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ORIGINAL PAPER

# Comparison of microbial hosts and expression systems for mammalian CYP1A1 catalysis

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**Abstract** Mammalian cytochrome P450 enzymes are of special interest as biocatalysts for fine chemical and drug metabolite synthesis. In this study, the potential of different recombinant microorganisms expressing rat and human *cyp1a1* genes is evaluated for such applications. The maximum specific activity for 7-ethoxyresorufin O-deethylation and gene expression levels were used as parameters to judge biocatalyst performance. Under comparable conditions, *E. coli* is shown to be superior over the use of *S. cerevisiae* and *P. putida* as hosts for biocatalysis. Of all tested *E. coli* strains, *E. coli* DH5α and *E. coli* JM101 harboring rat CYP1A1 showed the highest activities (0.43 and 0.42 U $g_{CDW}^{-1}$, respectively). Detection of active CYP1A1 in cell-free *E. coli* extracts was found to be difficult and only for *E. coli* DH5α, expression levels could be determined (41 nmol $g_{CDW}^{-1}$). The presented results show that efficient expression of mammalian *cyp1a1* genes in recombinant microorganisms is troublesome and host-dependent and that enhancing expression levels is crucial in order to obtain more efficient biocatalysts. Specific activities currently obtained are not sufficient yet for fine chemical production, but are sufficient for preparative-scale drug metabolite synthesis.

**Keywords** Cytochrome P450 1A1 · 7-Ethoxyresorufin O-deethylation · Whole-cell biocatalysis · Gene expression · *Escherichia coli*

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## Introduction

Mammalian cytochrome P450 monooxygenases (CYPs) are of exceptional interest for the synthesis of fine chemicals, pharmaceuticals, and drug metabolites. The main reasons are the ability to selectively oxyfunctionalize unactivated C–H bonds, their prominent role in drug metabolism and degradation of xenobiotics, and the wide range of relatively large molecules that they accept as substrates [3, 17, 41]. Numerous systems for the heterologous expression of mammalian *cyp* genes have been developed [18, 76]. Recombinant microorganisms serve as an alternative source of enzymes next to microsomes derived from laboratory test animals and human organs. Additionally, they have great potential as catalysts for chemical biosynthesis. For such applications, whole microbial cells are often favored over the use of purified enzymes. As biocatalysts, whole cells are self-renewable and provide a stable environment for the oxygenase of interest. Membrane-bound CYPs are difficult to purify and are almost always dependent on additional proteins for electron transfer. In vitro reconstitution of such multicomponent systems requires additional purification of these proteins and may result in inefficient assembly. In contrast, the genes for such auxiliary redox partners can be co-expressed in whole cells, directly yielding a fully active catalyst [47]. Finally, living cells regenerate required reduced nicotinamide co-factors via cell metabolism, which circumvents the need for separate addition of expensive co-factors or co-factor regeneration systems [14, 33, 73].

In most cases, *Escherichia coli* strains are used as hosts for the expression of mammalian *cyp* genes. Advantages of *E. coli* include the ease of cultivation and manipulation, well-understood genetics, and low production costs. However, in order to achieve significant CYP production,

**Table 1** Different heterologous expression systems for *cyp1a1* in microorganisms

| Origin of *cyp1a1* gene | Host organism | CYP reductase | Tested in this study | References |
|---|---|---|---|---|
| Human | *E. coli* | Co-expressed | Yes | [47] |
| Human | *E. coli* | Fused to CYP | No | [10] |
| Rat | *E. coli* | Co-expressed | Yes | [64] |
| Rat | *S. cerevisiae* | Co-expressed | Yes | [37] |
| Rat | *S. cerevisiae* | Fused to CYP | Yes | [55] |
| Human | *Y. lipolytica* | Co-expressed | No | [40] |

alteration of the original gene sequence (e.g., N-terminal modifications [2, 47]) or a fusion of the gene to an inner-membrane targeting sequence [50] is usually required, as well as the addition of expensive $\delta$-aminolevulinic acid (ALA) to ensure heme incorporation [22]. Alternatively, yeast strains such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Yarrowia lipolytica*, and *Pichia pastoris* are used [3, 8, 31, 40]. These hosts do not require gene sequence modifications or ALA addition. Moreover, they constitute a eukaryotic background and possess organelles. These features can be beneficial for the expression of genes that encode membrane-bound, eukaryotic enzymes [49]. The use of *Pseudomonas* strains can also be of interest. Specific activities for limonene hydroxylation in whole cells of *P. putida*, harboring bacterial CYP153A6 were 30-fold higher than reported activities for the same enzyme in *E. coli*, without the need to supplement ALA to the medium [69]. Additionally, some *Pseudomonas* strains are solvent-tolerant, which might be a useful feature when toxic compounds are involved in the bioconversion of interest [23, 51].

Besides different choices for the host strain, strategies reported to enhance expression of mammalian *cyp* genes in microorganisms include directed evolution, chaperone co-expression, gene fusions, and codon optimization [28]. These different strategies yielded an overwhelming amount of different expression systems. For the selection of a suitable catalyst for application in biotechnological processes, it often remains unclear which system can be considered optimal. An evaluation of different CYP-based catalysts, based on literature data, is challenging because experiments are often carried out under different conditions, with different reaction substrates, and by different laboratories [53, 54]. Furthermore, inconsistent data presentation, e.g., differences in investigated parameters and units in which measured values are given, often complicates a meaningful comparison of reported data.

CYP1A1 (P4501A1, P450MC, P450c) is a prominent enzyme in mammalian metabolism and involved in the activation of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic amines/amides (HAAs), which can result in carcinogenesis [34]. Furthermore, CYP1A1 is an important enzyme for drug metabolite screening and synthesis. An efficient CYP1A1-based catalyst is of interest for synthetic applications (e.g., production of metabolic intermediates) as well as for bioremediation (e.g., degradation of pollutants) [47, 60, 64]. In literature, at least six different whole-cell expression systems have been described for *cyp1a1* genes, which may be considered for such applications (Table 1).

In the present study, the performance (in terms of achievable activity and gene expression level) of different microbial strains (*E. coli*, *S. cerevisiae*, and *P. putida*) harboring rat or human CYP1A1 was evaluated with the aim to determine the optimal system and its characteristics for biocatalytic applications. The gene for the auxiliary CYP-reductase (CPR) was either co-expressed with or fused to the *cyp1a1* gene.

