cell systems is the limited substrate/product transfer across the cell membrane [6]. Nevertheless, whole-cell systems have already been implemented successfully in industry. Amongst others, Novartis is employing recombinant *Escherichia coli* for CYP metabolite synthesis in the mg scale [7].

Suitable expression systems are a prerequisite for efficient whole-cell biocatalysts. Human P450s are challenging targets for recombinant production, since they constitute membrane-based proteins that require the presence of heme (protoporphyrin IX) as cofactor [8]. Nevertheless, CYP2D6 was already expressed heterologously in a vast range of different hosts including bacteria and yeasts with *E. coli* and *Saccharomyces cerevisiae* as the most prominent ones as well as insect and mammalian cells (see Tabel 1 and references therein). Although a lot of data is available in the literature, differences in the expression strategies and in the employed assays prevent a direct comparison of different CYP expression systems and hamper the decision which system might be considered optimal for a biotechnological application.

Therefore, the aim of our study is to evaluate the potential of four different expression hosts as whole-cell biocatalysts in CYP2D6 mediated reactions under comparable conditions. The microbial systems under investigation were the commonly used P450 expression hosts *E. coli* and *S. cerevisiae* as well as *Pichia pastoris* and *Yarrowia lipolytica*. Compared to other yeasts the latter two species offer features, which might be especially beneficial for whole-cell biotransformations. *P. pastoris* can easily be grown to very high cell densities on cheap media, yielding up to 130 g CDW/L [9] and high titers of recombinant protein are often achieved. This allows the simple production of the biocatalyst in large quantities. *Y. lipolytica* would be an interesting P450 expression host, because of its ability to grow in biphasic systems. This feature could be exploited for the biotransformation of largely water-insoluble compounds as represented by many P450 substrates. The full length cDNAs of CYP2D6 and CPR including the signal peptide and hydrophobic N-terminal sequence were used for expression without any modification to ensure the same starting point for all expression systems. Levels of P450 enzyme based on determination by differential CO spectroscopy and monooxygenase activities were set as parameters to compare the performance of the tested hosts.

**Table 3.** Reported expression levels and expression strategies for CYP2D6 production in different recombinant hosts.

| Expression host | Expression strategy | Remarks | Expression Level [pmol/ mg protein] | Expression Level [nmol/ L culture] | Reference |
|---|---|---|---|---|---|
| *E. coli* DH5α | Co-expression of CPR, separate plasmids | N-terminus modified (17α-hydroxylase sequence) | 371.44 ± 44.57 | - | [44] |
| *E. coli* JM109 | | N-terminus modified (17α-hydroxylase sequence) | 310 ± 50 | 546 ± 49 | [45] |
| *E. coli* JM109 | Co-expression of CPR, separate plasmids | N-terminus modified (17α-hydroxylase sequence) | 140 ± 70 | 381± 42 | [45] |
| *E. coli* JM109 | | N-terminal ompA leader | 490 ± 100 | 481 ± 58 | [45] |
| *E. coli* JM109 | Co-expression of CPR, separate plasmids | N-terminal ompA leader | 210 ± 40 | 365 ± 73 | [45] |
| *E. coli* JM109 | Co-expression of CPR, separate plasmids | N-terminal ompA leader | 370 ± 120 | 235 ± 48 | [46] |
| *E. coli* DH5α | CYP-CPR fusion | N-terminal ompA leader | 60-90 | - | [47] |
| *E. coli* DH5α | Co-expression of CPR, one plasmid, two promoters | N-terminal truncation | - | 91± 44 | [39] |
| *E. coli* BL21(DE3) | Co-expression of CPR, bicistronic | | n.d.[a] | n.d.[a] | This study |
| *E. coli* LEMO21(DE3) | Co-expression of CPR, bicistronic | | n.d.[a] | n.d.[a] | This study |
| *S. cerevisiae* AH 22 | | | 67 ± 31 | - | [48] |
| *S. cerevisiae* AH 22 | Co-expression of yeast CPR | | 83.1 | - | [49] |
| *S. cerevisiae* 2805 | | peptidase-deficient strain | 250 ± 30 | - | [50] |
| *S. cerevisiae* INVSc1-HR | Co-expression of CPR, genomic integration | | 22 | - | [37] |
| *S. cerevisiae* W(R) | Over-expression of yeast CPR | | 11.6 ± 5.4 | - | [51] |
| *S. cerevisiae* W303a | Co-expression of CPR, genomic integration | | n.d.[a] | n.d.[a] | This study |
| *S. cerevisiae* W303a | Co-expression of CPR, one plasmid, same promoter | | n.d.[a] | n.d.[a] | This study |
| *S. cerevisiae* W303a | Co-expression of CPR, one plasmid, two promoters | | n.d.[a] | n.d.[a] | This study |
| *P. pastoris* X-33 | Co-expression of CPR | | 120 | - | [15] |
| *P. pastoris* CBS7435 mut$^{s}$ | Co-expression of CPR, separate plasmids | 5 copies CYP2D6, 1 copy CPR | 409-656 | 580-617 | This study |
| *P. pastoris* CBS7435 mut$^{s}$ | Co-expression of CPR, one plasmid | 3 copies each CYP2D6 and CPR | 85-161 | 241-280 | This study |
| *Y. lipolytica* H222-S4 | Co-expression of CPR | codon optimization, multicopy integration (>40 copies) | 92.3 ± 9.2 | - | [18] |
| *Y. lipolytica* H222-S4 | Co-expression of CPR | multicopy integration (~30 copies) | n.d.[a] | n.d.[a] | This study |
| Sf9 cells | | | 330 ± 60 | - | [30] |
| TN5B1-4 cells | | | 700 ± 120 | - | [30] |
| HepG2 | | | 35-45 | - | [52] |

[a] n.d., not detectable

# 2 Materials and Methods

## *2.1 Chemicals*

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) or Carl Roth (Karlsruhe, Germany) with the highest purity available. Bufuralol and 1´-hydroxybufuralol were purchased from BD Bioscience (Becton, Dickinson and Company, Sparks, USA). Zeocin was purchased from InvivoGen (San Diego, USA).

## *2.2 Microorganisms, plasmids and media*

*E. coli* Top10 (Invitrogen, Carlsbad, USA) was used for all cloning steps and plasmid propagation. For cytochrome P450 expression, the *E. coli* strains BL21(DE3) and LEMO21(DE3) from New England BioLabs (Ipswich, USA) were employed. The plasmid pET-26b was purchased from Novagen (Merck, Darmstadt, Germany). Cells were cultivated in Terrific Broth (TB) medium containing 1 mM thiamin and trace elements [10], supplemented with 30 µg/L chloramphenicol or/and 30 µg/L kanamycin for *E. coli* LEMO21(DE3) and *E. coli* BL21(DE3) respectively.

The *S. cerevisiae* strain W303 (MATa, *ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3-1, GALs*$^{+}$) was used for the expression study. The expression constructs were based on the plasmids pYES2 (Invitrogen, Carlsbad, USA) and pESC-URA (Agilent Technologies, Santa Clara, USA) carrying galactose-inducible promoters. YPD medium was made of 10 g/L Bacto™ yeast extract (Becton, Dickinson and Company), 20 g/L Bacto™ peptone and 20 g/L glucose. SC minimal medium contained 6.7 g/L Difco™ yeast nitrogen base (Becton, Dickinson and Company) and 167 mg/L of each adenine, lysine, tyrosine, histidine, leucine and tryptophan. The minimal medium was supplemented with 2 % (w/v) glucose for cell growth and with 2 % (w/v) galactose and 1 % (w/v) raffinose for induction of protein expression.

Cytochrome P450 expression in *P. pastoris* was carried out in a CBS7435 mut$^{s}$ strain [11]. Plasmids pPp_B1 and pPp_Kan_opt_S (unpublished; similar to plasmid pPpB1_S with NCBI accession number JQ519685) carrying zeocin and kanamycin/geneticin resistance markers, respectively, were used. *Pichia* cultures were grown in buffered minimal dextrose (BMD) or buffered mineral methanol (BMM) medium containing 200 mM $KP_i$, pH 6.0, 13.4 g/L yeast nitrogen base and 0.4 mg/L biotin supplemented with 2 % (w/v) glucose or 5 % (v/v) methanol, respectively.

The *Y. lipolytica* strain H222-S4 (*MATA ura3-302*) and the plasmid p64ICL1 were provided by Dr. Stefan Mauersberger (Institute for Microbiology, TU Dresden, Germany). Cultivations were carried out in buffered minimal medium containing 6.7 g/L yeast nitrogen base supplemented with glucose and 200 mM $KP_i$, pH 6.5 (YNBG).

cDNA clones of human CYP2D6 (IMAGE Clone ID: 30915411) and human CPR (IMAGE Clone ID: 7262313) were purchased from BioCat GmbH (Heidelberg, Germany).

### *2.3 Plasmid and strain construction*

A list of plasmids and strains generated during this study is shown in Supplemental Table 1. Vector maps of the expression constructs are available as "Supporting Information". Standard molecular cloning technologies were used [12]. For cytochrome P450 expression in *E. coli*, bicistronic constructs were assembled (Supplemental Figure 1). The genes for CYP2D6 and CPR were amplified using either primers *Xba*I_rbs_2D6_fw, *Bam*HI_CPR_rev, 2D6_Linker_rev and CPR_Linker_fw; (see Supplemental Table 2) or primers *Xba*I_rbs_CPR_fw, *Bam*HI_2D6_rev, CPR_Linker_rev and 2D6_Linker_fw. PCR fragments were joined by overlap-extension PCR (oe-PCR) via a linker as described in [13]. The oe-PCR products harboring the CYP2D6/CPR or CPR/CYP2D6 fragments were subsequently cloned into pET-26b via *Xba*I and *Bam*HI restriction sites. *E. coli* BL21(DE3) and LEMO21(DE3) cells were transformed according to the manual provided by the supplier.

A *S. cerevisiae* strain harboring an integrated cassette of the human CPR gene was generated. Therefore, a linear cassette containing the CPR gene under the control of the *GAL1* promoter and *CYC1* terminator and a selection marker for geneticin resistance was assembled by oe-PCR (Supplemental Figure 2A). In a first step, the CPR gene was amplified using primers *Eco*RI_CPR_WT_fw and *Not*I_CPR_WT_rev, and *Eco*RI/*Not*I cloned into pYES2. Using the resulting vector as template, the CPR coding region was amplified applying primers Ura_Gal_CPR_fw and CPR_CyC _rev. In parallel, the kanMX6 cassette was amplified from pPp_Kan_opt using primers Tef1_Kan_fw and Ura_Kan_rev. The CPR and kanamycin resistance cassette fragments were joined by oe-PCR and thus directed for insertion at the *ura3* locus. *S. cerevisiae* was transformed with the CPR cassette employing the lithium acetate method [14]. Positive transformants were selected on YPD agar plates containing 300 mg/L geneticin. The correct integration of the CPR expression cassette at the *ura3* locus was confirmed by colony PCR. The resulting strain *S. c*. W303a hCPR was used for further transformation with the plasmid pYES2 carrying the CYP2D6 gene cloned via *Eco*RI/*Not*I. Transformants were selected on SC minimal medium for uracil prototrophy.

Furthermore, co-expression plasmids based on pESC-URA and pYES2 were constructed (Supplemental Figure 2B and 2C). The plasmid pESC-2D6-CPR was generated by cloning the CPR gene amplified using primers *Not*I_hCPR_fw and *Bgl*II_hCPR_rev into the multiple cloning site (MCS) 1 and the CYP2D6 gene amplified using primers *Bam*HI_2D6_fw and *Hind*III_2D6_rev into the MCS 2. For the pYES2-based construct, the CPR gene was amplified (*Hind*III_hCPR_fw, *Bam*HI_hCPR_rev) and cloned into the MCS of pYES2. The resulting plasmid was modified by introducing the restriction sites for *Bgl*II and *Asc*I using primers pYES2_AscI_fw and pYES2_*Bgl*II_rev, and was re-circularized by ligation with the CYP2D6 expression cassette. The latter was constructed by cloning *Hind*III_2D6_fw and *Bam*HI_2D6_rev amplified CYP2D6 fragment into pYES2 and by amplifying the whole expression cassette using primers *Bgl*II_Gal1_fw and *Asc*I_CYC1_rev.

A co-expression plasmid for the production of CYP2D6 and CPR in *P. pastoris* was generated as described by Dietrich et al. [15] (Supplemental Figure 3A). Shortly, both genes were separately cloned into the plasmid pPp_B1 via *Eco*RI/*Not*I resulting in pPp_B1_2D6 and pPp_B1_CPR. Primers *Eco*RI_2D6_WT_fw, *Not*I_2D6_WT_rev, *Eco*RI_CPR_WT_fw and *Not*I_CPR_WT_rev used for this task are described in the Supplemental table. The CYP2D6 expression cassette was isolated from pPp_B1_2D6 by digestion with *Bgl*II and *Bam*HI and, subsequently, cloned into the *Bgl*II site of pPp_B1_CPR. Electrocompetent *P. pastoris* cells were prepared and transformed with 1-2 µg of *Bgl*II linearized plasmid as described recently [16]. Positive transformants were selected on YPD agar plates containing 100 mg/L zeocin. For the two-plasmid strategy (Supplemental Figure 3B), the CPR gene was cloned into pPp_Kan_opt_S via *Eco*RI/*Not*I restriction sites. The plasmid was integrated into *P. pastoris* and the resulting strain was, subsequently, transformed with pPp_B1_2D6 (selection on YPD agar plates containing 100 mg/L zeocin and 300 mg/L geneticin). Copy numbers of integrated expression cassettes in the *Pichia* strains of interest were determined using quantitative real-time PCR (qRT-PCR) [17].

To generate a co-expression plasmid for *Y. lipolytica* (Supplemental Figure 4), the starting plasmid p64ICL1 was modified by replacing the isocitric lyase 1 (*ICL1*) gene with a linker containing the restriction sites for *Spe*I and *Asc*I as described by Braun *et al*. [18]. These sites were used to clone a fragment consisting of the CPR gene followed by the *ICL1* terminator, the *ICL1* promoter and the CYP2D6 gene assembled by oe-PCR. The resulting plasmid was digested with *Sac*II prior to transformation into *Y. lipolytica* by the lithium acetate method [19]. Positive transformants were selected on minimal media agar plates for $Ura^+$ phenotype. Copy number determination was accomplished by qRT-PCR.

## 2.4 Recombinant protein production

### 2.4.1 CYP2D6 and CPR expression in E. coli

500 mL modified TB-medium (in a 2 L flask) were inoculated with 5 mL of an *E. coli* over-night culture. Cells were grown at 37°C to an $OD_{600}$ of 0.6-0.8. Protein expression was induced by the addition of 0.5 mM and 1 mM IPTG for *E. coli* LEMO21(DE3) and *E. coli* BL21(DE3), respectively. Concomitantly, 0.5 mM □-aminolevulinic acid was added and induction was performed for 48 h at 28°C.

Preliminary experiments in 10 mL scale had been conducted to determine the optimal expression level for CYP2D6 production in *E. coli* LEMO21(DE3). Therefore, L-rhamnose was added at different concentrations (0 – 2000 µM) to the main culture as described in the LEMO21(DE3) manual.

### 2.4.2 CYP2D6 and CPR expression in S. cerevisiae

Over-night cultures of *S. cerevisiae* were used to inoculate 200 mL of minimal glucose medium in 2 L baffled flasks to a final $OD_{600}$ of 0.1. Cultures were incubated at 30°C and 120 rpm. After 48 h, the cells were harvested by spinning 5 min at 1000g and resuspended in 200 mL induction medium. Protein production was conducted for 24 h at 30°C.

### 2.4.3 CYP2D6 and CPR expression in P. pastoris

Protein expression in *P. pastoris* was performed essentially as described in [20]. Briefly, 200 mL of BMD1% medium in a baffled 2 L flask were inoculated with a single colony and shaken at 28°C and 120 rpm for 60 h. Induction of expression was maintained by daily addition of methanol to 0.5% for 72 h.

### 2.4.4 CYP2D6 and CPR expression in Y. lipolytica

*Y. lipolytica* clones were grown in YNBG (1% glucose) over night at 28°C. These precultures were used to inoculate 250 mL YNBG (0.6% glucose) in a 1 L flask to an $OD_{600}$ of 0.5. Protein production was induced after 24 h by the addition of ethanol to a final concentration of 1%. At 8 h of induction, further ethanol was added to 1% and cells were incubated for 16 h.

## 2.5 Preparation of membrane fractions

Cell disruption of *P. pastoris* was performed essentially as described in [21]. Cells were harvested by 10 min centrifugation at 3000g and 4°C. The pellet was washed once with water before being resuspended in 15-20 mL homogenization buffer (50 mM $KP_i$, pH 7.9) containing 5 % glycerol, 1 mM EDTA, 2 mM DTT and 1 mM phenylmethylsulfonyl fluoride. Cell suspensions were mixed with an equal amount of acid-washed glass beads of 0.5 mm diameter and broken in a mechanical homogenizer (B. Braun Biotech International GmbH, Melsungen, Germany). The same protocol was applied for *S. cerevisiae* and *Y. lipolytica*.

*E. coli* cells were harvested for 10 min at 4000g and 4°C and resuspended in homogenization buffer supplemented with 1 mg/mL lysozyme. After short incubation on ice, cells were disrupted by sonication at 80% power for 6 min.

The crude cell lysates were then separated from cell debris by 10 min centrifugation at 10,000g and 4°C. To recover the membrane fractions from yeasts and *E. coli*, the cleared lysates were ultra-centrifuged at 180,000g and 4°C for 1 h and 16 h, respectively. Total membranes were resuspended in homogenization buffer and stored at -80°C.

Total protein concentrations of membrane preparations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Germany), according to the manufacturer´s instructions, using BSA as standard.

## 2.6 SDS-PAGE/Western blotting

Five µg of total protein per lane were separated by SDS-PAGE under reducing conditions using NuPAGE® 4-12% Bis-Tris gel (Invitrogen). Protein bands were transferred onto a nitrocellulose membrane (GE Healthcare, Chalfont St Giles, UK) electrophoretically in a wet blotting system. Immunoblot detection was performed using a CYP2D6-specific antibody (BD Biosciences) according to the manual provided by the supplier. The presence of CYP2D6 was visualized by staining with NBT/BCIP (Merck).

## 2.7 Quantification of cytochrome P450

Cytochrome P450 content in membrane preparations was determined by CO-difference spectroscopy as described by Omura and Sato [22]. Two mL aliquots of isolated membranes in 100 mM $KP_i$, pH 7.4, containing 20% glycerol were supplemented with 100 µL of 200 mM KCN, pH 7.7, to mask the negative peak of cytochrome oxidases at 445 nm [23]. A few milligrams of the reductant sodium dithionite were added and the reaction mixture was split into two cuvettes. A difference spectrum was measured between 400 to 500 nm using a dual-beam spectrophotometer (Specord 205 UV/Visible spectrophotometer, Analytik Jena). The sample cuvette was aerated with carbon monoxide for 1 min and reduced once more with some sodium dithionite. Upon 1 min incubation time, the difference spectrum was recorded again. For determining CYP content in whole cells, cell suspensions with an $OD_{600}$ of 35-70 were prepared. CYP content was calculated based on a molar extinction coefficient of $\varepsilon_{450\text{-}490nm} = 91\ mM^{-1}\ cm^{-1}$.

## 2.8 Determination of CYP2D6 and CPR activity in membrane preparations

CYP2D6 activity in membrane preparations was determined applying the bufuralol 1´-hydroxylation assay essentially as described [24]. Shortly, 20 µL of membrane preparation were mixed with 1 mM NADPH in assay buffer (100 mM $KP_i$, pH 7.4). The reaction was started by the addition of bufuralol to a final concentration of 50 µM in 200 µL final volume. After incubation for 10 min at 37°C, the enzymatic reaction was stopped by the addition of 20 µL of 70% (v/v) perchloric acid and incubation on ice. Ten µL of 1 mM prednisolone were added as internal standard for later metabolite quantification. The reaction mixture was centrifuged for 5 min at maximum speed in a table top centrifuge. The supernatant was subjected to analysis by HPLC-MS. Reactions were carried out in triplicate.

CPR activity was estimated by its ability to reduce bovine heart cytochrome c [25]. Twenty-five µL of (diluted) membrane preparations were mixed with 125 µL of a 300 µM cytochrome c solution and made up to a final volume of 650 µL with 50 mM Tris-HCl buffer, pH 7.5. Fifty µL of 50 mM KCN solution, pH 7.7, were added to yeast preparations to mask endogenous oxidase activites. The enzymatic reaction was started by the addition of 50 µL of 1.5 mM NADPH. The increase in absorption at 550 nm was recorded for 2 min using an UV/Vis DU 800 spectrophotometer (Beckman Coulter, USA). Reductase activity was calculated based on a molar extinction coefficient of 21 $mM^{-1}\ cm^{-1}$.

### 2.9 Bufuralol 1´-hydroxylation by whole cells

For whole-cell conversions, preinduced cells were resuspended in reaction buffer (100 mM $KP_i$, pH 7.4, 1% glucose) to yield cell suspensions with an $OD_{600}$ of ~ 5 for *P. pastoris* strains and an $OD_{600}$ of ~20 for *E. coli*, *S. cerevisiae* and *Y. lipolytica* strains. Two-hundred µL of cell suspension were transferred into an Eppendorf tube and mixed with 10 µL of 1 mM bufuralol. Reactions were carried out at 30°C under vigorous shaking for 30 min in triplicate. Reactions were stopped by spinning out cells at full speed and 4°C for 10 min. Prednisolone was added to a final concentration of 50 µM to the supernatants as internal standard prior to analysis by HPLC-MS.

To determine the temperature profile, whole-cell conversions of bufuralol were set up as described above. Reactions were carried out at 20°C, 30°C, 35°C, 40°C and 50°C in triplicate. The pH dependency was investigated in reaction buffers at pH 5.0, pH 5.5, pH 6.0, pH 6.5, pH 7.0, pH 7.5 and pH 8.0.

### 2.10 Determination of CYP2D6 stability in whole-cell conversions

Fifty mL of cells resuspended in reaction buffer as desribed above were incubated with and without 200 µM propranolol at 30°C and 120 rpm. One ml aliquots were drawn at time 0, 0.5, 1, 1.5, 2, 4, 6, 8, 9, 24, 28, 32 and 48 h of reaction. Cells were harvested by 5 min centrifugation at 3000g and resuspended in 1 mL of fresh reaction buffer. $OD_{600}$ measurements were done at this point to account for changes in biomass. The residual CYP2D6 activity of the thus obtained cell suspensions was determined by bufuralol 1´-hydroxylation as described above

### 2.11 Bufuralol 1´-hydroxylation analysis by HPLC-MS

Analysis was performed by HPLC (1200 series, Agilent technologies) equipped with a MSD SL detector with an electrospray ionization unit. Metabolites were separated on a XDB-C18, 1.8 µm, 4.6 x 50 mm column (Agilent technologies) using 10 mM ammonium acetate, pH 5.0, and acetonitrile (ACN) as mobile phase. By applying a gradient of 0-1.6 min, 20% ACN; 1.6-3.0 min, 40% ACN; 3.01-4.0 min, 20% ACN, 1´-hydroxybufuralol (m/z 276), prednisolone (m/z 361) and bufuralol (m/z 262) were eluting after 1.5, 2.8 and 2.9 min, respectively. 1´-hydroxybufuralol was quantified by external calibration using the reference metabolite.

# 3 Results

## *3.1 CYP2D6 and CPR expression in Escherichia coli*

*E. coli* is the bacterial workhorse for recombinant protein production. The expression of mammalian CYPs in *E. coli* is often achieved by modification of the N-terminal sequence of these proteins (see Table 1 and references therein). These modifications include, amongst others, the removal of hydrophobic segments, which serve as membrane-anchors. In this study, however, the expression of the full-length protein was investigated. Since the expression of membrane proteins can be problematic in *E. coli*, the *E. coli* strain Lemo21(DE3) was evaluated for the production of CYP2D6 and CPR. Lemo21(DE3), a derivative of BL21(DE3), offers the special feature to tune T7 expression by modulating the level of lysozyme, the natural inhibitor of the T7 RNA polymerase through the external addition of L-rhamnose to the expression media.

Although in other studies the productivity of membrane proteins was improved by weaker expression [26], the highest CYP2D6 production levels were achieved when no L-rhamnose was added, i.e. when the T7 promoter was at its full strength. This indicates that the CYP2D6 expression level is not exceeding the capacity of the cell to insert the built proteins into the membrane. On the contrary, adding 100 µM L-rhamnose to the media resulted in only 3.03 % of CYP2D6 activity compared to the activity at full promoter strength. By further increasing the L-rhamnose concentration, no more CYP2D6 activity was observed (data not shown). Although the fine tuning of CYP2D6 expression seems to be not strictly required in *E. coli* LEMO21(DE3), this host provided more CYP2D6 compared to its parental strain *E. coli* BL21(DE3) (Figure 1). Although Western blot analysis clearly showed CYP2D6 expression in both *E. coli* strains, the produced amounts were too low to result in reduced CO difference spectra for CYP quantification.

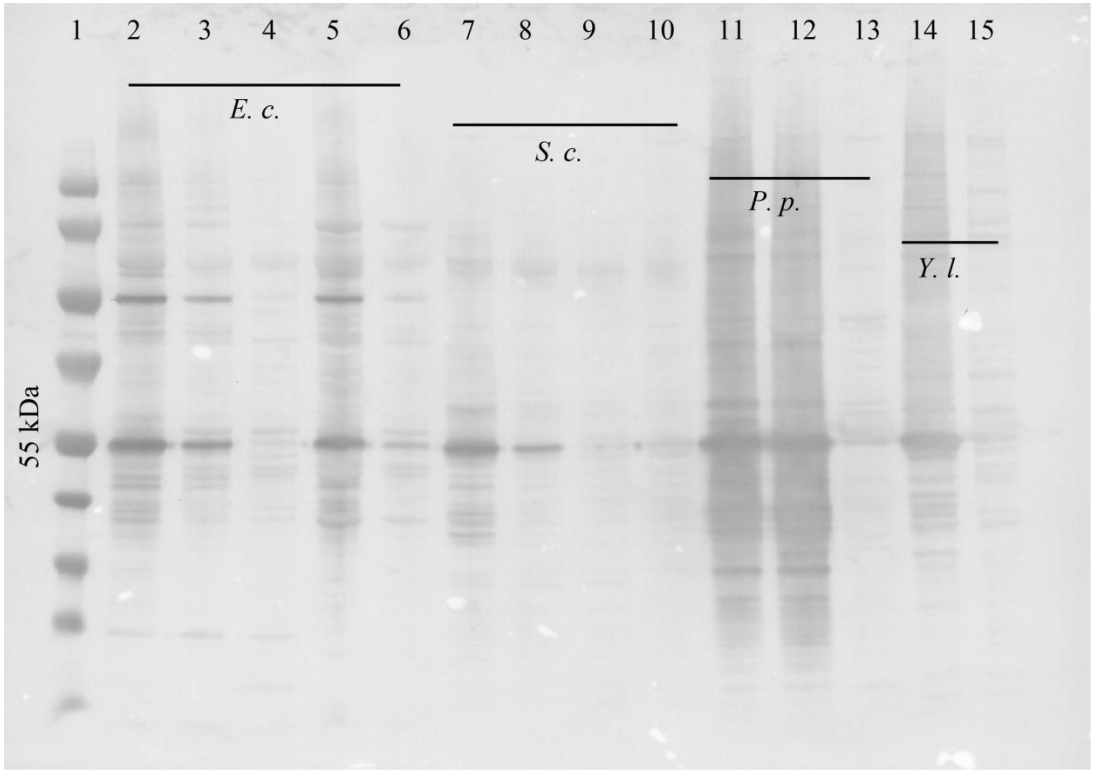


**Figure 3:** Western blot analysis of over-expressed CYP2D6 at expected molecular weight of 55.75 kDa. Beside 5 µL of PageRuler Standard (1), 5 µg of total protein in membrane fractions of *E.c.* LEMO-2D6-CPR (2), *E.c.* LEMO-CPR-2D6 (3), *E.c.* LEMO (4), *E.c.* BL21-2D6-CPR (5), *E.c.* BL21-CPR-2D6 (6), *S.c.* pESC-2D6-CPR (7), *S.c.* pYES2-2D6-CPR (8), *S.c.* hCPR-2D6 (9), *S.c.* W303a (10), *P.p.* 5x2D6/1xCPR (11), *P.p.* 3x2D6/CPR (12*), P.p.* CBS7435 mut$^{s}$ (13), *Y.l.* 2D6-CPR (14), *Y.l.* H222-S4 (15).

To obtain a functional monooxygenase system, CYP2D6 was co-expressed with CPR in a bicistronic format. Two different constructs were generated in which either the CYP2D6 or the CPR gene was directly placed behind the T7 promoter, while the second gene trailed another ribosomal binding site. As can be seen from Figures 1 and 2 the relative location to the promoter had an effect on the achieved CYP2D6 and CPR levels. If CYP2D6 was placed right behind the T7 promoter, an activity of 0.05 mU/mg total membrane protein could be detected, while the corresponding CPR activity was not significantly increased compared to the negative control, i.e. *E.c.* LEMO empty strain. CPR was expressed, if placed first in the cistron, yielding in a CPR activity of 0.45 U/mg total membrane protein. Concomitantly, the CYP2D6 activity was decreased by a factor of ~3. The same trend was observed when using *E. coli* BL21(DE3) as expression host.

### *3.2 CYP2D6 and CPR expression in Saccharomyces cerevisiae*

A prominent expression system for mammalian P450 is *S. cerevisiae*. A feature of this system is that the yeast endogenous CPR can take over the required electron supply, making it thus catalytically self-

sufficient [27]. It was shown, however, that the limited amount of yeast CPR is responsible for rather low monooxygenase activites and that over-expression of CPR can overcome this problem [28, 29]. Therefore, a *S. cerevisiae* strain harboring an expression cassette of human CPR controlled by the *GAL1* promoter at the *ura3* locus was generated and transformed with pYES2-2D6 resulting in *S.c*. CPR-2D6. This strain did only display very minor CYP2D6 activity and the produced CYP2D6 levels were below the detection limit of Western blot analysis as well as of differential CO spectroscopy (Figure 1). Cytochrome P450 expression could be improved by employing co-expression constructs. Clear signals were obtained in the Western blot, but also in these cases CYP2D6 levels were not quantifiable. Co-expressing the genes for CYP2D6 and CPR under the control of the same promoter P(*GAL1*) (*S.c*. pYES2-2D6-CPR) resulted in significantly lower production levels and enzyme activities than co-expressing both genes under the control of two individual different promoters (*S.c*. pESC-2D6-CPR). With the latter strain CYP2D6 activities of 0.035 mU/mg total membrane protein were achieved, which is in the same range as the activities observed for *E. coli* (E.c. LEMO21-2D6-CPR) and *Y. lipolytica* (Figure 2A). In comparison to the other two yeast species, *S. cerevisiae* showed the lowest CPR activities. It was already reported that mammalian CPRs are poorly expressed in this yeast compared to the endogenous CPR [29].

### *3.3 CYP2D6 and CPR expression in Pichia pastoris*

Two different expression strategies have been employed to investigate the potential of *P. pastoris* as heterologous expression host for a functional monooxygenase system. In both cases, expression plasmids have been constructed which were linearized prior to transformation. These linear expression cassettes were then integrated in the genome of *P. pastoris*. One *Pichia* strain was set up by using a co-expression construct as described by Dietrich *et al.* [15]. Both genes, CYP2D6 and CPR, were placed under the separate control of the *AOX1* promoter on the same construct. With this strategy, an equal gene dosage of both genes is assured. Variable ratios of copy numbers of the CYP and CPR gene can be achieved by using two separate expression cassettes with different selection markers, which are used for simultaneous or consecutive *P. pastoris* transformations. For example a platform strain harboring the CPR gene can be generated and used for further transformation with different CYPs. This strategy is also useful in protein engineering experiments, since the use of rather large co-expression plasmids can be avoided, which otherwise might cause problems in diversity generation and reduce transformation efficiency.

These two strategies resulted in one strain harboring three copies each of CYP2D6 and CPR (*P.p.* 3x2D6/CPR) and in one strain harboring only one copy of CPR, but five copies of CYP2D6 (*P.p.* 5x2D6/1xCPR). Multiple copies of the CYP2D6 gene had a positive effect on its expression level.

According to CO-difference spectra, the strain *P.p.* 5x2D6/1xCPR produced up to ~660 pmol CYP2D6 per mg total membrane protein, which is - to our knowledge - the highest CYP2D6 level reported for any microbial expression system and the first report about successful CYP2D6 and CPR expression from different plasmids. The expression levels of *P.p.* 3x2D6/CPR were approximately four times lower (up to ~160 pmol CYP2D6 per mg total membrane protein) and comparable with the value reported in the literature for *P. pastoris* (Table 1). The differential CO spectra of CYP2D6 produced in *P. pastoris* clearly showed only one peak at 450 nm, indicating that correctly folded holo-enzyme was built. By comparing the CYP2D6 expression levels on the Western blot, the difference between the two *Pichia* strains is not as apparent as for the CO spectra. This might be explained by a saturation of the corresponding signal or correctly folded and active hydroxylase makes only a small fraction of the totally expressed enzyme,

Although the expression levels of CYP2D6 apparently differ in the membrane preparations of the two strains, the hydroxylation rates of bufuralol were only slightly different (Figure 2A). Furthermore, the specific CYP2D6 activity in the microsomal preparation of *P.p.* 5x2D6/1xCPR was 1.15 nmol/min/nmol, while it was 3.15 nmol/min/nmol in that of *P.p.* 3x2D6/CPR. This finding might be explained by the different CPR expression levels observed for these two strains. The three copies of CPR result in a CPR activity of 2.76 U/mg total membrane protein, while only 0.33 U/mg total membrane protein can be observed for the strain with one CPR copy (Figure 2B). The low CPR amount might be the limiting factor, resulting only in a fraction of the CYP2D6 activity that might be reached by sufficient electron supply. This result underlines the importance of CPR, whose adequate expression is as important as the cytochrome P450 enzyme in order to obtain an efficient monooxygenase system.

In *P. pastoris*, differential CO spectra of CYP2D6 were also recorded in whole cells. Shake flask cultivation of *P.p.* 5x2D6/1xCPR and *P.p.* 3x2D6/CPR yielded up to 77 and 29 nmol P450/gCDW, respectively. As in shake flask cultivations of *P. pastoris* $OD_{600}$ values of >15 are easily reached, which is equivalent to >8 gCDW/L, >610 nmol CYP2D6/L culture can be produced without much effort. Protein production under controlled conditions in the bioreactor should result in even higher yields.

### *3.4 CYP2D6 and CPR expression in Yarrowia lipolytica*

To co-express CYP2D6 and CPR, a plasmid carrying both genes under the independent control of the *ICL1* promoter was generated and used to transform *Y. lipolytica* after linearization. The linearized construct was chromosomally integrated in the rDNA. This resulted in the strain *Y.l.* 2D6/CPR,

harboring ~ 30 copies of the co-expression construct. Western blot analysis showed quite extensive CYP2D6 expression levels in the membrane fraction (Figure 1), but the characteristic P450 peak could not be detected by differential CO spectroscopy for quantification. However, codon optimization of the CYP2D6 gene along with high gene dosage (>40 copies) was shown to yield a *Y. lipolytica* strain producing 92.3 ± 9.2 pmol CYP2D6/mg membrane protein [18]. CYP2D6 activity in the membrane fraction of *Y.l.* 2D6/CPR was ~ 0.03 mU/mg total membrane protein and was thus ranking below the activities found in the preparations from both *P. pastoris* strains and *E. coli* Lemo-2D6-CPR (Figure 2A). CYP2D6 activity displayed in whole cells of *Y. lipolytica* was higher than for *E. coli*, but not than *P. pastoris* (Figure 3). CPR activity for the 30 copies containing strain of this study was determined to be 0.86 U/mg membrane protein, which is only 2.6 times higher than the activity observed with the *Pichia* strain containing a single CPR copy. Generally, it seems that a high gene dosage is mandatory to obtain adequate P450 expression in *Y. lipolytica*.

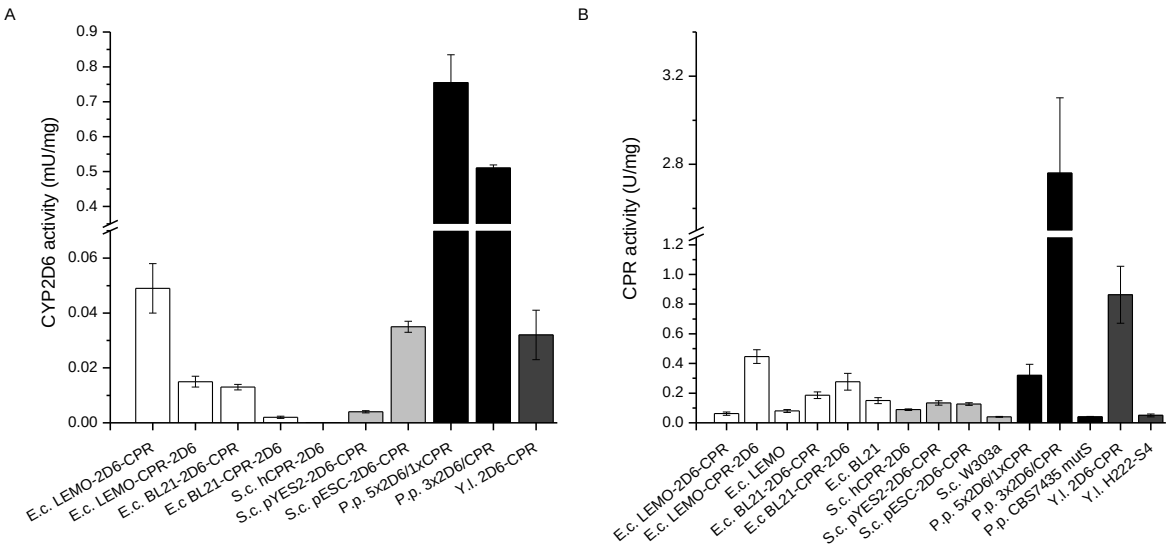


**Figure 4:** CYP2D6 (A) and CPR (B) activities in membrane preparations of different recombinant hosts. (A) CYP2D6 activity was determined in the bufuralol hydroxylation assay. Membrane preperations were incubated with 50 µM bufuralol and 1 mM NADPH in 100 mM $KP_i$, pH7.4, for 10 min at 37°C. Supernatants were analyzed for 1´-hydroxybufuralol production by HPLC-MS. (B) CPR activity was determined spectrophotometrically in the cytochrome c assay. The increase in absorption at 550 nm was recorded for 2 min. A representative result of three biological replicates is shown. Values are shown as mean ± SD of three technical replicates.

## *3.5 Whole-cell biotransformations*

Being aware of the advantages of using recombinant whole cells rather than isolated enzymes or microsomal preparations in biotransformations, we evaluated our different host systems as whole-cell biocatalysts. Whole-cell conversions of bufuralol were conducted using resting cells, since conversions with growing cells showed reduced hydroxylation rates in the case of *P. pastoris* and *Y.*

*lipolytica* (data not shown). The reaction buffer was supplemented with 1% glucose to ensure NADPH regeneration. The highest activities were achieved with the *P. pastoris* strains *P.p.* 5x2D6/1xCPR and *P.p.* 3x2D6/CPR, yielding in 9.6 and 14.3 mU/gCDW. Almost one order of magnitude lower were the activities using recombinant *Y. lipolytica* (*Y.l.* 2D6-CPR; 2.4 mU/gCDW), *E. coli* (*E.c.* Lemo 2D6-CPR; 1.8 mU/gCDW) and *S. cerevisiae* (*S.c.* pESC 2D6-CPR; 0.74 mU/gCDW) (Figure 3).

