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Research Article

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

Martina Geier[1], Andreas Braun[1], Anita Emmerstorfer[2], Harald Pichler[1,2] and Anton Glieder[2]

[1] Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria
[2] Austrian Centre of Industrial Biotechnology (ACIB), Graz, Austria

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* was the most efficient at expressing CYP2D6. Without additional over-expression of chaperons, the achieved yield of CYP2D6 was the highest of 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.

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1 Introduction

Cytochromes P450 (CYPs) constitute a super-family 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 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 is among the most important. Although CYP2D6 is expressed only weakly in the human liver, that is, <2% of total CYP liver enzyme content, it is involved in the metabolism of about 25% of drugs currently in use [2]. Furthermore, CYP2D6 is of pharmacological interest because it displays a very high degree of inter-individual variability. This variability is mainly caused by extensive genetic polymorphism: over 80 alleles and allele variants have been described [3]. Consequently, a variety of phenotypes arise, displaying, in extreme cases, no or even greater than normal CYP2D6 activity; thus referred to as a "poor metabolizer" and "ultra-rapid metabolizer", respectively. The consequences for a patient might therefore be 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 testing of drug metabolites has become an important issue in the drug discovery and development process. In 2008, the US Food and Drug Administration stated that metabolites present at >10% of the parent compound in 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 rarely achieved by standard chemical means. Whole-cell biotransformations

Correspondence: Dr. Anton Glieder, Austrian Centre of Industrial Biotechnology (ACIB), Petersgasse 14, 8010 Graz, Austria
E-mail: anton.glieder@acib.at

Abbreviations: CDW, cell dry weight; **CPR**, cytochrome P450 reductase; **CYP2D6**, cytochrome P450 2D6; **CYP**, cytochrome P450; **oe-PCR**, overlap-extension PCR

employing recombinant microorganisms are an elegant and scalable possibility for employing native enzymes in metabolite syntheses. Using whole cells as biocatalysts is advantageous in many aspects [5]. First, enzyme isolation and purification steps can be circumvented, which saves time and costs. Whole cells can also be regarded as covers to protect enzymes from shear forces or organic solvents and thus increase their stability. Mammalian CYPs almost always rely on the presence of cytochrome P450 reductase (CPR); the enzyme required for 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, 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 limited substrate/product transfer across the cell membrane [6]. Nevertheless, whole-cell systems have already been implemented successfully in industry. Amongst others, Novartis employs recombinant *Escherichia coli* for CYP metabolite synthesis on 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 a cofactor [8]. Nevertheless, CYP2D6 was 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 Table 1 and references therein). Although much data is available in the literature, differences in expression strategies and assays employed prevent a direct comparison of different CYP expression systems and hamper the decision of which system might be optimal for a biotechnological application.

Therefore, the aim of our study was 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 with other yeasts, the last two species offer features that might be beneficial for whole-cell biotransformations. *P. pastoris* can easily be grown to very high cell densities on cheap media, yielding up to 130 g cell dry weight (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 hosts tested.

2 Materials and Methods

2.1 Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) or Carl Roth (Karlsruhe, Germany) at 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 CYP 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 and/or 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) and pESC-URA (Agilent Technologies, Santa Clara, USA) carrying galactose-inducible promoters. YPD medium was made of 10 g/L Bacto^{TM} yeast extract (Becton, Dickinson and Company), 20 g/L Bacto^{TM} peptone, and 20 g/L glucose. SC minimal medium contained 6.7 g/L Difco^{TM} yeast nitrogen base (Becton, Dickinson and Company) and 167 mg/L each of adenine, lysine, tyrosine, histidine, leucine, and tryptophan. The minimal medium was supplemented with 2 % (w/v) glucose for cell growth and 2 % (w/v) galactose and 1 % (w/v) raffinose for induction of protein expression.

CYP 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

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Table 1. Reported expression levels and expression strategies for CYP2D6 production in different recombinant hosts

Expression host	Expression strategy	Remarks	Expression Level		Reference
			(pmol/mg protein)	(nmol/L culture)	
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.