## Materials and methods

### Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (Munich, Germany) or Carl-Roth (Karlsruhe, Germany) with the highest purity available. All chemicals were used without further purification.

### Microbial strains and plasmids and related cultivation and transformation methods

The strains and plasmids used in this study are listed in Table 2. *E. coli* strains were grown on Luria–Bertani (LB) medium [58], Terrific Broth (TB) [58] or M9* mineral medium [46] with either 0.5% (w/v) glucose or glycerol as growth substrate. Mineral media were supplemented with 1 mM thiamine and 100 μg $ml^{-1}$ ampicillin (for pCW vectors) or 50 μg $ml^{-1}$ gentamicin (for pCom8 vectors). *P. putida* was grown on E2 medium [32] with 0.5% (w/v) citrate as growth substrate and 50 μg $ml^{-1}$ gentamicin. *S. cerevisiae* was grown on yeast extract-peptone-dextrose (YPD) medium [58] or Verduyn mineral medium [71] with

**Table 2** List of strains and plasmids used in this study

| Strain or plasmid | Remarks | References |
|---|---|---|
| *E. coli* DH5α | *supE44* Δ*lac*U169 (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | [20] |
| *E. coli* JM101 | *supE thi* Δ(*lac-proAB*) *F'*[*traD36 proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15] | [36] |
| *E. coli* E609 | *Hfrc pps* | [75] |
| *E. coli* E609L | *lpp*:*Tn10* derivative of *E. coli* E609, Tc$^r$ | [38, 75] |
| *P. putida* KT2440 | *P. putida* mt-2 cured of the TOL plasmid | [1] |
| *S. cerevisiae* BY4741 | MATa, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* | [6] |
| *S. cerevisiae* AH22 | *a leu2, his4, can1 cir*$^+$ | [24] |
| pCWori+ | pHSe5 derivative with *cheW* chemotaxis gene, double *tac* promoter, *trpA* terminator, phage M13 ori, *lacI*$^q$, Amp$^r$ | [2] |
| pCWh1A1 | pCWori+ containing a N-terminally modified human CYP1A1 gene and the full-length human CPR gene, does not contain *cheW* gene, Amp$^r$ | [47] |
| pCWr1A1 | pCWori+ containing a N-terminally truncated rat CYP1A1 gene and a slightly modified human CPR gene (Gly2Ala), does not contain *cheW* gene, Amp$^r$ | [64] |
| pCom8 | Broad-host-range expression vector, *alk* promoter, *oriT, alkS* regulator gene, Gm$^r$ | [65] |
| pCom8r1A1 | pCom8 containing a N-terminally truncated rat CYP1A1 gene and a slightly modified human CPR gene (Gly2Ala), Gm$^r$ | This study |
| pAMR2 | pAAH5 derivative containing the rat CYP1A1 gene (ADH promoter/terminator) and a yeast CPR gene (yeast reductase promoter/terminator), carries gene for leucine selection | [37] |
| pAFCR1 | pAAH5 derivative containing the rat CYP1A1 gene linked to a yeast CPR gene by an Arg-Ala linker (ADH promoter/terminator), carries gene for leucine selection | [55] |

1% (w/v) glucose as growth substrate. Verduyn medium was supplemented with histidine for *S. cerevisiae* AH22 or uracil, histidine, and methionine for *S. cerevisiae* BY4741 (20 mg $l^{-1}$ of each amino acid). All strains were incubated in screw-capped, baffled Erlenmeyer flasks in horizontal shakers at 30°C. Cell densities were monitored by measuring the optical density at 450 nm ($OD_{450}$), using a spectrophotometer (Libra S11, Biochrom Ltd., Cambridge, U.K.). The correlation factor between $OD_{450}$ and cell dry weight (CDW) concentration was determined as described elsewhere [5] whereby 1 $OD_{450}$ unit corresponds to 0.166, 0.152, and 0.223 $g_{CDW}$ $l^{-1}$ for *E. coli*, *S. cerevisiae*, and *P. putida*, respectively. Plasmids were introduced into *E. coli* by heat-shock transformation, into *P. putida* by electroporation (2,500 V, Equibio EasyjecT Prima, Kent, UK), and into *S. cerevisiae* by lithium acetate transformation. All transformation methods were performed as described [58].

## Molecular biology techniques

For the construction of the bicistronic plasmid pCom8r1A1, the genes for truncated rat CYP1A1 and human CPR were isolated from pCWr1A1 by PCR [58] using Phusion high-fidelity polymerase (Finnzymes Oy, Espoo, Finland) and the following primers (Eurofins MWG Operon, Ebersberg, Germany): 5′-CCGGAATTCCATATGGCTACAAGAACATG-3′ (forward) and 5′-CGCGGATCCCTAGCTCCACACGTCCAG-3′ (reverse). The underlined sequences represent *Eco*RI (forward primer) and *Bam*HI (reverse primer) restriction sites. The resulting PCR product (3,558 bp), encoding the genes for rat CYP1A1 and human CPR as two separate open reading frames, and pCom8 were digested with *Eco*RI/*Bam*HI (Fermentas GmbH, St. Leon-Rot, Germany), purified (PeqGOLD Microspin Cycle-Pure Kit, Peqlab Biotechnologie GmbH, Erlangen, Germany) and ligated (T4-ligase, Fermentas GmbH, St. Leon-Rot, Germany) according to the supplier's recommendations. The product was introduced into *E. coli* DH5α and after overnight growth on LB-agar plates containing gentamicin, the desired plasmid was isolated from obtained transformants. The correct insertion of the genes was verified by digestion with different restriction enzymes and PCR amplification of the insert.