Interestingly, *P.p.* 3x2D6/CPR was performing better in whole-cell conversions than *P.p.* 5x2D6/1xCPR, which had displayed the highest CYP2D6 activity *in vitro* (Figure 2A). This might be due to low CYP2D6 stability in the *in vitro* system, which was compensated in the case of *P.p.* 5x2D6/1xCPR by the high enzyme level. In whole-cell conversions CYP2D6 stability is not an issue, whereas efficient electron supply is required as provided in the strain *P.p.* 3x2D6/CPR. The specific activities of CYP2D6 in whole cells were 0.13 nmol/min/nmol and 0.58 nmol/min/nmol for *P.p.* 5x2D6/1xCPR and *P.p.* 3x2D6/CPR, respectively, which is approximately one order of magnitude lower than the specific activities found in the corresponding microsomal preparations. The decrease might reflect the impact of the cell wall and the plasma membrane, which act as a barrier between the extracellular substrate and the intracellular enzyme. This result clearly indicates that substrate uptake is a limiting step in the performed whole-cell conversions and, thus, represents a point of action for optimization and engineering. A parameter for optimization is the pH at which the biotransformations are conducted. Generally, in all four expression systems the protonation state, and therefore the charge of the substrate appeared to influence its uptake (data not shown).

A detailed summary of expression levels and activities of CYP2D6 and CPR obtained with the four expression systems under investigation can be found in the Supporting Information (Supplemental Table 3).

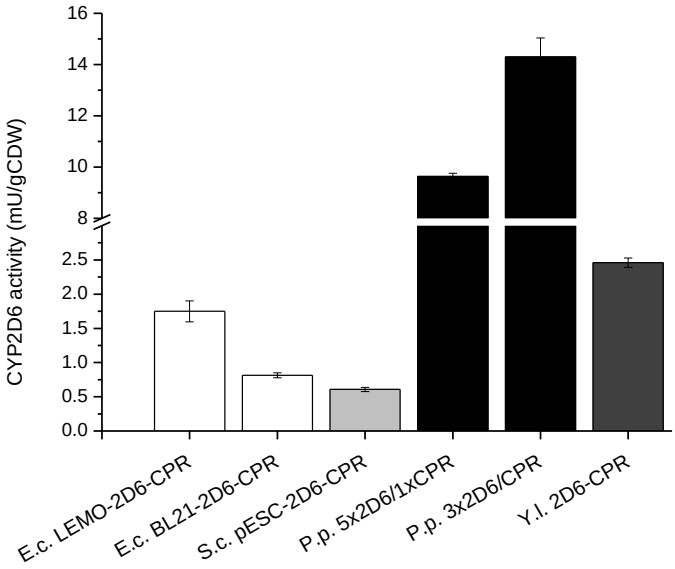


**Figure 5:** Whole-cell conversions of bufuralol by different recombinant expressions hosts. Conversions were conducted at 30°C in 100 mM $KP_i$, pH7.4, containing 1% glucose. After 30 min, the supernatants were analyzed for 1´-hydroxybufuralol production by HPLC-MS. A representative result of three biological replicates is shown. Values are shown as mean ± SD of three technical replicates. Whole-cell conversions of bufuralol by different recombinant expressions hosts. Conversions were conducted at 30°C in 100 mM $KP_i$, pH7.4, containing 1% glucose. After 30 min, the supernatants were analyzed for 1´-hydroxybufuralol production by HPLC-MS. A representative result of three biological replicates is shown. Values are shown as mean ± SD of three technical replicates.

## *3.6 Temperature dependence of whole-cell biotransformations*

To determine the influence of the temperature on biotransformations, whole-cell conversions were conducted at temperatures ranging from 20°C to 50°C (Figure 4). The profiles obtained for the three yeast species looked similar. Increasing the reaction temperature resulted in higher whole-cell activities, yielding in an approximately two fold improvement at elevated temperature as compared to 30°C. Being beneficial for biotransformations employing yeast cells, higher temperatures did not have the same effect for reactions carried out by recombinant *E. coli*. On the contrary, *E. coli* cells displayed the highest activity in a temperature range from 30°C to 35°C (~ 140%), while at 50°C only ~20% of the activity at standard conditions (37°C, pH 7.4) was observed. At this point it can only be speculated whether the substrate uptake is hampered at elevated temperature or the cytochrome P450 system is not stable in *E. coli* under these conditions.

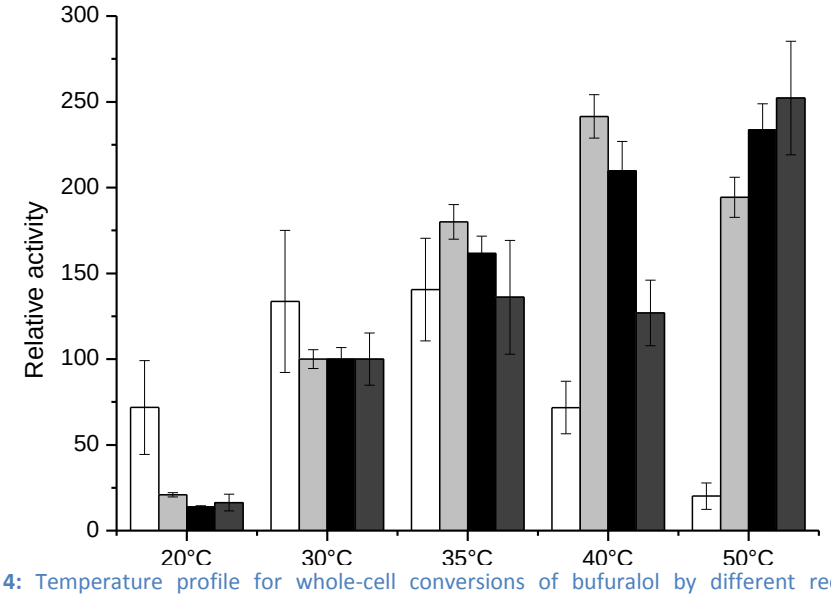


**Figure 4:** Temperature profile for whole-cell conversions of bufuralol by different recombinant expression hosts. Conversions were conducted in 100 mM $KP_i$, pH 7.4, containing 1% glucose at temperatures ranging from 20°C to 50°C. Activities at standard conditions (30°C and pH 7.4 for yeast species, 37°C and pH 7.4 for *E. coli*) were defined as 100%. Values are shown as mean ± SD of three technical replicates. White bars - *E.c.* LEMO 2D6-CPR, light grey bars - *S.c.* pESC-2D6-CPR, black bars - *P.p.* 3x2D6/CPR, dark grey bars - *Y.l.* 2D6-CPR.

## *3.7 Stability of whole-cell biotransformations*

Another important process parameter is the stability of the catalyst used in the biotransformation. In this study, we evaluated on the one hand the stability of CYP2D6 in resting cells of the tested recombinant host. Therefore, cells were incubated in reaction buffer at 30°C for 48 h. Samples were drawn at certain time points and the residual CYP2D6 activity was determined by the bufuralol hydroxylation assay (Figure 5). On the other hand, to test the stability of CYP2D6 doing biocatalysis, the same experimental set up was used, but the cells were incubated in reaction buffer containing 200 µM propranolol. Propranolol is another CYP2D6 substrate, which was chosen as substrate to avoid interference in the analysis of the residual activity. Propranolol was metabolized by CYP2D6 in whole-cell conversions with all expression hosts used (data not shown).

In the absence of propranolol, the *Pichia* strain *P.p.* 3x2D6/CPR was still displaying ~90% of the residual activity after 6 h of incubation and even after 48 h there was still detectable activity (Figure 5C). During whole-cell conversions of propranolol, the CYP2D6 activity dropped significantly already after 30 min to ~27% residual activity. Whether the regeneration of the biocatalyst CYP2D6 or the regeneration of the cofactor is the limiting step is not yet known. The stability of CYP2D6 in *S. cerevisiae* (S.c. pESC 2D6-CPR) was lower than observed for *P. pastoris* (Figure 5B). After 6 h of incubation without propranolol, ~27% of the starting activity was retained and after 48 h activity was

completely lost. In the presence of propranolol, only ~16% residual CYP2D6 activity was left after 30 min. Whole cells of *E. coli* (*E.c.* LEMO 2D6-CPR) were more or less inactive after 6 h of incubation without propranolol (Figure 5A). It seems that *E. coli* is not providing a favorable environment for CYP2D6 stability although expression is relatively simple and efficient. Interestingly, the stability of this enzyme was slightly higher during whole-cell conversion of propranolol. The residual activity was only reduced by half after 30 min and ~18% of the activity was retained after 6 h of incubation. It should be noted that *E. coli* showed by far the lowest propranolol hydroxylation rate during this time course. In contrast to the other microbial systems, the activity of CYP2D6 in *Y. lipolytica* increased by incubating the cells in reaction buffer. The activity was rising during the first 8 h of incubation, before it started to drop. The same trend was found by incubating the cells with propranolol. In the *Yarrowia* system, CYP2D6 was not inactivated by the incubation with propranolol, displaying the same activities during the first 6 h as in resting cells alone. Even after 48 h of reaction time and full conversion of propranolol, cells were as active as without propranolol (Figure 5D).

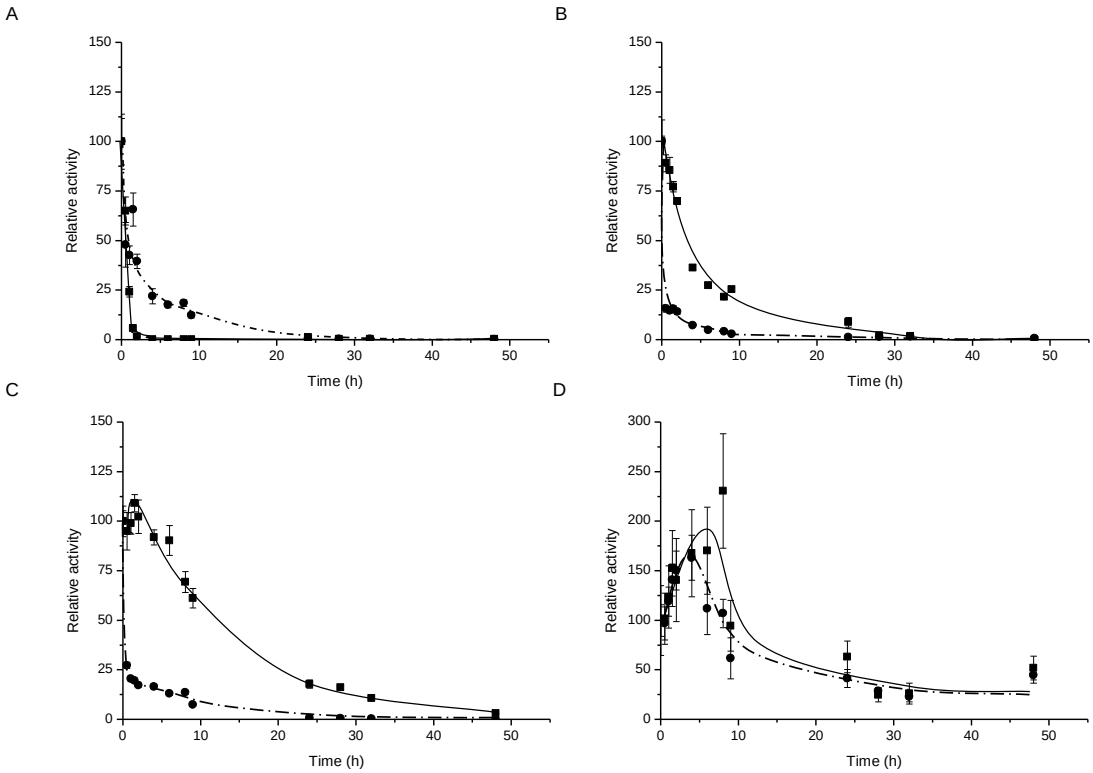


**Figure 5:** Stability of CYP2D6 in whole-cell biotransformations. Cells were incubated in 100 mM $KP_i$, pH 7.4, containing 1% glucose in the absence (full line) and in the presence of 200 µM propranolol (dashed line). Samples were drawn at certain time points and residual CYP2D6 activity was determined in the bufuralol hydroxylation assay. Activity at time = 0 h was defined as 100%. Values are shown as mean ± SD of three technical replicates. (A) *E.c.* LEMO 2D6-CPR, (B) *S.c.* pESC-2D6-CPR, (C) *P.p.* 3x2D6/CPR, (D) *Y.l.* 2D6-CPR. The course of CYP2D6 activitiy was manually interpolated.

## 4 Discussion

In our hands, *P. pastoris* showed the best expression of CYP2D6/CPR. CYP2D6 amounts detectable by CO difference spectroscopy in whole cells as well as in membrane preparations have been produced. The expression levels were amongst the highest reported in the literature (Table 1). Only insect cells were reported to produce higher CYP2D6 levels [30]. However, working with insect cell cultures is technically demanding and expensive, which lowers their attractiveness as industrial biocatalysts [7]. Furthermore, the reported levels were achieved by solely expressing the CYP2D6 gene, while in our study the CPR gene was functionally co-expressed. The highest activities of the CYP2D6 monooxygenase system were also achieved when employing *P. pastoris*. So far, this was not repeated with other human cytochrome P450s. Besides CYP2D6 only one further human isoform, namely CYP17, was functionally expressed in *P. pastoris* [31]. CYP3A4, another important drug metabolizing enzyme was even not functionally expressed in *P. pastoris* so far, while its functional expression was reported for *E. coli* [32], *S. cerevisiae* [33] and *Y. lipolytica* [18]. Therefore, the proven versatility of *Pichia* as expression host for P450 enzymes is still low in comparison to other systems like *E. coli* and *S. cerevisiae.*

The two expression strategies presented in this study for *P. pastoris* might be used for different purposes. A *P. pastoris* strain harboring multiple copies of the CYP2D6 gene might be useful for the production of large cytochrome P450 quantities for further isolation and purification. If employed as biocatalyst, an optimal ratio between CYP2D6 and CPR is essential to obtain optimal activity.

The high CYP2D6 expression levels reported in literature for *E. coli* were not reached with the strains constructed in this study. This might mainly be due to the fact that the N-terminal sequence of CYP2D6 remained unmodified. These N-terminal truncations are regarded as key elements to achieve high-level expression of mammalian CYPs in bacteria [10, 27]. The thus altered P450 enzymes, however, might be problematic in drug metabolite production. A recent study showed that N-terminal modifications of human CYP1A2 changed its active site surrounding and may affect the product spectrum of the enzyme [34]. Van *et al.* [35] investigated the sequential metabolism of dextromethorphan by CYP2D6 from different recombinant sources. They showed that CYP2D6 from Supersomes™ (membrane preparations from insect cells) and Bactosomes™ (bacterial membranes) showed different selectivities in dextromethorphan metabolism. It was suggested, that the modifications in the bacterial system might account for the observed differences. This highlights the importance of expressing the CYP proteins in a membrane environment.

Our *S. cerevisiae* strains did not turn out to be efficient CYP2D6 expression strains as might have been expected from the reports in literature. The apparent discrepancy might be explained by the use of different yeast strains. It is reported that strains although displaying similar genotypes produce either

active or no P450 proteins under identical conditions [27]. In addition, CYP2D6 seems to be a difficult to express cytochrome P450 in baker's yeast. By comparing the expression levels of various human CYPs in *S. cerevisiae*, CYP2D6 levels were amongst the lowest [36, 37]. In a comparative study by Cornelissen *et al*., *S. cerevisiae* was inferior to *E. coli* in the expression of rat and human CYP1A1 [38].

Although *E. coli* and *S. cerevisiae* did not meet the expectations from literature in our comparative study these two systems are still valuable hosts for the recombinant production of CYP enzymes. All major human CYP isoforms have been successfully expressed in these hosts [8, 36,37, 39] highlighting their versatility and potential.

*Y. lipolytica* has already been shown to be a suitable expression host for mammalian cytochrome P450 systems [18, 40, 41]. Especially in whole-cell conversions, *Y. lipolytica* displayed positive properties such as the best stability of CYP2D6 during biotransformation in our hands. Its potential application in biphasic reaction setups makes this yeast interesting for industrial applications.

Generally, the yeast expression systems constitute a valuable platform as whole-cell biocatalysts. Among the yeasts, the fission yeast *Schizosaccharomyces pombe* would be a further alternative. However, although Bureik *et al.* successfully used this yeast as host for whole-cell biotransformations with human CYPs, no CYP concentrations were reported [42, 43].

While whole cell activities described in this comparative study still remained in the previously observed mU/gCDW range, significantly higher long-term catalyst stability of the applied yeasts compared to *E. coli* provide an advantage for efficient metabolite production. Furthermore even low enzymatic activities are generally regarded as useful for the selective, simple and quick production of high value drug metabolites [6]. This study provides knowledge and tools for an optimal starting point for whole-cell membrane bound CYP catalyst construction and the functional two-plasmid strategy facilitates human CYP library construction in *P. pastoris* for future enzyme engineering. Protein engineering to increase CYP activity, strain engineering to improve cell permeability and co-factor regeneration and process engineering are some of the possible strategies to obtain higher productivities for future applications, if needed.

# 5 References

[1] Crespi, C. L., Miller, V. P., The use of heterologously expressed drug metabolizing enzymes-state of the art and prospects for the future. *Pharmacol Ther*. 1999, *84*, 121-31.

[2] Zhuge, J., Yu, Y., Wu, X., Stable expression of human cytochrome P450 2D6*10 in HepG2 cells. *World J Gastroenterol*. 2004, *10*, 234-237.

[3] Zanger, U. M., Raimundo, S., Eichelbaum, M., Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. *Naunyn Schmiedebergs Arch Pharmacol*. 2004, *369*, 23-37.

[4] Smith, D. A., Obach, R. S., Metabolites in safety testing (MIST ): considerations of mechanisms of toxicity with dose, abundance, and duration of treatment. *Chem Res Toxicol*. 2009, *22*, 267-279.

[5] Duetz, W. A., van Beilen, J. B., Witholt, B., Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis. *Curr Opin Biotechnol*. 2001, *12*, 419-425.

[6] Julsing, M., Cornelissen, S., Bühler, B., Schmid, A., Heme-iron oxygenases: powerful industrial biocatalysts? *Curr Opin Chem Biol*. 2008, *12*, 177-186.

[7] Schroer, K., Kittelmann, M., Lütz, S., Recombinant human cytochrome P450 monooxygenases for drug metabolite synthesis. *Biotechnol Bioeng*. 2010, *106*, 699-706.

[8] Gonzalez, F. J., Korzekwa, K. R., Cytochromes P450 expression systems. *Annu Rev Pharmacol Toxicol*. 1995, *35*, 369-90.

[9] Lin-Cereghino, J., Cregg, J. M., Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev*. 2000, *24*, 45-66.

[10] Guengerich, F., Martin, M. V., Gua, Z., Chun, Y.-J., Purification of functional recombinant P450s from bacteria. *Methods Enzymol*. 1996, *272*, 35-44.

[11] Näätsaari, L., Mistlberger, B., Ruth, C., Hajek T., *et al*., Deletion of the *Pichia pastoris* KU70 homologue facilitates platform strain generation for gene expression and synthetic biology. *PLoS One*. 2012, 7, e39720.

[12] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., *et al.* (Eds.), *Current protocols in molecular biology*, John Wiley and Sons, New York, 2007.

[13] Ahn, T., Yun, C.-H., High-level expression of human cytochrome P450 3A4 by co-expression with human molecular chaperone HDJ-1 (Hsp40). *Arch Pharm Res*. 2004, *27*, 319-323.

[14] Adams, A., Gottschling, D. E., Kaiser, C. A., Stearns, T. (Ed.), *Methods in yeast genetics*, Cold Spring Harbor Laboratory Press, 1998.

[15] Dietrich, M., Grundmann, L., Kurr, K., Valinotto, L., *et al.*, Recombinant production of human microsomal cytochrome P450 2D6 in the methylotrophic yeast *Pichia pastoris*. *Chembiochem*. 2005, *6*, 2014-22.

[16] Lin-Cereghino, J., Wong, W. W., Xiong, S., Giang, W., *et al.*, Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. *BioTechniques*. 2005, *38*, 44-48.

[17] Abad, S., Kitz, K., Hörmann, A., Schreiner, U., *et al.*, Real-time PCR-based determination of gene copy numbers in *Pichia pastoris*. *Biotechnol J*. 2010, *5*, 413-20.
[18] Braun, A., Geier, M., Bühler, B., Schmid, A., et al., Steroid biotransformations in biphasic systems with *Yarrowia lipolytica* expressing human liver cytochrome P450 genes. *Microb. Cell Fact*. 2012, in press.

[19] Barth, G., Gaillardin, C., *Yarrowia lipolytica*, in: Wolf, K. (Ed.), *Nonconventional yeasts in biotechnology*, Springer-Verlag, Berlin 1996, pp. 313-388.

[20] Weis, R., Luiten, R., Skranc, W., Schwab, H., *et al.*, Reliable high-throughput screening with *Pichia pastoris* by limiting yeast cell death phenomena. *FEMS Yeast Res*. 2004, *5*, 179-89.

[21] Andersen, M. D., Møller, B. L., Use of methylotropic yeast *Pichia pastoris* for expression of cytochromes P450. *Methods Enzymol*. 2002, *357*, 333-342.

[22] Omura, T., Sato, R., The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem*. 1964, *239*, 2370-2378.

[23] Schenkman, J. B., Jansson, I., Spectral analyses of cytochromes P450, in: Phillips, I. R., Shephard, E. A. (Eds.), *Cytochrome P450 protocols*, Humana Press Inc., 2004, pp. 11-18.

[24] Crespi, C. L., Chang, T. K. H., Waxman, D. J., CYP2D6-dependent bufuralol 1´-hydroxylation assayed by reverse-phase ion-pair high-performance liquid chromatography with fluorescence detection, in: Phillips, I. R., Shephard, E. A. (Eds.), *Cytochrome P450 protocols*, Humana Press Inc., 2006, pp. 121-125.

[25] Phillips, A. H., Langdon, R. G., Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. *J Biol Chem*. 1962, *237*, 2652-60.

[26] Wagner, S., Klepsch, M. M., Schlegel, S., Appel, A., *et al.*, Tuning *Escherichia coli* for membrane protein overexpression. *Proc Natl Acad Sci U S A*. 2008, *105*, 14371-6.

[27] Gonzalez, F. J., Korzekwa, K. R., Cytochromes P450 expression systems. *Annu Rev Pharmacol Toxicol*. 1995, *35*, 369-390.

[28] Murakami, H., Yabusaki, Y., Ohkawa, H., Expression of rat NADPH-cytochrome P-450 reductase cDNA in *Saccharomyces cerevisiae*. *DNA*. 1986, *5*, 1-10.

[29] Truan, G., Cullin, C., Reisdorf, P., Urban, P., *et al.*, Enhanced *in vivo* monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b5. *Gene*. 1993, *125*, 49-55.

[30] Evert, B., Eichelbaum, M., Haubruck, H., Zanger, U. M., Functional properties of CYP2D6 1 (wild-type) and CYP2D6 7 (His324Pro) expressed by recombinant baculovirus in insect cells. *Naunyn Schmiedebergs Arch Pharmacol*. 1997, *355*, 309-18.

[31] Kolar, N. W., Swart, A. C., Mason, J. I., Swart, P., Functional expression and characterisation of human cytochrome P45017alpha in *Pichia pastoris*. *J Biotechnol*. 2007, *129*, 635-44.

[32] Blake, J. A., Pritchard, M., Ding, S., Smith, G. C., *et al.*, Coexpression of a human P450 (CYP3A4) and P450 reductase generates a highly functional monooxygenase system in *Escherichia coli*. *FEBS Lett*. 1996, *397*, 210-4.

[33] Renaud, J. P., Cullin, C., Pompon, D., Beaune, P., *et al.*, Expression of human liver cytochrome P450 IIIA4 in yeast. A functional model for the hepatic enzyme. *Eur J Biochem*. 1990, *194*, 889-896.

[34] Kim, D.-H., Kim, K.-H., Isin, E. M., Guengerich, F. P., *et al.*, Heterologous expression and characterization of wild-type human cytochrome P450 1A2 without conventional N-terminal modification in *Escherichia coli*. *Protein Expr Purif*. 2008, *57*, 188-200.

[35] Van, L. M., Sarda, S., Hargreaves, J. A., Rostami-Hodjegan, A., Metabolism of dextrorphan by CYP2D6 in different recombinantly expressed systems and its implications for the *in vitro* assessment of dextromethorphan metabolism. *J Pharm Sci*. 2009, *98*, 763-771.

[36] Imaoka, S., Yamada, T., Hiroi, T., Hayashi, K., *et al.*, Multiple forms of human P450 expressed in *Saccharomyces cerevisiae*. Systematic characterization and comparison with those of the rat. *Biochem Pharmacol*. 1996, *51*, 1041-50.

[37] Masimirembwa, C., Otter, C., Berg, M., Jönsson, M., *et al.*, Heterologous expression and kinetic characterization of human cytochromes P-450: validation of a pharmaceutical tool for drug metabolism research. *Drug Metab Dispos*. 1999, *27*, 1117-1122.

[38] Cornelissen, S., Julsing, M. K., Comparison of microbial hosts and expression systems for mammalian CYP1A1 catalysis. *J Ind Microbiol Biotechnol*. 2011.

[39] Iwata, H., Fujita, K., Kushida, H., Suzuki, A., *et al.*, High catalytic activity of human cytochrome P450 co-expressed with human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Biochem Pharmacol*. 1998, *55*, 1315-1325.

[40] Novikova, L. a., Faletrov, Y. V., Kovaleva, I. E., Mauersberger, S., *et al.*, From structure and functions of steroidogenic enzymes to new technologies of gene engineering. *Biochemistry (Mosc)*. 2010, *74*, 1482-1504.

[41] Nthangeni, M. B., Urban, P., Pompon, D., Smit, M. S., *et al.*, The use of *Yarrowia lipolytica* for the expression of human cytochrome P450 CYP1A1. *Yeast*. 2004, *21*, 583-92.

[42] Peters, F., Dragan, C., Kauffels, A., Schwaninger, A., *et al.*, Biotechnological synthesis of the designer drug metabolite 4'-hydroxymethyl-alpha-pyrrolidinohexanophenone in fission yeast heterologously expressing human cytochrome P450 2D6--a versatile alternative to multistep chemical synthesis. *J Anal Toxicol*. 2009, *33*, 190-197.

[43] Drăgan, C., Peters, F., Bour, P., Schwaninger, A., *et al.*, Convenient gram-scale metabolite synthesis by engineered fission yeast strains expressing functional human P450 systems. *Appl Biochem Biotechnol*. 2011, *163*, 965-80.

[44] Pan, Y., Abd-Rashid, B. A., Ismail, Z., Ismail, R., *et al.*, Heterologous expression of human cytochromes P450 2D6 and CYP3A4 in *Escherichia coli* and their functional characterization. *Protein J*. 2011, *30*, 581-91.

[45] Pritchard, M., Glancey, M., Blake, J., Gilham, D., *et al.*, Functional co-expression of CYP2D6 and human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Pharmacogenetics*. 1998, *8*, 33-42.

[46] Li, D. N., Pritchard, M. P., Hanlon, S. P., Burchell, B., *et al.*, Competition between cytochrome P-450 isozymes for NADPH-cytochrome P-450 oxidoreductase affects drug metabolism. *J Pharmacol Exp Ther*. 1999, *289*, 661-7.

[47] Deeni, Y., Paine, M., Ayrton, A., Clarke, S., *et al.*, Expression, purification, and biochemical characterization of a human cytochrome P450 CYP2D6-NADPH cytochrome P450 reductase fusion protein. *Arch Biochem Biophys*. 2001, *396*, 16-24.

[48] Ellis, S., Ching, M., Watson, P., Henderson, C., *et al.*, Catalytic activities of human debrisoquine 4-hydroxylase cytochrome P450 (CYP2D6) expressed in yeast. *Biochem Pharmacol*. 1992, *44*, 617-620.

[49] Fukuda, T., Nishida, Y., Imaoka, S., Hiroi, T., *et al.*, The decreased *in vivo* clearance of CYP2D6 substrates by CYP2D6*10 might be caused not only by the low-expression but also by low affinity of CYP2D6. *Arch Biochem Biophys*. 2000, *380*, 303-8.

[50] Krynetski, E., Drutsa, V., Kovaleva, I., Luzikov, V., High yield expression of functionally active human liver CYP2D6 in yeast cells. *Pharmacogenetics*. 1995, *5*, 103-109.

[51] Oscarson, M., Hidestrand, M., Johansson, I., Ingelman-Sundberg, M., A combination of mutations in the CYP2D6*17 (CYP2D6Z) allele causes alterations in enzyme function. *Mol Pharmacol*. 1997, *52*, 1034-40.

[52] Waxman, D., Lapenson, D., Aoyama, T., Gelboin, H., *et al.*, Steroid hormone hydroxylase specificities of eleven cDNA-expressed human cytochrome P450s. *Arch Biochem Biophys*. 1991, *290*, 160-166.

# Chapter III

# Screening of the EUROSCARF knockout collection or the history of finding *ICE2*

# 1 Introduction

In this chapter, the experimental background leading to the publication presented in chapter IV is delineated in detail. The identification of *ICE2* and its connection to cytochrome P450 reductases (CPR) is described (Emmerstorfer et al, 2014, manuscript in preparation). Applicability of cytochrome P450 enzymes (CYPs) for industrial, fine chemical synthesis often suffers from diverse limitations like poor activity, reduced stability and low amounts of recombinant protein. Therefore, finding a way to increase CYP activity levels is a major interest to render this highly useful class of enzymes more attractive. As model enzymes, *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO) (Takahashi et al., 2007) co-expressed with a cytochrome P450 reductase (CPR) from *Arabidopsis thaliana* were selected. HPO and CPR catalyze the conversion of (+)-valencene to trans-nootkatol (Figure 1).

Diverse single knockout mutants were selected from the EUROSCARF knockout collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) to be tested for their potential to improve (+)-valencene conversion with resting cells (Table 1). Different cellular processes possibly affecting CYP functionality were considered. Due to the fact that CYPs strongly depend on supply of the cofactor NADPH, gene deletions coding for ER-localized NADPH depleting enzymes were selected. The same strategy was used to augment intracellular iron supply by knocking out FeS cluster proteins. Iron can be a limiting factor for CYP activity, because it is important for building the porphyrin ring located to the HPO active site (Denisov et al., 2005). Heterologous membrane proteins often underlie ubiquitination processes leading to degradation via the yeast ER-associated protein degradation (ERAD) pathway (Hampton, 2002; Kostova and Wolf, 2003). This was shown to be avoided for diverse human CYPs by knocking out different proteinases, kinases and ER specific ubiquitin ligases (Wang et al., 2012, Wang et al., 2011, Wang et al., 2009). Incontestably, phase transfer plays a huge role as soon as hydrophobic compounds are meant to be converted. Therefore, selected single knockouts of ABC transporters were also chosen to be examined for better intracellular substrate suppy (Bauer et al., 1999; Ernst et al., 2005; Nishida et al., 2013a; Nishida et al., 2013b; Piecuch and Obłąk, 2013).

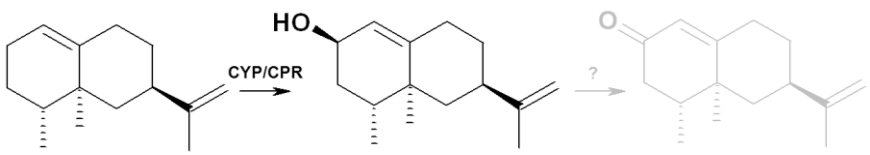


**Figure 1: (+)-valencene (1) biohydroxylation by CYP/CPR activity recombinantly expressed in *S. cerevisiae*. trans-nootkatol (2) formed by cytochrome P450 enzymes may be further oxidized to (+)-nootkatone (3) by an unidentified intrinsic activity of baker's yeast.**

# 2 Materials and Methods

## *2.1. Cloning and strain construction*

Single-gene knockout mutants from the EUROSCARF strain collection (Winzeler et al., 1999) were kindly provided by Professor Günther Daum (Graz University of Technology; Table 1). Single genes had been knocked out by inserting a KanMX cassette directly into the locus (Brachmann et al., 1998; Winzeler et al., 1999). The correct knockout of genes was confirmed by colony PCR (Kwiatkowski et al., 1990) with one specific forward primer Fw(*gene*) designed to bind about 100 bp upstream of the deleted gene and a universal reverse primer binding directly in the KanMX cassette, Rv_KanMX (Table 2).

**Table 1: Single-gene knockout mutants chosen from the EUROSCARF collection to be tested for improved (+)-valencene conversion in resting cells assays**

| ID | Gene | Cellular function | ID | Gene | Cellular function |
|---|---|---|---|---|---|
| **1** | Δ*ubc7* | ER-associated, ubiquitination | **19** | Δ*cho1* | phosphatidylserine synthase |
| **2** | Δ*ubc5* | Ubiquitination of abnormal protein | **20** | Δ*yor1* | ABC transporter |
| **3** | Δ*pka1* | protein kinase, ubiquitination of P450 | **21** | Δ*tul1* | ubiquitination (E3) |
| **35** | Δ*pka2* | protein kinase, ubiquitination of P450 | **22** | Δ*pex10* | peroxisomal protein transport |
| **5** | Δ*pep4* | proteinase A | **23** | Δ*pex4* | peroxisomal protein transport |
| **7** | Δ*pdr1* | pleiodropic drug resistance | **25** | Δ*rip1* | FeS clustering |
| **8** | Δ*pdr3* | PDR-transcriptional activator | **26** | Δ*isu1* | FeS clustering |
| **9** | Δ*pdr5* | PDR plasma membrane ABC transporter | **27** | Δ*isu2* | FeS clustering |
| **10** | Δ*snq2* | ABC transporter | **28** | Δ*ayr1* | NADPH-dependent 1-acyl-P reductase |
| **11** | Δ*stb5* | PDR regulator | **29** | Δ*zta1* | NADPH-dependent quinone reductase |
| **12** | Δ*yap1* | regulation of ABC transporter | **30** | ΔYMR315W | NADP(H) oxidoreductase activity |
| **13** | Δ*dap1* | heme-binding protein | **31** | Δ*ypr1* | NADPH-dependent aldo-keto reductase |
| **14** | Δ*ydj1* | Hsp40 | **32** | Δ*ice2* | ER-membrane protein, ER inheritance |
| **16** | Δ*hlj1* | Co-chaperone of Hsp40 | **33** | Δ*sur1* | sphingolipid biosynthesis |
| **17** | Δ*erg6* | ERGosterol biosynthesis | **34** | Δ*fen2* | plasma membrane pantothenate transporter |

To generate the pYES2 based co-expression construct (Figure 2), the *CPR* gene was amplified using primers Fw_CPR_*Hind*III and Rv_CPR-myc_*Bam*HI (Table 2) for *Hind*III/*Bam*HI cloning into pYES2. The resulting plasmid was modified by introducing the restriction sites for *Bgl*II and *Asc*I using primers pYES2_*Asc*I_fw and pYES2_*Bgl*II_rev, and was re-circularized by ligation with the HPO-Flag expression cassette. The latter was constructed by *Hind*III/*Bam*HI cloning the HPO-Flag fragment amplified with Fw_HPO*Sc*_*Hind*III and Rv_HPO-Flag_*Sc* into pYES2 and, subsequently, by amplifying the whole expression cassette using primers *Bgl*II_Gal1_fw and *Asc*I_CYC1_rev. For exact description of recombinant genes see chapter IV. Transformation of plasmids was done

following the lithium acetate method (Gietz and Schiestl, 1995). Transformants were selected on synthetic defined plates lacking uracil (Botstein and Davis, 1982).

**Table 2: Primers used for colony PCR and cloning of constructs**

| Name | Sequences 5‘-3‘ |
|---|---|
| **Rv(KanMX4)** | CCCATATAAATCAGCATCCA |
| **Fw(*fen2*)** | GATCTTGAAAGGCATTTTTG |
| **Fw(*ubc7*)** | CATCTACCTCACGAAGATGC |
| **Fw(*ubc5*)** | AAAGATAGCCGACCCAAAAT |
| **Fw(*pka1*)** | AAACATCATAGAAGCTGCGA |
| **Fw(*pka2*)** | ATTGGACGGTTTTAAAGGAG |
| **Fw(*pep4*)** | CACGTAAGGGAAGAATAACA |
| **Fw(*pdr1*)** | CTTCAAAACAACCTTATTCG |
| **Fw(*pdr3*)** | TTT CCGCGGAATAATAAATG |
| **Fw(*pdr5*)** | TCCAAAAGAAAAAAGTCACG |
| **Fw(*snq2*)** | ATTACATTCTCAGTGCATCC |
| **Fw(*stb5*)** | CGAGCGGAAATACTAAAAGA |
| **Fw(*yap1*)** | TTTAGCTTTTTTTCCTGAGC |
| **Fw(*dap1*)** | TTTTCTTGTTGCAGTAAGCT |
| **Fw(*ydj1*)** | GTCACAAAAAGTCCTTTTCC |
| **Fw(*hlj1*)** | AGATTCGTATATAGCGGACC |
| **Fw(*erg6*)** | CTGCTCCACTTCGTCTCAATGG |
| **Fw(*yor1*)** | TACGGAGGTAGAACAGCTCT |
| **Fw(*cho1*)** | GGACCCATCTAAAGATGAAG |
| **Fw(*tul1*)** | TCTTTCAATTATCACCCCA |
| **Fw(*pex10*)** | GCTGTCCATATGTGCATCTT |
| **Fw(*pex4*)** | TCTCTTTTCGAAATCCTGAA |
| **Fw(*rip1*)** | TCCCTTATAAGGACTGGAAA |
| **Fw(*isu1*)** | GATGCAAAAATTGAGAGGAA |
| **Fw(*isu2*)** | ATTGTTTCAGAGTCCGGTTC |
| **Fw(*ayr1*)** | TTTTTTCCCCCTACTTCCTC |
| **Fw(*zta1*)** | TAATATAGTACGTGCCGCTC |
| **Fw(YMR315W)** | GGGGAATAAACGGAGTTATC |
| **Fw(*ypr1*)** | TATTGTCACTTTTCATCACG |
| **Fw(*ice2*)** | AAGGATCGAGGTGTAGTGAT |
| **Fw(*sur1*)** | CTTCGCATGTTTTGTTTTCT |
| **Fw_CPR_*Hind*III** | CCCAAGCTTCGAAACGCATATGACTTCTG |
| **Rv_CPR-myc_*Bam*HI** | CGCGGATCCTTATTACAAATCCTCTTCAGAAATCAATTTTTGTTCCCAGACATCTCTCAAGTATCTACC |
| **pYES2_*Asc*I_fw** | TTGGCGCGCCTTAATTAAACGGATTAGAAGCCGCCGAG |
| **pYES2_*Bgl*II_rev** | TTAGATCTGGCGCGCCCGATTCATTAATGCAGGGCC |
| **Fw_HPOSc_*Hind*III** | CCCAAGCTTCGAAACGCATATGCAATTCT |
| **Rv_HPO-Flag_*Sc*** | CGCGGATCCCTCGAGTTATTACTTATCGTCGTCATCCTTGTAATCCTCTCGGGAAGGTTGGTAA |
| ***Bgl*II_Gal1_fw** | TTGGCGCGCCTTAATTAAACGGATTAGAAGCCGCCGAG |
| ***Asc*I_CYC1_rev** | TTAGATCTGGCGCGCCCGATTCATTAATGCAGGGCC |

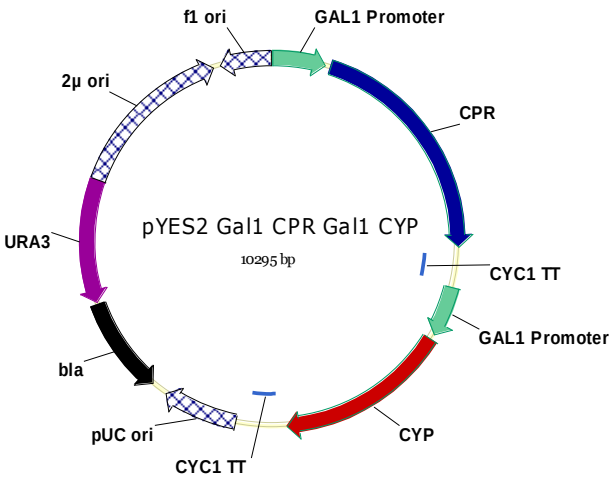


**Figure 2: Co-expression construct cloned for HPO (CYP) and CPR (pYES2-HPO-CPR).**

## 2.2. Recombinant protein expression, Western Blot analysis

For expression of recombinant proteins, 300 mL baffled shake flasks containing 50 mL of synthetic defined growth media (6.7 g yeast nitrogen base w/o amino acids; 1 g drop-out powder consisting of equal amounts of adenine, lysine, tyrosine, histidine, leucine and tryptophane; 2% glucose) were inoculated to an $OD_{600}$ of 0.1. Cell suspensions were shaken for 48 h at 130 rpm and 30 °C. After centrifugation for 5 min at 1,062 x g, cell pellets were resuspended in 50 mL of synthetic defined induction media containing 2% galactose and 0.7% raffinose instead of 2% glucose. Induction was carried out for 6 h at 130 rpm and 30 °C.