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 the Supporting information, Table S1. Vector maps of the expression constructs are also available as Supporting information. Standard molecular cloning technologies were used [12]. For CYP expression in *E. coli*, bicistronic constructs were assembled (Supporting information, Fig. S1). The genes for CYP2D6 and CPR were amplified using primers *Xba*I_rbs_2D6_fw, *Bam*HI_CPR_rev, 2D6_Linker_rev, and CPR_Linker_fw; (Supporting information, Table S2) 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 (Supporting information, Fig. S2A). In the 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 a template, the CPR coding region was amplified by 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 by 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. cerevisiae* (*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 (Supporting information, Fig. S2B and S2C). 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 the *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] (Supporting information, Fig. S3A). 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 Supporting information, table S2. 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 (Supporting information, Fig. S3B), 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* (Supporting information, Fig. S4), 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 transforma-

tion 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* overnight 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 and 1 mM IPTG for *E. coli* LEMO21(DE3) and *E. coli* BL21(DE3), respectively. Concomitantly, 0.5 mM δ-aminolevulinic acid was added and induction was performed for 48 h at 28°C.

Preliminary experiments on the 10 mL scale were 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

Overnight 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 for 5 min at 1000*g* 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 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) overnight 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 as described in [21]. Cells were harvested by 10 min centrifugation at 3000*g* 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 4000*g* and 4°C and resuspended in homogenization buffer supplemented with 1 mg/mL lysozyme. After brief 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 10 000*g* and 4°C. To recover the membrane fractions from yeasts and *E. coli*, the cleared lysates were ultra-centrifuged at 180 000*g* and 4°C for 1 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 a standard.

2.6 SDS-PAGE/western blotting

5 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 CYP

CYP content in membrane preparations was determined by CO difference spectroscopy as described by Omura and Sato [22]. 2 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 and 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 $\varepsilon_{450-490\ nm}$ = 91 $mM^{-1}\ cm^{-1}$.

2.8 Determination of CYP2D6 and CPR activity in membrane preparations

CYP2D6 activity in membrane preparations was determined by applying the bufuralol 1´-hydroxylation assay as described [24]. In brief, 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. 10 µL of 1 mM prednisolone were added as an internal standard for later metabolite quantification. The reaction mixture was centrifuged for 5 min at maximum speed in a tabletop 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]. 25 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. 50 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 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 about 5 for *P. pastoris* strains and an OD_{600} of about 20 for *E. coli*, *S. cerevisiae*, and *Y. lipolytica* strains. 200 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 an 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, 30, 35, 40, and 50°C in triplicate. The pH dependency was investigated in reaction buffers at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0.

2.10 Determination of CYP2D6 stability in whole-cell conversions

50 mL of cells resuspended in reaction buffer as described 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 3000*g* 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) using an instrument equipped with an MSD SL detector with an electrospray ionization unit. Metabolites were separated on an XDB-C18, 1.8 µm, 4.6 × 50 mm column (Agilent technologies) using 10 mM ammonium acetate, pH 5.0, and acetonitrile (ACN) as the 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 eluted 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 *E. 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 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 of tuning T7 expression by modulating the level of lysozyme, which is 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, that is, when the T7 promoter was at its full strength. This indicates that the CYP2D6 expression level does not exceed the capacity of the cell to insert the constructed proteins into the membrane. On the contrary, adding 100 µM L-rhamnose to the media resulted in only 3.03% CYP2D6 activity compared with the activity at full promoter strength. By further increasing the L-rhamnose concentration, the CYP2D6 ac-

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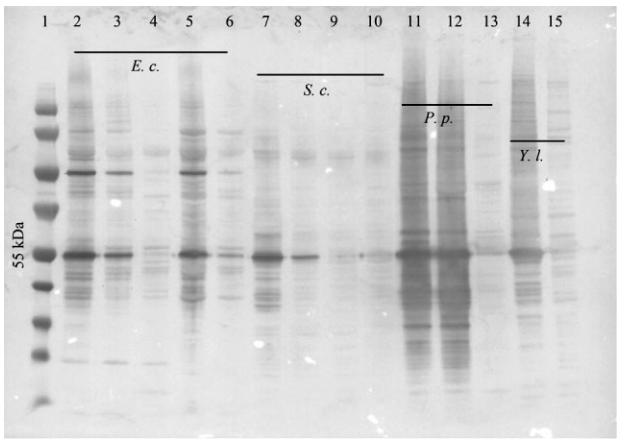