## Ethoxyresorufin O-deethylation (EROD) with resting cells

Bacteria and yeast strains were grown on mineral media to an $OD_{450}$ of 0.5. Then, cells were induced by the addition

of 1 mM isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) (*E. coli* containing pCW vectors) or 0.1% (v/v) octane (*E. coli* and *P. putida* containing pCom8 vectors). Genes encoded on vectors for yeast are constitutively expressed. For cultures with *E. coli* strains, 0.5 mM ALA was added upon induction of the cells. During growth, cells were sampled from the shake flasks and centrifuged (10 min, 4,600 × *g*, 4°C). The pellet was resuspended in 100 mM Tris–HCl buffer (pH 7.8) to a biomass concentration of 2 $g_{CDW}$ $l^{-1}$. Of this cell stock, 10 μl were added to 170 μl Tris-buffer (100 mM, pH 7.8) in a well of a black 96-well microtiter plate (Nunc, Roskilde, Denmark). After adding 10 μl of a 20% (w/v) glucose stock, EROD was started by adding 5 μM 7-ethoxyresorufin from a 0.1 mM stock in dimethyl sulfoxide. Fluorescence, resulting from resorufin formation by CYP1A1 ($\lambda_{ex} = 530$ nm, $\lambda_{em} = 585$ nm) [54] was followed in a microtiter plate reader (Infinite M200, Tecan, Männedorf, Switzerland) for 30 min. The output signal of the microtiter plate reader was correlated to product formation using a calibration curve based on commercially available resorufin (Sigma–Aldrich). The initial specific activity (given in U $g_{CDW}^{-1}$, with 1 U = 1 μmol formed product $min^{-1}$) was determined for the time interval in which product formation was linear. All specific activities reported in this study were determined in triplicate.

CO-difference spectra

Active CYP1A1 in cell-free extracts was quantified by CO-difference spectra. Induced cells were harvested by centrifugation (10 min, 4,600 × *g*, 4°C) and resuspended in 100 mM potassium phosphate buffer (pH 7.4, 5% (w/v) glycerol, 1 mM dithiothreitol) to obtain a suspension with a biomass concentration of 15 $g_{CDW}$ $l^{-1}$. Cells were disrupted by three passages through a pre-cooled French press (5.5 MPa, SLM-Aminco, Rochester, NY, USA). Whole cells and cell debris were removed from the extracts by centrifugation (5 min, 17,000 × *g*, 4°C). Then, 0.5 ml of cell-free extract was mixed with 0.5 ml of a 20 mM sodium dithionite solution and a baseline was recorded with a UV–visible spectrophotometer (Varian Type CARY 300, Palo Alto, CA, USA). Subsequently, the sample was gassed with carbon monoxide (Linde AG, Munich, Germany) for 30 s and a CO-difference spectrum was recorded between 400 and 600 nm. The CYP concentration was calculated using a value of 91 $mM^{-1}$ $cm^{-1}$ for the molar extinction increment between 450 and 490 nm (active CYP) and a value of 110 $mM^{-1}$ $cm^{-1}$ for the molar extinction increment between 420 and 490 nm (inactive CYP and/or non-P450 cytochromes) [43]. Alternative methods to disrupt cells such as sonification, lysozyme treatment, and glass bead disruption resulted in lower EROD activities and were therefore not considered for the determination of *cyp* expression levels.

## Results

Specific CYP1A1 activity of resting *E. coli* JM101 grown on different media

In this study, the biocatalytic performance of different microorganisms expressing human or rat *cyp1a1* genes is investigated. The low catalytic activities of recombinant microorganisms harboring mammalian CYPs are often regarded as a major factor limiting the implementation of such systems in biotechnological processes [28]. Therefore, the specific activity was chosen as the main parameter to evaluate biocatalyst performance.

EROD was used as a model reaction, as very low product concentrations can be detected fluorometrically (detection limit: ~10 nM). Reported turnover numbers for CYP1A1-catalyzed EROD range from 0.8 to 44.8 nmol $min^{-1}$ $nmol^{-1}$ [10, 26, 47, 68] and are comparable to values reported for other CYP1A1-catalyzed reactions using substrates, such as benzo[a]pyrene (from 0.97 to 1.40 nmol $min^{-1}$ $nmol^{-1}$), phenacetin (from 0.2 to 36.9 nmol $min^{-1}$ $nmol^{-1}$), 2,3,7-trichloro-dibenzo-*p*-dioxin (27.5 nmol $min^{-1}$ $nmol^{-1}$), and theophylline (3.5 nmol $min^{-1}$ $nmol^{-1}$) [10, 47, 64, 68]. EROD, therefore, can be considered a suitable model reaction.

In order to adequately evaluate the performance of the different biocatalysts, they were tested under comparable reaction conditions. In this regard, the selection of a suitable medium is important. In many studies, complex media (e.g., TB medium) are used as standard medium. However, for bioprocesses, mineral media are favored over complex media as they are generally cheaper, comprise more defined reaction conditions, and simplify downstream processing [77]. Moreover, auxotrophy selection markers present on the yeast expression vectors for *cyp1a1* genes require the use of mineral media. Hence, the use of mineral media for *E. coli* strains was investigated in order to increase the comparability of activities measured for yeast and bacterial strains.

The specific activity of *E. coli* JM101 harboring rat CYP1A1 and human CPR grown in mineral media was compared to the activities obtained using complex TB medium. For this purpose, the specific activity of resting cells of *E. coli* JM101 (pCWr1A1) was measured at regular time intervals during growth on different media (Fig. 1). IPTG-induced *E. coli* JM101 (pCWr1A1) grown on TB medium reached a maximum specific activity of