For Western blot analysis, 5 $OD_{600}$ units of induced cells were harvested and prepared for SDS-PAGE according to Riezman et al. (1983). Ten µL of the resulting supernatants were separated under reducing conditions on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, USA), and were then transferred electrophoretically onto nitrocellulose membrane (GE Healthcare, Chalfont St Giles, UK) in a wet blotting system. Protein loading was assessed by PonceauS staining of the membrane. Immunodetection was performed by incubation with commercially available rabbit anti-Flag and anti-*myc* (Thermo Scientific, St. Leon-Rot, Germany) followed by an anti-rabbit (for myc) or anti-mouse (for Flag and HIS) secondary antibody linked to horse radish peroxidase purchased from Sigma-Aldrich® (Steinheim, Germany). Western blots were developed by SuperSignal West Pico chemoluminescent substrate (Thermo Scientific, St. Leon-Rot, Germany) and chemiluminescent bands were detected using a G:BOX Chemi HR16 bioimaging system (Syngene, Cambridge, UK).

## 2.3. Resting cells activity assay

For resting cells activity assay, six-hundred $OD_{600}$ units of galactose-induced cells were harvested and resuspended in 2 mL of 50 mM $KP_i$ buffer, pH 7.4. Cell suspensions were split into two equal aliquots in Pyrex tubes and 20 µL of 100 mM (+)-valencene in DMSO as well as 0.1% of TRITON® X-100 (Amresco, Solon, Ohio) were added. Pyrex tubes were only sealed loosely with screw covers to balance between air supply for cells and low substrate volatilization. Conversions were carried out for 16 h at 170 rpm and 30 °C. Terpenoids were extracted with 500 µL of ethyl acetate using a VXR basic Vibrax® (IKA®, Staufen, Germany) at room temperature and maximum speed for 30 min. Vigorous vortexing was important for quantitative extraction, as yeast cells have been shown to accumulate trans-nootkatol intracellularly (Kaspera et al., 2005). After phase separation by centrifugation for 15 min at 2,720 x g, organic layers were analyzed by GC-MS or GC-FID.

### *2.3 Product analysis by GC-MS and GC-FID*

Terpenoid extracts in organic solvents, i.e. ethyl acetate as well as n-dodecane, were initially analyzed by GC-MS for identification of compounds using reference standards and comparing the derived mass fragmentation spectra. A 30 m HP column (0.25 mm x 0.25 µm) was used on a Hewlett-Packard 5890 Series II plus GC equipped with a 5972 series mass selective detector (MSD). Sample aliquots of 1 µL were injected in split mode (split ratio 20:1) at 220°C injector and 280°C detector temperatures with helium as carrier gas at constant flow rate of 32 cm/s. The oven temperature program was as follows: 70 °C for 1 min, 10°C/min ramp to 200°C, and 30°C/min ramp to 290°C (2 min). MSD was operated in a mass range of 40-250 amu with 3.5 scans/s and at electron multiplier voltage of 1635 V. A GC-FID method was developed for routine analyses of terpenoid samples. Therefore, we used a HP-5 column (crosslinked 5 % Ph-Me Siloxane; 10 m x 0.10 mm x 0.10 µm) on a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID). Sample aliquots of 1 µL were injected in split mode (split ratio 30:1) at 250°C injector temperature and 320°C detector temperature with hydrogen as carrier gas and a flow rate set to 0.4 mL/min in constant flow mode (49 cm/s linear velocity). The oven temperature program was as follows: 100°C for 1 min, 20°C/min ramp to 250°C, and 45°C/min ramp to 280°C (0.5 min). The use of a high-speed/high-resolution column reduced the total run time to 9 min per sample, without any loss of chromatographic resolution.

## 3 Results

### *3.1 Western Blot analysis of strains co-expressing HPO and CPR*

Being identified as foreign substances, recombinant proteins often face rapid degradation by the respective host machinery. Therefore, analysis of expression levels and possible degradation products of heterologous proteins is a powerful tool to investigate and evaluate different expression hosts. Western Blot analysis revealed expression levels to strongly depend on the strain background (Figure 3). In the single-gene knockout mutants Δ*yap1*, Δ*ypr1* and Δ*sur1* no significant amounts of HPO and CPR could be detected. Nevertheless, these strains did convert (+)-valencene to a similar extent as the wild type strain co-expressing HPO and CPR (Figure 4). Also, other strains have shown reduced expression of either HPO or CPR. For example, Δ*ubc5*, Δ*hlj1*, Δ*pdr1/3/5*, Δ*pex10/*4 andΔ*fen2* did have reduced expression of the CYP enzyme as compared to the wild type strain background. Four of these strains, Δ*ubc5*/*pdr1*/*pdr3* and Δ*fen2* also showed reduced trans-nootkatol formation (highlighted in light-grey in Figure 3). This indicates that balanced amounts of HPO and CPR are important

prerequisites for optimal activity. Strains Δ*pka1*/*pep4*/*snq1*/*cho1*/*ayr1* showed better conversion of (+)-valencene, although expression levels of HPO and CPR were not altered remarkably (highlighted in dark-grey, Figure 3). Another highly interesting phenomenon was seen in the Δ*ice2* knockout strain. Stability of the reductase was compromised, whereas substrate conversion remained unchanged. For a more precise investigation, please see the follow-up study presented in Chapter IV.

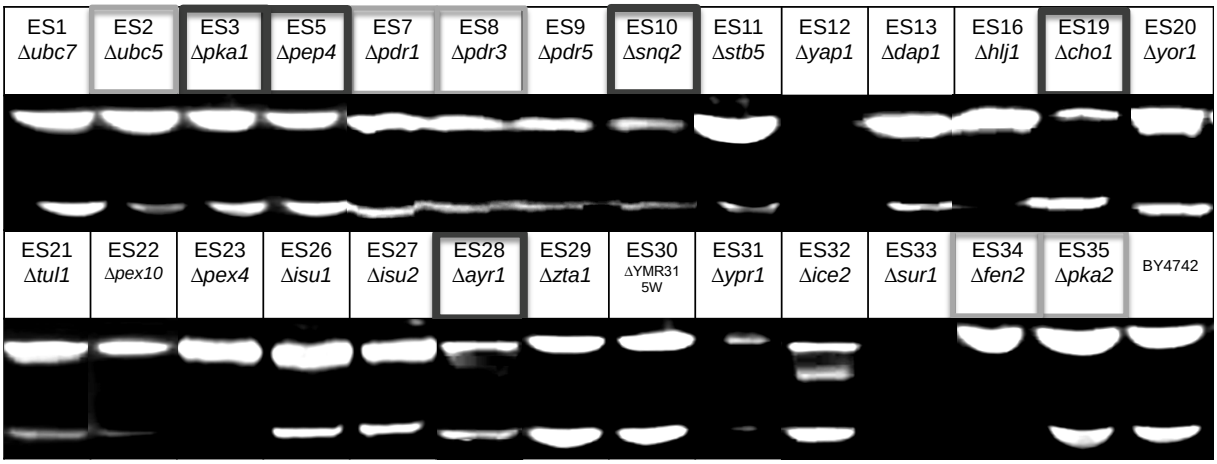


**Figure 3: Western Blot analysis of strains from the EUROSCARF knockout collection co-expressing CPR-*myc* (upper band, 72 kDa) and HPO-Flag (lower band, 53 kDa) from the pYES2 plasmid. Strain BY4742 is the wild type strain harboring pYES2-HPO-CPR. Strains showing improved (+)-valencene conversion were highlighted in dark-grey, those performing significantly worse than the positive control were highlighted in light-grey (see Figure 4).**

## *3.2 Resting cells activity assay*

In general, cell growth was not hampered by any of the genes knocked out. All strains grew to similar optical densities (data not shown). Results of (+)-valencene conversions reached with different mutant strains were subdivided into categories of cellular functions. Interestingly, no functional category has shown to generally improve (+)-valencene conversion. Obviously, the lack of single, ER-associated and NADPH requiring enzymes did either not enhance overall NADPH amounts significantly or NADPH supply is not rate limiting for (+)-valencene conversion. The absence of diverse ABC transporter did rather negatively affect product formation. The mechanism of import and export of hydrophobic substrates has not been fully clarified yet (Brennan et al., 2013). Therefore, it is not easy to predict how single genes involved in pleiotropic drug resistance influence transport of substrates and products. However, the mutant *snq2* did reach highest levels of trans-nootkatol of all strains tested. This effect was not seen for strains producing (+)-valencene intracellularly by co-expression of (+)-valencene synthase and t*HMG1* in the Δ*snq2* strain background (data not shown) suggesting the effect to be based on substrate accumulation in cells.

Knocking out genes responsible for ubiquitination even showed a tendency to negatively affect conversion, although expression levels of HPO and CPR were unchanged compared to the control strain. However, knocking out *PKA* genes has been shown to reduce ubiquitination and, therefore, degradation of heterologous membrane proteins (Wang et al., 2011), which is a possible explanation for better (+)-valencene conversion in the Δ*pka1* strain. Also, deletion of the master proteinase *pep4* is an established strategy to drastically reduce turnover of (recombinant) proteins (Murray et al., 2002; Rao et al., 2010; Teichert et al., 1989). In our case, this did not lead to significantly increased signals in Western blot analyses, but improved (+)-valencene conversion (Figure 3).

Changing ER membrane composition by knocking out genes involved in synthesis of diverse compounds did reveal better conversion for cells lacking *cho1*, a phosphatidylserine synthase (Letts et al., 1983). In case of the *fen2* background, a significantly decreased amount of the product trans-nootkatol was detected.

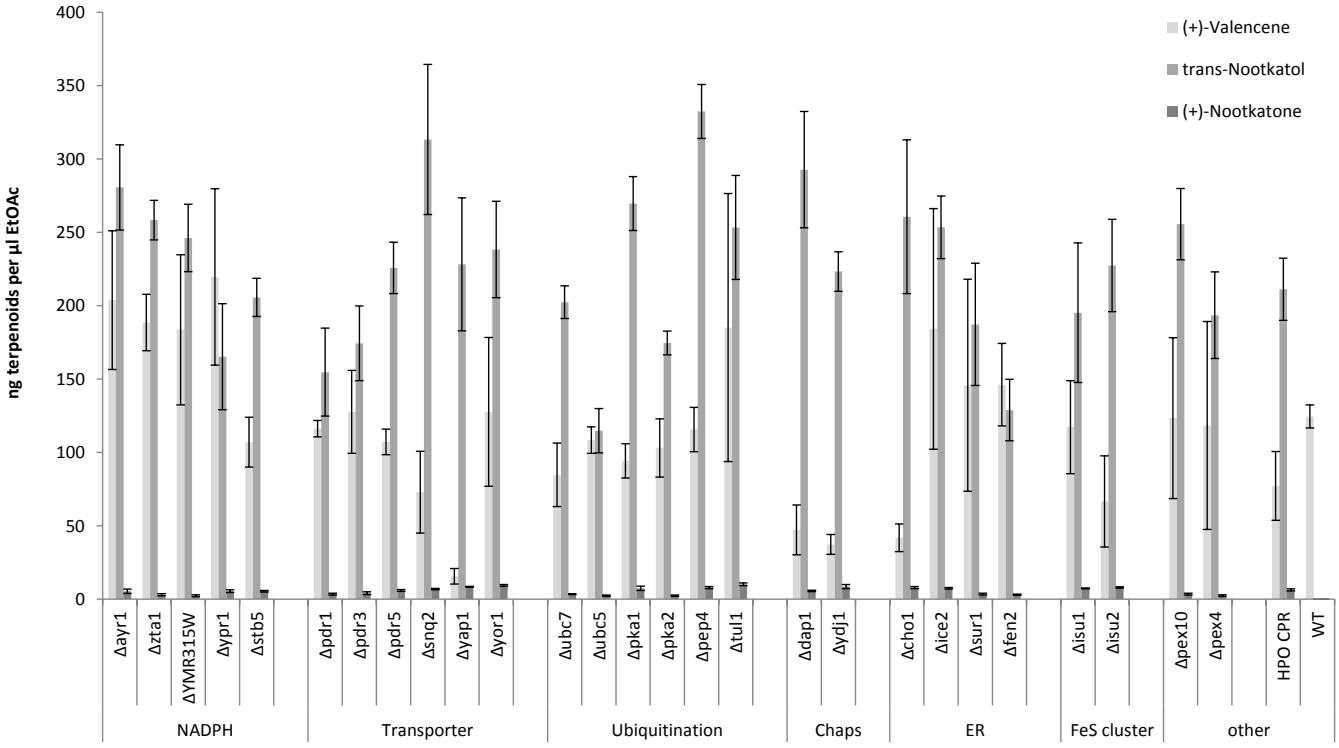


**Figure 4: Different EUROSCARF knockout strains transformed with the pYES2-$P_{Gal1}$-CPR-*myc* $P_{Gal1}$-HPO-FLAG co-expression plasmid and tested for (+)-valencene conversion. Resting cells assays were performed with a theoretically re-extractable amount (TRA) of 817 ng of (+)-valencene per µL of ethyl acetate. Genes that were knocked out are listed in the graph according to their cellular functionality. NADPH requirement: Δ*ayr1*, Δ*zta1*, ΔYMR315W, Δ*ypr1*, Δ*stb5*; ABC transporter proteins: Δ*pdr1*, Δ*pdr3*, Δ*pdr5*, Δ*snq2*, Δ*yap1*, *yor1*; ubiquitin E3 ligases, kinases and peptidases: Δ*ubc7*, Δ*ubc5*, Δ*pka1*, Δ*pka2*, Δ*pep4*, Δ*tul1*; ER chaperones: Δ*dap1*, Δ*ydj1*; proteins involved in the synthesis of ER compounds: Δ*cho1*, Δ*ice2*, Δ*sur1*, Δ*fen2*; sulfur-iron cluster enzymes: Δ*isu1*, Δ*isu2*; others: Δ*pex10*, Δ*pex4*; HPO CPR: positive control BY4742 pYES2-$P_{Gal1}$-CPR-*myc* $P_{Gal1}$-HPO-FLAG, WT: negative control BY4742**

## Discussion

Considering the fact, that the genome of *S. cerevisiae* consists of 5596 annotated genes whereof almost 5000 are non-essential (Otero et al., 2010), the idea that one single gene deletion might affect a whole cellular process seems rather unlikely. However, the screening of 30 different knockout mutants covering different cellular functions has shown to considerably influence CYP activity and expression. This finding suggests that testing more knockout mutants would be highly interesting. However, the development for a high throughput screening system is a prerequisite, as the currently established resting cells assay is highly elaborate and time-consuming. Only few examples exist for successful implementation of screening systems dealing with hydrophobic substrates. However, most of them are based on the existence of color development, like producing carotenoids (Dietrich et al., 2010; Kirby and Keasling, 2008). For example, the whole EUROSCARF knockout collection was screened by introducing genes encoding for carotenoid biosynthesis to find deletions improving isoprenoid production (Özaydın et al., 2013). Screening based on selection, i.e. leading to highest throughput, could not be realized for terpenoids so far. Additionally, drawbacks of colorless, volatile substances are given by their tedious handling and their need to be extracted from the aqueous phase prior to analysis.

However, the established assay worked with the accuracy required to find better converting strain backgrounds. We were able to identify interesting gene targets for further investigation and application for production of other terpenoids and conversion of other hydrophobic compounds. Still, many possibilities for optimization of CYP activity like combining different gene deletions or overexpression of different target genes, e.g. chaperones, exists. Finally, the results of the EUROSCARF strain collection screening drew our attention to the highly interesting Ice2p (see Chapter IV).

# References


Bauer, B.E., Wolfger, H., Kuchler, K, 1999. Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochimica et biophysica acta 1461, 217–36.

Botstein, D., and Davis, R.W., 1982. The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 607–636.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J, Hieter, P., Boeke, J.D., 1998. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast (Chichester, England) 14, 115–32.

Brennan, T.C.R., Krömer, J.O., Nielsen, L.K., 2013. Physiological and transcriptional responses of *Saccharomyces cerevisiae* to d-limonene show changes to the cell wall but not to the plasma membrane. Applied and environmental microbiology 79, 3590–600.

Denisov, Ilia G, Makris, T.M., Sligar, Stephen G, Schlichting, I., 2005. Structure and chemistry of cytochrome P450. Chemical reviews 105, 2253–77.

Dietrich, J.A., McKee, A.E., Keasling, J.D., 2010. High-throughput metabolic engineering: advances in small-molecule screening and selection. Annual review of biochemistry 79, 563–590.

Ernst, R., Klemm, R., Schmitt, L., Kuchler, Karl, 2005. Yeast ATP-binding cassette transporters: cellular cleaning pumps. Methods in enzymology 400, 460–84.

Gietz, R.D., Schiestl, R.H., 1995. Transforming yeast with DNA. Methods in Molecular and Cellular Biology 5, 255–269.

Hampton, R.Y., 2002. ER-associated degradation in protein quality control and cellular regulation. Current Opinion in Cell Biology 14, 476–482.

Kaspera, R., Krings, U., Nanzad, T., Berger, R.G., 2005. Bioconversion of (+)-valencene in submerged cultures of the ascomycete *Chaetomium globosum*. Applied Microbiology and Biotechnology 67, 477–83.

Kirby, J., Keasling, J.D., 2008. Metabolic engineering of microorganisms for isoprenoid production. Natural product reports 25, 656–661.

Kostova, Z., Wolf, D.H., 2003. For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. the The European Molecular Biology Organization Journal 22, 2309–2317.

Kwiatkowski, T.J., Zoghbi, H.Y., Ledbetter, S.A., Ellison, K.A., Chinault, A.C., 1990. Rapid identification of yeast artificial chromosome clones by matrix pooling and crude lysate PCR. Nucleic acids research 18, 7191–2.

Letts, V.A., Klig, L.S., Bae-Lee, M., Carman, G.M., Henry, S A, 1983. Isolation of the yeast structural gene for the membrane-associated enzyme phosphatidylserine synthase. Proceedings of the National Academy of Sciences of the United States of America 80, 7279–83.

Murray, B.P., Zgoda, V.G., Correia, M.A., 2002. Native CYP2C11: heterologous expression in *Saccharomyces cerevisiae* reveals a role for vacuolar proteases rather than the proteasome system in the degradation of this endoplasmic reticulum protein. Molecular pharmacology 61, 1146–53.

Nishida, N., Jing, D., Kuroda, K., Ueda, M., 2013a. Activation of signaling pathways related to cell wall integrity and multidrug resistance by organic solvent in *Saccharomyces cerevisiae*. Current genetics.

Nishida, N., Ozato, N., Matsui, K., Kuroda, K., Ueda, M., 2013b. ABC transporters and cell wall proteins involved in organic solvent tolerance in *Saccharomyces cerevisiae*. Journal of biotechnology 165, 145–52.

Otero, J.M., Vongsangnak, W., Asadollahi, M.A., Olivares-Hernandes, R., Maury, J., Farinelli, L., Barlocher, L., Osterås, M., Schalk, M., Clark, A., Nielsen, J., 2010. Whole genome sequencing of *Saccharomyces cerevisiae*: from genotype to phenotype for improved metabolic engineering applications. BMC genomics 11, 723.

Özaydın, B., Burd, H., Lee, T.S., Keasling, J.D., 2013. Carotenoid-based phenotypic screen of the yeast deletion collection reveals new genes with roles in isoprenoid production. Metabolic engineering 15, 174–183.

Piecuch, A., Obłąk, E., 2013. Yeast ABC proteins involved in multidrug resistance. Cellular & molecular biology letters.

Rao, K.N.P., Suman, S.K., Kiran, Y.R., Kuanr, A.R., Gupta, A.K., Bhalla, K., Kumar, V., Kundu, P., Arora, K., Soni, R., 2010. Co-expression of recombinant human CYP2C9 with human cytochrome P450 reductase in protease deficient *S. cerevisiae* strain at a higher scale yields an enzyme of higher specific activity. Drug metabolism letters 4, 246–53.

Riezman, H, Hase, T., Van Loon, A.P., Grivell, L.A., Suda, K., Schatz, G., 1983. Import of proteins into mitochondria: a 70 kilodalton outer membrane protein with a large carboxy-terminal deletion is still transported to the outer membrane. The EMBO journal 2, 2161–8.

Takahashi, S., Yeo, Y.-S., Zhao, Yuxin, O´Maille, P.E., Greenhagen, B.T., Noel, J.P., Coates, R.M., Chappell, J., 2007. Functional characterization of premnaspirodiene oxygenase, a cytochrome P450 catalyzing regio- and stereo-specific hydroxylations of diverse sesquiterpene substrates. The Journal of Biological Chemistry 282, 31744–31754.

Teichert, U., Mechler, B., Müller, H., Wolf, D H, 1989. Lysosomal (vacuolar) proteinases of yeast are essential catalysts for protein degradation, differentiation, and cell survival. The Journal of biological chemistry 264, 16037–16045.

Wang, Y.Q., Guan, S., Acharya, P., Koop, D.R., Liu, Y., Liao, M., Burlingame, A.L., Correia, M.A., 2011. Ubiquitin-dependent proteasomal degradation of human liver cytochrome P450 2E1: identification of sites targeted for phosphorylation and ubiquitination. The Journal of biological chemistry 286, 9443–56.

Wang, Y.Q., Guan, S., Acharya, P., Liu, Y., Thirumaran, R.K., Brandman, R., Schuetz, E.G., Burlingame, A.L., Correia, M.A., 2012. Multisite phosphorylation of human liver cytochrome P450 3A4 enhances Its gp78- and CHIP-mediated ubiquitination: a pivotal role of its Ser-478 residue in the gp78-catalyzed reaction. Molecular & cellular proteomics: MCP 11, M111.010132.

Wang, Y.Q., Liao, M., Hoe, N., Acharya, P., Deng, C., Krutchinsky, A.N., Correia, M.A., 2009. A role for protein phosphorylation in cytochrome P450 3A4 ubiquitin-dependent proteasomal degradation. The Journal of biological chemistry 284, 5671–84.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., et al., 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science (New York, N.Y.) 285, 901–6.

# Chapter IV

# Over-expression of *ICE2* improves biohydroxylation of (+)-valencene and stabilizes cytochrome P450 reductase levels in *Saccharomyces cerevisiae* and *Pichia pastoris*

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## Abstract

Membrane-attached cytochrome P450 enzymes (CYPs) are a versatile and interesting class of enzymes for industrial applications as they are capable of regio- and stereoselectively hydroxylating hydrophobic molecules. However, CYPs require the balanced co-expression of suitable cytochrome P450 reductases for regeneration of their catalytic capacity. We have employed the premnaspirodiene oxygenase from *Hyoscyamus muticus* (HPO) and the cytochrome P450 reductase from *Arabidopsis thaliana* (CPR) for the specific hydroxylation of the orange flavor (+)-valencene to form trans-nootkatol in *Saccharomyces cerevisiae*. Searching for effector proteins of HPO/CPR function we transformed and screened selected strains from a *S. cerevisiae* knockout collection for HPO/CPR levels and activity. Thereby, we realized that in yeast cells lacking the type III membrane protein Ice2p, CPR was destabilized as compared to the control strains. Over-expression of *ICE2* improved (+)-valencene conversion in resting cells assays 1.4-fold. Upon co-overexpression of (+)-valencene synthase from *Callitropsis nootkatensis* and truncated endogenous *HMG1* beside HPO/CPR, *S. cerevisiae* produced 20 mg of trans-nootkatol per liter of cell culture *de novo*. Over-expression of *ICE2* in this whole-cell biocatalyst enhanced trans-nootkatol formation by 40%. Time-resolved Western blot analysis and cytochrome c reductase activity assays revealed that *ICE2* up-regulation stabilized CPR levels over 48-72 h of bioconversion, thus most likely contributing to enhanced trans-nootkatol formation. The beneficial effect of *ICE2* overexpression was confirmed for other CYP/CPR combinations and the alternative expression host *P. pastoris*.

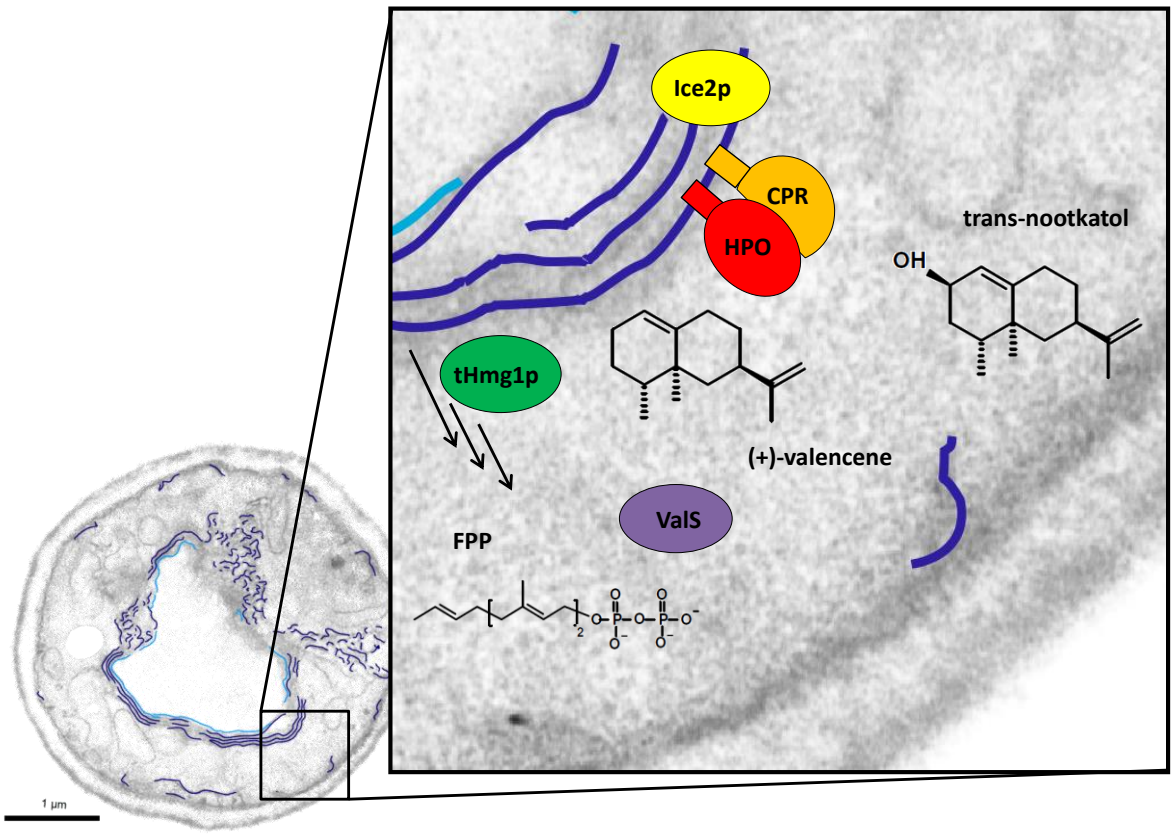
## Highlights

- Biosynthesis of trans-nootkatol in metabolically engineered *S. cerevisiae* cells
- *ICE2* overexpression stabilized cytochrome P450 reductase (CPR) during bioconversion
- Beneficial effect of Ice2p confirmed for alternative CPRs and *P. pastoris*

## Keywords

Yeast, cytochrome P450, cytochrome P450 reductase, whole-cell biotransformation, terpenoid, *ICE2*

## Graphical Abstract



# 1 Introduction[1]

To circumvent phase transfer issues, an efficient *S. cerevisiae* strain capable of reaching relevant intracellular titers of (+)-valencene was constructed. First, (+)-valencene synthase (ValS), a soluble enzyme originating from Alaska cedar heartwood (*Callitropsis nootkatensis*) was introduced into yeast (Beekwilder et al., 2014). ValS forms (+)-valencene from farnesyl pyrophosphate (FPP), a product of the mevalonate pathway. Extensive withdrawal of the essential FPP building block can lead to impaired cell growth, because under these conditions cells may become auxotrophic for ergosterol (Chambon et al., 1991). In order to increase the FPP pool in *S. cerevisiae*, a truncated version of *HMG1* was overexpressed. In numerous related projects, over-expression of t*HMG1* has successfully enhanced production of terpenoids (Asadollahi et al., 2008; Farhi et al., 2011; Ro et al., 2006; Scalcinati et al., 2012). Expression and activity of Hmg1p is tightly regulated including feedback- and cross-regulations. A highly conserved part of the protein, the membrane anchoring domain - also referred to as sterol sensing domain (SSD) - was shown to be responsible for regulation phenomena (Brown and Goldstein, 1980; Hampton and Rine, 1994; Hampton et al., 1996). Truncation of the SSD and over-expression of t*HMG1* leads to a constitutively active, non-membrane bound enzyme provoking cellular accumulation of squalene, the coupling-product of two FPP molecules (Donald et al., 1997; Polakowski et al., 1998). An engineered strain over-expressing ValS and t*HMG1* from genomically integrated cassettes and HPO/CPR from a multicopy vector was evaluated through cultivation in biphasic systems using n-dodecane for trapping the synthesized terpenoids (Asadollahi et al., 2008; Girhard et al., 2009; Scalcinati et al., 2012). This elegant method prevents toxicity or inhibitory effects of high concentrations of terpenoids on yeasts providing an on-line extraction step. High concentrations of terpenoids have recently been described to hamper (+)-valencene biohydroxylation in *S. cerevisiae* (Gavira et al., 2013). Very much like in the resting cells assays, co-expression of *ICE2* resulted in 50% increased formation of trans-nootkatol yielding 30 mg $L^{-1}$ of culture. Ice2p obviously stabilized CPR over the period of bioconversion. However, the molecular details thereof remain to be unveiled. To test for general applicability of *ICE2* overexpression in stabilizing CYP/CPR activities for biocatalytic application, we have extended our analysis to alternative CYP/CPR combinations and *P. pastoris* as expression host and have detected

[1] **Abbreviations:** HPO, *Hyoscymaus muticus* premnsapirodiene oxygenase; CPR, cytochrome P450 reductase; t*HMG1*, truncated 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase 1; ValS, (+)-valencene synthase; CYP, cytochrome P450 enzyme; FPP, farnesyl pyrophosphate; ER, endoplasmic reticulum; PM17, *Piperita mentha* (-)-limonene-3-hydroxylase; hCPR, human cytochrome P450 reductase; CYP2D6, human cytochrome P450 2D6; *Pp*, *Pichia pastoris*

CPR stabilization throughout. Heterologous expression of membrane-anchored cytochrome P450 enzymes (CYPs) in microbial hosts for application in biocatalytic processes is challenging. CYP activity is irrevocably linked to a finely balanced system of NADPH cofactor recycling, to oxygen supply, to correct integration of heme iron into the active site of the CYP and, of course, to perfect interaction with their corresponding cytochrome P450 reductase (CPR) that functions as electron donor (Gu et al., 2003; Henderson et al., 2003; Omura, 2010). Most of ER-linked CYPs convert poorly water-soluble substrates and, therefore, are needed for functionalization and/or detoxification of hydrophobic compounds (Danielson, 2002). In this study, we have employed the cytochrome P450 enzyme premnaspirodiene oxygenase CYP71D55 from *Hyoscyamus muticus* (HPO) and cytochrome P450 reductase from *Arabidopsis thaliana* (CPR). Two amino acid exchanges, V482I and A484I, shifting the substrate preference of HPO from premnaspirodiene to (+)-valencene (Takahashi et al., 2007) were introduced into HPO. (+)-Valencene is the most abundant aroma component of oranges, a side product of orange juice production and, thus, of relatively low market value. HPO/CPR hydroxylate the C2-atom of (+)-valencene forming trans-nootkatol. Through further oxidation caused by intrinsic *S. cerevisiae* activity, small amounts of the high-value organoleptic product (+)-nootkatone can be obtained (Gavira et al., 2013). The microbial synthesis of terpenoidic flavors and fragrances has become attractive because their extraction from natural resources is hampered by low terpenoid concentrations, slow plant growth, seasonal variations and tedious purification processes. For lack of alternatives, terpenoids were lately mainly produced via chemical synthesis that frequently involved toxic heavy metals, highly flammable compounds or strong oxidants (Majetich and Behnke M., 1985; Salvador and Clark, 2002). Therefore, more attention is being paid to the development of safe methods and innovative biotechnology solutions reducing the ecological footprint (Chang and Keasling, 2006; Fraatz et al., 2009; Kirby and Keasling, 2009). Searching for protein effectors of HPO/CPR function, we have transformed and screened *S. cerevisiae* single-gene knockout mutants from the EUROSCARF collection (Brachmann et al., 1998; Winzeler et al., 1999) for HPO/CPR activity and protein levels. The most interesting finding was that CPR was prone to degradation in the Δ*ice2* knockout strain. Over-expressing Ice2p increased HPO/CPR-mediated bioconversion of (+)-valencene up to 1.4-fold in resting cells assays. Ice2p is a type III membrane protein with eight predicted transmembrane domains and an essential role in ER distribution, localization and inheritance in budding yeast (Estrada De Martin et al., 2005; Tavassoli et al., 2013). The Δ*ice2* knockout strain was shown to be devoid of peripheral ER membranes close to the plasma membrane (Estrada De Martin et al., 2005).Moreover, Ice2p influenced intracellular $Zn^{2+}$ homeostasis possibly functioning as $Zn^{2+}$ transporter (North et al., 2012).

# 2 Materials and Methods

## 2.1. Chemicals

Unless stated otherwise, standard laboratory reagents were obtained from Sigma-Aldrich® (Steinheim, Germany) or Carl Roth GmbH & Co. KG (Karlsruhe, Germany) with the highest purity available. The pYES2 expression vector was purchased from Invitrogen (Carlsbad, USA). The pESC-URA expression vector was obtained from Agilent Technologies (Santa Clara, USA). Restriction enzymes were acquired from Thermo Scientific (St. Leon-Rot, Germany). Difco™ yeast nitrogen base w/o amino acids (YNB), Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson and Company (Schwechat, Austria). Geneticin sulfate (G418), Hygromycin B and Kanamycin monosulfate were ordered from Formedium™ (Norfolk, United Kingdom). Zeocin™ was purchased from InvivoGen (Eubio, Vienna, Austria). Sterile water was purchased from Fresenius Kabi (Graz, Austria). Terpenoid standards were supplied by DSM Innovative Synthesis B.V. (Geleen, The Netherlands).

## 2.2. Microorganisms, Plasmids and Media

*For all cloning steps and plasmid replication, E. coli* Top10 F' (F'[lacI$^q$ Tn10(tet$^R$)] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str$^R$) endA1 λ$^-$) from Laife technologies (Vienna, Austria) was used. *S. cerevisiae* strains were constructed either in the BY4742 (*MAT*α, *his3-1*, *leu2-0*, *lys2-0*, *ura3-0*) or in the W303 (*MAT*a and *MAT*α, *ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3-1, GALs*$^+$) background. BY4742 and single gene knockout strains thereof were obtained from the EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/).

*S. cerevisiae* strains were cultivated in synthetic defined media (6.7 g yeast nitrogen base w/o amino acids; 1 g drop-out powder consisting of equal amounts of adenine, lysine, tyrosine, histidine, leucine and tryptophane; 2% glucose). Synthetic defined induction media containing 2% galactose and 0.7% raffinose instead of 2% glucose was used for induction.

*P. pastoris* cultures were either grown in YPD (1 % yeast extract, 2 % peptone and 2 % glucose) or buffered complex glycerol medium, BMGY (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 4 × 10$^{-5}$ % biotin, 1 % glycerol). BMMY (1 % yeast extract, 2 %

peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 4 × $10^{-5}$ % biotin, 1 % methanol) was used as induction medium.

## *2.3. Cloning of expression vectors and yeast strain generation*

A list of strains generated during this study is given in Table 1. Standard molecular cloning technologies were used in strain generation (Ausubel et al., 2003). For gene amplification, Phusion® High Fidelity DNA polymerase (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany) was utilized in accordance with the recommended PCR protocol. Codon optimized gene variants of HPO (*H. muticus* premnaspirodiene oxygenase), CPR (*A. thaliana* cytochrome P450 reductase), and ValS (*C. nootkatensis* terpene synthase), were designed manually and purchased from GeneArt® (Wriessnegger et al., manuscript in revision).

*Saccharomyces cerevisiae*

For generation of the pYES2 based co-expression construct, the *CPR* gene was amplified using primers Fw_CPR_*Hind*III and Rv_CPR-myc_*Bam*HI (Table 2) for *Hind*III/*Bam*HI cloning into pYES2. The resulting plasmid was modified by introducing the restriction sites for *Bgl*II and *Asc*I using primers pYES2_*Asc*I_fw and pYES2_*Bgl*II_rev, and was re-circularized by ligation with the HPO-Flag expression cassette. The latter was constructed by *Hind*III/*Bam*HI cloning the HPO-Flag fragment amplified with Fw_HPOSc_*Hind*III and Rv_HPO-Flag_*Sc* into pYES2 and, subsequently, by amplifying the whole expression cassette using primers *Bgl*II_Gal1_fw and *Asc*I_CYC1_rev. Knock-out of *ICE2* was achieved through integration of a kanMX cassette amplified with F1(*ICE2*) and R1(*ICE2*) primers from pFA6a-KanMX (Longtine et al., 1998). For expression of chromosomally His-tagged Ice2p, *ICE2* (GenBank number: NM_001179438.1) was amplified from genomic DNA of *S. cerevisiae* W303 using primers Fw(*ICE2*_*Xho*I) and Rv(*ICE2*-6His_*Xba*I) and was *Xho*I/*Xba*I cloned into pYES2. The *ICE2* gene placed under the control of the $P_{Gal1}$ promoter and CYC1 termination sequence was amplified using *Bgl*II_Gal1_fw and *Asc*I_CYC1_rev primers and was inserted into pFA6a-*TRP1* (Longtine et al., 1998) via *Bgl*II/*Asc*I restriction sites. This construct served as template to generate cassettes for *ICE2*-$His_6$ expression from the endogenous promoter using primers F1(*ICE2*start) and R1(*ICE2*), or for over-expression from the $P_{Gal1}$ promoter using primers F1(*ICE2*) and R1(*ICE2*). Both expression cassettes were integrated into the genomic *ICE2* locus. The codon-optimized (+)-valencene synthase gene was subcloned from the Geneart® delivery vector into pYES2 via *EcoR*I and *Bam*HI restriction sites. The ValS integration cassette was amplified with *Bgl*II_Gal1_fw and *Asc*I_CYC1_rev primers and *Bgl*II/*Asc*I

cloned into the vector pFA6a-*HIS3kanMX6* (Longtine et al., 1998). Then, the ValS expression cassette was amplified using primers F1(trp1) and R1(trp1), and was transformed into yeast. For over-expression of truncated *HMG*1 from the $P_{PGK1}$ promoter, the *PGK1* promoter region was amplified from genomic DNA of *S. cerevisiae* W303 using Fw($P_{PGK1}$_*Xma*I) and Rv($P_{PGK1}$_*BamH*I) primers to be inserted into pFL36-*LEU2* (Bonneaud et al., 1991) via *Xma*I and *Bam*HI restriction sites. Truncated *HMG1* (GenBank number: NM_001182434.1) devoid of its sterol sensing domain was amplified from genomic DNA of *S. cerevisiae* with primers Fw(t*HMG1_Pst*I) and Rv(t*HMG1_Hin*dIII) and was cloned into pFL36-*LEU2*-$P_{PGK1}$ via *Pst*I and *Hind*III sites. Primers Fw(ex_*leu2*_t*HMG1*) and Rv(ex_leu2_t*HMG*1) were applied to amplify the t*HMG1* expression cassette including the $P_{PGK1}$ promoter and *LEU2* selection marker for integration into the W303 *leu2* locus. Transformations of plasmids as well as DNA cassettes was done following the lithium acetate method (Gietz and Schiestl, 1995). Transformants were selected either on synthetic defined plates lacking histidine, uracil or leucine or on YPD plates containing 300 mg $L^{-1}$ geneticin (Botstein and Davis, 1982). Correct integration of cassettes into the specific loci was routinely confirmed by colony PCR (Kwiatkowski et al., 1990). For generating triple knockin/knockout mutants, strain W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{GAL1}$-ValS was mated with single mutants W303 *MAT*α *Δice2*::KanMX and W303 *MAT*α $P_{GAL1}$-*ICE2*-6His to be sporulated and dissected for single spores harboring all three genes (Amberg et al., 2006).