Figure 1. Western blot analysis of over-expressed CYP2D6 at the expected molecular weight of 55.75 kDa. (1) 5 µL of PageRuler Standard, (2) 5 µg of total protein in membrane fractions of *E. coli* (*E.c.*) LEMO-2D6-CPR, (3) *E.c.* LEMO-CPR-2D6, (4) *E.c.* LEMO, (5) *E.c.* BL21-2D6-CPR, (6) *E.c.* BL21-CPR-2D6,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

tivity was completely abolished (data not shown). Although the fine-tuning of CYP2D6 expression is not strictly required in *E. coli* LEMO21(DE3), this host provided more CYP2D6 than its parental strain *E. coli* BL21(DE3) (Fig. 1). Although Western blot analysis clearly showed CYP2D6 expression in both *E. coli* strains, the amounts produced were too low to result in reduced CO difference spectra for CYP quantification.

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 CPR gene was directly placed behind the T7 promoter, while the second gene trailed another ribosomal binding site. As can be seen from Figs. 1 and 2, the relative location to the promoter had an effect on the CYP2D6 and CPR levels achieved. 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 relative to the negative control, *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 about 3. The same trend was observed when using *E. coli* BL21(DE3) as the expression host.

3.2 CYP2D6 and CPR expression in *S. cerevisiae*

A prominent expression system for mammalian P450 is *S. cerevisiae*. A feature of this system is that the endogenous yeast CPR can take over the required electron supply; thus making it catalytically self-sufficient [27]. It was shown, however, that the limited amount of yeast CPR was responsible for rather low monooxygenase activities and that over-expression of CPR could overcome this problem [28, 29]. Therefore, a *S. cerevisiae* strain harbor-