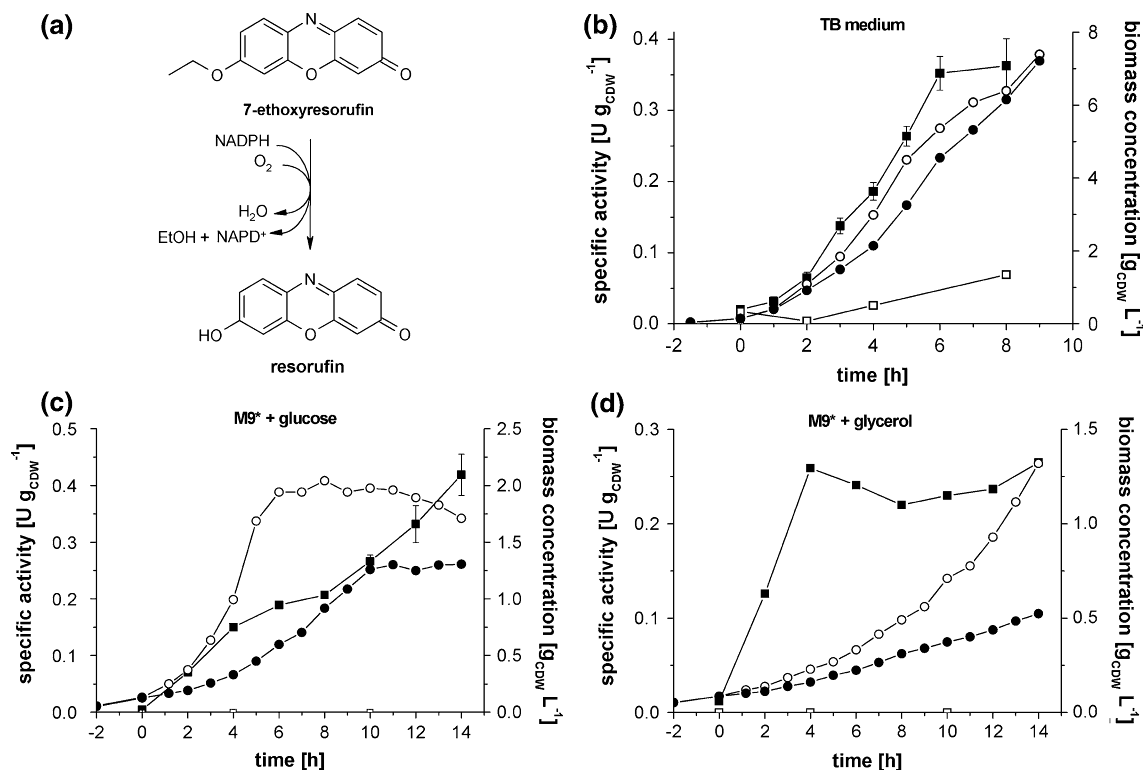


**Fig. 1** Ethoxyresorufin O-deethylation using CYP1A1 (**a**). Specific activity and growth behavior of *E. coli* JM101 (pCWr1A1) growing on different media: TB complex medium (**b**), M9* mineral medium with 0.5% (w/v) glucose as carbon source (**c**), M9* mineral medium with 0.5% (w/v) glycerol as carbon source (**d**). *Closed symbols* show growth (*circles*) and specific activity (*squares*) of induced cells (induction at $t = 0$ h). *Open symbols* show growth (*circles*) and specific activity (*squares*) of non-induced cells. For details see the "Materials and methods" section

$0.36 \pm 0.04$ U $g_{CDW}^{-1}$. Uninduced cells also showed significant activity towards ethoxyresorufin (maximum 0.07 U $g_{CDW}^{-1}$) (Fig. 1b). This indicates leaky expression from the *tac* promoter present on pCW vectors when complex TB medium is used for growth. Cells growing on M9* mineral medium with glucose as carbon source reached higher activities than TB-grown cells (up to $0.42 \pm 0.04$ U $g_{CDW}^{-1}$). Moreover, the *tac* promoter was tightly regulated and no activity was detected for uninduced cells (Fig. 1c). Long induction times are required to reach the maximum specific activity, especially in case of glucose-grown cells (>10 h). When glycerol was used as alternative growth substrate, maximum specific activities ($0.27 \pm 0.01$ U $g_{CDW}^{-1}$) were already reached after 4 h of induction (Fig. 1d) and remained stable for at least 24 h (data not shown).

In conclusion, the use of mineral M9* medium with glucose allowed similar specific activities as the use of complex TB medium. Catabolite repression of the *tac* promoter by glucose can be prevented by using glycerol as an alternative growth substrate. However, this also results in lower maximal activities. As *E. coli* containing rat CYP1A1 showed the highest activity when grown in mineral M9* medium with glucose as carbon source, this medium was used for further investigations.

## Catalytic activity of rat and human CYP1A1 in different *E. coli* strains

For expression of mammalian *cyp* genes in bacteria, *E. coli* DH5α is used as standard host [10, 26, 47, 59, 64, 70]. However, for technical applications, the use of genetically less modified and faster-growing strains, such as *E. coli* JM101, is often preferred [7, 45].

Mammalian CYPs are typically involved in the functionalization of complex substrates with relatively high molecular weight (>200 g $mol^{-1}$) [28]. Especially for such compounds, substrate mass transfer across the cell membranes can limit the bioconversion when using whole-cell catalysts [14]. To alleviate this limitation, the use of *E. coli* strains with enhanced outer membrane permeability, e.g., *E. coli* E609L, may be helpful [38, 39].

Taking the above-mentioned aspects into account, the catalytic activity of human and rat CYP1A1 was tested in different *E. coli* strains (Fig. 2). In all cases, the pCW expression vector was used for the co-expression of a truncated rat *cy1a1* gene together with a gene for human CPR (pCWr1A1) [64] or a modified (Leu2Ala) human *cyp1a1* gene together with a gene for human CPR (pCWh1A1) [47]. For both plasmids, *E. coli* DH5α, *E. coli*

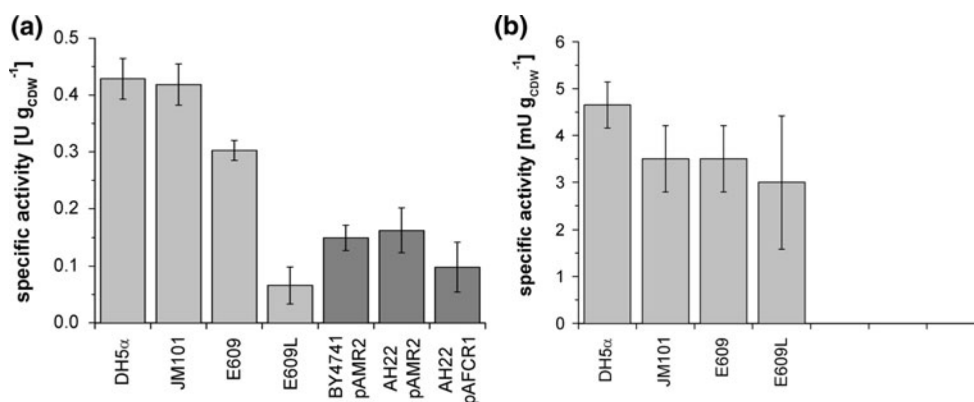


**Fig. 2** **a** Maximum specific activities of different *E. coli* (*light gray*) and *S. cerevisiae* (*dark gray*) strains harboring rat CYP1A1. **b** Maximum specific activities of different *E. coli* strains harboring human CYP1A1. All *E. coli* strains carry plasmid pCWr1A1 (**a**) or pCWh1A1 (**b**)

JM101, *E. coli* E609L, and *E. coli* E609 (isogenic parent of *E. coli* E609L) were tested as host strains.