Codon optimized gene variants of the following genes were designed manually by applying the *P. pastoris* codon usage: limonene-3-hydroxylase (PM17 isoform, CYP71D13, GenBank number of native gene: AF124816), human cytochrome P450 2D6 (GenBank number of native gene: NM_000106) and human cytochrome P450 reductase (GenBank number of native gene: NM_000941) (Table S1).

Optimized hCPR was cloned into pESC-URA with *Eco*RI and *Not*I by cutting the synthetic gene directly out of the delivered vector. Afterwards, CYP2D6 was cloned into pESC-URA-hCPR with *Hind*III and *BamH*I. To construct the co-expression vector for PM17 and CPR, CPR was amplified with Fw(CPR_*Not*I) and Rv(CPR*myc_Bgl*II) and cloned into pESC-URA. PM17 was cut out with *BamH*I and *Hind*III from the synthetic vector to be ligated with pESC-URA-CPR.

*Pichia pastoris*

The *P. pastoris* strain CBS7435 *his4* (Näätsaari et al., 2012) was used as host strain for the construction of strains *Pp*PM17/CPR and *Pp*2D6/hCPR, respectively. Generation of strain *Pp*HCV was recently described in detail by Wriessnegger et al. (manuscript in revision). Codon optimized PM17, CYP2D6, CPR and hCPR encoding genes with desired *Eco*RI/*Not*I restriction sites for cloning, and C-terminal FLAG- and *myc*-tags on the CYP450s and *CPR*s, respectively, were purchased from GeneArt® (Supplemental Table S1). For creation of the *P. pastoris PM17*/*AtCPR* co-expression vector, the *AtCPR*

and the *PM17* genes were subcloned into the *EcoR*I and *Not*I digested expression vector p*Pp*B1 containing a synthetic variant of the *AOX1* promoter and a Zeocin™ resistance marker cassette for selection. The generated p*Pp*B1[*CPR*] vector was cut with *Bgl*II and *Bam*HI to obtain the *AtCPR* gene flanked by *AOX1* promoter and terminator regions. The purified fragment was ligated into the *Bam*HI digested vector p*Pp*B1[*PM17*] to obtain the p*Pp*B1[*PM17*/*AtCPR*] co-expression vector. The same strategy was applied for the generation of the p*Pp*B1[*2D6*/*hCPR*] co-expression vector. Expression vectors were checked by sequencing the expression cassette and were linearized with *Bgl*II for integration into the genome of *P. pastoris.*

*PpICE2* (PAS_chr2-2_0195) was identified by blasting the *ScICE2* protein sequence against the *P. pastoris* GS115 genome database. *PpICE2* was amplified from genomic DNA of *P. pastoris* CBS7435 using primer pairs Fw*Pp*ICE2 and Rv*Pp*ICE2 containing restriction sites *EcoR*I and *Not*I, respectively, for cloning into the p*Pp*Kan expression vector harboring the *AOX1* promoter and the kanamycin/geneticin selection cassette (Table 2). After linearization of the expression vector p*Pp*Kan[*Pp*ICE2] with *Bgl*II, for transformation into the genome of CYP/CPR co-expressing *P. pastoris* strains *Pp*HCV, *Pp*PM17/*At*CPR and *Pp*2D6/hCPR, respectively. Routinely, competent *P. pastoris* cells were transformed with ~2 µg of linearized plasmids according to the protocol of Lin-Cereghino (Cereghino and Cregg, 2000). After transformation, aliquots were plated on YPD plates containing 100 mg/L Zeocin™ or 400 mg/L geneticin.

**Table 1: Strains used in this study**

| Strain ID | Genotypes of strains (harboring plasmids) | Source |
|---|---|---|
| BY4742 pYES2 (empty vector control, evc) | *MAT*α, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0* (pYES2) | This study |
| BY4742 pYES2-HPO-CPR | BY4742 (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| BY4742 Δ*ice2* pYES2-HPO-CPR | BY4742 *ice2*::KanMX (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| BY4742 Δ*ayr1* pYES2-HPO-CPR | BY4742 *ayr1*::KanMX (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| BY4742 Δ*yor1* pYES2-HPO-CPR | BY4742 *yor1*::KanMX (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| W303 pYES2 (evc) | *MAT*a or *MAT*α, *ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3-1, GALs*$^{+}$ *(pYES2)* | This study |
| W303 Δ*ice2* pYES2-HPO-CPR | W303 *MAT*α Δ*ice2*::KanMX (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| W303 Δ*ice2* (evc) | W303 *MAT*α Δ*ice2*::KanMX (pYES2) | This study |
| W303 $P_{GAL1}$-*ICE2* pYES2-HPO-CPR | W303 *MAT*α $P_{GAL1}$-*ICE2-His*$_6$ (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| W303 $P_{GAL1}$-*ICE2* (evc) | W303 *MAT*α $P_{GAL1}$-*ICE2-His*$_6$ (pYES2) | This study |
| W303 t*HMG1* ValS (evc) | W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{GAL1}$-ValS (pYES2) | This study |
| W303 t*HMG1* ValS pYES2-HPO-CPR | W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{GAL1}$-ValS (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| W303 t*HMG1* ValS Δ*ice2* pYES2-HPO-CPR | W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{GAL1}$-ValS Δ*ice2* (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| W303 t*HMG1* ValS $P_{GAL1}$-*ICE2*-His$_6$ pYES2-HPO-CPR | W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{GAL1}$-ValS $P_{GAL1}$-*ICE2-His*$_6$ (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| W303 t*HMG1* ValS $P_{GAL1}$-*ICE2*-His$_6$ (evc) | W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{GAL1}$-ValS $P_{GAL1}$-*ICE2-His*$_6$ (pYES2) | This study |
| W303 t*HMG1* ValS $P_{ICE2}$-*ICE2*-His$_6$ pYES2-HPO-CPR | W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{ICE2}$-ValS $P_{GAL1}$-*ICE2-His*$_6$ (pYES2 $P_{GAL1}$-HPO-Flag $P_{GAL1}$-CPR-*myc*) | This study |
| W303 t*HMG1* ValS $P_{ICE2}$-*ICE2*-His$_6$ (evc) | W303 *MAT*a $P_{PGK1}$-t*HMG1* $P_{ICE2}$-ValS $P_{GAL1}$-*ICE2-His*$_6$ (pYES2) | |
| *Pp*CBS7435 (wild type) | CBS7435 *his4* | (Näätsaari et al., 2012) |
| *Pp*[HPO/CPR]ValS (HCV) | CBS7435 *his4 ku70*, p*Pp*HIS4[HPO/CPR], p*Pp*B1-Zeocin[ValS] | Wriessnegger et al., manuscript in revision |
| *Pp*[HPO/CPR]ValS/*PpICE2* (HCV/*Pp*ICE2) | CBS7435 *his4 ku70*, p*Pp*HIS4[HPO/CPR], p*Pp*B1-Zeocin[ValS], pPpKan[*PpICE2*] | This study |
| *Pp*[PM17/*At*CPR] | CBS7435 *his4,* p*Pp*B1[PM17/*At*CPR] | This study |
| *Pp*[PM17/*At*CPR]*PpICE2* | CBS7435 *his4,* p*Pp*B1[PM17/*At*CPR], pPpKan[*PpICE2*] | This study |
| *Pp*[2D6/hCPR] | CBS7435 *his4,* p*Pp*B1[2D6/hCPR] | This study |
| *Pp*[2D6/hCPR]*PpICE2* | CBS7435 *his4,* p*Pp*B1[2D6/hCPR], pPpKan[*PpICE2*] | This study |

**Table 2: Primers used in this study (restriction sites underlined)**

| Primer name | Primer sequence |
|---|---|
| **Fw_CPR_*Hind*III** | CCCAAGCTTCGAAACGCATATGACTTCTG |
| **Rv_CPR-myc_*BamH*I** | CGCGGATCCTTATTACAAATCCTCTTCAGAAATCAATTTTTGTTCCCAGACATCTCTCAAGTATCTACC |
| **pYES2_*Asc*I_fw** | TTGGCGCGCCTTAATTAAACGGATTAGAAGCCGCCGAG |
| **pYES2_ *Bgl*II _rev** | TTAGATCTGGCGCGCCCGATTCATTAATGCAGGGCC |
| **Fw_HPOSc_*Hind*III** | CCCAAGCTTCGAAACGCATATGCAATTCT |
| **Rv_HPO-Flag_Sc** | CGCGGATCCCTCGAGTTATTACTTATCGTCGTCATCCTTGTAATCCTCTCGGGAAGGTTGGTAA |
| ***Bgl*II_Gal1_fw** | TTGGCGCGCCTTAATTAAACGGATTAGAAGCCGCCGAG |
| ***Asc*I_CYC1_rev** | TTAGATCTGGCGCGCCCGATTCATTAATGCAGGGCC |
| **F1(trp1)** | GTGAGTATACGTGATTAAGCACACAAAGGCAGCTTGGAGTCGGATCCCCGGGTTAATTAA |
| **R1(trp1)** | TGCACAAACAATACTTAAATAAATACTACTCAGTAATAACGAATTCGAGCTCGTTTAAAC |
| **Fw($P_{PGK1}$_*Xma*I)** | GTACCCGGGGATTATTTTAGATTCCTGACTTC |
| **Rv($P_{PGK1}$_*BamH*I)** | TATGGATCCTCTTGTTTTTATATTTGTTGTAAA |
| **Fw(t*HMG1_Pst*I)** | GACCTGCAGGCACCCTGCAGACCAATTGGTGAAAACTG |
| **Rv(t*HMG1_ Hin*dIII)** | TGCAAGCTTGGCCTAACACATGGTGCTGTTGTGCTT |
| **Fw(ex_*leu2*_t*HMG1*)** | AGCAATATATATATATATATTTCAAGGATATACCATTCTATGTAAAACGACGGCCAGT |
| **Rv(ex_leu2_t*HMG*1)** | TAAAGTTTATGTACAAATATCATAAAAAAAGAGAATCTTTCCGATTCATTAATGCAGC |
| **F4(*ICE2*)** | CGTAAAGTGTTGGTGGATCTTATAGTATTCGTGAAGAATTCGAGCTCGTTTAAAC |
| **R2(*ICE2*)** | CTGCATGAAGCTTTTGGACAAACTGGTCATTTTGAGATCCGGGTTTT |
| **F1(*ICE2*)** | GTGGCCGATCACGCTAAAGATTAGGCAACGCGGATCCCCGGGTTAATTAA |
| **R1(*ICE2*)** | GTATTTCACCTTCCTTTTTGTCTTCGCGTATTTGGCAAAGGAATTCGAGCTCGTTTAAAC |
| **F1(*ICE2*start)** | AGAGAGGTGCTGTTTGTGGCCGATCACGCTAAAGATTAGGCAACGATGACCAGTTTGTCCAAAAG |
| **Fw(CDC73_qRT)** | GAAAGGCGAGACATCCGATA |
| **Rv(CDC73_qRT)** | TTGTTTCCACCACAACTGGA |
| **Fw(HPO_qRT)** | ACATTGCGTTTTGCCCTTAC |
| **Rv(HPO_qRT)** | GCAACACTTCATCGCGTCTA |
| **Fw(CPR_qRT)** | GGTTGCTGGTTTCGTTGTCT |
| **Rv(CPR_qRT)** | ACCCAAGTCCAAGTCGTCAT |
| **Fw(*ICE2*_qRT)** | CGTCTGGCAGAAACATCAAA |
| **Rv(*ICE2*_qRT)** | AAGGACCCCCATAACACCTC |
| **Fw(CPR_*Not*I)** | CCTCACTAAAGGGCGGCCGCAACAAAATGACTTCTGCTTTGTACGC |
| **Rv(CPRmyc_*Bgl*II)** | TTAATTAAGAGCTCAGATCTTTATTACCAGACATCTCTCA |
| **FwIce2Pp** | CGGAATTCCGAAACGATGCCCAAGATACGCTCC |
| **RvIce2Pp** | ATAAGAATGCGGCCGCTTAGTGATGGTGATGGTGGTGTGTCTCCCAATTACTAGTCAAATTATC |

## *2.4. Expression of recombinant proteins*

For expression of recombinant proteins in *S. cerevisiae*, 300 mL baffled shake flasks containing 50 mL of synthetic defined growth media (6.7 g yeast nitrogen base w/o amino acids; 1 g drop-out powder consisting of equal amounts of adenine, lysine, tyrosine, histidine, leucine and tryptophane; 2% glucose) were inoculated to an $OD_{600}$ of 0.1. Cell suspensions were shaken for 48 h at 130 rpm and 30°C. After centrifugation for 5 min at 1,062 x g, cell pellets were resuspended in 50 mL of synthetic defined induction media containing 2% galactose and 0.7% raffinose instead of 2% glucose. Induction was carried out for 6 h at 130 rpm and 30 °C.

Randomly chosen *P. pastoris* transformants were screened in 96-DWPs as previously described (Weis et al., 2004). In brief, cells were cultivated in 250 µL of BMGY medium for 24 h at 28°C, 320 rpm and 80 % humidity. Induction was started by addition of 250 µL of BMMY (2 % methanol). Methanol was added every 12 h to a final concentration of 1 % until 48 h of induction. Cultivated transformants from DWPs were pinned onto plates containing up to 2 mg $mL^{-1}$ Zeocin™ for screening of potential

multi-copy gene integration events of CYP/CPR. Transformants growing at high Zeocin concentration were picked for further Western Blot analyses for detection of Flag-tagged CYP and *myc*-tagged CPR proteins. Overexpression of *PpICE2* was determined by Western Blot analysis using an antibody against the C-terminal 6xHis-tag ($His_6$).

## *2.5. SDS-PAGE/Western blotting (Fiji quantification)*

Five $OD_{600}$ units of induced *S. cerevisiae* or *P. pastoris* cells were harvested and prepared for SDS-PAGE according to (Riezman et al., 1983). Ten µL of the resulting supernatants were separated under reducing conditions on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen), and were then transferred onto nitrocellulose membrane (GE Healthcare, Chalfont St Giles, UK) in a wet blotting system. Protein loading was assessed by PonceauS staining of the membrane. Immunodetection was performed with commercially available rabbit anti-Flag, anti-*myc* or anti-polyHIS primary antibodies (Thermo Scientific, St. Leon-Rot, Germany) , and with anti-rabbit (for *myc*) or anti-mouse (for Flag and HIS) secondary antibodies linked to horse radish peroxidase purchased from Sigma-Aldrich® (Steinheim, Germany). Western blots were developed by Super Signal West Pico chemiluminescent substrate (Thermo Scientific, St. Leon-Rot, Germany) and protein bands were detected using a G:BOX Chemi HR16 bioimaging system (Syngene, Cambridge, UK). Quantification of Western blot signal intensities was done with Fiji as biological image analysis platform (Schindelin et al., 2012). Samples were loaded in triplicates and average signal intensities of HPO and CPR of the control strain were set to be 100%. The other signal intensities were normalized to those of the control strain.

## *2.6. Quantitative RT-PCR*

Three-hundred µL of galacatose-induced cell culture were harvested in a table top centrifuge and resuspended in RNAlater® in amounts recommended by the supplier (Life technologies, Vienna, Austria). Purified RNA extracts were prepared using ZR Fungal/Bacterial RNA MiniPrep™ kit (ZymoResearch, Germany, Freiburg). RNA quality and concentrations were determined via NanoDrop ND2000 (Thermo Scientific, St. Leon-Rot, Germany) and ethidium bromide gel electrophoresis. qRT-PCR was performed in a 2-step procedure. The Revert Aid Premium First Strand cDNA Synthesis Kit from Thermo Scientific (St. Leon-Rot, Germany) was applied on 600 ng of total RNA as described in the manual for reverse transcription (RT). Reaction mixtures were incubated at 25 °C for 10 min, at

50 °C for 15 min and RT was terminated by heating at 85 °C for 5 min. For the quantitative PCR step, Maxima SYBR Green PCR MM (2x) from Thermo Scientific (St. Leon-Rot, Germany) was used. Then, 2.5 µL of cDNA template solution were mixed with 12.5 µL of Maxima SYBR Green qPCR Mix (2x, ROX added) followed by the addition of 1 µL of respective forward and reverse primers (qRT-labeled primers; Table 2). Primers had been constructed with the help of the Primer3 program comprising the recommended guidelines (frodo.wi.mit.edu) (Koressaar and Remm, 2007; Untergasser et al., 2012). The reaction mixtures were filled up with nuclease free water to a final volume of 25 µL. A no-template control and a Reverse Transcriptase Minus (RT-) control were performed and tested negative for impurities or contaminant DNA. Following the two-step cycling protocol, mixtures were initially denatured at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/elongation at 60 °C for 60 s. Results were evaluated based on the $\Delta C_t$ method via normalization of the observed values onto the house-keeping gene *CDC73* (Reed et al., 1988).

## *2.7. Resting cells assay for (+)-valencene, (-)-limonene and bufuralol conversion*

*(+)-Valencene and (-)-limonene hydroxylation assay*

For bioconversions with *S. cerevisiae*, six-hundred $OD_{600}$ units of galactose-induced cells were harvested and resuspended in 2 mL of 50 mM $KP_i$ buffer, pH 7.4. Cell suspensions were split into two equal aliquots in Pyrex tubes and 20 µL of 100 mM (+)-valencene or 300 mM (-)-limonene in DMSO as well as 1% of Triton® X-100 (Amresco, Solon, Ohio) were added, respectively. Pyrex tubes were only sealed loosely with screw caps to strike a balance between air supply for cells and substrate volatilization. Conversions were carried out for 16 h at 170 rpm and 30 °C. Terpenoids were extracted with 500 µL of ethyl acetate using a VXR basic Vibrax® (IKA®, Staufen, Germany) at room temperature and maximum speed for 30 min. After phase separation by centrifugation for 15 min at 2,720 x g, organic layers were verified via GC-MS and quantified via GC-FID.

Pre-selected *P. pastoris* transformants co-expressing PM17/CPR/*Pp*Ice2 were cultivated in shake flasks as described, but without addition of n-dodecane. After 48 h of induction, $OD_{600}$ of the cell cultures was determined and culture volumes corresponding to 300 $OD_{600}$ units were transferred to sterile PYREX® tubes. The cells were pelleted at 3220 x g for 5 min in an Eppendorf 5810R centrifuge and the supernatants were discarded. Limonene substrate solution (300 mM (-)-limonene in DMSO, 1% Triton X-100) was added to the cell suspension to a final concentration of 6 mM. The reaction was

carried out at 28 °C for 24 h at 170 rpm. Monoterpenoids were extracted with 500 µl ethyl acetate to be analyzed by GC-FID.

*Bufuralol 1'-hydroxylation assay*

For *S. cerevisiae*, bufuralol conversion assays were conducted exactly as described by Geier et al. (2012). *P. pastoris* transformants were cultivated in 96-DWPs as described. After 48 h of induction, $OD_{600}$ values of cultures in each well were determined followed by centrifugation of the DWP at 3,220 x g for 5 min. The supernatants were discarded and the cell pellets were resuspended in 100 mM potassium phosphate buffer, pH 7.4. Prednisolone was added to the supernatants as an internal standard to a final concentration of 50 ng $\mu l^{-1}$. The reaction mix was measured by HPLC-MS according to the method described by (Geier et al., 2012).

## *2.8. Biphasic (+)-valencene synthesis and bioconversion assay*

Adapting the protocol of Cankar et al. (2011), *S. cerevisiae* cells co-expressing HPO, CPR and ValS were cultivated in 50 mL synthetic defined growth media in 100 mL shake flasks, starting at an $OD_{600}$ of 0.1. After 24 h of shaking at 30°C and 170 rpm, 1 mL of induction solution (20% galactose and 7% raffinose) and 1 mL of supplement solution, i.e. 0.167 g yeast nitrogen base and 25 mg dropout powder dissolved in sterile $ddH_2O$, were added to the cell suspension. Five mL of n-dodecane were added directly to the flasks to form second, organic phases. Cells were cultivated as before for 24 h, followed by a second addition of 1 mL each of induction and supplement solutions. Cells were induced for up to 72 h, before the n-dodecane phases were subjected to GC-MS and GC-FID analyses.Cultivation of *P. pastoris* strains expressing HPO/CPR and ValS in combination with *Pp*Ice2 was scaled up to 50 ml total volume in shake flasks. Therefore, 25 mL of pre-cultures in BMGY medium were grown for 48 h at 28 °C, 140 rpm, followed by the addition of 25 ml of induction medium BMMY containing 2 % methanol and n-dodecane at a final concentration of 10%. Induction was performed as for DWP cultivations. After 48 h of induction, the cultures were transferred to 50 mL plastic tubes, spun at 3220 x g for 5 min and the organic layers were transferred into GC vials for GC-FID analysis.

## 2.9. Cytochrome c reductase assay

CPR activity was estimated by its ability to reduce bovine heart cytochrome c (Phillips and Langdon, 1962) .

*P. pastoris* and *S. cerevisiae* cell lysates were prepared by glass bead lysis according to the "*Pichia* Expression Kit" manual (Life technologies, Vienna, Austria) with minor modifications. In brief, cell pellets were disrupted with equal volumes of glass beads in 1.5 mL reaction tubes with 200 µL of breaking buffer (50 mM $NaH_2PO_4$, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5 % glycerol), respectively. The cell suspensions were intermittently vortexed for 30 s and incubated on ice for 30 s, which was repeated for 10 cycles. After cell disruption, total cell lysates were centrifuged at 1500 x g in a tabletop centrifuge to remove unbroken cells and cell debris. Protein amounts in cell lysates were quantified using the Bio-Rad (Bradford) protein assay with bovin serum albumin as a standard.

Twenty-five µL of the supernatants were mixed with 125 µL of 300 µM cytochrome c solutions and made up to a final volume of 650 µL with 50 mM Tris-HCl buffer, pH 7.5. Fifty µL of 50 mM KCN solution, pH 7.7, were added to yeast preparations to mask endogenous oxidase activities. The enzymatic reaction was started by the addition of 50 µL of 1.5 mM NADPH. The increase in absorption at 550 nm was recorded for 2 min using an UV/Vis DU800 spectrophotometer (Beckman Coulter, Brea, California). Reductase activity was calculated based on a molar extinction coefficient of 21 $mM^{-1}$ $cm^{-1}$ for the reduced cytochrome c (Phillips and Langdon, 1962).

## 2.10. Product analysis by GC-MS and GC-FID

Terpenoid extracts in organic solvents, i.e. ethyl acetate as well as n-dodecane, were initially analyzed by GC-MS for identification of compounds using reference standards and comparing the derived mass fragmentation spectra. A 30 m HP column (0.25 mm x 0.25 µm) was used on a Hewlett-Packard 5890 Series II plus GC equipped with a 5972 series mass selective detector (MSD). Sample aliquots of 1 µL were injected in split mode (split ratio 20:1) at 220°C injector and 280 °C detector temperatures with helium as carrier gas at constant flow rate of 32 cm $s^{-1}$. The oven temperature program was as follows: 70 °C for 1 min, 10°C/min ramp to 200 °C, and 30 °C $min^{-1}$ ramp to 290 °C (2 min). MSD was operated in a mass range of 40-250 amu with 3.5 scans $s^{-1}$ and at electron multiplier voltage of 1635 V.

GC-FID methods were developed for routine analyses of terpenoid samples. Therefore, we used a HP-5 column (crosslinked 5 % Ph-Me Siloxane; 10 m x 0.10 mm x 0.10 µm) on a Hewlett-Packard 6890 GC equipped with a flame ionization detector (FID). Sample aliquots of 1 µL were injected in split mode (split ratio 30:1) at 250 °C injector temperature and 320°C detector temperature with hydrogen as carrier gas and a flow rate set to 0.4 mL $min^{-1}$ in constant flow mode (49 cm $s^{-1}$ linear velocity). For analysis of (+)-valencene and its products, the oven temperature program was as follows: 100°C for 1 min, 20°C $min^{-1}$ ramp to 250°C, and 45°C $min^{-1}$ ramp to 280°C (0.5 min). The use of a high-speed/high-resolution column reduced the total run time to 9 min per sample, without any loss of chromatographic resolution (Wriessnegger et al., manuscript in revision). For analysis of (-)-limonene and isopiperitenol, the following oven temperatures were used: 40°C for 1 min, 4 °C $min^{-1}$ ramp to 90 °C and 30 °C $min^{-1}$ ramp to 280°C (0.5 min). Total run time was reduced to 22 min per sample.

## *2.11. Electron microscopy*

*S. cerevisiae* strains were cultivated as described for expression of heterologous proteins. After 6 h of induction with galactose, cells were harvested at 2,500 rpm for 5 min in an Eppendorf 5810R centrifuge and the cell pellets were washed with distilled $H_20$. The cells were fixed for 5 min in 1% aqueous $KMnO_4$ at room temperature, washed with distilled $H_20$, and fixed in 1% aqueous $KMnO_4$ for 20 min. Fixed cells were washed four times in distilled water and incubated in 0.5% aqueous uranyl acetate over night at 4 °C. The samples were dehydrated for 20 min, each, in a graded series of ethanol (50 %, 70 %, 90 %, and 100 %). Pure ethanol was then exchanged by propylene oxide, and specimen were gradually infiltrated with increasing concentrations (30 %, 50 %, 70 % and 100 %) of Agar 100 epoxy resin mixed with propylene oxide for a minimum of 3 h per step. Samples were embedded in pure, fresh Agar 100 epoxy resin and polymerized at 60 °C for 48 h. Ultra-thin sections of 80 nm were stained for 3 min with lead citrate and viewed with a Philips CM 10 transmission electron microscope.

# 3 Results

## *3.1. Screening for effectors of CYP/CPR function in recombinant S. cerevisiae strains*

(+)-valencene is a side-product of orange juice production and of low commercial impact. However, there are numerous attempts to convert (+)-valencene into the attractive flavor and fragrance compound (+)-nootkatone by diverse biocatalytic approaches (reviewed by Fraatz et al., 2009). As highly stereo- and regioselective catalysts, soluble and membrane-attached cytochrome P450 enzymes (CYPs) have been tested for performing this reaction (Cankar et al., 2011; Gavira et al., 2013; Girhard et al., 2009; Takahashi et al., 2007). There is solid data underscoring that CYPs do hydroxylate (+)-valencene to yield nootkatol (Figure 1), but that oxidation of the latter compound to nootkatone is performed by endogenous activities of *S. cerevisiae* (Gavira et al., 2013) or *P. pastoris* (Wriessnegger et al., manuscript in revision). An important feature of CYPs is their requirement for cytochrome P450 reductase (CPR) activities regenerating the catalytically active CYPs. There are reports suggesting mutual stabilization of membrane-associated CYPs and CPRs necessitating balanced stoichiometry upon heterologous expression of such CYP/CPR (Geier et al., 2012; Gonzalez and Korzekwa, 1995; Murakami et al., 1986). Searching for protein effectors of CYP/CPR function, we have functionally co-expressed *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO) variant V482I A484I (Takahashi et al., 2007) and *Arabidopsis thaliana* cytochrome P450 reductase (CPR) from a multicopy vector (pYES2-HPO-CPR) in *S. cerevisiae*. Recombinant yeast strains hydroxylated (+)-valencene as expected from literature (Figure 1B).

Compounds were verified by GC-MS (Figure 2). Co-expression of HPO/CPR from a single vector allowed for screening available yeast strain collections for effector proteins of HPO/CPR levels and activities. Selected single-gene knockout mutants of the EUROSCARF collection were transformed with the pYES2-HPO-CPR plasmid and were tested for altered (+)-valencene conversion in resting cells assays. Most transformants showed conversion rates indistinguishable from the reference strain BY4742 pYES2-HPO-CPR (data not shown). A huge drawback of external (+)-valencene addition in resting cells assays was the high volatility of (+)-valencene in aqueous environment. After 20 h of conversion, only 20% of the initially added (+)-valencene was re-extractable from control cells harboring the empty pYES2 vector (Figure 1B). Because side-products of (+)-valencene hydroxylation

were not detected in GC-MS, we ascribe the loss to evaporation. In equivalent experiments, only 30% of the added trans-nootkatol evaporated within 20 h of shaking at 30°C (data not shown).

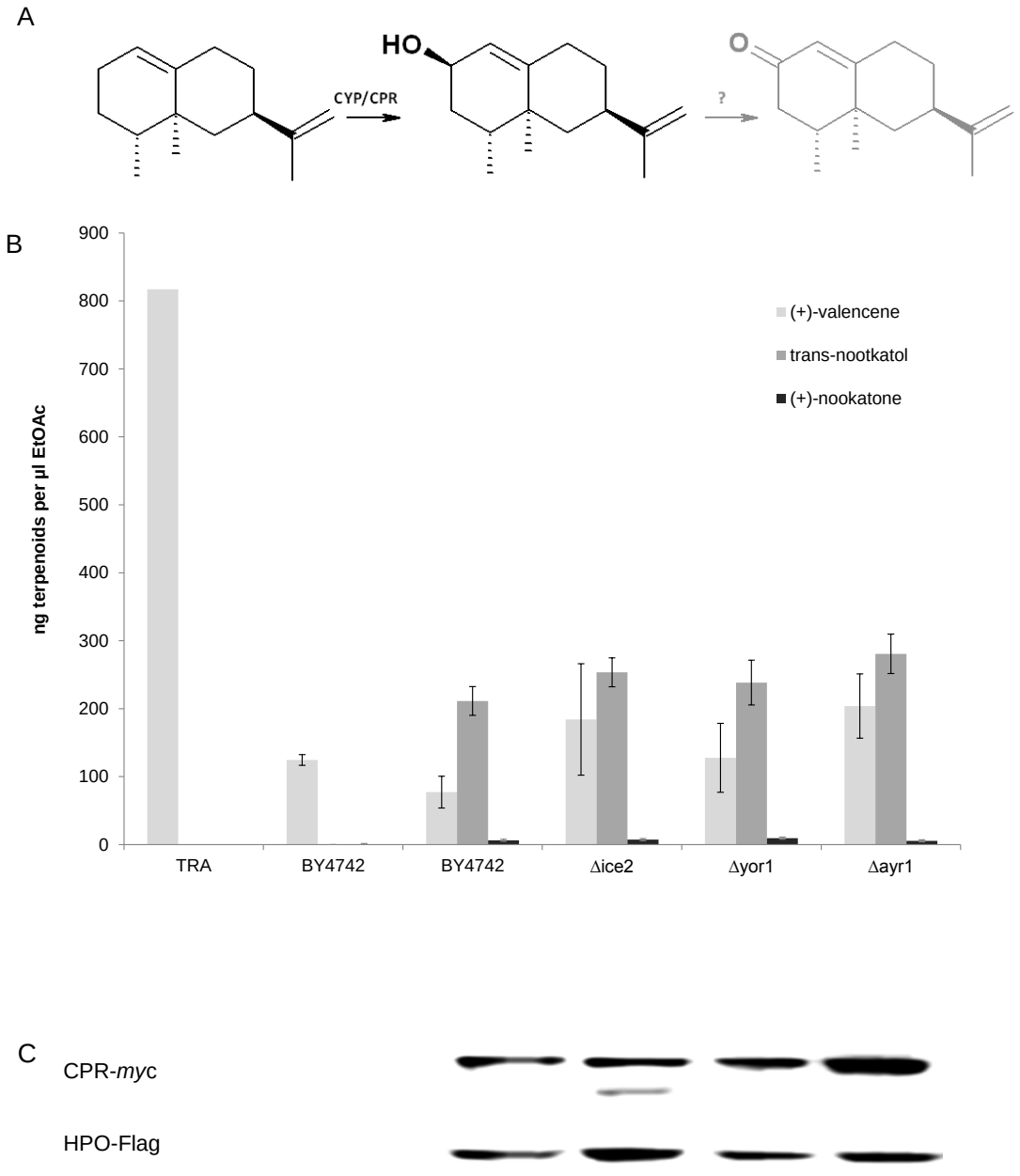



**Figure 1. (+)-valencene (1) biohydroxylation by CYP/CPR activity recombinantly expressed in *S. cerevisiae*. trans-nootkatol (2) formed by cytochrome P450 enzymes may be further oxidized to (+)-nootkatone (3) by an unidentified intrinsic activity of baker's yeast (A). Effectors of HPO/CPR activity in *S. cerevisiae* were screened by resting cells assays with a theoretically re-extractable amount (TRA) of 817 ng of (+)-valencene per µL of ethyl acetate (B). BY4742 (evc) indicates substrate loss within 20 h of assay. BY4742 and single knockout strains disrupted for Δ*ice2*, Δ*yor1* and Δ*ayr1* in the same background were transformed with pYES-HPO-CPR to be assayed for (+)-valencene conversion. Results are given as mean values and standard deviations of resting cells assays performed in technical quadruplicate for two biological replicas. Western blot analyses were performed to reveal HPO/CPR levels after 6 h of galactose induction (C).**

Aliquots of galactose-induced cells were routinely checked for HPO (53 kDa) and CPR (72 kDa) levels by Western blotting. Thereby, we observed that CPR-*myc* appeared to be partially degraded in BY4742 Δ*ice2*, which was not observed in most other single knockout strains we evaluated (Figure 1C and data not shown). However, trans-nootkatol formation by HPO/CPR activity was not significantly different in BY4742 Δ*ice2* as compared to the reference strain (Figure 1C).

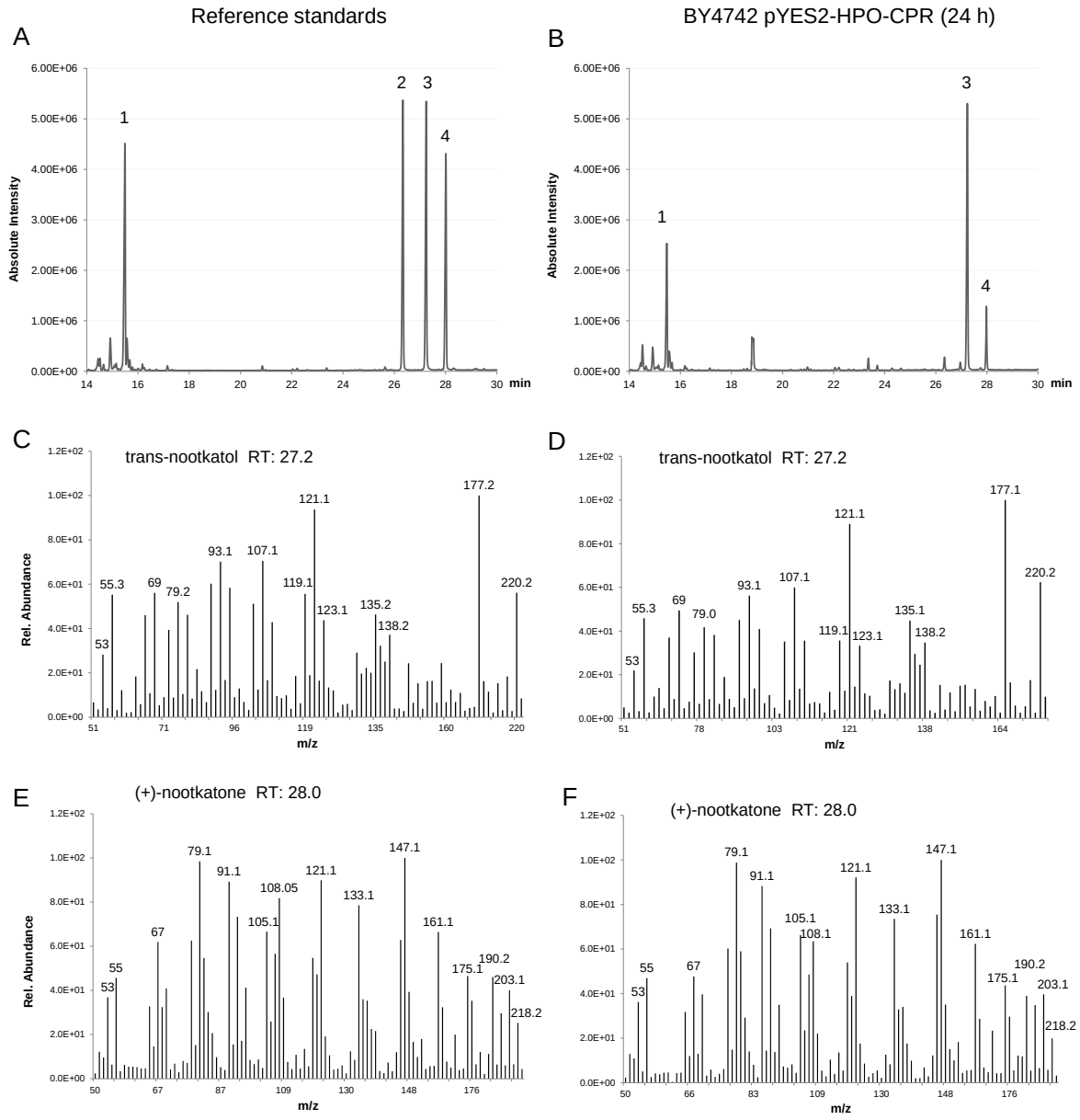



**Figure 2. GC-MS chromatograms of reference standards (A) and terpenoids produced by resting cells assay (B); 1, (+)-valencene; 2, cis-nootkatol; 3, trans-nootkatole; 4, (+)-nootkatone. Mass spectra of trans-nootkatol and (+)-nootkatone are shown for reference standards (C, E) and biotransformation products with resting cells (D, F).**

### *3.2. Over-expression of ICE2 in S. cerevisiae W303*

Among the various *S. cerevisiae* host strains used for CYP/CPR expression and CYP-mediated bioconversions, it has particularly been the W303 strain that turned out to be the most applicable host (Loeper et al., 1998; Pompon et al., 1996; Truan et al., 1993; P. Urban et al., 1994). Many of the standard lab strains of *S. cerevisiae*, but not the W303 strain, do carry a deletion in the *HAP1* gene. Hap1p is a transcription factor responsible for regulation of genes governing intracellular heme abundance in response to changes in available oxygen levels (Davies and Rine, 2006; Ihrig et al., 2010). A Δ*hap1* genotype strain is supposedly less favorable for CYP expression and function as these are strongly dependent on optimal heme and oxygen supply. To test for the host strain effect, we performed HPO/CPR-mediated resting cells conversion of (+)-valencene in parallel in the BY4742 and the W303 yeast strains harboring pYES-HPO-CPR. As expected, (+)-valencene conversion levels were clearly higher in the W303 strain background - yielding 400 ng trans-nootkatol per µL of ethyl acetate (Figure 3B) - than in the BY4742 strain background at 200 ng trans-nootkatol per µL of ethyl acetate (Figure 1B). In both strain backgrounds, the knockout of Δ*ice2* did not significantly influence the conversion of (+)-valencene to trans-nootkatol as compared to the corresponding reference strains. Since (+)-valencene conversion was substantially more efficient in the W303 strain background, we focused our further efforts on this host strain.