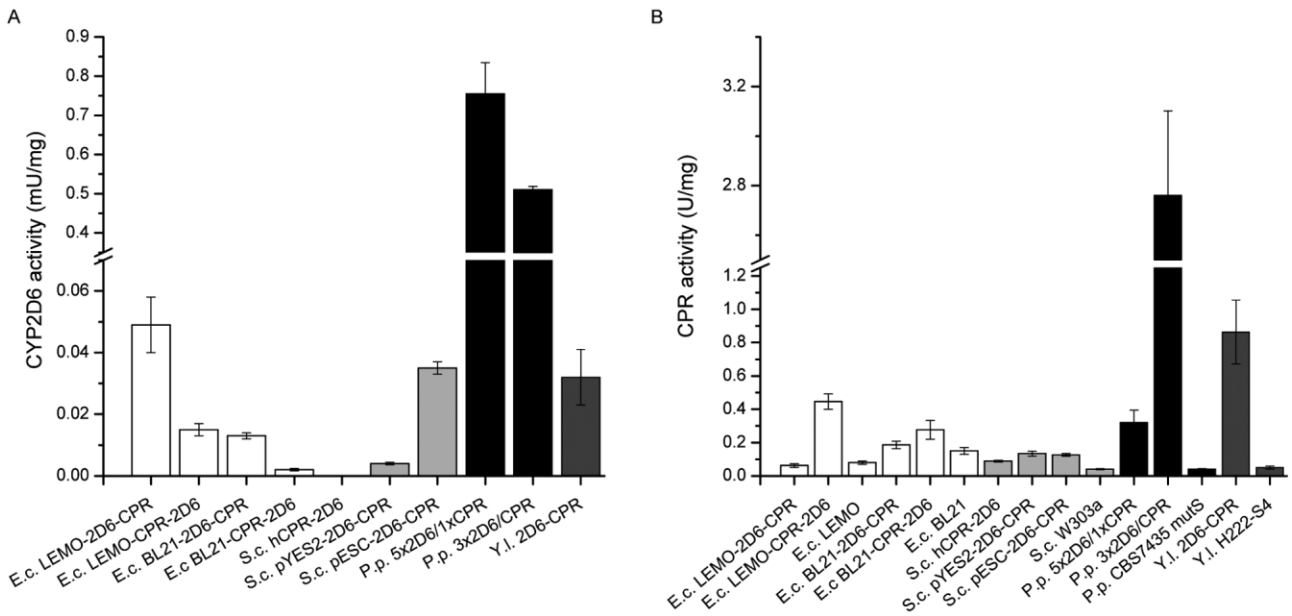

Figure 2. (**A**) CYP2D6 and (**B**) CPR activities in membrane preparations of different recombinant hosts. (**A**) CYP2D6 activity was determined in the bufuralol hydroxylation assay. Membrane preparations were incubated with 50 µM bufuralol and 1 mM NADPH in 100 mM KP_i, pH7.4, for 10 min at 37°C. 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.

ing 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 only displayed very minor CYP2D6 activity and the CYP2D6 levels produced were below the detection limit of western blot analysis and differential CO spectroscopy (Fig. 1). CYP expression could be improved by employing co-expression constructs. Clear signals were obtained in the western blot, but 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; this is in the same range as the activities observed for *E. coli* (E.c. LEMO21-2D6-CPR) and *Y. lipolytica* (Fig. 2A). In comparison with the other two yeast species, *S. cerevisiae* showed the lowest CPR activities. It was already reported that mammalian CPRs were poorly expressed in this yeast relative to the endogenous CPR [29].

3.3 CYP2D6 and CPR expression in *P. pastoris*

Two different expression strategies were employed to investigate the potential of *P. pastoris* as a heterologous expression host for a functional monooxygenase system. In both cases, expression plasmids were constructed and linearized prior to transformation. These linear expression cassettes were then integrated into 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 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 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 about 660 pmol of CYP2D6 per mg of total membrane protein, which is, to the best of our knowledge, the highest CYP2D6 level reported for any microbial expression system and the first report of 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* (see Table 1). Differential CO spectra of CYP2D6 produced in *P. pastoris* clearly showed only one peak at 450 nm, indicating that a correctly folded holo-enzyme was produced. 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 saturation of the corresponding signal or correctly folded and active hydroxylase making up only a small fraction of the total 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 (Fig. 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 (Fig. 2B). The low CPR amount might be the limiting factor, resulting in only a fraction of the CYP2D6 activity that might be reached by sufficient electron supply. This result underlines the importance of CPR, the adequate expression of which is as important as that of the CYP enzyme 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/g CDW, respectively. As in shake-flask cultivations of *P. pastoris*, OD_{600} values of >15 are easily reached, which is equivalent to >8 g CDW/L; >610 nmol CYP2D6 per L of 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 *Y. 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 into the rDNA. This resulted in strain *Y.l.* 2D6-CPR, which harbored about 30 copies of the co-expression construct. Western blot analysis showed fairly extensive CYP2D6 expression levels in the membrane fraction (Fig. 1), but the characteristic P450 peak could not be detected by differential CO spectroscopy for quan-

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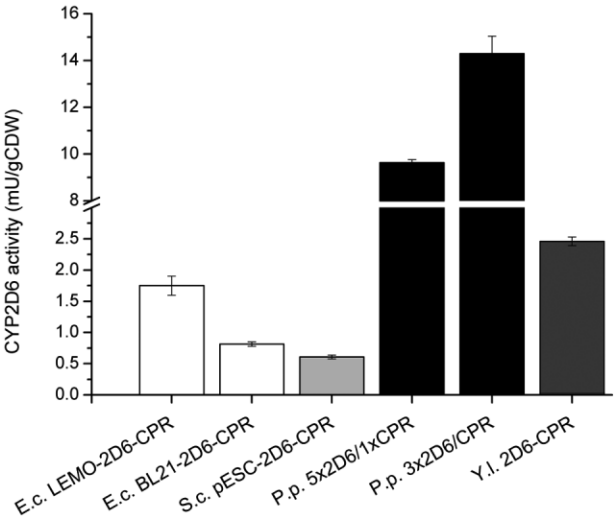