For rat CYP1A1, the highest maximum activities were found for *E. coli* DH5α (0.43 ± 0.04 U $g_{CDW}^{-1}$) and *E. coli* JM101 (0.42 ± 0.04 U $g_{CDW}^{-1}$) as hosts, whereas *E. coli* E609 showed lower activities (0.30 ± 0.02 U $g_{CDW}^{-1}$) (Fig. 2a). The Braun's lipoprotein deficiency of *E. coli* E609L negatively influenced EROD, as this strain showed a maximum activity of only 0.07 ± 0.03 U $g_{CDW}^{-1}$, which is 22% of the activity reached by the parental strain.

Specific whole-cell activities with human CYP1A1 for EROD are roughly 100 times lower than activities found with rat CYP1A1. For human CYP1A1, *E. coli* DH5α is the most active host with a maximum specific activity of 4.7 ± 0.5 mU $g_{CDW}^{-1}$. The activities of the other three tested strains are in a similar range, but slightly lower (3–3.5 mU $g_{CDW}^{-1}$). As the specific EROD activities with human CYP1A1 were very low compared to the activities displayed by rat CYP1A1, human CYP1A1 was not considered for further investigations.

It can be concluded that both *E. coli* DH5α as well as *E. coli* JM101 are suitable host strains for CYP1A1. For biotechnological applications, the use of faster-growing *E. coli* JM101 is preferred over *E. coli* DH5α. Rat CYP1A1-catalyzed EROD is 100-fold faster in comparison to human CYP1A1-catalyzed EROD.

### Catalytic activity of rat CYP1A1 in *S. cerevisiae*

In order to characterize the catalytic performance of CYP1A1-containing yeast strains and to enable a comparison with the results obtained for the tested *E. coli* strains, the maximum specific activity of *S. cerevisiae* BY4741 and AH22 containing plasmid pAMR2 (for co-expression of the genes for rat CYP1A1 and yeast CPR) was investigated (Fig. 2a). *S. cerevisiae* AH22 harboring CYP1A1 fused with CPR (*S. cerevisiae* AH22 (pAFCR1)) was also tested. Microsomal fractions of *S. cerevisiae* AH22 containing the fusion protein have been reported to hydroxylate zoxazolamine at a higher rate than microsomal fractions containing non-fused rat CYP1A1 and yeast CPR [55].

*S. cerevisiae* AH22 is considered a standard host for mammalian *cyp* gene expression [35, 55, 57], whereas BY4741 is, in general, frequently used as a host for biotechnological and biochemical studies [44]. Both strains have a leucine auxotrophic phenotype, which was used to select for plasmid-containing strains. Similar maximum activities for EROD were found for *S. cerevisiae* BY4741 and AH22 harboring plasmid pAMR2 (0.15–0.16 U $g_{CDW}^{-1}$). However, these activities were approximately three times lower than the activities reached by *E. coli* (Fig. 2a). The maximum specific activity of *S. cerevisiae* AH22 (pAFCR1) containing fused rat CYP1A1 and CPR is 1.5 to 1.7-fold lower than that of strains co-expressing the genes for CYP1A1 and CPR (Fig. 2a). Extracts of *S. cerevisiae* AH22 (pAMR2) showed a maximum activity of 0.03 U $g_{CDW}^{-1}$ (normalized to the amount of cells used for preparation of the extract), which is 5-fold lower than the activity achieved with whole cells.

It is concluded that *E. coli* strains, in general, display higher specific activities than *S. cerevisiae* for EROD catalyzed by rat CYP1A1. Next to that, the use of a fusion protein of CYP1A1 and CPR does not enhance specific whole-cell EROD activities in *S. cerevisiae* and the use of whole cells is favorable over the use of cell-free extracts.

### Expression levels of rat CYP1A1 in tested host strains

CYPs are unique enzymes as they can be specifically quantified in whole cells or cell-free extracts using CO-difference spectra [43]. This allows the determination of turnover numbers (expressed in nmol product $min^{-1}$ (nmol enzyme)$^{-1}$), which is, for other enzymes, usually only possible after enzyme purification. The *cyp* expression levels for all recombinant microorganisms tested in this study were determined by CO-difference spectra of cell-free extracts (Table 3).

**Table 3** P420 and P450 concentrations in cell-free extracts, maximum specific activities, and turnover numbers of different recombinant microorganisms harboring rat CYP1A1

| Strain | Plamid | P420 (nmol/gCDW) | P450 (nmol/gCDW) | Max. activity (mU/gCDW) | Turnover number (nmol $min^{-1}$ $nmol^{-1}$) |
|---|---|---|---|---|---|
| *E. coli* DH5α | pCWori+ | 360 ± 51 | n.d. | n.d. | – |
| | pCWr1A1 | 71 ± 4 | 41 ± 2 | 429 ± 36 | 10 |
| | pCom8-r1A1 | 48 ± 1 | 7 ± 1 | 47 ± 3.3 | 7 |
| *E. coli* JM101 | pCWr1A1 | 18 ± 6 | n.d. | 419 ± 36 | – |
| *E. coli* E609 | pCWr1A1 | 130 ± 11 | n.d. | 303 ± 18 | – |
| *E. coli* E609L | pCWr1A1 | 56 ± 15 | n.d. | 66 ± 33 | – |
| *P. putida* KT2440 | – | n.d. | n.d. | n.d. | – |
| | pCom8-r1A1 | n.d. | n.d. | n.d. | – |
| *S. cerevisiae* AH22 | – | n.d. | n.d. | n.d. | – |
| *S. cerevisiae* AH22 | pAMR2 | n.d. | 2 ± 1 | 163 ± 39 | 82 |
| | pAFCR1 | n.d. | 2 ± 1 | 98 ± 44 | 49 |
| *S. cerevisiae* BY4741 | pAMR2 | n.d. | 2 ± 1 | 150 ± 22 | 71 |

n.d., not detectable

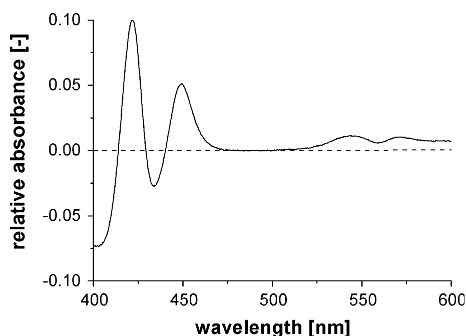


**Fig. 3** CO-difference spectrum of a cell-free extract of *E. coli* DH5α (pCWr1A1). For preparation of the extract a cell stock with a biomass concentration of 25 $g_{CDW}$ $l^{-1}$ was used. The measured ΔA at 450 nm is 0.05. For more details, see the "Materials and methods" section