Triggered by the degradation products of CPR-*myc* observed in the Δ*ice2* knock-out strain (Figure 1C), we created strains expressing *ICE2* from the $P_{GAL1}$ promoter inserted in front of the gene and used the engineered strains to be tested for HPO/CPR expression and (+)-valencene conversion, which were ideally controlled by the same promoter element. Interestingly, expressing *ICE2* from $P_{GAL1}$ resulted in 1.4-fold improved (+)-valencene conversion independent of the host strain background (Figure 1B and Figure 3B). The manipulations of Ice2 expression levels did not significantly affect cellular growth characteristics, as all strains grew to almost identical optical densities without and upon induction with galactose/raffinose (data not shown). To quantify expression of HPO, CPR and *ICE2* on the mRNA level, quantitative real-time PCR was performed on all strains with modulated Ice2 expression. RNA was extracted after 6 h of galactose/raffinose induction and normalized against the housekeeping gene *CDC73* in each sample. *ICE2* appeared to be expressed at low levels in wild type cells (Figure 3A), which is consistent with previous studies ascribing a total number of 606 molecules of Ice2p per cell growing exponentially in YPD (Ghaemmaghami et al., 2003). In the same study, 538 molecules of *CDC73* were predicted under the same cultivation conditions. Strikingly, another ER-membrane spanning protein, Sec61p was found to be present in roughly 24,800 copies per cell. Expressing *ICE2* from the $P_{GAL1}$ promoter resulted in 100-fold up-regulation of the gene as compared

to the strains with *ICE2* under the control of its own promoter (Fig. 3A). Interestingly, mRNA levels of HPO and CPR were largely independent of Ice2 modulation. Thus, we speculated that the enhanced biohydroxylation of (+)-valencene observed for the W303 $P_{GAL1}$-*ICE2* pYES2-HPO-CPR strain might indeed be elicited by posttranscriptional effects of elevated Ice2 expression on HPO/CPR protein levels and/or activity.

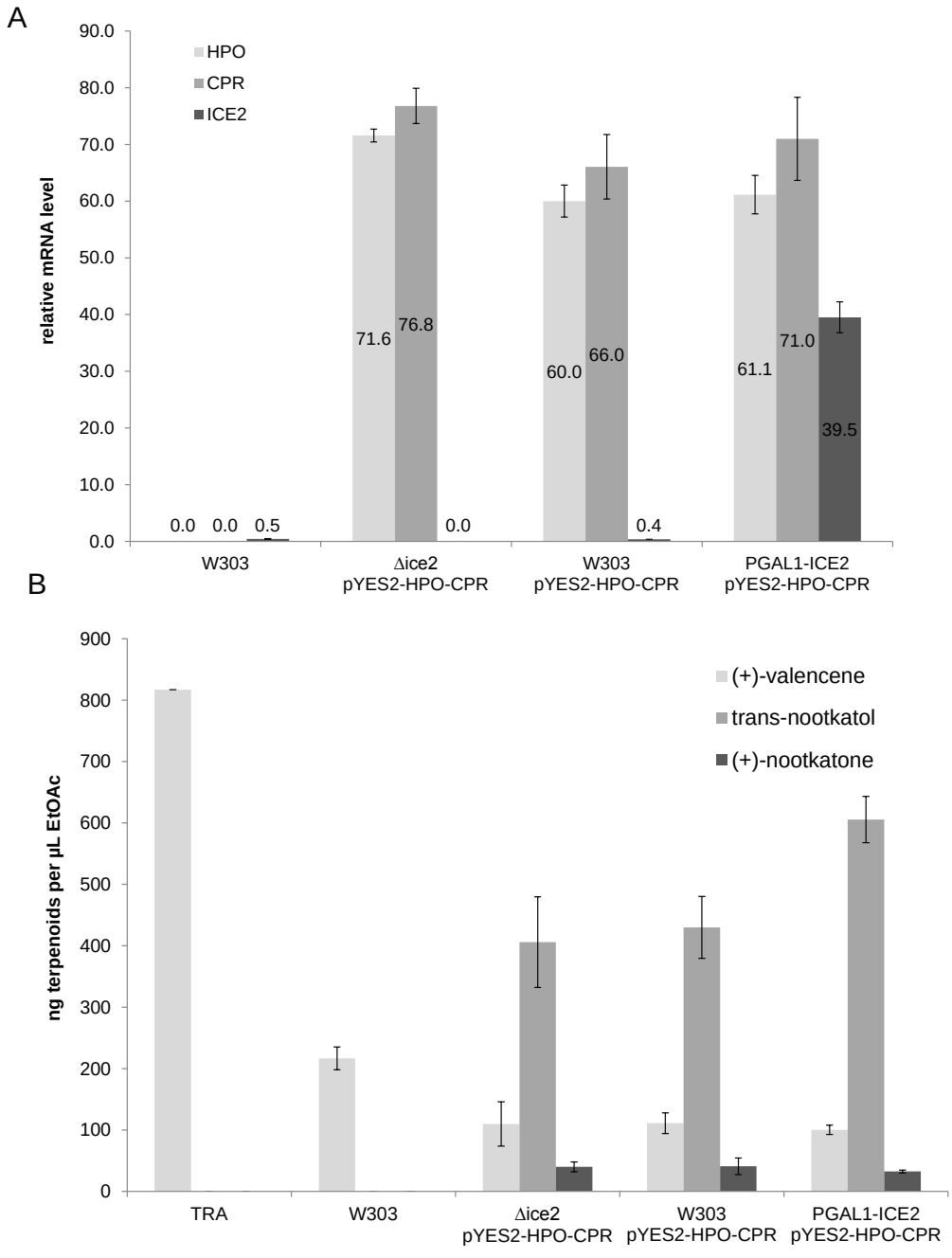



**Figure 3. Assessing the effect of modulated *ICE2* expression levels on HPO/CPR-mediated conversion of (+)-valencene. Quantitative real-time PCR analysis (A). Control strain W303 and control strain, Δ*ice2* and $P_{GAL1}$-*ICE2* strains co-expressing HPO and CPR from the pYES2 vector. mRNA levels were determined according to the $\Delta C_t$ method normalizing expression levels to the housekeeping gene *CDC73*. Same strains were used for resting cells activity assays performed in three biological and four technical replicas each (B).**

### *3.3. In vivo bioconversion of (+)-valencene in S. cerevisiae*

Assessing HPO/CPR-driven (+)-valencene hydroxylation in resting cells and quantifying the effect of $P_{GAL1}$-*ICE2* expression thereon is subject to an important uncertainty factor, which is the availability of the (+)-valencene substrate. It is not only the evaporation of the terpene in aqueous environment that needs to be taken into account (Figure 1B and Figure 3B), but also diverse transport processes into and out of *S. cerevisiae* cells. To a pure hydrocarbon compound like (+)-valencene, the plasma membrane of yeast should not pose a stringent barrier for entry into the yeast cell. However, there are plenty of export mechanisms described for *S. cerevisiae* efficiently expelling hydrophobic compounds from the cell interior, e.g. diverse ABC transporters (Nishida et al., 2013b; Wawrzycka, 2011), Pry proteins (Choudhary and Schneiter, 2012; Jacquier and Schneiter, 2012), and others. Thus, the intracellular (+)-valencene concentration, i.e. the substrate pool for HPO/CPR-mediated bioconversion, in resting cells assays is influenced by multiple factors that may be beyond the control of the experimenter. This situation obviously leads to the relatively high standard deviations in resting cells assays (Figure 1B and Figure 3B). Moreover, cell engineering, as for example over-expression of *ICE2*, might have an impact on the equilibrium of (+)-valencene transport across the yeast plasma membrane and, therefore, on intracellular availability of the compound. In order to avoid these uncertainties, we generated strains producing (+)-valencene intracellularly from FPP. Expression of sequence optimized valencene synthase (ValS) from *C. nootkatensis* in *S. cerevisiae* required co-expression of truncated Hmg1 in contrast to published work (Beekwilder et al., 2014). Strains expressing ValS without concomitant over-expression of t*HMG1* grew extremely slowly, probably due to massive withdrawal of FPP from ergosterol biosynthesis (Parks et al., 1995; L. Song, 2003). Cells co-expressing ValS, t*HMG1*, HPO and CPR were initially cultivated according to the protocol described by Cankar et al. (2011), but cell growth was rather poor under these conditions. Conditions were adapted to pre-cultivating cells in 50 mL of glucose containing media for 24 h, followed by 24-72 h of induction via direct addition of galactose/raffinose to shake flasks. Overlaying the cell broth with 10% n-dodecane simultaneously with induction entrapped all terpenoids formed during bioconversions. Thereby, the W303 *tHMG1* ValS pYES2-HPO-CPR strain produced roughly 20 mg of trans-nootkatol per liter of culture broth (Figure 4). Remarkably, production of trans-nootkatol was increased by ~ 50% upon expressing Ice2p-$His_6$ from the $P_{GAL1}$ promoter resulting in a maximum productivity of 30 mg trans-nootkatol per liter of cell culture after 72 h of induction for the W303 *tHMG1* ValS $P_{GAL1}$-*ICE2* pYES2-HPO-CPR strain. Knocking out *ICE2*, on the other hand, did not influence the amount of terpenoids formed as compared to the reference strain (Figure 4). Consequently, modulation of *ICE2* expression did have equivalent effects upon HPO/CPR-mediated

(+)-valencene hydroxylation in resting cells with externally added substrate (Figure 3B) and in cells producing the substrate *in vivo* (Figure 4). These results underscore that the beneficial effect of Ice2 overexpression on HPO/CPR function was rather not due to altering bioavailability of the (+)-valencene substrate, but was probably due to altering HPO/CPR enzyme activity.

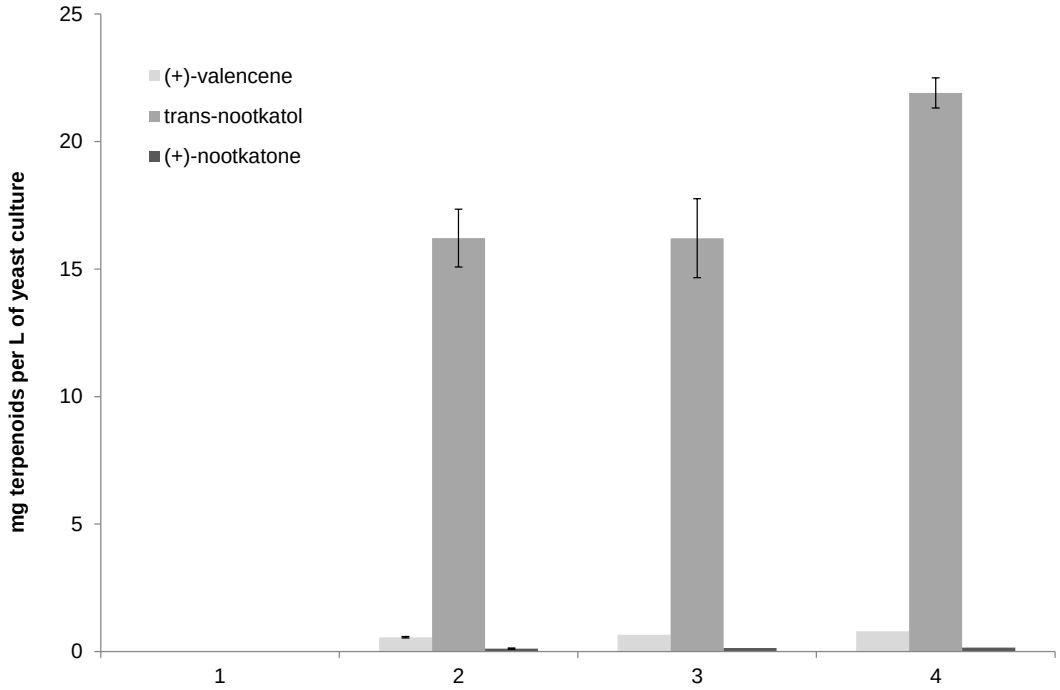



**Figure 4. *In vivo* production and bioconversion of (+)-valencene in cells co-expressing ValS, t*HMG1*, HPO and CPR (2) and strains additionally harboring an deletion of *ice2* (3) or overexpressing *ICE2* from the $P_{GAL1}$ promoter (4). Strains were compared to the control strain W303 (1). Conversions were done in triplicates and consistently repeated for more than three independent experiments.**

## *3.4. ICE2 overexpression stabilizes CPR levels and activity*

In an attempt to characterize the effects of *ICE2* overexpression in more detail, we created strains that expressed Ice2p-$His_6$ either from its endogenous promoter or from $P_{GAL1}$ and co-expressed HPO/CPR, ValS and t*HMG1*. These strains were subjected to time course bioconversion studies comparing HPO-Flag, CPR-*myc* and Ice2p-$His_6$ levels by Western blotting besides quantifying terpenoid formation by GC (Figure 5). Samples taken from the n-dodecane phase after 24, 48 and 72 h of galactose/raffinose induction revealed that for the first 48 h of bioconversion there was only little difference in trans-nootkatol productivity (≤ 10%) between the strains that differed in the expression of Ice2p-$His_6$ (Figure 5A). However, after 72 h of bioconversion, the strain expressing

Ice2p-$His_6$ from the galactose-inducible promoter formed 300 ng trans-nootkatol $\mu L^{-1}$ of n-dodecane (equivalent to 30 mg $L^{-1}$ of yeast culture), which was about 40% more than the 220 ng trans-nootkatol $\mu L^{-1}$ n-dodecane produced by the strain expressing Ice2p-$His_6$ from its native promoter. Western blot analysis showed that $P_{GAL1}$-driven ICE2 over-expression was not only detectable on the mRNA level (Figure 3A), but also translated into higher protein levels at each time point (Figure 5C). The Ice2-$His_6$ signals were hardly detectable when under the control of its native promoter in 24 h samples and further decreased at later time points. However, Ice2p-$His_6$ levels were much higher with a slight decrease in 72 h samples when the protein was expressed from the galactose-inducible promoter. Most interestingly, HPO-Flag and, particularly, CPR-*myc* protein levels were clearly more stable over 72 h of bioconversion in the strain expressing higher levels of Ice2p-$His_6$ (Figure 5B). It is reasonable to assume that $P_{GAL1}$-driven Ice2p-$His_6$ expression enhanced (+)-valencene bioconversion by stabilizing CPR levels, because HPO action requires CPR for regeneration. Thus, the strain harboring elevated levels of Ice2p-$His_6$ and detectable amounts of CPR-*myc* will have further hydroxylated (+)-valencene until 72 h of conversion, in contrast to the strain that basically lacked CPR-*myc* protein at the last time point. HPO/CPR protein levels were quantified by densitometric scanning with the Fiji program (Schindelin et al., 2012). However, HPO/CPR protein levels might not be representative of HPO/CPR activity required for specific hydroxylation of (+)-valencene. Unfortunately, the active fraction of HPO could not be characterized by CO-difference spectroscopy (Omura and Sato, 1964) due to low signal intensities in whole cells and cell homogenates. Yet, we quantified the activities of cytochrome P450 reductase at different time points of bioconversion using the cytochrome c reductase assay. The evaluation of CPR activity is based on the reduction of oxidized cytochrome c and detecting reduced cytochrome at 550 nm (Phillips and Langdon, 1962). Reference strains without co-expression of HPO and CPR, i.e. strains $P_{ICE2}$-*ICE2*-$His_6$ (evc) and $P_{GAL1}$-*ICE2*-$His_6$ (evc) were found to have background reductase activities of 0.030 ± 0.004 and 0.042 ± 0.007 U per mg of total protein for the W303 and $P_{GAL1}$-*ICE2*-6His strain, respectively. This was in good agreement with values reported for the W303 strain (Geier et al., 2012). Upon subtracting the background activity values, we realized that CPR activity was virtually independent of *ICE2* expression at 24 h of terpenoid bioconversion experiments, but strongly decreased at low Ice2p levels after 48 h of galactose/raffinose induction (Figure 5D). While overexpression of Ice2 from the inducible promoter stabilized ≥75% of CPR activity over 72 h of assay time, CPR activity was not detectable in the strain expressing *ICE2* from its native promoter. These values corresponded very well to the CPR-*myc* levels detected by Western blotting (Figure 5C). Thus, we propose that the positive effect of *ICE2* overexpression on HPO/CPR-based (+)-valencene hydroxylation is mediated - at least partially - by stabilization of CPR activity during *in vivo* terpenoid formation.

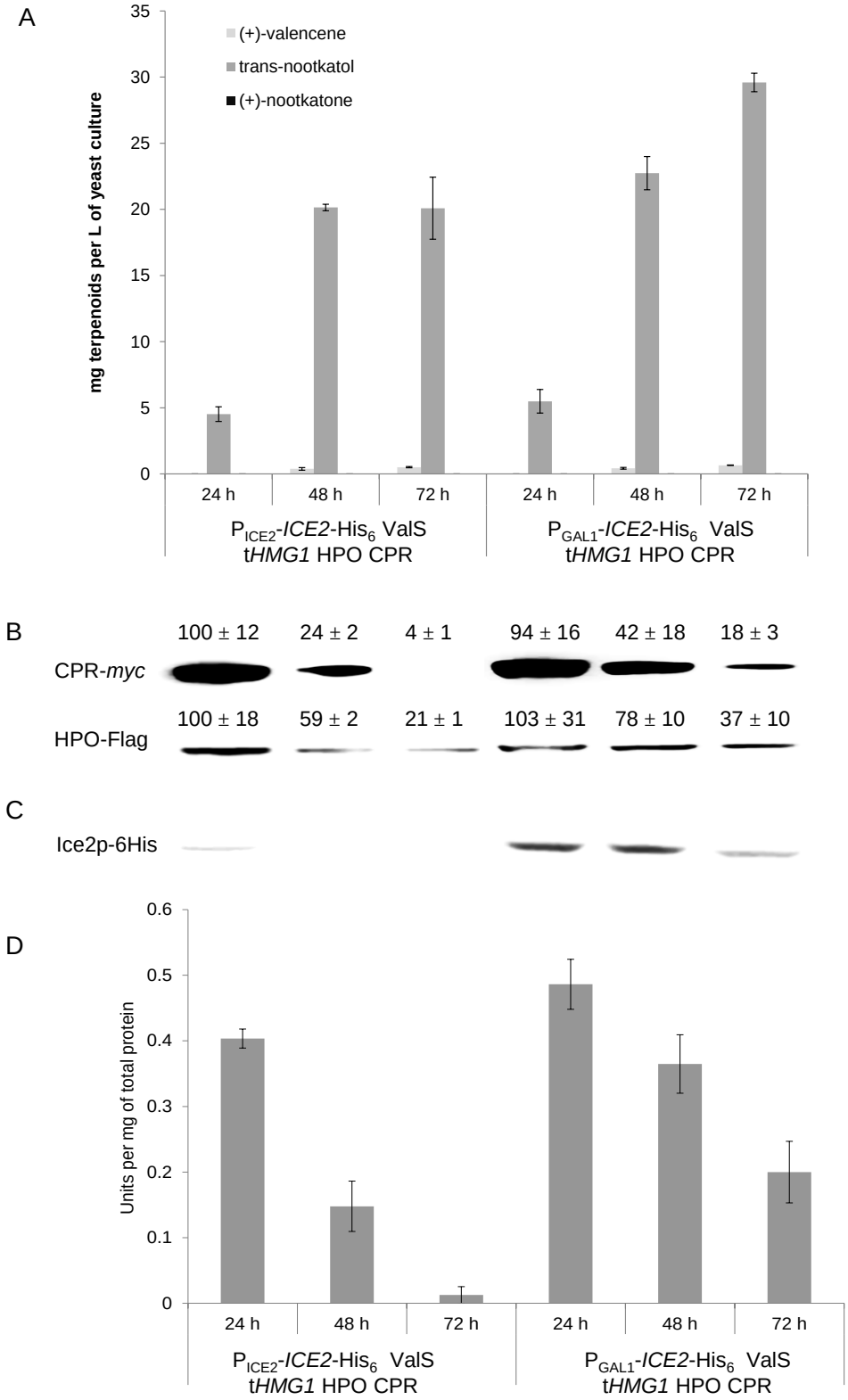



**Figure 5. Time-dependent analysis of *in vivo* production of terpenoids of the reference strain W303 t*HMG1* ValS HPO CPR and the strain over-expressing *ICE2* (A). Quantification of Western blot signals was done with the Fiji program from samples taken at time points 24 h, 48 h and 72 h. Intermediate band intensities were calculated in percentages from four samples loaded per strain and timepoint (B). At the same time points, expression of Ice2p-His$_6$ was tracked either from the endogenous promoter or from the P$_{GAL1}$-promoter (1 and 2 OD$_{600}$ units loaded, respectively) (C). Cytochrome c reductase activity assay was done with the control strain W303 t*HMG1* ValS HPO CPR and the one over-expressing *ICE2*-His$_6$.**

**Background reductase activities of strains W303 *MATa* and W303 *MATα* $P_{Gal1}$-*ICE2*-$His_6$ were subtracted. Measurements were done in quadruplicates with samples taken from two different cultivations (D).**

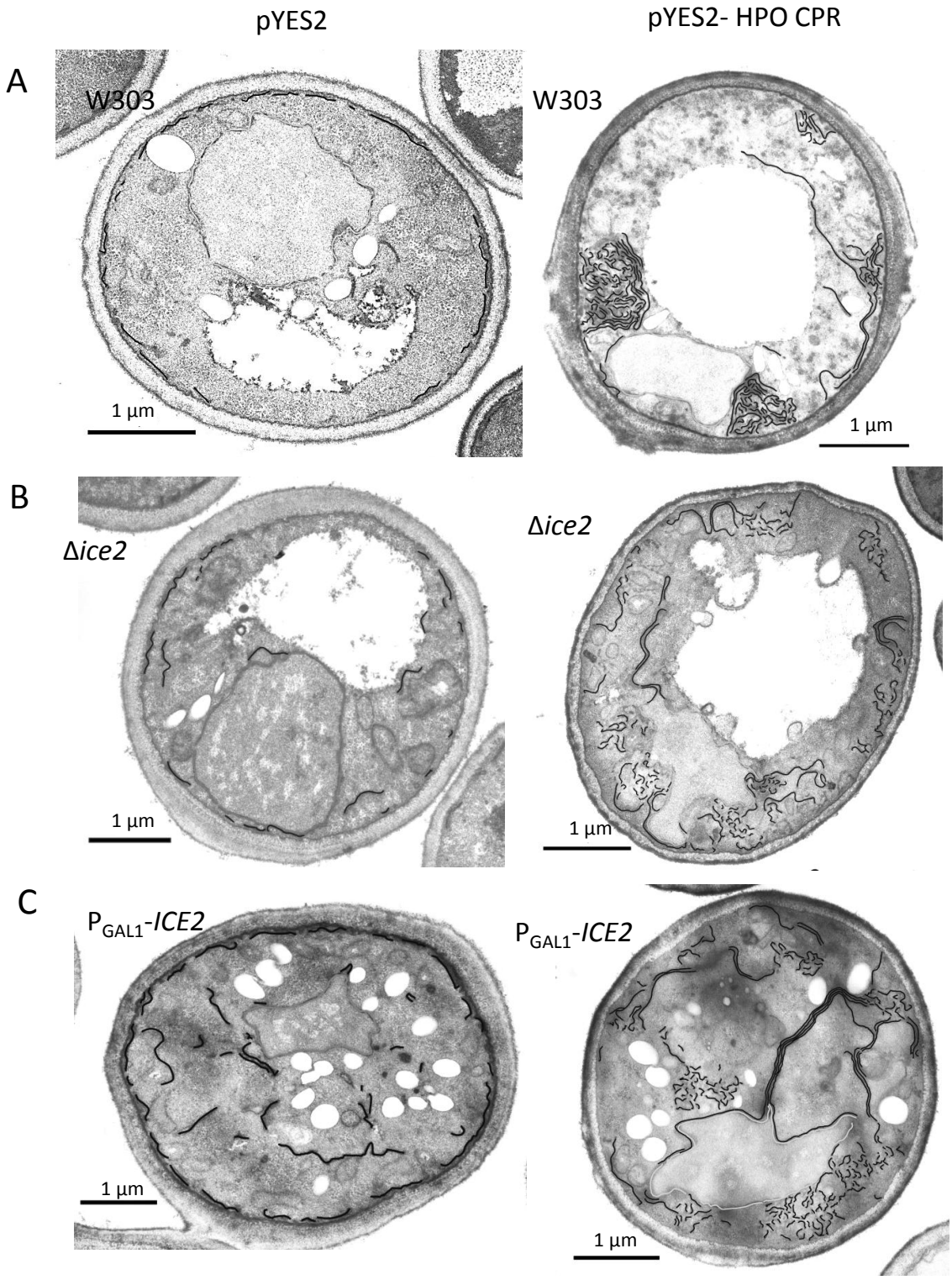



**Figure 6. Electron micrographs of *S. cerevisiae* strains. Subcellular compartments were traced in different colors for better visualization. Nuclear membrane, light grey (N, nucleus); peripheral ER, dark grey. The wild type strain W303 (A), the Δ*ice2* (B) and the strain overexpressing *ICE2*(C) with and without co-expression of HPO and CPR are shown.**

On the basis of previous studies reporting an altered cellular ER membrane distribution in *S. cerevisiae* cells disrupted for *ice2* (Estrada De Martin et al., 2005; Tavassoli et al., 2013), electron microscopy images of engineered strains were generated (Figure 6). In general, overexpression of membrane proteins results in formation of staggered ER-membranes, also called karmellae (Wright et al., 1988). The same effect was observed for W303 wild type, *Δice2* and $P_{GAL1}$-ICE2 strains overexpressing HPO and CPR (Figure 6). As expected, knocking out *ice2* did slightly reduce amounts of peripheral ER membranes (Figure 6B), although the effect was not as explicit as shown by Tavassoli et al. (2013) who presented electron microscopy pictures of the *ice2 scs2* double knockout mutant. Interestingly, overexpression of *ICE2* did lead to a significant increase in ER membranes adjacent to the yeast plasma membrane (Figure 6C).

### *3.5. A general effect of ICE2 overexpression on CPR stability*

To test whether stabilization of CPR levels and/or activity is a general effect of *ICE2* overexpression in *S. cerevisiae* and other yeast hosts, we extended our analysis to alternative CYP/CPR combinations in *S. cerevisiae* and *P. pastoris*. Therefore, we have chosen to express (-)-limonene-3-hydroxylase from *Mentha piperita* (PM17) together with *At*CPR as well as human cytochrome P450 2D6 (CYP2D6) combined with human reductase (hCPR). PM17 and CPR catalyze the conversion of the monoterpene (-)-limonene to (-)-trans-isopiperitenol (Karp et al., 1990; Lupien et al., 1995). CYP2D6 and hCPR act as main detoxifying system of drugs in the human liver (Zhuge et al., 2004). CYP2D6 activity is assessed by hydroxylation of the model substrate bufuralol to 1-hydroxybufuralol (Geier et al., 2012).

While (-)-limonene conversion was not enhanced in *S. cerevisiae* by over-expression of *ICE2* (Figure 7A), hydroxylation of bufuralol, however, was improved 1.5-fold (Figure 7B). Western blot analysis did not reveal any strain-dependent alterations of HPO and CPR levels (7C). In contrast, analyses of CPR activities in Ice2 co-expression strains showed a clear stabilisation effect, particularly after 24 h of induction (Table 3). After 6 h of induction, the reductases showed constantly higher activities if expressed in the Ice2-upregulated strain. This effect was even more striking if cells were induced for 24 h leading to 4-fold higher reductase activities in strains with higher Ice2p levels compared to the wild-type situation (Table 3).

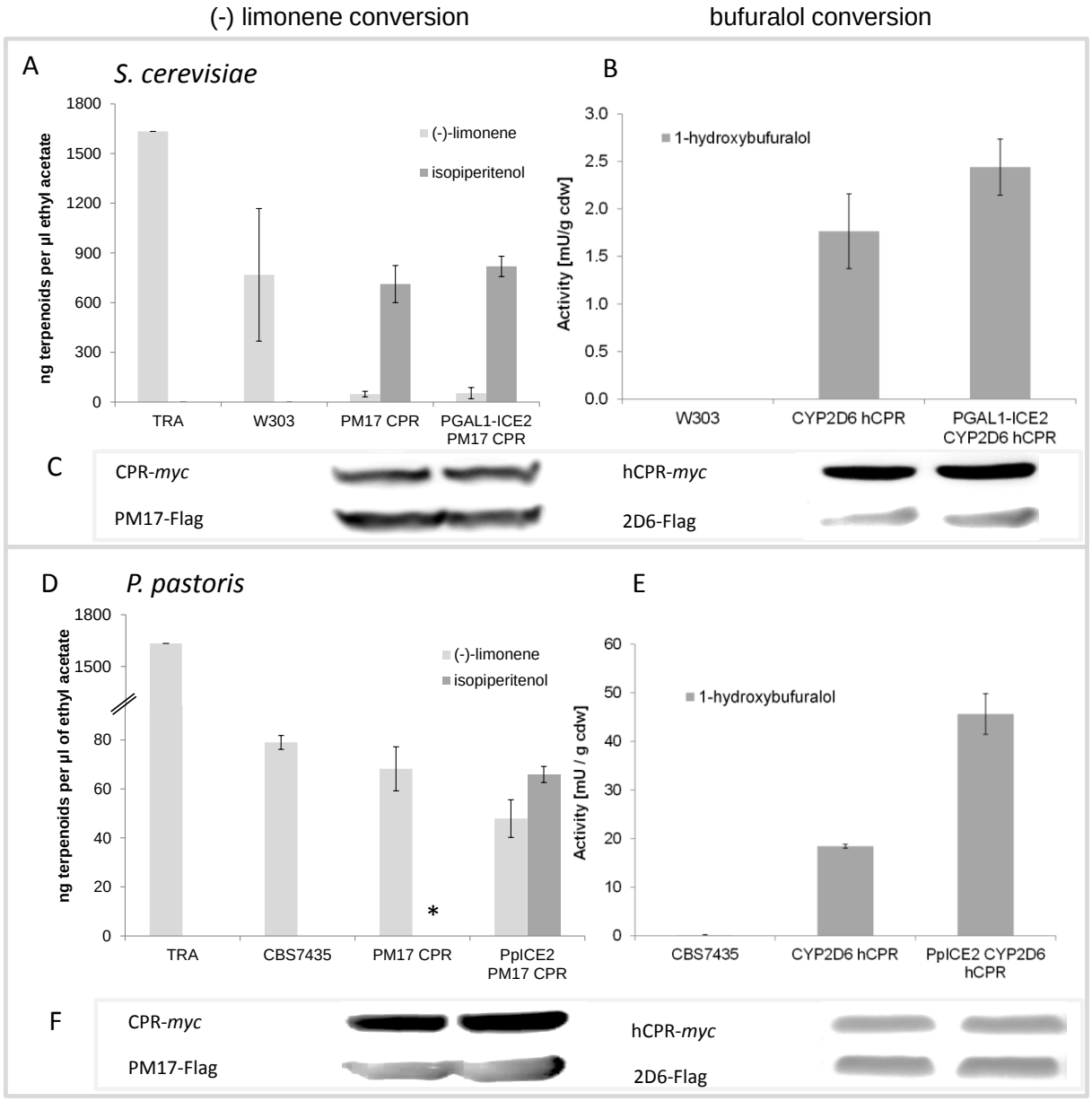



**Figure 7. Alternative CYP/CPR systems tested for substrate conversion in *S. cerevisiae* and *P. pastoris* strains overexpressing *ICE2*. *S. cerevisiae* resting cells assays for conversion of (-)-limonene (PM17/CPR) (A) and bufuralol (CYP2D6/hCPR) (B). Western blot analysis of *S. cerevisiae* strains expressing alternative pairs of CYPs and reductases were performed (C). As alternative expression host, *P. pastoris* was tested for both, (-)-limonene (D) and bufuralol (E) conversion. Strains were analyzed for expression of HPO and CPR by Western blot (F).**

**Table 3: Reductase activities in U mg$^{-1}$ of total protein of CYP/CPR pairs in *S. cerevisiae* and *P. pastoris*. *S. cerevisiae* resting cells were induced for 6 h, which is the time-point when resting cells assays where set up. Induction was prolonged to 24 h for analysis of reductase activities. *P. pastoris* strains were induced for 48 h and samples were drawn for cytochrome c reductase activity assays.**

***S. cerevisiae***

| Plasmid | pYES2 HPO CPR | | pESC-URA PM17 CPR | | pESC-URA CYP2D6 hCPR | |
|---|---|---|---|---|---|---|
| $P_{GAL1}$-*ICE2* | - | + | - | + | - | + |
| 6 h | 1.10 ± 0.09 | 1.55 ± 0.02 | 2.38 ± 0.23 | 3.26 ± 0.22 | 7.13 ± 0.03 | 8.37 ± 0.49 |
| 24 h | 0.36 ± 0.02 | 1.06 ± 0.02 | 0.22 ± 0.01 | 0.82 ± 0.07 | 0.28 ± 0.01 | 0.92 ± 0.14 |

***P. pastoris***

| Strain | HCV | | PM17/CPR | | CYP2D6/hCPR | |
|---|---|---|---|---|---|---|
| *PpICE2* | - | + | - | + | - | + |
| 48 h | 0.26 ± 0.02 | 0.37 ± 0.01 | 0.26 ± 0.01 | 0.34 ± 0.01 | 0.52 ± 0.00 | 0.69 ± 0.01 |

A *P. pastoris* strain producing (+)-valencene, trans-nootkatol and (+)-nootkatone was constructed by Wriessnegger et al. (manuscript in revision). Co-expression of *PpICE2* did neither improve yields of terpenoids (Figure 8A) nor could an effect on CPR and HPO protein levels be detected (Figure 8B). (-)-limonene conversion was troublesome in *P. pastoris* although PM17 and CPR proteins were strongly detected in Western blots (Figure 7D and 7F). There are different studies describing toxic effects of (-)-limonene on yeast cells (Brennan et al., 2013; Liu et al., 2013). We assume that *P. pastoris* might have more efficient mechanisms to get rid of potentially cell-toxic substances such as monoterpenoids than *S. cerevisiae* (own unpublished results). However, conversion of (-)-limonene was only detected if *PpICE2* was co-expressed (7D). *Pp*Ice2p improved conversion of bufuralol 2.5-fold (7E). Interestingly, after 48 h of induction we observed a higher activity of reductases in all three *P. pastoris* co-expression systems (Table 3). Employing two different yeast expression hosts, *S. cerevisiae* and *P. pastoris*, we could document that over-expression of *ICE2* was beneficial for the conversion of two of three tested substrates per strain. Most remarkably, in all CYP/CPR systems tested a significant stabilization of cytochrome P450 reductases was observed in strains with up-regulated Ice2p levels suggesting that Ice2p is involved in stabilizing membrane protein levels and/or activities.

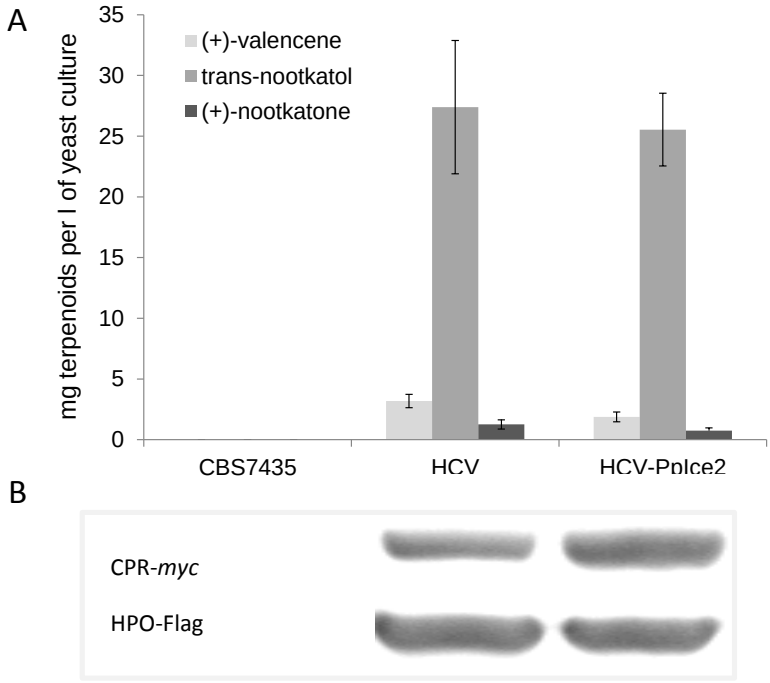



**Figure 8. Results of biphasic (+)-valencene whole-cell hydroxylation assay using *P. pastoris* strains HCV (expressing ValS, HPO and CPR) and HCV-*Pp*Ice2, additionally co-expressing *Pp*Ice2 (A). Immunological detection of HPO-flag and CPR-*myc* proteins in HCV and HCV-*Pp*Ice2 strains.**

# 4 Discussion

Regio- and stereoselective functionalization of (hydrophobic) compounds is of central interest in biocatalytic and metabolic engineering approaches. Highly specific hydroxylation reactions are performed by cytochrome P450 enzymes (CYPs). Therefore, cytochrome P450 activities have been in the focus of research over the last decades and are still heavily investigated. On the one hand, the discovery and characterization of novel CYPs with superior properties is ongoing and on the other hand, strategies to improve the applicability of CYPs and the yield of their applications are desired. Previous studies haven proven *S. cerevisiae* to be a suitable host for either conversion of externally added (+)-valencene (Gavira et al., 2013) or *in situ* production of the substrate combined with intracellular biotransformation to the desired products trans-nootkatol and (+)-nootkatone (Cankar et al., 2011). However, in these works terpenoid yields were rather low compared to other terpenoids produced with baker's yeast. For example, production of artemisinic acid and α-santalene, was enhanced by strain engineering including over-expression of truncated *HMG1* combined with a down-regulation of the *ERG9* gene to enable a redirected carbon flux towards the desired terpenoid products (Asadollahi et al., 2008; Asadollahi et al., 2009; Ro et al., 2006; Scalcinati et al., 2012). Based on these strategies, industrially relevant yields of g $L^{-1}$ of artemisinic acid and α-santalene were obtained.

In this study, we focused on improving activities of the cytochrome P450 enzyme HPO and reductase CPR pair. High amounts of terpenoids, in particular monoterpenoids and sesquiterpenoids, have been discussed to be detrimental for cell viability causing alterations of cell wall stability, intracellular accumulation of reactive oxygen species and wielding bad influence on membrane enzyme activities (Brennan et al., 2013; Gavira et al., 2013; Liu et al., 2013). Therefore, the optimal amount of (+)-valencene added to resting cells assays was found to be 2 mM. Higher amounts of 5 mM to 10 mM (+)-valencene did not lead to higher conversion rates, but on the contrary reduced product formation, which has also been shown lately by Gavira et al. (2013). Handling of fragrants and, therefore, extremely volatile hydrophobic substrates in whole-cell conversions in the laboratory is delicate. Especially in case of resting cells bioconversions a huge loss of (+)-valencene and (-)-limonene has to be taken into account. To find a balance between lowering substrate loss and keeping cells sufficiently aerated, Pyrex tubes were only sealed loosely with caps during resting cells bioconversions. As soon as the substrate was hydroxylated to the product trans-nootkatol (HPO/CPR) or isopiperitenol (PM17/CPR), volatility was lowered drastically. Gavira et al. (2013) managed to produce 11 mg $L^{-1}$ trans-nootkatol upon external addition of (+)-valencene to cell cultures. In this study, we extracted up to 606 ng $\mu L^{-1}$ trans-nootkatol in 500 µL of ethyl acetate from resting cells of 25 mL of $P_{GAL1}$-*ICE2* cell cultures (Figure 3B). This gives a calculated total amount of 12.1 mg of trans-

nootkatol per liter of yeast culture. However, self-sufficiently producing and converting cells yielded up to 30 mg $L^{-1}$ trans-nootkatol underscoring that phase transfer and product toxicity is a main issue in terpenoid formation and conversion. Additionally, intracellular production of terpenoid backbones is much easier to handle as it is less tedious and more efficient. Recently, the same (+)-valencene synthase we used in our work was published to yield 1.34 mg (+)-valencene per L of yeast culture (Beekwilder et al., 2014). In 2011, Cankar et al. managed to produce 0.93 mg trans-nootkatol per L *in vivo*. Inspired by the cultivation protocol for *P. pastoris*, we optimized cultivation conditions by introducing a prolonged growth phase followed by milder induction conditions using less of the inducing sugar galactose. This seemed to strengthen cells, letting them grow to higher initial cell densities and, hence, redirecting the metabolic flux to terpenoid products after induction. Thus, we obtained 30 mg $L^{-1}$ trans-nootkatol, which is the highest value reported for *S. cerevisiae* so far.