Figure 3. Whole-cell conversions of bufuralol by different recombinant expressions hosts. Conversions were conducted at 30°C in 100 mM KP_i, pH7.4, containing 1% glucose. After 30 min, the supernatants were analyzed for 1′-hydroxybufuralol production by HPLC–MS. A representative result of three biological replicates is shown. Values are shown as mean ± SD of three technical replicates.

tification. However, codon optimization of the CYP2D6 gene along with high gene dosage (>40 copies) yielded a *Y. lipolytica* strain producing 92.3 ± 9.2 pmol CYP2D6 per mg membrane protein [18]. CYP2D6 activity in the membrane fraction of *Y.l.* 2D6-CPR was about 0.03 mU/mg total membrane protein, and thus was lower than the activities found in the preparations from both *P. pastoris* strains and *E. coli* Lemo-2D6-CPR (Fig. 2A). CYP2D6 activity displayed in whole cells of *Y. lipolytica* was higher than that for *E. coli*, but not that of *P. pastoris* (Fig. 3). CPR activity for the 30-copy-containing strain of this study was 0.86 U/mg membrane protein, which was 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*.

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 9.6 and 14.3 mU/g CDW. The activities were almost one order of magnitude lower with recombinant *Y. lipolytica* (*Y.l.* 2D6-CPR; 2.4 mU/g CDW), *E. coli* (*E.c.* Lemo 2D6-CPR; 1.8 mU/g CDW), and *S. cerevisiae* (*S.c.* pESC 2D6-CPR; 0.74 mU/g CDW) (Fig. 3).

Interestingly, *P.p.* 3x2D6/CPR performed better in whole-cell conversions than *P.p.* 5x2D6/1xCPR, which displayed the highest CYP2D6 activity in vitro (Fig. 2A). This might be due to low CYP2D6 stability in the in vitro system, which was compensated for in the case of *P.p.* 5x2D6/1xCPR by the high enzyme level. In whole-cell conversions, CYP2D6 stability is not an issue, but 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 was 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 extracellular substrate and intracellular enzyme. This result clearly indicates that substrate uptake is a limiting step in the whole-cell conversions performed, 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, which were obtained with the four different expression hosts, can be found in the Supporting information, Table S3.

3.6 Temperature dependence of whole-cell biotransformations

To determine the influence of temperature on biotransformations, whole-cell conversions were conducted at temperatures ranging from 20 to 50°C (Fig. 4). The profiles obtained for the three yeast species looked similar. Increasing the reaction temperature resulted in higher whole-cell activities, yielding an approximately two-fold improvement at elevated temperature relative to that at 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 the temperature range from 30 to 35°C (≈140%), while at 50°C only about 20% of the activity recorded under standard conditions (37°C, pH 7.4) was observed. At this point, it can only be speculated as to whether substrate uptake is hampered at elevated temperature or if the CYP system is unstable in *E. coli* under these conditions.