For all microorganisms, it was found that CYP1A1 was hard to detect by analysis of CO-difference spectra, as achieved expression levels were low. *E. coli* DH5α was the only *E. coli* strain that allowed accurate determination of the CYP concentration (Fig. 3). Presence of rat CYP1A1 in *E. coli* DH5α could not be detected by SDS-PAGE analysis (data not shown). All tested *E. coli* strains, including *E. coli* DH5α (pCWori+) not expressing any *cyp* gene, display a significant peak at 420 nm, obviously originating from the host itself. A peak at 420 nm was absent in case *S. cerevisiae* strains were tested. For the tested yeast strains, very low concentrations of CYP1A1 were detectable in cell-free extracts (Table 3). The results show that expression of rat *cyp1a1* in *E. coli* or *S. cerevisiae* results in very low oxygenase concentrations. Turnover numbers achieved with yeast strains carrying pAMR2 are 7–8-fold higher than those obtained with *E. coli* DH5α (pCWr1A1).

### Catalytic activity of rat CYP1A1 in *P. putida*

To test the performance of rat CYP1A1 in *P. putida*, the genes for rat CYP1A1 and human CPR were inserted in plasmid pCom8. This is a broad-host-range vector that can be applied for gene expression in both *E. coli* as well as *P. putida* [65]. Remarkably, no EROD by rat CYP1A1 was observed with *P. putida* KT2440. The data were confirmed by CO-difference spectra, which showed no active CYP in *P. putida* KT2440 (pCom8r1A1) (Table 3). However, activity and gene expression could be detected for *E. coli* DH5α carrying the same plasmid. Although lower maximum specific activities were obtained when the pCom8 vector was used instead of pCW, the biocatalytic efficiency (given by the turnover number) of CYP1A1 was comparable in both cases. From the presented data it can be concluded that *P. putida* is not a suitable host for the functional expression of the genes for rat CYP1A1 and human CPR.

## Discussion

### Rat and human CYP1A1 activities within whole cells

The achieved specific activities of *E. coli* DH5α and *E. coli* JM101 containing rat CYP1A1 are roughly 100-fold higher than the maximum achievable activities of the same strains harboring human CYP1A1 (Fig. 2). The difference may be caused by inter-species variability. Rat and human CYP1A1 show 80% homology, based on the amino acid sequences. This implies that catalysis by rat CYP1A1 may differ from human CYP1A1-catalysis. Inter-species differences have been reported before, e.g., the main

metabolite found during paclitaxel metabolism catalyzed by human CYP2C8 is 6-hydroxypaclitaxel, whereas rat CYP2C8 is not capable of paclitaxel hydroxylation at this position [11, 27]. Inter-individual variability, caused by genetic polymorphism, may also explain the observed differences in activity [25], i.e., the *cyp1a1* gene of a different human being may encode a more active CYP1A1 oxygenase. In general, a screening among different species and different individuals may yield more active variants of mammalian CYPs.

### *E. coli* as most suitable strain for mammalian *cyp* expression

*E. coli* DH5α and *E. coli* JM101 show 2.5-fold higher maximum activities than the best-performing *S. cerevisiae* strain (Fig. 2). Yeast, as a eukaryotic host, does not ensure higher expression levels compared to *E. coli*, which may explain the corresponding low whole-cell activities. However, the low activities obtained with yeast may also result from a less-efficient interaction of rat CYP1A1 with yeast CPR as used for *S. cerevisiae* in comparison to the interaction of rat CYP1A1 with human CPR as used for *E. coli*.

The use of a fusion protein in *S. cerevisiae* or the use of a Braun's lipoprotein-deficient *E. coli* strain were both not favorable for EROD. The latter result indicates that transfer of 7-ethoxyresorufin over the outer membrane of *E. coli* does not limit EROD at the conversion rates observed in this study. However, the use of *E. coli* E609L may be of interest when pronounced mass transfer limitations over cell membranes occur [39].

*P. putida* is not suitable as a host for CYP1A1 as neither EROD activity nor expression could be detected for *P. putida* KT2440 (pCom8r1A1). The reason for the lack of *cyp1a1* expression in *P. putida* is unclear but some factors can be ruled out. Expression of the *cyp1a1* gene in *E. coli* using pCom8r1A1 resulted in the formation of functionally active CYP1A1, showing that the plasmid is functioning properly. Furthermore, the use of pCom8 for the functional expression of bacterial *cyp* genes in *P. putida* has been demonstrated previously [69]. On this basis, functional expression of the *cyp1a1* gene using pCom8 and *P. putida*, in principle, can be expected. Differences in codon-usage between *P. putida* and rat can also be ruled out as a reason, since codon-usage in *P. putida* is very similar to that of *E. coli*, which showed functional expression of the rat *cyp1a1* gene. It may be speculated that incorrect protein folding in *P. putida* (e.g., because of a lack of the correct chaperones) is the reason for the absence of rat CYP1A1 in this strain.

Interestingly, CYP1A1 displays 7–8-fold higher turnover numbers in *S. cerevisiae* than in *E. coli* DH5α (Table 3), indicating that yeast provides a favorable reaction environment for CYP1A1. However, expression levels in *S. cerevisiae* are so low that these turnover numbers do not result in higher specific whole-cell activities. Thus, the higher expression levels achieved with *E. coli* make this strain more suitable as a host for CYP1A1 biocatalysis. This conclusion is further supported by literature data. For example, CYP3A4-containing *E. coli* extracts were reported to show a 115-fold higher catalytic activity for testosterone hydroxylation than respective yeast extracts [21]. Furthermore, CYP concentrations achieved with *E. coli* range from 0.8 to 4,500 nmol $l^{-1}$, whereas concentrations achieved with *S. cerevisiae* are generally lower ($\leq$88 nmol $l^{-1}$; Table 4). This indicates that *E. coli*, in general, enables higher *cyp* expression levels. However, it is important to note that although expression levels are generally reported in nmol $l^{-1}$, these values strongly depend on the applied biomass concentration, which is not given in most cases. For this reason, expression levels in this study are reported in nmol $g_{CDW}^{-1}$.