Previous studies have shown that *ice2* knockout cells have changed patterns of cortical and peripheral ER membrane distribution compared to wild type cells. Peripheral ER membranes close to the plasma membrane were reduced (Estrada De Martin et al., 2005). We confirmed this effect by electron microscopy, but also showed reversion of this phenotype upon over-expression of HPO and CPR in the Δ*ice2* knockout strain. Obviously, forcing ER membranes to proliferate and form staggered layers due to higher abundance of ER linked membrane proteins (Wright et al., 1988) causes a wild-type like ER distribution in the Δ*ice2* strain (Figure 6).

A time dependent analysis of samples revealed that the $P_{Gal1}$-*ICE2*-$His_6$ strain still produced trans-nootkatol after 48 h of conversion, which was not the case for the control strain. Proven by Western blot analysis and activity assays the CPR proteins turned out to be more stable at later time-points, in both, *S. cerevisiae* and *P. pastoris* strains. Of course, stabilization of the reductase will not sustain hydroxylation activity on its own, because hydroxylation is undoubtedly performed by the cytochrome P450 enzyme itself. Yet, it is assumed that the reductase is responsible for higher mRNA stability of P450 enzymes (Dong et al., 2013) and better stabilization of P450 enzymes (Murataliev et al., 2008) and, therefore, is counteracting cellular degradation pathway.

Previously, the Δ*ice2* knockout mutant was shown to have severe growth defects under low $Zn^{2+}$ conditions assuming Ice2p to play a role in $Zn^{2+}$ pumping (North et al., 2012). No direct link could be found explaining the connection of endoplasmic reticulum $Zn^{2+}$ level and properties of the reductase, neither on stability nor on activity. However, the question hovers, if high abundance of Ice2p itself influences stability and/or activity of the reductase, or maybe acts on another protein causing indirectly the stabilizing effect. The real function of Ice2p on stability of CPR remains to be clarified.

# 5 Acknowledgement

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# 6 References


Amberg, D.C., Burke, D.J., Strathern, J.N., 2006. Tetrad dissection. CSH protocols 2006, pdb.prot4181.

Asadollahi, M.A., Maury, J., Møller, K., Nielsen, K.F., Schalk, M., Clark, A., Nielsen, J., 2008. Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: effect of *ERG9* repression on sesquiterpene biosynthesis. Biotechnology and bioengineering 99, 666–77.

Asadollahi, M.A., Maury, J., Patil, K.R., Schalk, M., Clark, A., Nielsen, J., 2009. Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through in silico driven metabolic engineering. Metabolic Engineering 11, 328–334.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Wiley, C.J., Allison, R.D., Bittner, M., Blackshaw, S., 2003. Current Protocols in Molecular Biology Current Protocols in Molecular Biology, Molecular Biology. John Wiley & Sons, Inc.

Beekwilder, J., Van Houwelingen, A., Cankar, K., Van Dijk, A.D.J., De Jong, R.M., Stoopen, G., Bouwmeester, H., Achkar, J., Sonke, T., Bosch, D., 2014. Valencene synthase from the heartwood of Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene. Plant biotechnology journal 12, 174–182.

Bonneaud, N., Ozier-Kalogeropoulos, O., Li, G.Y., Labouesse, M., Minvielle-Sebastia, L., Lacroute, F., 1991. A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae/E. coli* shuttle vectors. Yeast (Chichester, England) 7, 609–15.

Botstein, D., and Davis, R.W., 1982. The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 607–636.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J, Hieter, P., Boeke, J.D., 1998. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast (Chichester, England) 14, 115–32.

Brennan, T.C.R., Krömer, J.O., Nielsen, L.K., 2013. Physiological and transcriptional responses of *Saccharomyces cerevisiae* to d-limonene show changes to the cell wall but not to the plasma membrane. Applied and environmental microbiology 79, 3590–600.

Brown, M.S., Goldstein, J.L., 1980. Multivalent feedback regulation of *HMG* CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. Journal of lipid research 21, 505–17.

Cankar, K., Van Houwelingen, A., Bosch, D., Sonke, T., Bouwmeester, H., Beekwilder, J., 2011. A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene. FEBS Letters 585, 178–182.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*, FEMS Microbiology Reviews.

Chambon, C., Ladeveze, V., Servouse, M., Blanchard, L., Javelot, C., Vladescu, B., Karst, F., 1991. Sterol pathway in yeast. Identification and properties of mutant strains defective in mevalonate diphosphate decarboxylase and farnesyl diphosphate synthetase. Lipids 26, 633–6.

Chang, M.C.Y., Keasling, Jay D, 2006. Production of isoprenoid pharmaceuticals by engineered microbes. Nature chemical biology 2, 674–681.

Choudhary, V., Schneiter, R., 2012. Pathogen-Related Yeast (PRY) proteins and members of the CAP superfamily are secreted sterol-binding proteins. Proceedings of the National Academy of Sciences of the United States of America 109, 16882–7.

Danielson, P.B., 2002. The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. Current drug metabolism 3, 561–97.

Davies, B.S.J., Rine, Jasper, 2006. A role for sterol levels in oxygen sensing in *Saccharomyces cerevisiae.* Genetics 174, 191–201.

Donald, K.A., Hampton, R Y, Fritz, I.B., 1997. Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. Applied and environmental microbiology 63, 3341–4.

Dong, M.-S., Lee, S.-B., Kim, H.-J., 2013. Co-expression of human cytochrome b5 increases expression of cytochrome P450 3A4 in *Escherichia coli* by stabilizing mRNA. Protein expression and purification 89, 44–50.

Estrada De Martin, P., Du, Y., Novick, P., Ferro-Novick, S., 2005. Ice2p is important for the distribution and structure of the cortical ER network in *Saccharomyces cerevisiae*. Journal of Cell Science 118, 65–77.

Farhi, M., Marhevka, E., Masci, T., Marcos, E., Eyal, Y., Ovadis, M., Abeliovich, H., Vainstein, A., 2011. Harnessing yeast subcellular compartments for the production of plant terpenoids. Metabolic engineering 13, 474–81.

Fraatz, M.A., Berger, R.G., Zorn, H., 2009. Nootkatone-a biotechnological challenge. Applied microbiology and biotechnology 83, 35–41.

Gavira, C., Höfer, R., Lesot, A., Lambert, F., Zucca, J., Werck-Reichhart, Danièle, 2013. Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*. Metabolic engineering 18, 25–35.

Geier, M., Braun, A., Emmerstorfer, A., Pichler, H., Glieder, A., 2012. Production of human cytochrome P450 2D6 drug metabolites with recombinant microbes - a comparative study. Biotechnology journal 1–13.

Ghaemmaghami, S., Huh, W.-K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., Weissman, J.S., 2003. Global analysis of protein expression in yeast. Nature 425, 737–41.

Gietz, R.D., Schiestl, R.H., 1995. Transforming yeast with DNA. Methods in Molecular and Cellular Biology 5, 255–269.

Girhard, M., Machida, K., Itoh, M., Schmid, R.D., Arisawa, A., Urlacher, V.B., 2009. Regioselective biooxidation of (+)-valencene by recombinant E. coli expressing CYP109B1 from Bacillus subtilis in a two-liquid-phase system. Microbial cell factories 8, 36.

Gonzalez, F.J., Korzekwa, K.R., 1995. Cytochromes P450 expression systems. Annual review of pharmacology and toxicology 35, 369–90.

Gu, J., Weng, Yan, Zhang, Q.-Y., Cui, H., Behr, M., Wu, L., Yang, W., Zhang, Li, Ding, X., 2003. Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. The Journal of Biological Chemistry 278, 25895–25901.

Hampton, R Y, Rine, J, 1994. Regulated degradation of *HMG*-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. The Journal of cell biology 125, 299–312.

Hampton, R., Dimster-Denk, D., Rine, J, 1996. The biology of *HMG*-CoA reductase: the pros of contra-regulation. Trends in biochemical sciences 21, 140–5.

Henderson, C.J., Otto, D.M.E., Carrie, D., Magnuson, M.A., McLaren, A.W., Rosewell, I., Wolf, C.R., 2003. Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. The Journal of Biological Chemistry 278, 13480–13486.

Ihrig, J., Hausmann, A., Hain, A., Richter, N., Hamza, I., Lill, R., Mühlenhoff, U., 2010. Iron regulation through the back door: iron-dependent metabolite levels contribute to transcriptional adaptation to iron deprivation in *Saccharomyces cerevisiae*. Eukaryotic cell 9, 460–71.

Jacquier, N., Schneiter, R., 2012. Mechanisms of sterol uptake and transport in yeast. The Journal of steroid biochemistry and molecular biology 129, 70–8.

Karp, F., Mihaliak, C.A., Harris, J.L., Croteau, R., 1990. Monoterpene biosynthesis: specificity of the hydroxylations of (-)-limonene by enzyme preparations from peppermint (*Mentha piperita*), spearmint (*Mentha spicata*), and perilla (*Perilla frutescens*) leaves. Archives of biochemistry and biophysics 276, 219–26.

Kirby, J., Keasling, J. D., 2009. Biosynthesis of plant isoprenoids: perspectives for microbial engineering. Annual review of plant biology 60, 335–355.

Koressaar, T., Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics (Oxford, England) 23, 1289–91.

Kwiatkowski, T.J., Zoghbi, H.Y., Ledbetter, S.A., Ellison, K.A., Chinault, A.C., 1990. Rapid identification of yeast artificial chromosome clones by matrix pooling and crude lysate PCR. Nucleic acids research 18, 7191–2.

Liu, J., Zhu, Y., Du, G., Zhou, J., Chen, Jian, 2013. Response of *Saccharomyces cerevisiae* to D-limonene-induced oxidative stress. Applied Microbiology and Biotechnology 97, 6467–75.

Loeper, J., Louérat-Oriou, B., Duport, C., Pompon, D., 1998. Yeast expressed cytochrome P450 2D6 (CYP2D6) exposed on the external face of plasma membrane is functionally competent. Molecular pharmacology 54, 8–13.

Longtine, M.S., McKenzie, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., Pringle, J.R., 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast Chichester England 14, 953–61.

Lupien, S., Karp, F., Ponnamperuma, K., Wildung, M., Croteau, R., 1995. Cytochrome P450 limonene hydroxylases of *Mentha* species. Drug metabolism and drug interactions 12, 245–60.

Majetich G., Behnke M., H.K., 1985. A Stereoselective Synthesis of (+/-)-Nootkatone and (+/-)-Valencene via an Intramolecular Sakurai Reaction. J. Org. Chem 50, 3615–3618.

Murakami, H., Yabusaki, Y., Ohkawa, H., 1986. Expression of rat NADPH-cytochrome P-450 reductase cDNA in *Saccharomyces cerevisiae.* DNA (Mary Ann Liebert, Inc.) 5, 1–10.

Murataliev, M.B., Guzov, V.M., Walker, F.A., Feyereisen, R., 2008. P450 reductase and cytochrome b5 interactions with cytochrome P450: effects on house fly CYP6A1 catalysis. Insect biochemistry and molecular biology 38, 1008–15.

Näätsaari, L., Mistlberger, B., Ruth, C., Hajek, T., Hartner, F.S., Glieder, A., 2012. Deletion of the *Pichia pastoris KU70* homologue facilitates platform strain generation for gene expression and synthetic biology. PloS one 7, e39720.

Nishida, N., Jing, D., Kuroda, K., Ueda, M. Activation of signaling pathways related to cell wall integrity and multidrug resistance by organic solvent in *Saccharomyces cerevisiae*. Current Genetics, Epub ahead of print Dec 31, 2013

Nishida, N., Ozato, N., Matsui, K., Kuroda, K., Ueda, M., 2013b. ABC transporters and cell wall proteins involved in organic solvent tolerance in *Saccharomyces cerevisiae*. Journal of biotechnology 165, 145–52.

North, M., Steffen, J., Loguinov, A. V, Zimmerman, G.R., Vulpe, C.D., Eide, D.J., 2012. Genome-Wide Functional Profiling Identifies Genes and Processes Important for Zinc-Limited Growth of Saccharomyces cerevisiae. PLoS Genetics 8, e1002699.

Omura, T., 2010. Structural diversity of cytochrome P450 enzyme system. Journal of Biochemistry 147, 297–306.

Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. The Journal of biological chemistry 239, 2370–8.

Parks, L.W., Smith, S.J., Crowley, J.H., 1995. Biochemical and physiological effects of sterol alterations in yeast-a review. Lipids 30, 227–30.

Phillips, A.H., Langdon, R.G., 1962. Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. The Journal of biological chemistry 237, 2652–60.

Polakowski, T., Stahl, U., Lang, C., 1998. Overexpression of a cytosolic hydroxymethylglutaryl-CoA reductase leads to squalene accumulation in yeast. Applied Microbiology and Biotechnology 49, 66–71.

Pompon, D., Louerat, B., Bronine, A., Urban, P., 1996. Yeast expression of animal and plant P450s in optimized redox environments. Methods in enzymology 272, 51–64.

Reed, S.I., Ferguson, J., Jahng, K.Y., 1988. Isolation and characterization of two genes encoding yeast mating pheromone signaling elements: CDC72 and CDC73. Cold Spring Harbor symposia on quantitative biology 53 Pt 2, 621–7.

Riezman, H, Hase, T., Van Loon, A.P., Grivell, L.A., Suda, K., Schatz, G., 1983. Import of proteins into mitochondria: a 70 kilodalton outer membrane protein with a large carboxy-terminal deletion is still transported to the outer membrane. The EMBO journal 2, 2161–8.

Ro, D.-K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C.Y., Withers, S.T., Shiba, Y., Sarpong, R., Keasling, J. D., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940–943.

Salvador, J.A.R., Clark, J.H., 2002. The allylic oxidation of unsaturated steroids by tert-butyl hydroperoxide using surface functionalised silica supported metal catalysts. Green Chemistry 4, 352–356.

Scalcinati, G., Knuf, C., Partow, S., Chen, Y., Maury, J., Schalk, M., Daviet, L., Nielsen, J., Siewers, V., 2012. Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene α-santalene in a fed-batch mode. Metabolic engineering 14, 91–103.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nature methods 9, 676–82.

Song, L., 2003. Detection of farnesyl diphosphate accumulation in yeast *ERG9* mutants. Analytical biochemistry 317, 180–5.

Takahashi, S., Yeo, Y.-S., Zhao, Yuxin, O´Maille, P.E., Greenhagen, B.T., Noel, J.P., Coates, R.M., Chappell, J., 2007. Functional characterization of premnaspirodiene oxygenase, a cytochrome P450 catalyzing regio- and stereo-specific hydroxylations of diverse sesquiterpene substrates. The Journal of Biological Chemistry 282, 31744–31754.

Truan, G., Cullin, C., Reisdorf, P., Urban, P., Pompon, D., 1993. Enhanced in vivo monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b5. Gene 125, 49–55.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3-new capabilities and interfaces. Nucleic acids research 40, e115.

Urban, P., Werck-Reichhart, D, Teutsch, H.G., Durst, F., Regnier, S., Kazmaier, M., Pompon, D., 1994. Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway. European journal of biochemistry / FEBS 222, 843–50.

Wawrzycka, D., 2011. The ABC transporters of *Saccharomyces cerevisiae*. Postepy biochemii 57, 324–32.

Weis, R., Luiten, R., Skranc, W., Schwab, H., Wubbolts, M., Glieder, A., 2004. Reliable high-throughput screening with *Pichia pastoris* by limiting yeast cell death phenomena. FEMS yeast research 5, 179–89.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., et al., 1999. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science (New York, N.Y.) 285, 901–6.

Wright, R., Basson, M., D'Ari, L., Rine, J, 1988. Increased amounts of *HMG*-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. The Journal of Cell Biology 107, 101–114.

Zhuge, J., Yu, Y.-N., Wu, X.-D., 2004. Stable expression of human cytochrome P450 2D6*10 in HepG2 cells. World Journal of Gastroenterology 10, 234–237.

# Supplemental information

**Table S1: Sequences of codon optimized genes PM17, CYP2D6 and hCPR**

| PM17 |
| --- |
| atggaattgcaaatctcttctgccatcatcattttggttgtcacttacaccatttctttgttgatcatcaagcaatggagaaaaccaaagcctcaaga<br>aaacttgccaccaggtccaccaaagttgccattgattggtcacttgcatttgttatggggtaagttgcctcaacacgctttggcttccgttgctaagc<br>aatacggtccagttgctcacgttcaattgggtgaagttttctctgttgttttgtcctccagagaagctaccaaggaagctatgaaattggttgaccca<br>gcttgtgctgacagattcgaatccattggtaccaagatcatgtggtacgacaacgatgacattattttctctccatactccgtccactggagacaaa<br>tgagaaaaatctgtgtttctgaattactatctgccagaaacgtccgttctttcggtttcatcagacaagatgaagtttccagattattgggtcatttg<br>agatcctctgctgctgctggtgaagctgtcgatttgactgaaagaattgccactttgacctgttccatcatctgtcgtgctgctttcggttctgttatca<br>gagaccacgaagaattggttgaattggtcaaggacgctttgtccatggcttctggtttcgaattggctgacatgttcccatcttccaagttgttgaac<br>ttactatgttggaacaaatccaaattatggagaatgagaagaagagtcgatgccatcttggaagccattgtcgaagaacacaagttgaagaagt<br>ctggtgaatttggaggtgaagatatcattgatgtcttattcagaatgcaaaaggactctcaaatcaaggttccaatcaccaccaatgctatcaagg<br>ctttcatcttcgataccttctccgctggtactgaaacttcttccaccaccactttatgggttatggctgaattgatgagaaacccagaagtcatggcc<br>aaggctcaagctgaagtcagagctgctttgaagggtaagactgactgggatgttgacgatgtccaagaattgaaatacatgaagtctgttgtcaa<br>ggaaaccatgagaatgcacccaccaattccattgattccacgttcttgtcgtgaagaatgtgaagtcaacggttacaccattccaaacaaggcta<br>gaatcatgatcaacgtctggtccatgggtagaaacccattatactgggaaaagccagaaactttctggccagaaagattcgaccaagtttctcgt<br>gacttcatgggtaacgatttcgaatttattccattcggtgccggtagaagaatctgtccaggtttgaactttggtttggccaacgtcgaagttccatt<br>ggctcaattgttgtaccacttcgactggaaattggctgaaggtatgaacccatctgacatggacatgtctgaagctgaaggtttgactggtatcag<br>aaagaacaacttgttgttggttccaactccatacgacccttcctctgattacaaggatgacgacgataagtaataa |
| CYP2D6 |
| atgggtttggaagccttggtcccattagctgttatcgttgccatcttcttgttgttggttgacttgatgcacagaagacaaagatgggctgctcgttac<br>ccaccaggtccattaccattaccaggtttgggtaacttgttgcacgtcgatttccaaaacactccatactgtttcgaccaattgagaagaagattcg<br>gtgatgtcttctccttgcaattggcttggactccagttgttgtcttgaacggtttggctgccgttagagaagccttggttacccacggtgaagacact<br>gctgacagacctcctgttccaatcactcaaatcttaggtttcggtccacgttctcaaggtgttttcttggctcgttacggtccagcttggagagaaca<br>aagaagattctctgtttccactttgagaaacttgggtttaggtaagaagtctttggaacaatgggttactgaagaagctgcttgtttgtgtgctgcct<br>ttgctaaccactccggtagaccattcagaccaaacggtttgttggacaaggctgtctccaacgtcattgcttctttgacctgtggtagaagatttga<br>atacgatgacccaagattcttgagattgttggatttggctcaagaaggtttgaaggaagaatccggtttcttgagagaagtcttgaatgctgttcca<br>gtcttgctacatattccagctttggccggtaaggttttgagattccaaaaggctttcttgacccaattggacgaattgttgactgaacacagaatga<br>cctgggacccagctcaacctccaagagacttgactgaagccttcttggctgaaatggaaaaggccaagggtaacccagaatcctctttcaacgac<br>gaaaacttgtgtattgttgttgctgatttgttctccgctggtatggttaccacctctaccactttggcttggggtctattattgatgatcttgcacccag<br>atgtccaaagaagagttcaacaagaaattgacgatgtcatcggtcaagtccgtcgtccagaaatgggtgaccaagctcacatgccatacaccac<br>tgctgtcatccacgaagtccaaagatttggtgatatcgttccattaggtgtcacccacatgacctccagagacattgaagttcaaggtttccgtatt<br>ccaaagggtaccactttgatcaccaacttgtcttccgttttgaaggacgaagctgtctgggaaaagccattcagattccatccagaacacttcttgg |

| |
|---|
| atgctcaaggtcacttcgttaagccagaagccttcttgccattctctgctggtagaagagcttgtttgggtgaaccattggccagaatggaattatt<br>cttattcttcacttctctattacaacatttctctttctctgttccaactggtcaaccaagaccatctcaccacggtgtctttgctttcttggtcactccatc<br>tccatacgaattgtgtgctgtcccaagagattacaaggatgacgacgataagtaataa |
| hCPR |
| atgattaacatgggtgactcccacgttgacacctcttccactgtttctgaagctgttgctgaagaagtttctttgttctccatgactgacatgatctta<br>ttctctttgattgtcggtctattgacttactggttcttgtttagaaagaagaaggaagaagttccagaatttaccaagatccaaactttgacctcttct<br>gtcagagaatcttctttcgtcgaaaagatgaagaagactggtagaaacatcattgtcttctacggttctcaaaccggtactgctgaagaatttgct<br>aacagattatccaaggatgctcacagatacggtatgagaggtatgtccgctgacccagaagaatacgacttggctgatttgtcctctttgccagaa<br>atcgacaacgctttggttgttttctgtatggccacctacggtgaaggtgacccaactgacaacgctcaagatttctacgactggttacaagagactg<br>atgttgacttgtctggtgtcaagttcgctgtctttggtttgggtaacaagacctacgaacacttcaacgctatgggtaagtacgttgacaagagatt<br>agaacaattaggtgctcaaagaatcttcgaattgggtttgggtgatgatgacggtaatttggaggaagacttcatcacctggagagaacaattct<br>ggttagctgtctgtgaacacttcggtgttgaagccactggtgaagaatcctccatcagacaatacgaattggttgttcacactgacattgacgctgc<br>caaggtctacatgggtgaaatgggtagattgaaatcttacgaaaaccaaaagccaccattcgatgctaagaacccattcttggctgctgtcacca<br>ccaacagaaagttgaaccaaggtactgaaagacacttgatgcatttggaattggacatttctgactccaagatcagatacgaatctggtgaccac<br>gttgccgtctacccagccaacgattctgctttggtcaaccaattaggtaagattttgggtgctgatttggatgtcgttatgtccttgaacaacttgga<br>cgaagaatccaacaagaagcacccattcccatgtccaacttcttacagaaccgctttgacctactacttggatatcactaaccctccaagaaccaa<br>tgtcttatacgaattggctcaatacgcttctgaaccatctgaacaagaattgctaagaaagatggcttcctcttctggtgaaggtaaggaattgtac<br>ttgtcctgggttgtcgaagctcgtcgtcacattttggccattttgcaagactgtccatctttgagaccaccaattgaccatttgtgtgaattgttgcca<br>agattgcaagctagatactactccattgcttcttcttccaaggtccacccaaactccgttcacatctgtgccgttgttgtcgaatacgaaaccaagg<br>ctggtagaatcaacaagggtgttgccaccaactggttaagagccaaggaacctgttggtgaaaacggtggtagagctttggttccaatgtttgtca<br>gaaagtctcaattcagattaccattcaaggctaccactccagtcatcatggtcggtccaggtactggtgttgctccattcatcggtttcatccaaga<br>aagagcttggttaagacaacaaggtaaggaagtcggtgaaactttgttgtactacggttgtcgtcgttctgatgaagactatttgtacagagaaga<br>attggctcaattccaccgtgacggtgctttgactcaattgaacgttgctttctccagagaacaatctcacaaggtttacgttcaacatttgttgaagc<br>aagacagagaacatttgtggaaattgattgaaggtggtgctcacatctacgtctgtggtgatgctagaaacatggccagagatgtccaaaacact<br>ttctacgatattgttgctgaattgggtgccatggaacacgctcaagccgttgactacatcaagaaattgatgaccaagggtcgttactctttggatg<br>tctggagtgaacaaaaattgatttctgaagaggatttgtaataa |

# Concluding remarks and outlook

The overall goal of this thesis was to find ways to improve expression and, particularly, activity of cytochrome P450 monooxygenases for production of fine chemicals. Boosting cytochrome P450 expression and activities is important to enable their application in biotechnological production of high-value compounds. The challenge in this work was to heterologously express different CYPs in yeast hosts and establish suitable activity assays to screen for improved product formation. As illustrated in each of the chapters, different approaches were undertaken and evaluated for their potential in improving CYP function. For example, we have tested different expression hosts, made use of a *Saccharomyces cerevisiae* single-gene knockout collection and created yeast strains producing target compounds self-sufficiently from galactose as inducer and as carbon source.

*S. cerevisiae* was chosen as expression host because it is well established for production of recombinant proteins. An additional advantage is the availability of the EUROSCARF single-gene knockout collection, which was used as screening platform for elevated CYP activity. The inapplicability of an - at least partially - automated high-throughput screening procedure made testing of conversion efficiencies rather time consuming. However, different knockout targets were chosen from the strain deletion collection as background strains for HPO/CPR overexpression (see Chapter III). This approach led to the identification of several target gene knockout strains, which showed an increase in (+)-valencene conversion *in vitro*. One major result of the EUROSCARF knockout collection screening was that deletion of Δ*ice2* destabilizes CPR, whereas overexpression of *ICE2* supports stability of CPRs from human and plant sources. In case of HPO/CPR-mediated (+)-valencene hydroxylation, *ICE2* overexpression led to a 1.4-fold improvement.
Ice2p is a highly interesting protein with an as yet not fully understood cellular functionality. So far, it was found to play a major role in passing on ER membranes from the mother to the budding daughter cell. Deletion of *ice2* led to the deadlock of such ER transfers and the disappearance of peripheral ER membranes in general (Estrada De Martin et al., 2005; Tavassoli et al., 2013). Additional data suggest Ice2p to play a role in intracellular zinc homeostasis (North et al., 2012). Broad-range protein interaction studies (Gavin et al., 2006) revealed a lot of possible interaction partners for Ice2p, whereas the search in the string database gives totally different matches (http://string-db.org/newstring_cgi/show_network_section.pl) (Franceschini et al., 2013) These complementary results suggest that the Ice2p interaction network highly depends on outer factors such as cultivation conditions and cellular status. Starting own interaction studies with engineered strains cultivated under the own experimental conditions would be highly relevant to clarify Ice2p protein-protein interactions in the system presented in this thesis. In addition, the physiological cellular function of Ice2p under standard conditions needs to be investigated in more detail in the future.

Our engineered *S. cerevisiae* strain was capable of self-sufficiently producing 30 mg of trans-nootkatol per liter of cell broth. This is by far the highest level produced so far with baker's yeast (Beekwilder et al., 2014; Cankar et al., 2011). At the same time, we are aware of the fact that these production levels

represent a relatively low carbon efficiency compared to the theoretic maximum based on the total feedstock of carbon provided. This obvious gap exemplifies that there is still much room for improvement in terpenoid production efficiency.

Direct comparison of *S. cerevisiae* with *Pichia pastoris*, the second production platform established in our group, suggest that *P. pastoris* might be better suited for terpenoid production. We have chosen the two yeast strains because of their simple handling and convenient molecular engineering techniques. The huge advantage of working on two host systems in parallel is the possibility to compare efficiencies and determine differences. For example, the cultivation protocol for *in vivo* terpenoid production in *S. cerevisiae* could be optimized by adaptation of induction steps similar to those of the *P. pastoris* protocol. *P. pastoris* is a highly interesting production host due to its growth to extremely high cell densities and the availability of the highly inducible *AOX1* promoter (Gonçalves et al., 2013; Ramón and Marín, 2011). The overexpression of endogenous and *S. cerevisiae ICE2* in *P. pastoris* did not increase substrate conversion (data not shown), but the activity/stability of CPR was increased ~ 1.5-fold compared to the reference strain. This result indicates that *ICE2* has a similar effect on CPR stability in *P. pastoris* as shown for *S. cerevisiae*, which, however, appears not to be crucial for product formation. Literature and in-house work reveal *Yarrowia lipolytica* to be a highly attractive alternative host not only for expression of CYPs but also for conversion of hydrophobic substrates. This and maybe other yet unknown oleogenic microbes might be interesting hosts for the production of terpenoids and the metabolism of hydrophobic substances.

# References

Beekwilder, J., Van Houwelingen, A., Cankar, K., Van Dijk, A.D.J., De Jong, R.M., Stoopen, G., Bouwmeester, H., Achkar, J., Sonke, T., Bosch, D., 2014. Valencene synthase from the heartwood of Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene. Plant biotechnology journal 12, 174–182.

Cankar, K., Van Houwelingen, A., Bosch, D., Sonke, T., Bouwmeester, H., Beekwilder, J., 2011. A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene. FEBS Letters 585, 178–182.

Estrada De Martin, P., Du, Y., Novick, P., Ferro-Novick, S., 2005. Ice2p is important for the distribution and structure of the cortical ER network in *Saccharomyces cerevisiae*. Journal of Cell Science 118, 65–77.

Franceschini, A., Szklarczyk, D., Frankild, S., Kuhn, M., Simonovic, M., Roth, A., Lin, J., Minguez, P., Bork, P., Von Mering, C., Jensen, Lars J, 2013. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic acids research 41, D808–15.

Gavin, A.-C., Aloy, P., Grandi, P., Krause, R., Boesche, M., Marzioch, M., Rau, C., Jensen, Lars Juhl, Bastuck, S., Dümpelfeld, B., Edelmann, A., Heurtier, M.-A., Hoffman, V., Hoefert, C., Klein, K., Hudak, M., Michon, A.-M., Schelder, M., Schirle, M., Remor, M., Rudi, T., Hooper, S., Bauer, A., Bouwmeester, T., Casari, G., Drewes, G., Neubauer, G., Rick, J.M., Kuster, B., Bork, P., Russell, R.B., Superti-Furga, G., 2006. Proteome survey reveals modularity of the yeast cell machinery. Nature 440, 631–6.

Gonçalves, A.M., Pedro, A.Q., Maia, C., Sousa, F., Queiroz, J.A., Passarinha, L.A., 2013. *Pichia pastoris*: a recombinant microfactory for antibodies and human membrane proteins. Journal of microbiology and biotechnology 23, 587–601.

North, M., Steffen, J., Loguinov, A. V, Zimmerman, G.R., Vulpe, C.D., Eide, D.J., 2012. Genome-Wide Functional Profiling Identifies Genes and Processes Important for Zinc-Limited Growth of *Saccharomyces cerevisiae*. PLoS Genetics 8, e1002699.

Ramón, A., Marín, M., 2011. Advances in the production of membrane proteins in *Pichia pastoris*. Biotechnology journal 6, 700–706.

Tavassoli, S., Chao, J.T., Young, B.P., Cox, R.C., Prinz, W.A., De Kroon, A.I.P.M., Loewen, C.J.R., 2013. Plasma membrane-endoplasmic reticulum contact sites regulate phosphatidylcholine synthesis. EMBO reports 1–7.

# Appendix:

# Enzymatic Aerobic Alkene Cleavage Catalyzed by a $Mn^{3+}$-Dependent Proteinase A Homologue

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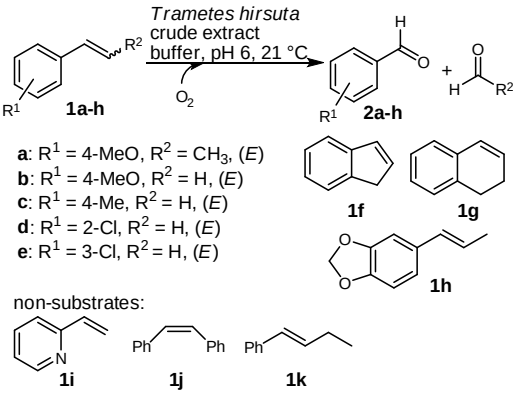
Mild, selective, safe and environmentally benign oxidation methods have gained increased importance.[1] However, the chemical cleavage of alkenes[2] to give aldehydes or ketones is still preferentially run by ozonization[3] or stoichiometric amounts of metal salts.[4] Alternative methods require for instance hypervalent iodine[5] or *N*-hydroxyphthalimide in the presence of molecular oxygen.[6] Although various enzymes have been described for alkene cleavage,[7] only few are applied for non-natural substrates. For instance, a crude preparation of the white-rot basidiomycete *Trametes hirsuta* FCC047 has been employed also on a preparative scale to cleave a C=C double bond adjacent to a phenyl moiety with molecular oxygen leading to benzaldehyde derivatives [8] (Scheme 1). For molecules bearing at least two alkene groups, the preparation is able to distinguish between different C=C double bonds within the same molecule. [9]

Studies of the mechanism of alkene cleavage revealed a novel mechanism involving radical intermediates and incorporating oxygen atoms of two different molecules of molecular oxygen.[10] However, the exact type of enzyme and the nature of the involved cofactor remained unknown. We report here the identification of this enzyme and the unexpected metal dependence.




**Scheme 1: Alkene cleavage catalyzed by the fungus *Trametes hirsuta* FCC047 at the expense of molecular oxygen as the sole oxidant.**

Freeze dried cells of the fungus showed an activity of 1 U/mg for the cleavage of *t*-anethole **1a**. First attempts to purify the enzyme from *T. hirsuta* were unsuccessful. After two purification steps (hydrophobic interaction chromatography followed by ion exchange chromatography) no activity was detected anymore, even for the very well accepted substrate *t*-anethole **1a**. Inductively coupled plasma mass spectrometry (ICP/MS) analysis of the active protein fraction after one purification step (Hydrophobic interaction, phenyl sepharose CL-4B) revealed the presence of three possible metal cofactors: Cu, Mn or Mo. Unfortunately, external addition of common metal salts of $Cu^{1+}$, $Cu^{2+}$ or $Mn^{2+}$ as well as of various Mo-salts or cofactors did not restore enzyme activity. Additionally $Fe^{2+}$ and $Fe^{3+}$ were tested for enzyme activation, but did not lead to positive results. By serendipity, assaying the enzyme in the presence of $Mn^{2+}$ and hydrogen peroxide showed increased activity, which was

subsequently attributed to the *in situ* formation of manganese in oxidation state three. Consequently, the alkene cleaving activity was restored by the addition of $Mn^{3+}$ salts [e.g. Mn(III) acetate, 0.4 mM]. Fresh Mn(III) acetate on its own, did not show alkene cleaving activity under assay conditions.

Alkene cleaving enzymes described so far[7] are either $Fe^{2+}$ or $Cu^{2+}$ dependent, some also show activity in the presence of $Mn^{2+}$, $Ni^{2+}$, $Co^{2+}$. Except for photosystem II, Mn(III) is only found in few types of enzymes like manganese superoxide dismutases (SODs), catalases and pseudocatalases,[11] as well as ribonucleotide reductases.[12] The SODs catalyze the decomposition of $O_2^-$ and catalases/pseudocatalases destroy $H_2O_2$. SODs contain one $Mn^{3+}$ ion per subunit, while catalases have two manganese ions in their center. The Mn(III) ion is in general bound to the protein via O- and N-ligands.

For identifying fractions containing the desired enzyme during purification, Mn(III) acetate (0.4 mM) was added to the assay mixture; consequently, a single protein band was identified on SDS-PAGE at ~40 kDa responsible for the observed activity after three purification steps (see Supporting information for details). Analyzing the protein band by MALDI-MS/MS led to the identification of short internal peptide sequences (Figure S2). The peptide sequences indicated homology to aspartic peptidase A1 from *Laccaria bicolor*. The DNA sequence of the alkene cleaving enzyme (AlkCE) could be deduced from cDNA (1236 bp) by primer walking (SI chapter 4). The corresponding gene was also amplified from genomic DNA leading to a sequence with 1552 bp. Alignment of the two DNA sequences obtained from cDNA and genomic DNA revealed six intron sequences in the gene isolated from genomic DNA (SI 4.4). The introns comprised 50 to 60 bp each. The two corresponding sequences encoding AlkCE were identical except for one silent mutation in position 842 (cDNA).

The deduced amino acid sequence consisting of 412 amino acids (44639 Da) indicated that AlkCE belongs to the fungal pepsin-retropepsin superfamily, showing high similarity to proteinase A from fungi. The highest sequence identity was noted for aspartic peptidase A1 from *Trametes versicolor* FP-101664 SS1 (90%), while 78% identity was found for aspartic peptidase A1 from *Laccaria bicolor* S238N-H82.

Alkene cleaving activity of the identified enzyme was confirmed by heterologous expression of AlkCE in *E. coli* with external addition of Mn(III) acetate to the assay, although no prominent band on SDS-PAGE was detectable. Consequently, a C-terminal $His_6$-tagged AlkCE was expressed in *E. coli* CodonPlus RP(DE3) and purified by affinity chromatography. Activity was also confirmed for the $His_6$-purified enzyme. Testing Mn(III) binding to a $His_6$-tagged alcohol dehydrogenase showed that potential complexation of Mn(III) by the tag did not contribute to activity. Active soluble enzyme could only be obtained in *E. coli* cell lines which either contained chaperone proteins [*E. coli* BL21(DE3).pTf16] or had a special machinery to overcome the codon discrepancy of heterologously expressed protein [*E. coli* BL21 CodonPlus RP(DE3)].

Proteinase A from *Saccharomyces cerevisiae* is the closest related enzyme for which a crystal structure is available (PDB ID: 1DP5). It shows 64% sequence identity with AlkCE and is expressed as a 405-amino acid residue precursor which is subsequently proteolytically cleaved to a 329-amino acid mature enzyme of 41.5 kDa[13] with 8.5% neutral sugar and 1% glucosamine attached to $Asn^{67}$ and $Asn^{266}$. Comparisons of the AlkCE sequence with proteinase A and other homologous enzymes suggested the presence of a pre-pro region. Consequently, an AlkCE gene was synthesized coding for a truncated protein (trunc-AlkCE) with the first 86 amino acid residues (the pre-pro-region) removed (35.6 kDa for 328 AAs). Additionally, the gene was codon-optimized for expression in *E. coli*. The truncated enzyme also fits the molecular mass observed for the wild type enzyme purified from *T. hirsuta* (~40 kDa considering glycosylation).

The truncated enzyme showed highest alkene cleaving activity if subcloned in the vector pET-28a(+) (N-terminal $His_6$-tag) and overexpressed in *E. coli* BL21(DE3).pTf16. Significantly improved overexpression was observed for the truncated version of the protein leading to a prominent band on SDS-PAGE. However, most of the protein was in the insoluble fraction (SI chapter 6). Nevertheless, the enzyme in the soluble fraction was purified by affinity chromatography and the activity was confirmed as well as the expression by Western blot analysis using an anti-His antibody.