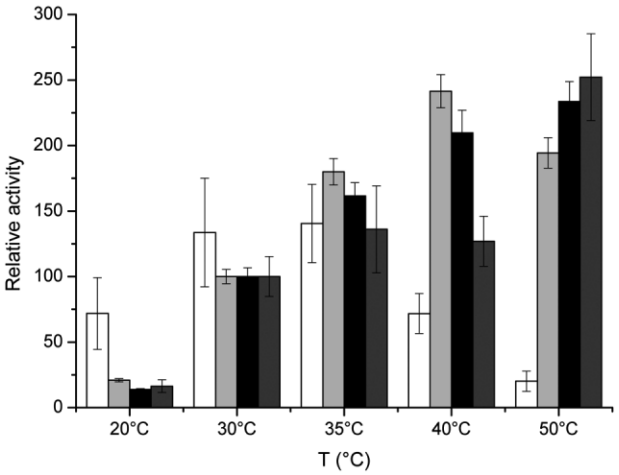

Figure 4. Temperature profile for whole-cell conversions of bufuralol by different recombinant expression hosts. Conversions were conducted in 100 mM KP_i, pH 7.4, containing 1% glucose at temperatures ranging from 20 to 50°C. Activities under standard conditions (30°C and pH 7.4 for yeast species, 37°C 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 one hand, the stability of CYP2D6 in resting cells of the recombinant host tested. 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 (Fig. 5). On the other hand, to test the stability of CYP2D6 during biocatalysis, the same experimental setup was used, but the cells were incubated in reaction buffer containing 200 μM propranolol. Propranolol is another CYP2D6 substrate and was chosen 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 still displayed about 90% of the residual activity after 6 h of incubation and even after 48 h there was still detectable activity (Fig. 5C). During whole-cell conversions of propranolol, the CYP2D6 activity dropped significantly after 30 min to about 27% residual activity. Whether regeneration of the biocatalyst CYP2D6 or that 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 that observed for *P. pastoris* (Fig. 5B). After 6 h of incubation without propranolol, about 27% of the starting activity was retained and after 48 h activity was completely lost. In the presence of propranolol, only about 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 (Fig. 5A). It seems that *E. coli* does not provide 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 about 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 increased during the first 8 h of incubation, before it started to drop. The same trend was found when incubating the cells with propranolol. In the *Yarrowia* system, CYP2D6 was not inactivated by incubation with propranolol and displayed the same activity during the first 6 h as that in resting cells alone. Even after 48 h of reaction time and full conversion of propranolol, cells were as active as those without propranolol (Fig. 5D).

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 were produced. The expression levels were amongst the highest reported in the literature (Table 1). Only insect cells produced 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, whereas 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 CYPs. Besides CYP2D6, only one further human isoform, namely CYP17, was functionally expressed in *P. pastoris* [31]. CYP3A4, another important drug metabolizing enzyme, was not functionally expressed in *P. pastoris* so far, whereas its functional expression was reported for *E. coli* [32], *S. cerevisiae* [33], and *Y. lipolytica* [18]. Therefore, the proven versatility of *Pichia* as an expression host for P450 enzymes is still low in comparison with other systems such as *E. coli* and *S. cerevisiae*.

The two expression strategies presented herein 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 CYP quantities for further isolation and purification. If employed as biocatalyst, an optimal ratio between CYP2D6 and CPR is essential to obtain optimal activity.

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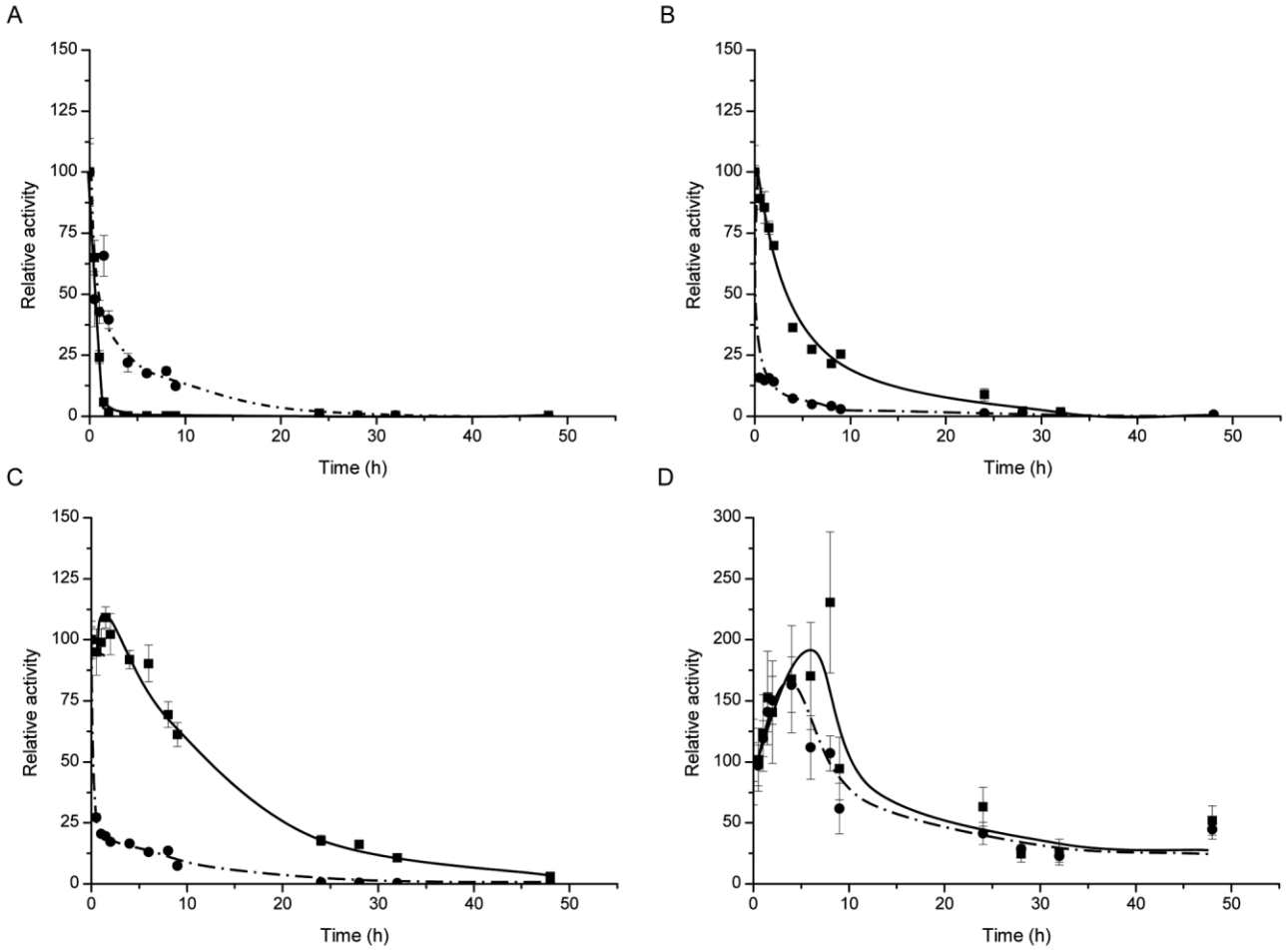