Table 4 shows that there is significant variation in reported turnover numbers in vivo. For example, turnover numbers for EROD range from 1.6 to 22.8 nmol $min^{-1}$ $nmol^{-1}$, for CYP1A1. One order of magnitude differences in turnover numbers can also be observed for CYP2D6 and CYP3A4 (Table 4). The variations probably result from different reaction conditions and show the importance of optimizing cell physiological conditions in order to exploit the full potential of CYPs in whole-cell catalysts. It furthermore highlights the importance of evaluating different *cyp*-expressing systems under comparable conditions, allowing more general conclusions on different expression systems.

### Expression of *cyp1a1* genes

Expression of rat *cyp1a1* was found to be difficult to detect. Only for *E. coli* DH5α and the tested *S. cerevisiae* strains, the presence of CYP1A1 could be demonstrated using CO-difference spectra (Table 3), although all of the tested *E. coli* and *S. cerevisiae* strains showed activity towards 7-ethoxyresorufin during separate activity assays. This indicates that active CYP1A1 was lost during preparation of the cell-free extracts, for which expression levels were measured, e.g., due to enzyme instability or protease activity. Activities of cell-free extracts of *S. cerevisiae* AH22 (pAMR2) also were lower compared to whole cells, which further supports that active CYP was lost during cell lysis. However, lower local concentrations of CYP and CPR might also have contributed to lower activities.

For all *E. coli* strains, significant peaks at 420 nm were detected. This was not observed for the tested yeast strains. The presence of a peak at 420 nm is often considered to indicate the presence of denatured or misfolded CYP [42, 53]. However, in agreement with a previous study [47], a

**Table 4** Reported in vivo activities and expression levels for different mammalian CYPs in *E. coli* and *S. cerevisiae*

| CYP | Substrate | Turnover number (nmol $min^{-1}$ $nmol^{-1}$) | | Expression level (nmol $l^{-1}$) | Redox partner(s) | References |
|---|---|---|---|---|---|---|
| | | *E. coli* | *S. cerevisiae* | | | |
| 1A1 | Benzo[a]pyrene | 0.82 | | 4,500 | Endogenous | [29] |
| | 7-Ethoxycoumarin | 34.6 | | 121 | Co-expression | [26] |
| | 7-Ethoxyresorufin | 1.6 | | 27 | Co-expression | [47] |
| | 7-Ethoxyresorufin | 22.8 | | 121 | Co-expression | [26] |
| | 7-Ethoxyresorufin | 0.46 | | 4,500 | Endogenous | [29] |
| | Naphthalene | | 23 μmol $g_{CDW}^{-1}$ $h^{-1}$ | 55–60 | Fused to CYP | [63] |
| | Phenacetin | 0.2 | | 27 | Co-expression | [47] |
| | 2,3,7-Trichloro-dibenzo-*p*-dioxin | 27.5 | | 62 | Co-expression | [64] |
| | 2,3,7-Trichloro-dibenzo-*p*-dioxin | | 3.6 | 56 | Co-expression | [57] |
| | Zoxazolamine | | Not given | – | Fused to CYP | [55] |
| 1A2 | 7-Ethoxycoumarin | 0.46 | | 172 | Co-expression | [26] |
| | 7-Ethoxyresorufin | 1.0 | | 350 | Co-expression | [47] |
| | 7-Ethoxyresorufin | 1.7 | | 172 | Co-expression | [26] |
| | Phenacetin | 0.29 | | 350 | Co-expression | [47] |
| | 2,3,7-Trichloro-dibenzo-*p*-dioxin | | 0.11 | 88 | Co-expression | [57] |
| 2A6 | Coumarin | 17.9 | | 66 | Co-expression | [26] |
| | Indole | 1.7–131[a] | | – | Co-expression | [74] |
| | 4-Chloro-indole | 1.2–54[a] | | – | Co-expression | [74] |
| | 4-Benzyl-oxo-indole | 0.45–5.3[a] | | – | Co-expression | [74] |
| | 5-Benzyl-oxo-indole | 0.28–3.0[a] | | – | Co-expression | [74] |
| 2C8 | Paclitaxel | Not given | | 381 | Co-expression | [26] |
| 2C9 | Tolbutamide | 1.1 | | 170 | Co-expression | [47] |
| | Tolbutamide | 4.6 | | 165 | Co-expression | [26] |
| 2C19 | *S*-mephenytoin | 6.4 | | 121 | Co-expression | [26] |
| 2D6 | Bufuralol | 0.22 | | 130 | Co-expression | [47] |
| | Bufuralol | 2.0 | | 91 | Co-expression | [26] |
| 2E1 | Aniline | 21.4 | | 92 | Co-expression | [26] |
| | Aniline | Not detected | | 0.8 | Co-expression | [12] |
| | Chlorozoxazone | 3.5 | | 160 | Co-expression | [47] |
| | 4-Nitrophenol | 22.6 | | 92 | Co-expression | [26] |
| | 4-Nitrophenol | Not detected | | 0.8 | Co-expression | [12] |
| 3A4 | 2,4-Dichlorophenol | | Not given | ~3 | Endogenous | [35] |
| | Nifedipine | 15.2 | | 200 | Co-expression | [4] |
| | Nifedipine | 59.8 | | 81 | Co-expression | [72] |
| | Testosterone | 17.3 | | 200 | Co-expression | [4] |
| | Testosterone | 6.3 | | 230 | Co-expression | [47] |
| | Testosterone | 71.8 | | 81 | Co-expression | [72] |
| | Testosterone | 59.7 | | 84 | Co-expression | [26] |
| 11A1 | Ergosta-5-eneol | | Not given | – | Co-expression | [16, 67] |
| | Pregnenolone | Not given | | – | Endogenous | [2] |
| | Progesterone | Not given | | – | Endogenous | [2] |
| 11B1 | 11-Deoxycortisol | | Not given | – | Co-expression | [67] |
| | 11-Deoxycortisol | | 0.53 μmol $l^{-1}$ $h^{-1}$ | – | Co-expression | [15] |
| | 11-Deoxycortisol | | 8.4 μmol $l^{-1}$ $h^{-1}$[b] | – | Endogenous | [13] |