Since sequence homology suggested AlkCE to have protease activity, the purified elution fractions of trunc-AlkCE were tested for protease activity using a commercial kit (SI chapter 6.5). Indeed, protease activity could be detected and was additionally confirmed by observed degradation products of the concentrated purified enzyme (Figure S8). This is likely the reason, why expression following standard protocols led only to low amounts of soluble protein making kinetic studies unreliable.

The homology model of AlkCE (Figure 1 and 2) based on the structure of proteinase A from *S. cerevisiae* suggested that in addition to the two aspartic acid residues ($Asp^{118}$ and $Asp^{300}$), which represent the common active site residues of aspartic proteases,[14] $Thr^{303}$ might also be involved in Mn-binding.

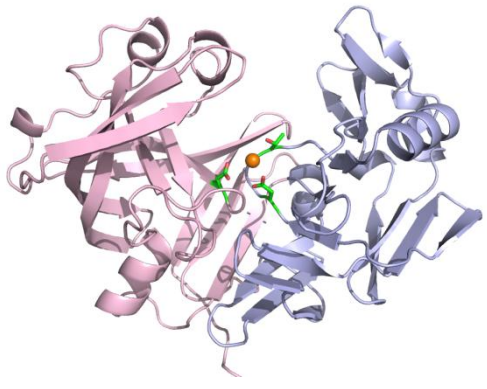


**Figure 1: Overall structure of the homology model of AlkCE based on proteinase A from *Saccharomyces cerevisiae* (sharing 64% sequence identity).**

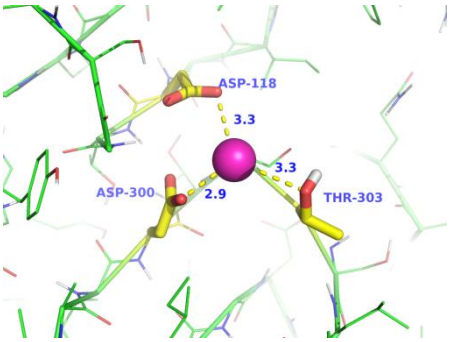



**Figure 2: Close-up of the active site region. Active site showing the two conserved Asp$^{118}$ and Asp$^{300}$ residues of aspartic peptidases. A manganese ion was placed into this site suggesting coordination to Asp$^{118}$, Asp$^{300}$ and Thr$^{303}$.**

To verify the role of these three residues in Mn(III)-binding, each residue was replaced by valine. The three single-point variants (D118V, D300V, T303V) were overexpressed in *E. coli* leading to clear visible bands in the soluble fraction on SDS-PAGE indicating the integrity of the expressed variant (Figure S9). None of the $His_6$-purified variants showed alkene cleavage activity (SI chapter 8, Table S6), supporting the role of the residues in forming a Mn(III)-binding site.

Interestingly, all $Mn^{3+}$-dependent enzymes described so far have at least one N-ligand, mostly His, for metal binding.[15] Binding of Mn(III) exclusively via O-ligands as found here for AlkCE was not described yet for an enzyme, to the best of our knowledge. To support that Mn(III) bound to O-ligands might be responsible for alkene cleavage activity, a metal complex Mn(III) acetylacetonate [$Mn(acac)_3$] was tested under similar conditions as the enzyme.[16] Acetylacetonate as a bidentate ligand is binding through two oxygen atoms. Obviously $Mn(acac)_3$ cannot be the active species in solution, since all its coordination sites are filled; however, it is in equilibrium with derived species loosing at a first stage at least one ligand thereby offering free coordination sites. As a control Mn(III) with an N-ligand [2,3,7,8,12,13,17,18-Octaethyl-21*H*,23*H*-porphine manganese(III) chloride] was tested. Indeed, employing the reaction conditions as in the enzymatic assay, $Mn(acac)_3$ transformed *t*-anethole **1a** and other alkenes **1b-1h** at the expense of molecular oxygen similar to the enzyme (Table S7); thus, the substrates transformed coincide with the substrates previously published for the crude enzyme preparation from *T. hirsuta*,[8a] also concerning non-substrates **1i-1k**. In contrast, Mn(III)-porphine transformed only *t*-anethole **1a** and indene **1f**, but none of the other substrates showing a clear difference in the substrate spectrum compared to $Mn(acac)_3$ and therefore supporting that Mn(III) needs the O-ligands for the observed substrate spectrum.

Although the substrate spectrum of $Mn(acac)_3$ and AlkCE was similar, the chemoselectivity of AlkCE for the transformation of the alkene to the aldehyde was in general higher compared to $Mn(acac)_3$, thus less side products were formed with AlkCE. A possible reason could be that the intermediate radicals are better shielded in the cavity of the enzyme leading to less side reactions than in the bulk medium. Comparing the activity of $Mn(acac)_3$ with the enzyme, the enzyme was at least

about 1500 times more active than $Mn(acac)_3$ under the conditions employed, which might indicate that the protein environment modulates the reactivity as already described for iron dependent enzymes.[17]

White-rot basidiomycetes like *T. hirsuta*, are well known to secrete a wide range of class II peroxidases, which traditionally include the lignin peroxidases and manganese peroxidases (MnP).[18] MnP preferentially oxidizes $Mn^{2+}$ ions, present in the wood and soil, into reactive $Mn^{3+}$. Hence, there is a potential source of $Mn^{3+}$ which can be bound by other enzymes in the fungus. The combination of Mn(III) and an existing protein backbone may provide the organism with a new promiscuous activity.[19]

Summarising, we have identified an unprecedented Mn(III) dependent enzyme cleaving alkenes at the expense of molecular oxygen, whereby $Mn^{3+}$ is bound exclusively via oxygen ligands which is in contrast to other known Mn(III)-dependent enzymes. The potential of the Mn(III) enzyme awaits further exploitation as well as elucidation of its detailed mode of oxygen activation[20] thereby expanding the toolbox for biocatalytic transformations.[21]

# Experimental Section

## 1. Materials and equipments

*t*-Anethole **1a** and reference compound *p*-anisaldehyde **2a** as well as **1b**-**k**, **2b**-**k** were obtained from commercial sources (Sigma-Aldrich, Lancaster and Acros, Austria) with highest purity available. 2,3,7,8,12,13,17,18-Octaethyl-21*H*,23*H*-porphine manganese(III) chloride and Mn(III) acetylacetonate were purchased from Sigma Aldrich. Phusion® High-Fidelity PCR Kit (NEB) and Thermal cycler PCR system (Eppendorf) was used for PCR. DNA ligase and restriction enzymes were purchased from Fermentas. Mass loading dye (6x) and GeneRuler™ DNA ladder mix from Fermentas, SYBR Safe DNA stain (10.000x conc. in DMSO) from Invitrogen and MINI-SUB® CELL GT agarose gel station from Bio-Rad were used for Agarose gel electrophoresis. Uvette® 220-1600 nm cuvettes and Biophotometer Plus from Eppendorf were used for photometric measurements. QIAprep® Spin Miniprep Kit (250) and QIAquick® Gel Extraction Kit (50) from Qiagen were used. Isopropyl-D-thiogalactopyranoside (IPTG) was purchased from PEQlab. Bio-Rad BioLogic DuoFlow Protein Purification System was used for protein purification at 4 °C. Bio-Rad Mini-Protean Tetra-cell system was used for SDS-PAGE. Precision Plus Protein™All Blue standard (pre-stained, Bio-Rad, 161-0373) was used as the protein standard for SDS-PAGE. VIVASPIN 6; Membrane: 50 000 MWCO PES (6 mL) from SARTORIUS was used for concentrating protein. Branson digital sonifier and Sorvall RC 5C PLUS cooling centrifuge were used for obtaining the cell-free extract for purification. Protein bands on SDS-PAGE were visualized by Coomassie blue staining (0.05% Coomassie R in 10% acetic acid) for 30 min followed by destaining (MeOH/HOAc/$H_2O$ = 3:1:6). The protein concentrations were determined using Bradford method (Bio-Rad protein assay reagent). The protein concentration was determined by calibration against BSA.

Primers were ordered at MWG Eurofins. Clontech SMART™ cDNA Library Kit (Clontech) was used for cDNA construction. CloneJET™ PCR Cloning kit (Thermo Scientific) was used for cloning. *DNA Walking SpeedUp™ Premix Kit* (Seegene) was used for primer walking. nitrocellulose membrane (GE Healthcare, Chalfont St Giles, UK), NuPAGE 4–12% Bis-Tris Gel (Invitrogen), rabbit anti-His primary antibody (Thermo Scientific), anti-rabbit secondary antibody linked to horse radish peroxidase (Sigma), Super Signal West Pico chemoluminescent substrate (Thermo Scientific) and Syngene G:BOX were used for Western blot analysis. QuantiCleave™ Protease Assay Kit (Thermo Scientific) was used for Protease assay. Quikchange XL Lightning site directed mutagenesis kit (Agilent technologies) was used for site directed mutagenesis. Chemically competent *E. coli* cell lines were purchased from Invitrogen.

## 2. Identification of AlkCE from Trametes hirsuta on SDS-PAGE

### 2.1. Assay for alkene cleavage during purification

Samples from purification steps (700-1000 µL) were placed in the wells of a RIPLATE LV (5 mL wells). Mn(III) acetate solution (0.4 mM, 40 µL of 10 mM solution prepared in DMSO) and the substrate *t*-anethol (5 µL) was added. The reaction was performed on a shaker (170 rpm) under oxygen pressure (2 bar) at 21 °C for 24 h. Control experiments with buffer (Bis-Tris buffer, 50 mM, pH 6) and substrate in the presence and absence of Mn(III) acetate were run in parallel. The reaction was stopped by extraction with ethyl acetate (2x 0.3 mL). The combined organic phases were dried ($Na_2SO_4$) and analyzed by GC.

### 2.2. Purification steps

Freeze-dried *Trametes hirsuta* cells (4 g) [i] were resuspended in Bis-Tris buffer [90 mL, 50 mM, pH 6, 1 M $(NH_4)_2SO_4$], disrupted by ultrasonication (1 s pulse on, 4 s pulse off, total time of 1 min 50 sec, 50% amplitude) and centrifuged (4 °C, 19,945 g, 30 min).

The cell free extract (80 mL) was applied to a phenyl sepharose column (phenyl sepharose CL-4B, 9.5 cm height, 5 cm diameter) equilibrated with buffer A [Bis-Tris, 50 mM, pH 6, 1 M $(NH_4)_2SO_4$]. Isocratic elution with buffer A (150 mL) was followed by a linear gradient to 100% water over 130 mL and finally isocratic elution with water (200 mL) (flow rate: 4 mL/min, fraction volume: 7 mL). The fraction of interest was eluted during the isocratic elution with 100% water. Samples (900 µL) were taken for determining protein concentration and activity.

Active fractions were pooled and applied to an IEC-column (HiTrap Q Sepharose FF column, 1 mL, GE Healthcare Life Sciences) equilibrated with buffer C (10 mM Bis-Tris, pH 6). After isocratic elution [10 mL, buffer C (10 mM Bis-Tris buffer pH 6)] the salt concentration was increased via a linear gradient (20 mL) to 100% buffer D (Bis-Tris, 50 mM, pH 6, 1 M NaCl) followed by isocratic elution with buffer D (20 mL) (flow rate: 1 mL/min, fraction volume: 4 mL). The desired activity was eluted before the gradient.

The pooled active fractions (80 mL, obtained from performing the first two purification steps twice) were applied to a commercial HIC column (Hitrap® Phenyl HP, 1 mL, GE Healthcare Life Sciences). Isocratic elution with buffer A (150 mL) was followed by a linear gradient to 100% water and finally isocratic elution with water (flow rate: 4 mL/min, fraction volume: 1 mL). The fraction of interest eluted at the beginning of 100% water.

SDS-PAGE (Fig. S1) showed three main bands for the active fractions. One at ~40 kDa, one at ~28 kDa and the last one at 20 kDa. By comparison of active and inactive fractions, the band at 40 kDa was identified as being responsible for activity, cut out and analysed by MALDI-MS/MS de-novo sequencing after Trypsin digestion (Protagen AG, Dortmund, Germany).

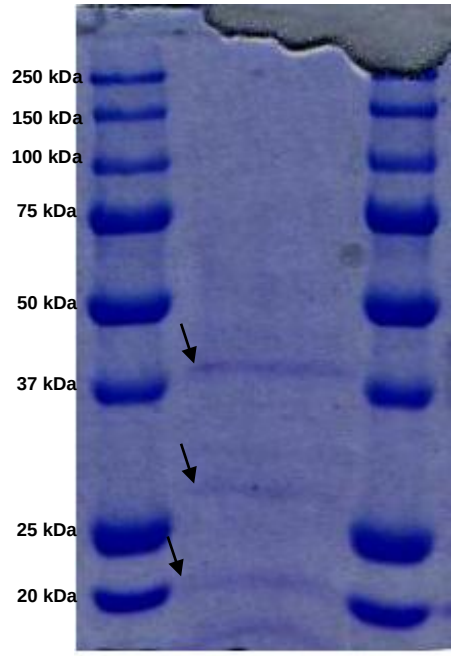



**Figure S1: SDS-PAGE used for protein sequencing. The protein band at 40 kDa was sent for MALDI-MS/MS analysis and de-novo sequencing.**

## 3. *Internal amino acid sequences of AlkCE*

This section is partly copied and adapted from the report received from Protagen AG.

### 3.1. In-Gel Digestion

The selected protein spot was cut out. The gel plug was washed alternately three times with $NH_4HCO_3$ (15 µL, 10 mM) and $NH_4HCO_3$ (5 mM)/50% acetonitrile. After drying the gel piece, Trypsin solution (33 ng/µL in 10 mM $NH_4HCO_3$, pH 7.8) was added to digest the protein for several hours at 37 °C.

### 3.2. MALDI Sample Preparation

The peptides were extracted from the gel piece with 0.1% trifluoro acetic acid (TFA) and purified using C18 material (ZipTip™, Millipore, Bedford, MA, USA) before spotting onto the MALDI target.

### 3.3. MALDI Spectrum Acquisition

The protein identification was performed using an Ultraflex III TOF/TOF Mass Spectrometer (Bruker Daltonics). For the acquisition of peptide mass fingerprint spectra (PMF, MS), 200 single shot spectra were averaged and peak picking was performed using the SNAP algorithm. The resulting mass list was sent to the ProteinScape™ database for protein identification. Peptide fragmentation spectra (PFF, MS/MS) were acquired where possible. The peaks for fragmentation were selected by the ProteinScape database based upon the results of the protein identification by PMF.

### 3.4. Protein Identification using a public database

Protein identification was performed by searching the mass spectra against the NCBI protein database (http://www.ncbi.nlm.nih.gov/) using several external search algorithms (ProFound™, Mascot™, Sequest™ For PMF spectra. The mass tolerance was set to 50 ppm. The protein identification was based on the metascore calculated from the individual search results by the ProteinScape™ database and on manual inspection of the data, if needed. PFF spectra were either used to confirm the protein already identified by PMF or for identification of proteins that eluded the PMF identification.

### *3.5. Automated de-novo sequencing*

The MS/MS data sets were automatically de-novo sequenced using the PEAKS 4.5 software package. The top scoring sequences are reported for each MS/MS spectrum.

### *3.6. Database Searching*

The following protein was identified by database search: gi|170091822|ref|XP_001877133.1| aspartic peptidase A1 [*Laccaria bicolor* S238NH82]. The peptide EPGLAFAFGK could be identified by database search and could be mapped to this protein.

### *3.7. De-novo Sequencing*

Sample PG379-U11-2010-001

The following peptide sequences (Fig. S2) were derived by de-novo sequencing. The amino acids, displayed in bold letters, are identified with very high confidence (PEAKS Score of this sequence part >90%).

| Parent mass | Peptide |
|---|---|
| 1036.5482 | **E P G L A F A F G K*** |
| 1166.6215 | **L V D S P V F S F R** |
| 1301.6235 | K **Y Y T V Y D H G R** |
| 2041.0100 | N Q **D F A E A T K E P G L A F A F G K** |
| 1173.5321 | Y Y **T V Y D H G R** |
| 1366.6850 | A Y W **E V E L E S I K** |
| 2375.0981 | L G **S S** E **E D G** G E **A L F G G V D E T A Y S G K** |
| 1023.4805 | N Q D F A E A T K |
| 1494.7802 | K A Y W E V E L E S I K |

* Also found by database searching

**Figure S2: Peptide sequences derived by de-novo sequencing. The amino acids displayed in bold letters were identified with high confidence.**

These peptides were identified by de-novo sequencing and could be mapped to gi|17009182 by high sequence homology between the peptide, identified by de-novo sequencing and a sequence region of gi|17009182. Thus, it was assumed that all peptides are part of the same protein.

# 4. Identification of the DNA sequence of the alkene cleaving enzyme (AlkCE)

## *4.1. Preparation of cDNA of Trametes hirsuta*

### *4.1.1. Isolation and purification of total RNA*

The isolation of the total RNA was performed using the RNeasy Plant Mini Kit from Qiagen. Frozen

cells (100 mg) that had been grown for one day were pulverized with a mortar and pestle under permanent addition of liquid nitrogen. All further steps were performed according to the protocol. For elution of total RNA, water (30 μL) was used.

A sample was analyzed via agarose gel electrophoresis. The successful extraction of RNA is indicated by both 28S and 18S ribosomal RNA bands (Fig. S3).

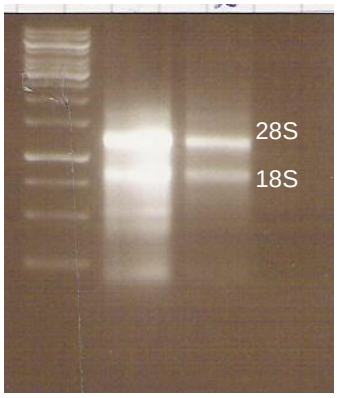



**Figure S3: RNA isolated from *Trametes hirsuta***

Treatment with DNaseI for 1.5 h destroyed remaining DNA residues in the RNA sample.

### *4.1.2. cDNA synthesis*

The cDNA synthesis was done using the Clontech SMART™ cDNA Library Kit. After the first strand synthesis 24 cycles of long distance PCR were performed to produce cDNA. The size fractionation of cDNA was performed according to protocol.

## *4.2. Overview of primer walking procedure used for DNA-sequence identification*

Based on the position and orientation of the peptide sequences (Fig. S2) in the sequence of aspartic peptidase A1 from *Laccaria bicolor*, degenerated forward and reverse primers were designed. The amplification was performed using standard PCR techniques. The created fragments were subcloned into a pJET1.2 blunt vector (CloneJET™ PCR Cloning kit). QIAprep(R) Spin Miniprep Kit (250) and QIAquick® Gel Extraction Kit (50) from Qiagen were used for isolating the plasmid DNA. DNA was sequenced by LGC Genomics, Berlin.

With the first internal DNA sequence, new target specific primers were designed. Additionally, with the aid of multiple sequence alignment with homologous proteins identified by pBLAST search, conserved regions were identified and new degenerated primers based on these sequences were designed. Finally, after four rounds of gene walking the *DNA Walking SpeedUp*™ *Premix Kit* (Seegene) was used in the last round leading to the complete sequence.

**Table S1: Overview of the primers used in successful sequencing and primer walking steps with cDNA as template.**

| Step | Primers |
|---|---|
| 1) initial sequencing | up:[a] 5'-AARGCNTAYTGGGARGTNGA-3'<br>down:[b] 5'-CRTGRTCRTANACNGTRTARTAYT-3' |
| 2) primer walking | up: 5'-TGTGGATCATTGGTGATGTCTTCCTGC-3'<br>down:[c] 5'-CGAGGCGGCCGACATGTTTTTTTT -3' |
| 3) primer walking | up:[d] 5'-AAYCARGAYTTYGCNGARGC-3'<br>down: 5'- AGTGTCGATGGCAGCGCCG -3' |
| 4) primer walking with nested PCR | up:[e] 5'-GTATCAACGCAGAGTGGCCATTACG-3'<br>down: 5'-CAGGCCGAGGATACCATC-3'<br>followed by nested PCR:<br>up:[d] 5'-AAYTWYATGAAYGCNCARTA-3'<br>down: 5'-CATCGAACTTGCCAAAG-3' |
| 6) primer walking, *DNA Walking SpeedUp*™ *Premix Kit* using two nested PCRs | up: DW2-ACP 1,2,3,4 primers from kit<br>down: 5'-GGGAGTGCCCAAGGTGA-3'<br>1st nested PCR:<br>up: DW2-ACPN primer from kit<br>down: 5'-GAGTGCCCAAGGTGATTTC -3'<br>2nd nested PCR:<br>up: UniP2 primer from kit<br>down: 5'-GGTGATTTCAGCGAAGTACT-3' |

a: degenerated primers were designed for the following AA sequence: KAYWEVE.

b: degenerated primers were designed for the following AA sequence:KYYTVYDHG.

c: this primer was based on the CDSIII primer of the SMART™cDNA library construction kit which was used to prepare the cDNA (incorporating the sequence of at the 3' end of all the cDNA fragments).

d: degenerated primer.

e: primer based on SMART IV primer of SMART™cDNA library construction kit.

## *4.3. Amplification of AlkCE gene from cDNA as well as genomic DNA*

The following primers were employed for the amplification of the AlkCE gene (nucleotides of the gene are in capital letters):

Up: 5'-aatta<u>catATG</u>ATACTCTCCAGATTCGCCCCC-3' (*Nde*I site is underlined)

Down: 5'-aatt*ctcGAG*TCACTTGGCAAGAGCGAAGCC-3' (*Xho*I site is underlined).

The gene was amplified from cDNA as well as genomic DNA. Genomic DNA of the fungus was isolated following the protocol of the peqGOLD Tissue DNA Mini Kit (Order no. 12-3396-00). Starting from 40 mg of lyophilized cells, 35 µg of genomic DNA was obtained.

PCR-conditions: Initial denaturation- 98 °C (3 min), Denaturation- annealing- extension (40 cycles) {98 °C [30 s], 65 °C[30 s], 72 °C[100 s]}, final extension- 72 °C °C(7 min).

Amplification from cDNA led to the following DNA sequence for AlkCE (GenBank accession number: KC905051):

5'-
ATGATACTCTCCAGATTCGCCCCCCTCGCCCTGCTCCCCTTCGTGGCCGCCGACGGCGTCCACAAGCTGAAGCT
CACCAAGCTTCCTCCCGCAACTTCCAACCCGTTGTTGGAGAGTGCTTACCTGGCTGAGAAGTATGGTGGTGGTT
CCCAGATGCCCCTTAGCGCGGGCATTGGCCGCAACGTCCGCGTGTCGCGCCCGACCGTCAAGGACGGCGAGGA
GCTCTTCTGGACTCAGGACGAGTTTTCGACCGAGGGCGGTCACAACGTTCCCTTGAGTAACTTCATGAACGCTC
AGTACTTCGCTGAAATCACCTTGGGCACTCCCCCCGCAATCGTTCAAGGTCATCCTGGACACTGGGTCGAGCAAC
CTCTGGGTTCCGAGCACCAAGTGTACCTCCATTGCGTGCTTCCTACACGCCAAGTATGACTCGACCGCTTCGTC
GACATACAAGGCGAACGGCTCCGAGTTCTCGATCCAGTATGGCTCTGGCTCCATGGAGGGCTTCGTCTCGCAA
GATGTCTTGACAATTGGTGACATCACCATCAAGAACCAAGATTTCGCAGAGGCCACCAAGGAGCCCGGCCTCG
CATTTGCCTTTGGCAAGTTCGATGGTATCCTCGGCCTGGGTTATGACACCATTTCCGTGAACCACATCACTCCTC
CCTTCTACCAGATGATGAACCAGAAGCTCGTCGATTCTCCTGTGTTCTCTTTCCGCCTCGGTAGCTCGGAAGAG
GACGGTGGTGAAGCCATCTTCGGAGGAGTCGATGAGACCGCGTACAGTGGCAAGATCGAATACGTCCCTGTCA
GGAGGAAGGCGTACTGGGAGGTGGAGCTGGAATCGATCAAACTCGGAGACGACGAGCTTGAGCTCGATAACA
CCGGCGCTGCCATCGACACTGGAACCTCGTTGATTGCTCTCCCCTCCGATCTGGCGGAGATGCTCAATGCGCAA
ATCGGTGCCAAGAAGTCCTGGAATGGCCAGTACACCGTCGACTGCGCGAAGGTCCCTACCCTCCCCGACCTCA
CCTTCTACTTCAGCGGCAAGCCTTACACTCTCAAGGGTACCGACTACGTCCTCGAAGTTCAGGGAACTTGCATG
TCCTCGTTCACCGGCATCGACATCAATCTGCCCGGCGGTGGTGCTCTGTGGATCATTGGTGATGTCTTCCTGCG
CAAGTACTACACTGTGTACGACCATGGCCGCGATGCCGTTGGCTTCGCTCTTGCCAAGTGA-3'

Corresponding amino acid sequence of AlkCE:

MILSRFAPLALLPFVAADGVHKLKLTKLPPATSNPLLESAYLAEKYGGGSQMPLSAGIGRNVRVSRPTVKDGEELFW
TQDEFSTEGGHNVPLSNFMNAQYFAEITLGTPPQSFKVILDTGSSNLWVPSTKCTSIACFLHAKYDSTASSTYKANGS
EFSIQYGSGSMEGFVSQDVLTIGDITIKNQDFAEATKEPGLAFAFGKFDGILGLGYDTISVNHITPPFYQMMNQKLVD
SPVFSFRLGSSEEDGGEAIFGGVDETAYSGKIEYVPVRRKAYWEVELESIKLGDDELELDNTGAAIDTGTSLIALPSDL
AEMLNAQIGAKKSWNGQYTVDCAKVPTLPDLTFYFSGKPYTLKGTDYVLEVQGTCMSSFTGIDINLPGGGALWIIG
DVFLRKYYTVYDHGRDAVGFALAK*

Analysis of the two PCR products employing cDNA and genomic DNA as template showed clearly a larger PCR product obtained from genomic DNA (Fig. S4) due to the presence of introns (see SI 4.4).

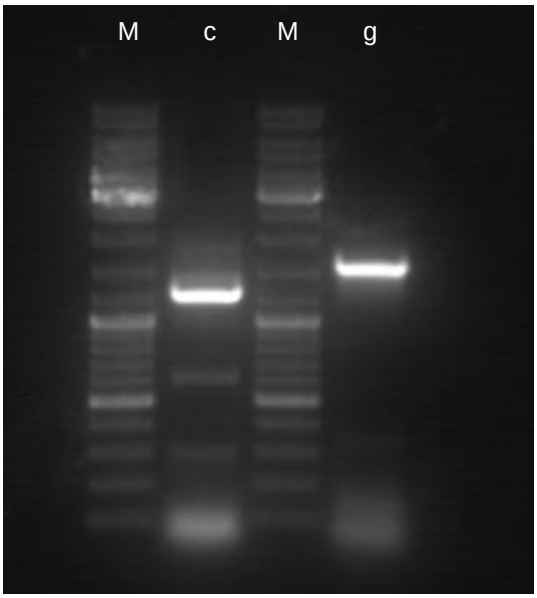



**Figure S4: The AlkCE gene amplified from cDNA (c) as well as genomic DNA (g). M indicates the DNA ladder (GeneRuler™ DNA Ladder Mix, Thermo Scientific)**

## *4.4. Alignment of the AlkCE gene amplified from cDNA and genomic DNA*

The alignment of the sequence of the AlkCE gene deduced from cDNA and genomic DNA (ClustalW2, EMBL-EBI) is given below. Introns and one deviation are marked in red.

CLUSTAL 2.1 multiple sequence alignment

```
genomic         ATGATACTCTCCAGATTCGCCCCCCTCGCCCTGCTCCCCTTCGTGGCCGCCGACGGCGTC 60
cDNA            ATGATACTCTCCAGATTCGCCCCCCTCGCCCTGCTCCCCTTCGTGGCCGCCGACGGCGTC 60
                ************************************************************

genomic         CACAAGCTGAAGCTCACCAAGCTTCCTCCCGCAACTTCCAACCCGTTGTTGGAGAGTGCT 120
cDNA            CACAAGCTGAAGCTCACCAAGCTTCCTCCCGCAACTTCCAACCCGTTGTTGGAGAGTGCT 120
                ************************************************************

genomic         TACCTGGCTGAGAAGTATGGTGGTGGTTCCCAGATGCCCCTTAGCGCGGGCATTGGCCGC 180
cDNA            TACCTGGCTGAGAAGTATGGTGGTGGTTCCCAGATGCCCCTTAGCGCGGGCATTGGCCGC 180
                ************************************************************

genomic         AACGTCCGCGTGTCGCGCCCGACCGTCAAGGACGGCGAGGAGCTCTTCTGGACTCAGGAC 240
cDNA            AACGTCCGCGTGTCGCGCCCGACCGTCAAGGACGGCGAGGAGCTCTTCTGGACTCAGGAC 240
                ************************************************************

genomic         GAGTTTTCGACCGAGGGCGGTCACAACGTTCCCTTGAGTAGTACGTCTCATTCCCTTACA 300
cDNA            GAGTTTTCGACCGAGGGCGGTCACAACGTTCCCTTGAGTA-------------------- 280
                ************************************

genomic         TAGTCGGTGCATCGTCTCATAACAACTGTCAGACTTCATGAACGCTCAGTACTTCGCTGA 360
cDNA            --------------------------------ACTTCATGAACGCTCAGTACTTCGCTGA 308
                                                 ****************************

genomic         AATCACCTTGGGCACTCCCCCGCAATCGGTACGTGACATATCTCTCTCTGGGGGTCTTCC 420
cDNA            AATCACCTTGGGCACTCCCCCGCAATCG-------------------------------- 336
                ****************************

genomic         TCACTCACTTCAGTTTAGTTCAAGGTCATCCTGGACACTGGGTACGTACAATACATGCAT 480
cDNA            ------------------TTCAAGGTCATCCTGGACACTGGGT----------------- 361
                                   *************************
```

```
genomic         TTACGCCGAACGATGACTCTGACAGTCGCCATTGTGTTCAGGTCGAGCAACCTCTGGGTT 540
cDNA            ---------------------------------------------CGAGCAACCTCTGGGTT 378
                                                              ****************

genomic         CCGAGCACCAAGTGTACCTCCATTGCGTGCTTCCTACACGCCAAGTATGACTCGACCGCT 600
cDNA            CCGAGCACCAAGTGTACCTCCATTGCGTGCTTCCTACACGCCAAGTATGACTCGACCGCT 438
                ************************************************************

genomic         TCGTCGACATACAAGGCGAACGGCTCCGAGTTCTCGATCCAGTATGGCTCTGGCTCCATG 660
cDNA            TCGTCGACATACAAGGCGAACGGCTCCGAGTTCTCGATCCAGTATGGCTCTGGCTCCATG 498
                ************************************************************

genomic         GAGGGCTTCGTCTCGCAAGATGTCTTGACAATTGGTGACATCACCATCAAGAACCAAGAT 720
cDNA            GAGGGCTTCGTCTCGCAAGATGTCTTGACAATTGGTGACATCACCATCAAGAACCAAGAT 558
                ************************************************************

genomic         TTCGCAGAGGCCACCAAGGAGCCCGGCCTCGCATTTGCCTTTGGCAAGTGAGTACAATCT 780
cDNA            TTCGCAGAGGCCACCAAGGAGCCCGGCCTCGCATTTGCCTTTGGCAAGT----------- 607
                ********************************************

genomic         CGTGTCTTCGCACTTACCTGTACTGAACTATCACGCAAAGGTTCGATGGTATCCTCGGCC 840
cDNA            ---------------------------------------------TCGATGGTATCCTCGGCC 625
                                                               ******************
genomic         TGGGTTATGACACCATTTCCGTGAACCACATCACTCCTCCCTTCTACCAGATGATGAACC 900
cDNA            TGGGTTATGACACCATTTCCGTGAACCACATCACTCCTCCCTTCTACCAGATGATGAACC 685
                ************************************************************

genomic         AGAAGCTCGTCGATTCTCCTGTGTTCTCTTTCCGCCTCGGTAGCTCGGAAGAGGACGGTG 960
cDNA            AGAAGCTCGTCGATTCTCCTGTGTTCTCTTTCCGCCTCGGTAGCTCGGAAGAGGACGGTG 745
                ************************************************************

genomic         GTGAAGCCATCTTCGGAGGAGTCGATGAGACCGCGTACAGTGGCAAGATCGAATACGTCC 1020
cDNA            GTGAAGCCATCTTCGGAGGAGTCGATGAGACCGCGTACAGTGGCAAGATCGAATACGTCC 805
                ************************************************************

genomic         CTGTCAGGAGGAAGGCGTACTGGGAGGTGGAGCTGGAGTCGATCAAACTCGGAGACGACG 1080
cDNA            CTGTCAGGAGGAAGGCGTACTGGGAGGTGGAGCTGGAATCGATCAAACTCGGAGACGACG 865
                ************************************ ***********************

genomic         AGCTTGAGCTCGATAACACCGGCGCTGCCATCGACACTGGTAAGGTGAACACCCAACCTC 1140
cDNA            AGCTTGAGCTCGATAACACCGGCGCTGCCATCGACACTGG-------------------- 905
                ****************************************

genomic         ATGCAGTATTCACAATTGACACCTCGATAGGAACCTCGTTGATTGCTCTCCCCTCCGATC 1200
cDNA            --------------------------------AACCTCGTTGATTGCTCTCCCCTCCGATC 934
                                                ****************************

genomic         TGGCGGAGATGCTCAATGCGCAAATCGGTGCCAAGAAGTCCTGGAATGGCCAGTACACCG 1260
cDNA            TGGCGGAGATGCTCAATGCGCAAATCGGTGCCAAGAAGTCCTGGAATGGCCAGTACACCG 994
                ************************************************************

genomic         TCGACTGCGCGAAGGTCCCTACCCTCCCCGACCTCACCTTCTACTTCAGCGGCAAGCCTT 1320
cDNA            TCGACTGCGCGAAGGTCCCTACCCTCCCCGACCTCACCTTCTACTTCAGCGGCAAGCCTT 1054
                ************************************************************

genomic         ACACTCTCAAGGGTACCGACTACGTCCTCGAAGTTCAGGGAACTTGCATGTCCTCGTTCA 1380
cDNA            ACACTCTCAAGGGTACCGACTACGTCCTCGAAGTTCAGGGAACTTGCATGTCCTCGTTCA 1114
                ************************************************************

genomic         CCGGCATCGACATCAATCTGCCCGGCGGTGGTGCTCTGTGGATCATTGGTACGCTAACTT 1440
cDNA            CCGGCATCGACATCAATCTGCCCGGCGGTGGTGCTCTGTGGATCATTGGT---------- 1164
                ************************************************

genomic         TATTTTGTGTGTTGTGGTAAGTGCTGATTTTTGTCTAGGTGATGTCTTCCTGCGCAAGTA 1500
cDNA            ---------------------------------------GATGTCTTCCTGCGCAAGTA 1184
                                                        ********************

genomic         CTACACTGTGTACGACCATGGCCGCGATGCCGTTGGCTTCGCTCTTGCCAAGTGA 1555
cDNA            CTACACTGTGTACGACCATGGCCGCGATGCCGTTGGCTTCGCTCTTGCCAAGTGA 1239
                ********************************************************
```

# 5. Overexpression of AlkCE

## 5.1. Cloning into pET-21a(+) vector

The PCR product obtained from cDNA (Fig. S4-c), was amplified in a pJET1.2 vector. The amplified gene was restricted with *Nde*I/*Xho*I (FastDigest® enzymes, Fermentas) and ligated into a pET-21a(+) vector using T4 DNA Ligase (Fermentas). The recombinant construct [AlkCE/pET-21a(+)] was then amplified in *E. coli* XL1-Blue cells.

## 5.2. Transformation into E. coli, enzyme overexpression & activity

The construct AlkCE/pET-21a(+) was transformed into different *E. coli* host cells. The cultures were grown in LB medium/ampicillin (100 µg/L) (300 mL in 1 L flask) and inoculated with 2 or 5 mL of overnight culture (shaking at 120 rpm at 37 °C). At an $OD_{600}$ of 0.6, enzyme expression was induced by IPTG (0.3 mM) and shaking was continued at 120 rpm at 20 °C for 16 h. The culture was centrifuged (19,945 g, 20 min, 4 °C) and the cell pellet was washed in Bis-Tris buffer (pH 6, 50 mM) and then resuspended in a minimal amount of the same buffer, frozen with liquid nitrogen and freeze-dried. For the first tests freeze-dried culture samples (50 mg/mL) were resuspended in Bis-Tris buffer (pH 6, 50 mM) and ultrasonicated at 4 °C (30% amplitude, 1 s on, 4 s off, 2 min 30 sec) and centrifuged (19,945 g for 20 min at 4 °C). For assaying, the supernatant (960 µL) was transferred into a well (RIPLATE, 10 mL), Mn(III) acetate solution (0.4 mM, 10 mM in DMSO, 40 µL) and substrate (34 mM, 5 µL of *t*-anethole) were added. After shaking (for 36 h at 170 rpm, 2 bar oxygen pressure, 21 °C), the reaction was stopped by extraction with ethyl acetate (2 x 500 µL). The combined organic layers were dried with $Na_2SO_4$ and analyzed by GC and GC/MS.

**Table S2: Overview of the different *E. coli* transformants tested for alkene cleavage activity and enzyme overexpression from AlkCE/pET-21a(+)**

| ***E. coli* host strain** | **Overexpression** | **Soluble overexpression** | **Alkene cleavage activity** |
|---|---|---|---|
| BL21(DE3) | **Yes** | - | No |
| Rosetta(DE3) | **Yes** | - | No |
| C43(DE3) | - | - | No |
| BL21(DE3).pG-KJE8 | - | - | No |
| BL21(DE3).pGro7 | - | - | No |
| BL21(DE3).pKJE7 | - | - | No |
| BL21(DE3).pTf16 | - | - | **Yes** |
| CodonPlus RP(DE3) | - | - | **Yes** |
| BL21(DE3).pLysS | - | - | No |

### 5.3. $His_6$-Tagging and enzyme purification & activity

The stop codon of the gene obtained from cDNA was mutated (primers: Up: AATTACATATGATACTCTCCAGATTCGCCCCC, Down: AATTCTCGAGTCTCTTGGCAAGAGCGAAGCC), the sequence verified by external sequencing and subcloned into pET-21a(+) vector in order to give it a C-terminal $His_6$-tag. After transformation into *E. coli* BL21 Codon Plus RP(DE3), the cells were cultivated as described in Section 5.2. The culture was centrifuged (19,945 g, 20 min, 4 °C) and the cell pellet (13.5 g) was washed in Bis-Tris buffer (pH 6, 50 mM) and then resuspended in lysis buffer (50 mL, 300 mM NaCl, 50 mM $NaH_2PO_4$, 10 mM imidazole). The suspension was ultrasonicated (30% amplitude, 1 s on, 4 s off, 3 min) and centrifuged (19,945 g, 20 min, 4 °C). Bio-Rad BioLogic DuoFlow Protein Purification System was used for the protein purification at 4 °C. The affinity chromatography column (HisTrap HP, 5 mL, GE Life Sciences) was washed with water (5 column volumes) and lysis buffer (5 column volumes). Cell-free extract (50 mL) was loaded, washed with wash buffer (5 column volumes, 300 mM NaCl, 50 mM $NaH_2PO_4$, 20 mM imidazole) and eluted with elution buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 300 mM imidazole). Fractions (3 mL) were collected during elution. After elution, the column was washed with water (degased) and stored in 20% EtOH (degased). Eluted purified enzyme fractions (960 µL) were tested for alkene cleavage activity as described in section 5.2 (see Table S3). Elution fractions were also concentrated by centrifuging in VIVASPIN tubes (Sartorius, 6 mL, Membrane: 50 000 MWCO PES) for SDS-PAGE analysis (Fig. S5).