Figure 5. Stability of CYP2D6 in whole-cell biotransformations. Cells were incubated in 100 mM KP_i, pH 7.4, containing 1% glucose in the absence (full line) and presence of 200 μM propranolol (dashed line). Samples were drawn at certain time points and residual CYP2D6 activity was determined by the 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, and (**D**) *Y.l.* 2D6-CPR. The course of CYP2D6 activity was manually interpolated.

The high CYP2D6 expression levels reported in the literature for *E. coli* were not reached with the strains constructed in this study. This might mainly be because the N-terminal sequence of CYP2D6 remained unmodified. These N-terminal truncations are regarded as key elements for achieving high-level expression of mammalian CYPs in bacteria [10, 27]. Thus the altered P450 enzymes might be problematic in drug metabolite production. A recent study showed that N-terminal modifications of human CYP1A2 changed the active-site surroundings 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 modifications in the bacterial system might account for the observed differences. This highlights the importance of expressing CYP proteins in a membrane environment.

Our *S. cerevisiae* strains were not efficient CYP2D6 expression strains as might have been expected from the reports in the 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 difficult to express in baker's yeast. By comparing the expression levels of various human CYPs in *S. cerevisiae*, CYP2D6 levels were amongst the lowest reported [36, 37]. In a comparative study by Cornelissen and Julsing [38], *S. cerevisiae* was inferior to *E. coli* in the expression of rat and human CYP1A1.

Although *E. coli* and *S. cerevisiae* did not meet expectations from the literature in our comparative study, these two systems are still valuable hosts for the recombinant production of CYP enzymes. All major human CYP iso-

forms have been successfully expressed in these hosts [8, 36, 37, 39], highlighting their versatility and potential.

Y. lipolytica is a suitable expression host for mammalian CYP 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 an alternative. However, although Bureik et al. successfully used this yeast as a host for whole-cell biotransformations with human CYPs, no CYP concentrations were reported [42, 43].

While whole-cell activities described in this comparative study remained in the previously observed mU/g CDW range, significantly higher long-term catalyst stability of the applied yeasts relative 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 available to obtain higher productivities for future applications, if needed.

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The authors declare no conflict of interest.

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