**Table 4** continued

| CYP | Substrate | Turnover number (nmol $min^{-1}$ $nmol^{-1}$) | | Expression level (nmol $l^{-1}$) | Redox partner(s) | References |
|---|---|---|---|---|---|---|
| | | *E. coli* | *S. cerevisiae* | | | |
| 11B2 | 11-deoxy corticosterone | | 0.39 nmol $l^{-1}$ $h^{-1}$[b] | – | Overexpression gene for endogenous redox partner | [8] |
| 17A1 | Progesterone | 1.3 | | 600–700 | Fused to CYP | [61] |
| | Progesterone | 50 | | 150–200 | Co-expression | [62] |
| | Progesterone | 8 | | 150–200 | Fused to CYP | [62] |
| | Progesterone | 0.16 | | 150–200 | Endogenous | [62] |
| | Progesterone | | Not given | – | Co-expression | [67] |
| | Progesterone | | 9 | – | Endogenous | [37] |
| | Progesterone | | 44 | – | Co-expression | [37] |
| 21A1 | 17-Hydroxy progesterone | | Not given | – | Co-expression | [67] |
| 21B1 | 25-Hydroxyvitamin D3 | Not given | | – | Endogenous | [56] |

[a] Different mutants tested

[b] *Schizosaccharomyces pombe* used as host strain

significant peak at 420 nm also was observed for *E. coli* DH5α carrying the pCW vector without the *cyp* gene. Thus, the peak at 420 nm most likely results from other hemoproteins in *E. coli*, such as cytochrome *o* [9].

Standard procedures to increase expression levels of mammalian *cyp* genes in *E. coli* include the replacement of the 5′-sequence of wild-type genes by a specific amino acid sequence (MALLLAVF-) or truncation of the hydrophobic N-terminus [76]. However, for human CYP1A1 used in this study, no truncation in the 5′-sequence of the gene has been made and only one amino acid has been modified. For human CYP1A2, a CYP that strongly resembles CYP1A1 (74% homology on the amino acid level), truncation of the N-terminal sequence allowed high expression levels (Table 4). It can be speculated that truncation of the N-terminal sequence of the human *cyp1a1* gene may also allow high expression levels.

For rat CYP1A1, the first 30 amino acids of the N-terminal protein sequence have been deleted and Arg31 and Val32 were replaced by Met and Ala, respectively [64]. These modifications also do not reflect the modifications that have led to high level expression of the human *cyp1a2* gene [30, 59]. Rigorous changes in the N-terminal sequence might have a negative influence on the interaction of the CYP oxygenase with the corresponding reductase [48]. Hence, also for rat CYP1A1, alternative truncation and modification strategies may result in high level expression of *cyp1a1*.

In conclusion, both in terms of achievable maximum activity and expression level, *E. coli* is superior to *S. cerevisiae* and *P. putida*. However, even for *E. coli*, expression of *cyp1a1* remains difficult. Optimization of *E. coli* as platform organism for mammalian CYP1A1-catalysis should focus on the improvement of expression levels, which may be achieved by optimization of N-terminal sequence modification as one possible strategy (Table 5).

## Industrial application of CYP1A1

Bioprocesses for industrial production of fine chemicals are considered to require a productivity of at least 0.1 g $l^{-1}$ $h^{-1}$ [52, 66]. Assuming the production of a fine chemical with a molecular weight of 200 g $mol^{-1}$ (roughly being the molecular weight of most products of CY1A1-catalyzed reactions) in a fed-batch process with an average biomass concentration of 15 $g_{CDW}$ $l^{-1}$, the minimum whole-cell activity needs to be ≥0.56 U $g_{CDW}^{-1}$ for several hours. For human CYP1A1, obtained activities were more than 119-fold lower than this minimum, whereas maximum activities obtained with *E. coli* DH5α or *E. coli* JM101 containing rat CYP1A1 approach this value (Fig. 2). However, for the latter, maximum activities are only obtained after long induction times, meaning that, in a biotransformation procedure, the overall productivity is expected to be much lower than 0.1 g $l^{-1}$ $h^{-1}$. Hence, the use of CYP1A1 for the production of fine chemicals with any of the tested *E. coli* expression systems does not appear to be feasible.

For the production of high-value pharmaceuticals and drug metabolites, process productivities may be as low as 0.001 g $l^{-1}$ $h^{-1}$ [28], representing a minimum required activity of 6 mU $g_{CDW}^{-1}$. This value was easily met by all rat CYP1A1-based systems and approached by human

**Table 5** N-terminal sequence modification of the *cyp1a1* and *cyp1a2* genes for heterologous expression in *E. coli*

| CYP | Sequence | N-terminal amino acid sequence [a] | Expression level (nmol $L^{-1}$) [b] |
|---|---|---|---|
| h1A2 | original [c] | MALSQSVPFSATELLLASAIFCLVFWVLKGLRPRVPKGL | - |
| | modified [c] | MA____________LLLAVFLFCLVFWVLKGLRPRVPKGL | 350 |
| h1A1 | original [c] | MLFPISMSATEFLLASVIFCLVFWVMRASRPQVPKGLKN | - |
| | modified [c] | MAFPISMSATEFLLASVIFCLVFWVMRASRPQVPKGLKN | 27 |
| r1A1 | original [d] | MPSVYGFPAFTSATELLLAVTTFCLGFWVVRVTRTWVPK | - |
| | modified [d] | ______________________________MATRTWVPK | 62 |

The original sequences are compared with the reported modified sequences

[a] Modifications compared to the original sequence are underlined

[b] Data from [47, 64]

[c] Modified and original sequences are from [19]

[d] Modified and original sequences are from [64]

CYP1A1-based *E. coli* (Fig. 2). Several pharmaceutical companies (e.g., Novartis, Hoffmann–La Roche, and Codexis) have already adopted *E. coli* as a standard host strain for expression of human *cyp* genes and use these strains for the synthesis of drug metabolites [21, 60]. Future research efforts should focus on the improvement of productivities and selectivities obtained with these systems [60]. Optimization of mammalian *cyp* expression in *E. coli* is crucial in order to reach this goal.

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