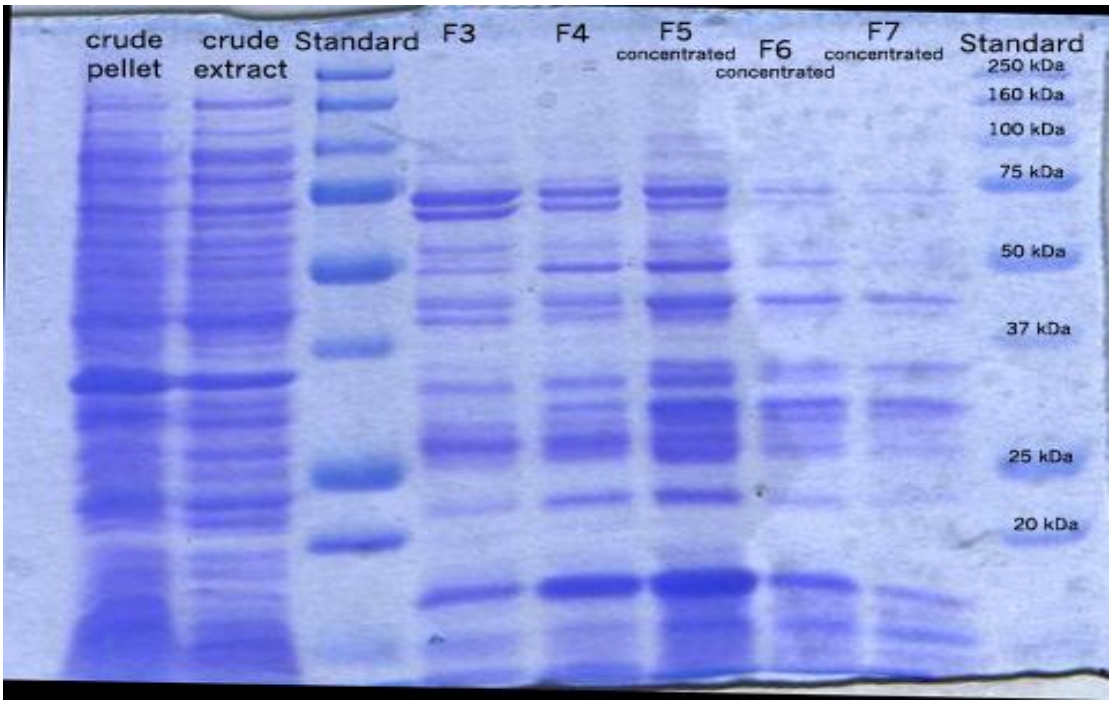



**Figure S5: SDS-PAGE gel of overexpression of AlkCE in *E. coli* CodonPlus RP after $His_6$-Tag purification. For this gel sample (10 µL) were mixed with Loading Dye (10 µL) and the total amount was loaded. F – elution fraction; crude means unpurified in this case.**

**Table S3: Conversion measured for purified fractions and crude extract.**

| Sample | Conversion[a] (%) |
|---|---|
| Crude extract | 13 |
| Elution fraction 3 | 6 |
| Elution fraction 4 | 38 |
| Elution fraction 5 | 10 |
| Negative control: $His_6$-tagged Ω-transaminase | <1 |

a: Conversion was measured by GC-FID. Background is subtracted.

# 6. Overexpression of the truncated enzyme, $His_6$-tag purification, Western blot & activity

## *6.1. Design of truncated gene*

Based on the comparison with the homologous enzyme proteinase A from *S. cerevisiae*, the first 84 amino acids were truncated (potential pre-pro region), a methionine was added and a gene was synthesized (GeneArt) which was codon optimized for expression in *E. coli*. The DNA sequence and protein sequence (329 AA residues including the stop codon) of the ordered gene is given below:

*Sac*I site

*gagctc***ATG**GGTGGTCATAATGTTCCGCTGAGCAATTTTATGAACGCACAGTATTTTGCCGAAATTACCCTGGGCA
CCCCTCCGCAGAGCTTTAAAGTTATTCTGGATACCGGTAGCAGCAATCTGTGGGTTCCGAGCACCAAATGTACC
AGCATTGCATGTTTTCTGCACGCCAAATATGATAGCACCGCAAGCAGCACCTATAAAGCAAATGGTAGCGAAT
TTAGCATTCAGTATGGTAGCGGTAGCATGGAAGGTTTTGTTAGCCAGGATGTTCTGACCATTGGTGACATTACC
ATTAAAAACCAGGATTTTGCAGAGGCAACCAAAGAACCGGGTCTGGCATTTGCATTTGGTAAATTTGATGGTA
TTCTGGGCCTGGGCTATGATACCATTAGCGTTAATCATATTACCCCACCGTTCTACCAGATGATGAATCAGAAA
CTGGTTGATAGTCCGGTGTTTAGCTTTCGTCTGGGTAGCAGCGAAGAGGATGGTGGTGAAGCAATTTTTGGTGG
TGTTGATGAAACCGCATATAGCGGCAAAATTGAATATGTTCCGGTTCGTCGTAAAGCCTATTGGGAAGTTGAA
CTGGAAAGCATTAAACTGGGTGATGATGAGCTGGAACTGGATAATACCGGTGCCGCAATTGATACCGGCACCA
GCCTGATTGCACTGCCGAGCGATCTGGCAGAAATGCTGAATGTTCAGATTGGTGCAAAAAAAAGCTGGAATGG
TCAGTATACCGTTGATTGTGCAAAAGTTCCGACCCTGCCGGATCTGACCTTTTATTTCAGCGGTAAACCGTATA
CCCTGAAAGGCACCGATTATGTTCTGGAAGTTCAGGGCACCTGTATGAGCAGCTTTACCGGTATTGATATTAAT
CTGCCTGGTGGTGGTGCACTGTGGATTATTGGTGATGTTTTTCTGCGCAAATACTACACCGTTTATGATCATGGT
CGTGATGCAGTTGGTTTTGCACTGGCAAAA**TAA***ttcgaa*

*Hind*III site

MGGHNVPLSNFMNAQYFAEITLGTPPQSFKVILDTGSSNLWVPSTKCTSIACFLHAKYDSTASSTYKANGSEFSIQYG
SGSMEGFVSQDVLTIGDITIKNQDFAEATKEPGLAFAFGKFDGILGLGYDTISVNHITPPFYQMMNQKLVDSPVFSFR
LGSSEEDGGEAIFGGVDETAYSGKIEYVPVRRKAYWEVELESIKLGDDELELDNTGAAIDTGTSLIALPSDLAEMLN
VQIGAKKSWNGQYTVDCAKVPTLPDLTFYFSGKPYTLKGTDYVLEVQGTCMSSFTGIDINLPGGGALWIIGDVFLR
KYYTVYDHGRDAVGFALAK*

The truncated gene (*Sac*I/*Hind*III) was subcloned into pET-28(a) (N-terminal $His_6$-tag) and overexpressed in *E. coli* BL21(DE3).pTf16.

## *6.2. Overexpression*

The truncated gene (*Sac*I/*Hind*III) was subcloned into pET-28(a) (N-terminal $His_6$-tag) and overexpressed in *E. coli* BL21(DE3).pTf16 [LB medium/ampicillin (100 µg/L), chloramphenicol (100 µg/L), trace element solution SL4 (1 mL/L)], induced with IPTG (0.3 mM) and arabinose (0.5 g/L) for the induction of the chaperone protein. SDS-PAGE showed significant overexpression, whereby most of the enzyme was in the insoluble fraction (pellet after ultrasonication). Highest conversion of *t*-anethole was obtained using crude cell extract (0.2 mg/mL freeze-dried cells, ultrasonicated, 2 min 30 s, 30% amplitude, 1s pulse on, 4 s pulse off).

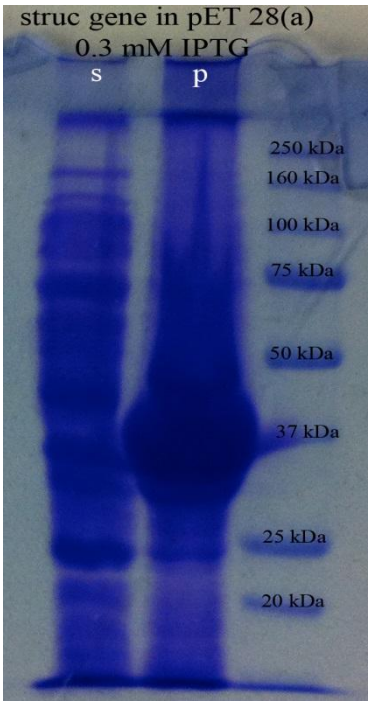



**Figure S6: Overexpression of trunc-AlkCE in *E. coli* BL21(DE3).pTf16/pET-28(a) (N-terminal $His_6$-tag). Most of the overexpressed trunk-AlkCE was in the insoluble fraction (p). s: soluble fraction, p: pellet after ultrasonication and centrifugation.**

## *6.3. $His_6$-tag purification & Western-Blot*

$His_6$-trunc-AlkCE was purified (4 L culture) by affinity column chromatography using the procedure given in section 4.3. Additionally, a Western blot analysis using anti-His antibody was performed. Protein samples (10 µL) were separated under denaturing conditions on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and then transferred electrophoretically onto a nitrocellulose membrane (GE Healthcare Life Sciences, Chalfont St Giles, UK) in a wet blotting system. According to the manual of the supplier, immunodetection was performed by incubation with a commercially available rabbit anti-His primary antibody (Thermo Scientific) followed by an anti-rabbit secondary antibody linked to horse radish peroxidase (Sigma). Western blots were developed by Super Signal West Pico

chemoluminescent substrate (Thermo Scientific) and $His_6$-tagged proteins were detected using a Syngene G:BOX (Figure S7).

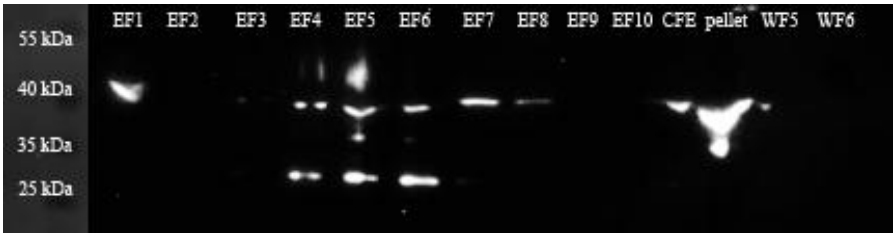



**Figure S7: SDS-PAGE and Western blot analysis of the $His_6$-tagged truncated enzyme. trunc-AlkCE was purified by affinity chromatography (Ni-NTA column). Western blot analysis with the anti-His antibody confirmed the presence of the overexpressed $His_6$-tagged enzyme in the elution fractions (EF) as well as the cell free extract (CFE) and cell pellet. Most of the overexpressed enzyme was detected in the insoluble pellet fraction. WF: washing fraction.**

## *6.4. Transformations employing the purified trunc AlkCE enzyme*

The purified fractions ($His_6$-tag purification) were tested for alkene cleavage activity in duplicates [500 µL sample + 490 µL Bis-Tris buffer (pH 6, 50 mM) +10 µL $Mn(OAc)_3$ solution (10 mM in DMSO) + 1.5 µL *t*-anethole in a RIPLATE 24 well vessel (10 mL volume each well)]. The reaction was performed at 21 °C, 2 bar $O_2$ pressure, 24 h, 170 rpm,.

## *6.5. Protease and alkene cleavage activity*

The purified elution fractions were tested for peptidase activity using the QuantiCleave™ Protease Assay Kit (Thermo Scientific), which uses succinylated casein as substrate. Initially a calibration curve was set up by the serial dilution of reference trypsin enzyme (500-0.05 µg/mL). The assay was performed according to the manual (all reactions in triplicates) and the protease activity of the purified elution fractions (EF 1-10) (50 µL of each purified fraction sample) were tested by subtracting the absorbance at 405 nm in the presence of the enzyme and substrate from the background absorbance.

Additionally, the same fractions were also tested for alkene cleavage activity in duplicates [500 µL sample + 490 µL Bis-Tris buffer (pH 6, 50 mM) +10 µL $Mn(OAc)_3$ solution (10 mM in DMSO) + 1.5 µL *t*-anethole in RIPLATE 24 well vessel (10 mL volume each well)]. The reaction was carried out at 170 rpm, 21 °C, 2 bar $O_2$ pressure, 24 h. The summary of the activity results for protease and alkene cleavage assays are given in Table S4.

**Table S4: Elution fractions tested for peptidase activity as well as for alkene cleavage.**

| Sample | Protease activity indicator | Alkene cleavage |
|---|---|---|
| | Δ A405 (*$10^{-3}$) | conversion[a] (%) |
| EF1 | 2 | <1 |
| EF2 | <2 | <1 |
| EF3 | <2 | 1 |
| EF4 | 5 | 14 |
| EF5 | 10 | 15 |
| EF6 | 12 | 29 |
| EF7 | 12 | 37 |
| EF8 | 12 | 16 |
| EF9 | 11 | n.d. |
| EF10 | 12 | 3 |

a: Conversion was measured by GC-FID for *t*-anethole to *p*-anisaldehyde. Background is subtracted. n.d. not determined

The positive purification fractions were pooled and concentrated by centrifuging in VIVASPIN tubes (Sartorius, 6 mL, Membrane: 50 000 MWCO PES) and analysed by SDS-PAGE. The gel was stained with InVision™ His-tag In-gel stain (Invitrogen) as well as by Coomassie blue stain. Fig. S8.

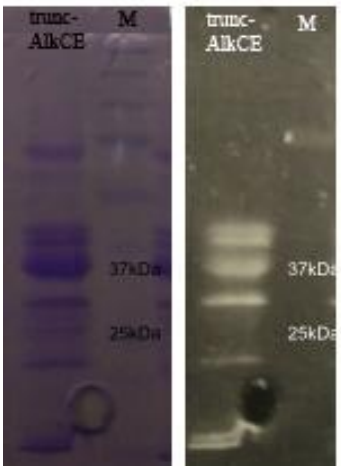



**Figure S8: Concentrated purified $His_6$-tagged trunc-AlkCE stained by Coomassie blue stain (left) as well as by InVision™ His-tag In-gel stain (Invitrogen) (right) showing degradation of the $His_6$-tagged protein. M: marker.**

## 6. Homology model, preparation of pictures

A homology model of AlkCE was built using the program Yasara[ii] based on structures of proteinase A from *Saccharomyces cerevisiae* (PDB-codes: 1DP5, 1DP7). The Mn-ion was placed manually into the proposed binding site, consisting of $Asp^{118}$, $Asp^{300}$ and $Thr^{303}$, using the program PyMOL (http://www.pymol.org/). This program was also used to analyze the structures and to prepare the figures.

## 7. Preparation of trunc-AlkCE variants

QuikChange XL Lightning site directed mutagenesis kit (Agilent technologies) was used for the preparation of variants. The trunc-AlkCE gene in pET-28a(+) vector having the *Sac*I/*Hin*dIII restrictions sites was used as the template DNA. The required primers were designed as detailed in the kit manual. Primers were designed for the following variants D118V, D300V & T303V (numbering based on whole protein sequence including the pre-pro region).

Amino acid sequence of trunc-AlkCE:

MGGHNVPLSNFMNAQYFAEITLGTPPQSFKVIL**D**TGSSNLWVPSTKCTSIACFLHAKYDSTASSTYKANGSEFSIQYG
SGSMEGFVSQDVLTIGDITIKNQDFAEATKEPGLAFAFGKFDGILGLGYDTISVNHITPPFYQMMNQKLVDSPVFSFR
LGSSEEDGGEAIFGGVDETAYSGKIEYVPVRRKAYWEVELESIKLGDDELELDNTGAAI**D**TG**T**SLIALPSDLAEMLN
VQIGAKKSWNGQYTVDCAKVPTLPDLTFYFSGKPYTLKGTDYVLEVQGTCMSSFTGIDINLPGGGALWIIGDVFLR
KYYTVYDHGRDAVGFALAK-

**Primers for mutation GAT -> GTT (D118 to V118)**
Up: 5'-GCTTTAAAGTTATTCTG**GTT**ACCGGTAGCAGCAATCTGTGGGTTC-3'
Down: 5'-GAACCCACAGATTGCTGCTACCGGT**AAC**CAGAATAACTTTAAAGC-3'

**Primers for mutation GAT -> GTT (D300 to V300)**
Up: 5'-GGTGCCGCAATT**GTT**ACCGGCACCAGCCTGATTGC-3'
Down: 5'-GCAATCAGGCTGGTGCCGGT**AAC**AATTGCGGCACC-3'

**Primers for mutation ACC -> GTC (T303 to V303)**
Up:5'-GCCGCAATTGATACCGGC**GTC**AGCCTGATTGC-3'
Down: 5'-GCAATCAGGCT**GAC**GCCGGTATCAATTGCGGC-3'

The sequences of the variants were confirmed by sequencing at LGC Genomics, Berlin, and alignment of the translated amino acid sequences.

The variants were overexpressed in *E. coli* BL21(DE3).pTf16 cells (section 7.1, 5 L culture) and purified by affinity column chromatography following the procedure in section 5.3 (Fig. S9). The

concentrated purified variants (200 µg protein) were tested for alkene cleavage activity [Bis-Tris buffer (pH 6, 50 mM), $Mn(OAc)_2$ (0.1 mM), DMSO (1%), *t*-anethole (10 mM) in RIPLATE 24 well, l70 rpm shaking, 21 °C, 2 bar $O_2$ pressure, 24 h]. The blank was buffer with Mn(III) without any enzyme (Table S5).

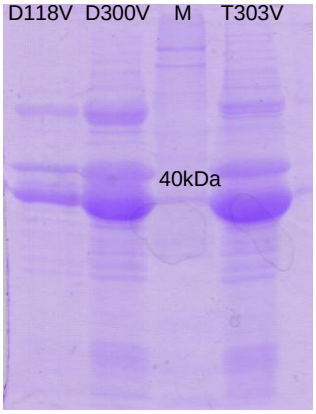



**Figure S9: The variants were overexpressed in *E. coli* BL21(DE3).pTf16 and purified using affinity column chromatography. The elution fractions were pooled and concentrated. Protein amounts loaded on the gel were: D118V (0.5 µg), D300V (4 µg) and T303V (8 µg)**

**Table S5: Testing for alkene cleavage of the purified variants**

| Variant | Conversion[a] (%) |
|---|---|
| **D**118**V** | <1 |
| **D**300**V** | <1 |
| **T**303**V** | <1 |

a: Conversion was measured by GC-FID for *t*-anethole. Background is subtracted.

# 9. Transformations employing Mn complexes catalyst

## *9.1 $Mn(acac)_3$ as catalyst*

The substrate (5 µL) was added to Mn(III) acetylacetonate (6 mM, 60 µL of a stock solution of 100 mM in methanol) in Bis-Tris buffer (940 µL, 10 mM, pH 6). The reaction was performed in glass vials (10 mL volume) covered with a septum with a hole at 2 bar oxygen pressure, 170 rpm, 24 h, 21 °C.

For determination of activity the same procedure as above was used but the catalyst loading was reduced (5 mM) and samples were taken after 3, 6 and 9 h.

## *9.2. Mn(III) porphyrin as catalyst*

A stock solution of 2,3,7,8,12,13,17,18-Octaethyl-21*H*,23*H*-porphine manganese(III) chloride was added (0.1 mM, 50 µL of a stock solution of 2 mM in DMSO) to Bis-Tris buffer (950 µL, 10 mM, pH 6) followed by the addition of the substrate (5 µL). The reaction was run in glass vials (4 mL volume) covered with a septum with a hole at 2 bar oxygen pressure, 170 rpm, 24 h, 21 °C.

**Table S6: Substrates transformed by Mn(III) acetylacetonate [$Mn(acac)_3$] in the presence of molecular oxygen[a] and comparison of the chemoselectivity to the enzyme catalyzed reaction.**

| | AlkCE[b] | $Mn(acac)_3$ | |
|---|---|---|---|
| Substrate | Chemoselectivity[c] [%] | Conversion[d] [%] | Chemosel.[c] [%] |
| *t*-anethole **1a** | >99 | 98 | 90 |
| 4-methoxystyrene **1b** | 90 | 22 | 88 |
| 4-methylstyrene **1c** | 94 | 14 | 78 |
| 2-chlorostyrene **1d** | 85 | 43 | 87 |
| 3-chlorostyrene **1e** | 80 | 29 | 82 |
| indene **1f** | 82 | 75 | 75 |
| 1,2-dihydronaphthalene **1g** | 69 | 48 | 50 |
| isosafrole **1h** | >99 | 3 | >99 |
| 2-vinylpyridine **1i** | n.a.[e] | n.c. | n.a.[e] |
| *cis*-stilbene **1j** | n.a.[e] | n.c. | n.a.[e] |
| 4-phenyl-1-butene **1k** | n.a.[e] | n.c. | n.a.[e] |

[a] Reaction conditions: Mn(III) acetylacetonate (6 mM), in Bis-Tris buffer (pH 6, 50 mM), DMSO (5%), 21 °C, substrate (30-50 mM), oxygen (2 bar), shaking (170 rpm), 24 h.
[b] Wild type preparation was employed. Data taken from literature.[iii]
[c] Chemoselectivity is the percentage of desired carbonyl product 2 over all products formed (based on GC-FID peak areas).
[d] Conversion measured by GC-FID based on peak areas.
[e] Not applicable due to no conversion.
n.c.: No conversion.

# 10. Analytics

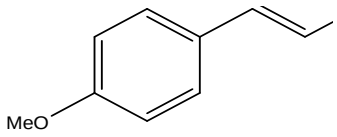
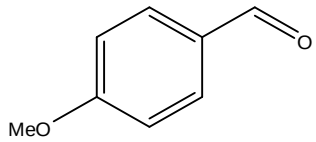
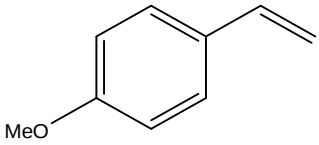
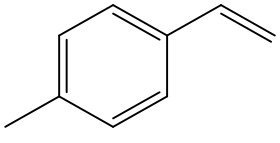
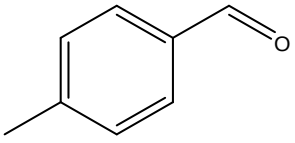
GC-FID analysis for the conversion determination was carried out in a Varian 3800 with a Varian chrompack 8200 autosampler. For the GC-MS analysis, Agilent technologies 7890 A GC system with a 5975 C inert XL MSD was used. DB-1701 capillary column (50 m, 0.2 mm, 0.33 µm film) and HP-5 MS capillary column (30 m, 0.25 mm, 0.25 µm film) were the columns used.

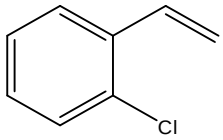
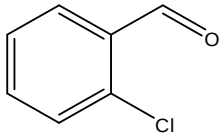
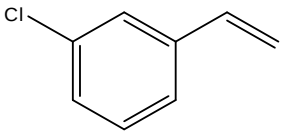
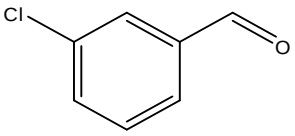
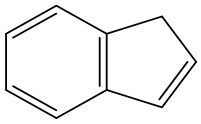
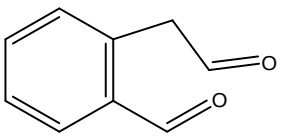
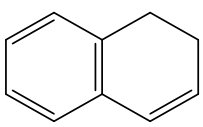
GC-FID method: 14.5 psi, 100 °C - 20 °C /min - 220 °C - hold 2 min, inlet temp: 220 °C.

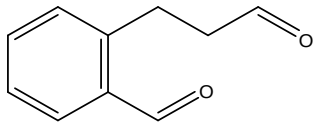
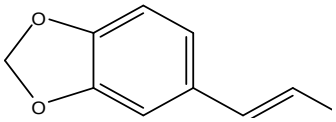
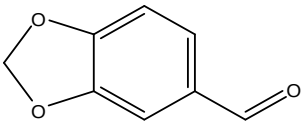
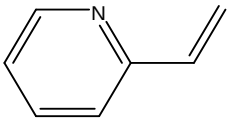
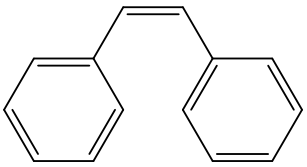
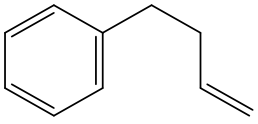
GC/MS method C: 32 psi, 100 °C-hold 0.5 min- 10 °C/min- 200 °C, inlet temp- 250 °C

GC/MS method D: 0.69 psi, 40 °C – hold 2 min - 10 °C/min- 180 °C, inlet temp- 250 °C

**Table S7: Summary of GC/GC-MS retention times of the tested substrates and formed products.**

| Compound | GC retention time (min) | GC-MS retention time (min) | GC-MS method (min) |
|---|---|---|---|
|  *t*-anethole (**1a**) | 4.6 | 6.9 | C |
|  *p*-anisaldehyde (**2a**) | 4.9 | 6.5 | C |
|  4-methoxystyrene (**1b**) | 3.7 | 5.3 | C |
|  4-methylstyrene (**1c**) | 2.7 | 7.3 | D |
|  | 3.5 | 8.0 | D |

| | | | |
|---|---|---|---|
| 4-methyl-benzaldehyde (**2c**) | 3.1 | 7.9 | D |
|  2-chlorostyrene (**1d**) | 3.7 | 8.4 | D |
|  2-chloro-benzaldehyde (**2d**) | 3.1 | 7.9 | D |
|  3-chlorostyrene (**1e**) | 3.8 | 8.4 | D |
|  3-chloro-benzaldehyde (**2e**) | 3.0 | 4.3 | C |
|  indene (**1f**) | 5.8 | 7.5 | C |
|  2-(2-oxo-ethyl)-benzaldehyde (**2f**) | 3.7 | 5.6 | D |
|  1,2-dihydronaphthalene(**1g**) | | | |

| | | | |
|---|---|---|---|
|  <br>2-(3-oxo-propyl-)benzaldehyde<br>(**2g**) | 6.5 | 8.7 | D |
|  <br>isosafrole (**1h**) | 5.3 | 8.1 | D |
|  <br>piperonal<br>(**2h**) | 5.5 | 7.6 | D |
|  <br>2-vinylpyridine (**1i**) | 2.5 | n.d | n.a. |
|  <br>*Cis*-stilbene (**1j**) | 6.2 | n.d | n.a. |
|  <br>4-phenyl-1-butene (**1k**) | 2.8 | n.d | n.a. |

n.d. not determined. n.a. not applicable.

# References


[1] a) F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmey, B. Bühler, *Green Chem.* **2011**, *13*, 226-265; b) Modern Oxidation Methods (Ed: J.-E. Baeckvall), Wiley, Weinheim, **2004**; c) G. J. ten Brink, I. W. C. E. Arends, R. A. Sheldon, *Chem. Rev.* **2004**, *104*, 4105-4123; d) R. Noyori, M. Aoki, K. Sato, *Chem. Commun.* **2003**, 1977-1986; e) M. Poliakoff, J. M. Fitzpatrick, T. R. Farren, P. T. Anastas, *Science* **2002**, *297*, 807-810; f) K. Sato, M. Hyodo, M. Aoki, X.-Q. Zheng, R. Noyori, *Tetrahedron* **2001**, *57*, 2469-2476.

[2] a) F. E. Kuhn, R. W. Fischer, W. A. Herrmann, T. Weskamp, in *Transition Metals for Organic Synthesis*, Vol. 2 (Eds: M. Beller, C. Bolm), Wiley-VCH, Weinheim, **2004**, pp 427-436; b) D. G. Lee, T. Chen, in *Comprehensive Organic Synthesis*, Vol. 7 (Eds: B. M. Trost, I. Fleming), Pergamon, Oxford, **1991**, pp. 541-591; c) M. Hudlicky, *Oxidation in Organic Chemistry*, ACS Monograph 186, American Chemical Society, Washington, DC, **1990**.

[3] a) S. G. Van Ornum, R. M. Champeau, R. Pariza, *Chem. Rev.* **2006**, *106*, 2990-3001; b) J. McGuire, G. Bond, P. J. Haslam, in *Handbook of Chiral Chemicals*, 2nd ed., Taylor & Francis, Boca Raton, FL, **2006**, pp 165-184; c) P. S. Bailey, *Ozonation in Organic Chemistry*, Vol. 1: *Olefinic Compounds*; Academic Press, New York, **1978**.

[4] One of the most widely used reagent is $OsO_4$-$NaIO_4$ (Lemieux-Johnson reagent): a) R. Pappo, D. S. Allen Jr., R. U. Lemieux, W. S. Johnson, *J. Org. Chem.* **1956**, *21*, 478-479; selected further reagents are: $OsO_4$/oxone: b) B. R. Travis, R. S. Narayan, B. Borhan, *J. Am. Chem. Soc.* **2002**, *124*, 3824-3825; cat. $RuCl_3$/($NaIO_4$ or Oxone): c) D. Yang, C. Zhang, *J. Org. Chem.* **2001**, *66*, 4814-4818; ruthenium nanoparticles/$NaIO_4$: d) C.-M. Ho, W.-Y. Yu, C.-M. Che, *Angew. Chem.* **2004**, *116*, 3365-3369, *Angew. Chem. Int. Ed.* **2004**, *43*, 3303-3307; other ruthenium derived catalysts: e) V. Kogan, M. M. Quintal, R. Neumann, *Org. Lett.* **2005**, *7*, 5039-5042; permanganate: h) D. Arndt, *Manganese Compounds as Oxidizing Agents in Organic Chemistry*, Open Court, LaSalle IL, **1981**, 241-246; peroxotungstate complexes with $H_2O_2$: f) A: Haimov, H. Cohen, R. Neumann, *J. Am. Chem. Soc.* **2004**, *126*, 11762-11763: Au(I)-catalysis with TBHP: g) H. Kitahara, H. Sakurai, *Chem. Lett.* **2012**, *41*, 1328-1330.

[5] K. Miyamoto, Y. Sei, K. Yamaguchi, M. Ochiai, *J. Am. Chem. Soc.* **2009**, *131*, 1382-1383.

[6] R. Lin, F. Chen, N. Jiao, *Org. Lett.* **2012**, *14*, 4158-4161.

[7] Selected examples: Tryptophan 2,3-dioxygenase and and indoleamine 2,3-dioxygenase: a) I. Efimov, J. Basran, S. J. Thackray, S. Handa, C. G. Mowat, E. L. Raven, *Biochemistry* **2011**, *133*, 16251-16257; b) L. W. Chung, X. Li, H. Sugimoto, Y. Shiro, K. Morokuma, *J. Am. Chem. Soc.* **2010**, *132*, 11993-12005; rubber oxygenase: c) R. Braaz, W. Armbruster, D. Jendrossek, *Appl. Environ. Microbiol.* **2005**, *71*, 2473-2478; carotenoid cleavage dioxygenases: d) D. P. Kloer, S. Ruch, S. Al-Babili, P. Beyer, G. E. Schulz, *Science* **2005**, *308*, 267-269; e) E. K. Marasco, C. Schmidt-Dannert, *ChemBioChem* **2008**, *9*, 1450-1461; lignostilbene-α-β-dioxygenase: f) S. Kamoda, T. Terada, Y. Saburi, *Biosci. Biotechnol. Biochem.* **2003**, *67***,** 1394-1396; β-diketone dioxygenase: g) G. D. Straganz, A. R. Diebold, S. Egger, B. Nidetzky, *J. Am. Chem. Soc.* **2010**, *49*, 996-1004; catechol dioxygenases: h) T. D. H. Bugg, *Tetrahedron* **2003**, *59*, 7075-7101; isoeugenol oxygenase: i) M. Yamada, Y. Okada, T. Yoshida, T. Nagasawa, *Arch. Microbiol.* **2007**, 187, 511–517.

[8] a) M. Lara, F. G. Mutti, S. M. Glueck, W. Kroutil, *Eur. J. Org. Chem.* **2008**, 3668-3672; b) H. Mang, J. Gross, M. Lara, C. Goessler, H. E. Schoemaker, G. M. Guebitz, W. Kroutil, *Angew. Chem.* **2006**, *118*, 5325-5328; *Angew. Chem. Int. Ed.* **2006**, *45*, 5201-5203.

[9] M. Lara, F. G. Mutti, S. M. Glueck, W. Kroutil, *J. Am. Chem. Soc.* **2009**, *131*, 5368-5369.

[10] C. E. Paul, A. Rajagopalan, I. Lavandera, V. Gotor-Fernández, W. Kroutil, V. Gotor, *Chem. Commun.* **2012**, *48*, 3303-3305.

[11] a) J. W. Whittaker, *Arch. Biochem. Biophys.* **2012**, *525*, 111-120; b) *Handbook of metalloproteins: Vol. 3* (Eds: A. Messerschmidt, W. Bode, M. Cygler), Wiley, Weinheim, **2004**; c) K. Wieghardt, *Angew. Chem.* **1989**, *101*, 1179-1198; *Angew. Chem. Int. Ed.* **1989**, *28*, 1153-1172.

[12] a) N. Cox, H. Ogata, P. Stolle, E. Reijerse, G. Auling, W. Lubitz, *J. Am. Chem. Soc.* **2010**, *132*, 11197-11213; b) J. A. Cotruvo, J. Stubbe, *Biochemistry* **2010**, *49*, 1297-1309; c) W. Jiang, D. Yun, L. Saleh, E. W. Barr, G. Xing, L. M. Hoffart, M.-A. Maslak, C. Krebs, J. M. Bollinger Jr., *Science* **2007**, *316*, 1188-1191.

[13] G. Ammerer, C. P. Hunter, J. H. Rothman, G. C. Saari, L. A. Vails, T. H. Stevens, *Mol. Cell. Biol.* **1986**, *6*, 2490-2499.

[14] B. M. Dunn, *Chem. Rev.* **2002**, *102*, 4431-4458.

[15] S. J. Smith, K. S. Hadler, G. Schenk, G. R. Hanson, N. Mitic, in *Metals in Biology: Applications of High-Resolution EPR to Metalloenzymes, Biological Magnetic Resonance*, Vol 29 (Eds: G. Hanson, L. Berliner), Springer, New York, **2010**, pp. 273-327.

[16] Only a single manganese porphyrin complex MnCl-TPP-$(PEO750)_4$ was reported for alkene cleavage requiring sodium periodate as oxidant. The reaction most likely involves epoxide and diol formation; the latter is finally cleaved by periodate: S. T. Liu, K. V. Reddy, R. Y. Lai, *Tetrahedron* **2007**, *63*, 1821-1825.

[17] a) G. I. Berglund, G. H. Carlsson, A. T. Smith, H. Szöke, A. Henriksen, and J. Hajdu, *Nature* **2002**, *417*, 463-468; b) I. Schlichting, J. Berendzen, K. Chu, A. M. Stock, S. A. Maves, D. E. Benson, R. M. Sweet, D. Ringe, G. A. Petsko, S. G. Sligar, *Science* **2000**, *287*, 1615-1622.

[18] A. Hatakka, *FEMS Microbiol. Rev*. **1994**, *13*, 125-135.

[19] a) K. Hult, P. Berglund, *Trends Biotechnol*. **2007**, *25*, 231-238; b) A. Taglieber, H. Höbenreich, J. D. Carballeira, R. J. G. Mondière, M. T. Reetz, *Angew. Chem.* **2007**, *119*, 8751-8754; *Angew. Chem. Int. Ed*. **2007**, *46*, 8597-8600; c) U. T. Bornscheuer, R. J. Kazlauskas, *Angew. Chem.* **2004**, *116*, 6156-6165; *Angew. Chem. Int. Ed.* **2004**, *43*, 6032-6040.

[20] V. L. Pecoraro, M. J. Baldwin, A. Gelasco, *Chem. Rev*. **1994**, *94*, 807-826.

[21] a) U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185-194; b) J. M. Woodley, *Trends Biotechnol.* **2008**, *26*, 321–327; c) H. E. Schoemaker, D. Mink, M. G. Wubbolts, *Science* **2003**, *299*, 1694–1697.

Supplemental

[i] A. Rajagopalan, B. Seisser, F. G. Mutti, M. Schober, W. Kroutil, *J. Mol. Cat. B: Enzym.* **2013**, *90*, 118-122.

[ii] E. Krieger, G. Koraimann, G. Vriend, *Proteins* **2002**, *47*, 393-402.

[iii] M. Lara, F. G. Mutti, S. M. Glueck, W. Kroutil, *Eur. J. Org. Chem*. **2008**, 3668–3672.

# Curriculum vitae

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| 2010 - 2014 onwards | Austrian Centre of Industrial Biotechnology (ACIB GmbH), Austria<br>PhD at the '*NAWI Graz Doctoral School of Molecular Biosciences and Biotechnology'* with the title 'Improving heterologous cytochrome P450 function in *S. cerevisiae*' |
| 2005 - 2010 | Graz University of Technology (TUG), Austria<br>Bachelor of Science in Chemistry, Master in Biotechnology.<br>Diploma thesis with the title '*Membrane Protein Expression in E.coli'*<br>Graduation with honours |
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| 2010 – 2012, 2014 | Graz University of Technology (TUG), Austria<br>Supervisor of the student laboratory courses 'Einführung in die Molekularbiologie' (MOL.501) and 'Einführung in die Biotechnologie' (CHE.177), teaching several molecular biological techniques |
| 2010, July - August | University of Novi Sad, Serbia<br>Scholarship at the Faculty of Science in the project 'Chemical synthesis of terpenoidic compounds' |
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| 2008, September | Graz University of Technology (TUG), Austria<br>Project laboratory at the *Research Centre Applied Biocatalysis* (Applied Biocatalysis, AB), research project focusing on the development of Enzyme-linked Immunosorbent Assays (ELISA) |
| **Awards and Prizes** | |
| 2013, July | Oral Presentation Award of 10th DocDay**,** NAWI Graz Doctoral School of Molecular Biosciences and Biotechnology |
| **Publications** | |
| 2013 | *EnzymaticAerobic Alkene Cleavage Catalyzed by a $Mn^{3+}$-Dependent Proteinase A Homologue.* Aashrita Rajagopalan, Markus Schober, Anita Emmerstorfer, Lucas Hammerer, Anna Migglautsch, Birgit Seisser, Silvia. M. Glueck, Frank Niehaus, Juergen Eck, Harald Pichler, Karl Gruber, Wolfgang Kroutil, 2013, ChemBioChem 14, 2427 – 2430 |
| 2012 | *Production of human cytochrome P450 2D6 drug metabolites with recombinant microbes – a comparative study*. Martina Geier, Andreas Braun, Anita Emmerstorfer, Harald Pichler, Anton Glieder, 2012, Biotechnology journal 7, 1346 – 135 |
| **Conference talks** | |
| 2013, June | *Saccharomyces cerevisiae as Fragrant Whole-Cell Biocatalyst.* Anita Emmerstorfer, Miriam Wimmer, Monika Müller, Daniel Mink, Harald Pichler, *30th INTERNATIONAL SPECIALISED SYMPOSIUM ON YEAST. Stara Lesna, Slovakia* |
| **Poster Presentations** | |
| June, 2013 | Emmerstorfer, A.; Wimmer, M.; Müller, M.; Mink, D.; Pichler, H.: *Saccharomyces cerevisiae as Fragrant Whole-Cell Biocatalyst.* 30th INTERNATIONAL SPECIALISED SYMPOSIUM ON YEAST. Stara Lesna, Slovakia |
| August, 2012 | Emmerstorfer, A.; Müller, M.; Kaluzna, I.; Macheroux, P.; Schwab, H.; Pichler, H.: *Saccharomyces cerevisiae as whole-cell biocatalyst for terpenoid production.* Yeast genetics & molecular biology. Princeton, New Jersey, USA |
| September, 2011 | Emmerstorfer, A.; Wriessnegger, T.; Augustin, P.; Müller, M.; Kaluzna, I.; Macheroux, P.; Schwab, H.; Pichler, H.: *Heterologous Expression of CYP71D55 in S. cerevisiae for (+)-Nootkatone Production.* Biotrans. Giardini Naxos, Sicily |

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