Expression of human liver cytochrome P450 enzymes in the non-conventional yeast *Yarrowia lipolytica* for the evaluation of two-liquid biphasic whole-cell biotransformation of steroids

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1. Abbreviations

ADH	alcoholdehydrogenase
Alk	alkane
AmpR	ampicillin resistence gene coding for beta-lactamases
AOX	alcoholoxidase
BM3 CYP102A1	cytochrome P450 enzyme from *Bacillus megaterium*
cDNA	complementary DNA, is DNA synthesized from a messenger RNA
CDW	cell dry weight
CYP	cytochrome P450
CYP2D6	human cytochrome P450 2D6
CYP3A4	human cytochrome P450 3A4
DAS	dihydroxyacetone synthase
DBP	dibutylphthalate
ddH_2O	double distilled H_2O
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	desoxyribonukleosidtriphosphate
E. coli	*Escherichia coli*
ESI	electron spray ionization
ET	electron transfer
EtOH or Eth	ethanol
FAD	flavine adenine dinucleotide
FDA	food and drug administration
FMN	flavine mononucleotide
G / C	guanidine, cytosine
GC	gas chromatography
Glu	glucose
GRAS	generally regarded as safe
H. polymorpha	*Hansenual polymorpha*
HCPR	human cytochrome P450 reductase
HF	high fidelity
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ICL1	isocitrate lyase 1 gene
IS	internal standard
K. lactis	*Kluyveromyces lactis*
LB	Luria-Bertani
LiAc	lithium acetate
Lip	lipide
log P	logarithm of partition coefficient
LTR	long terminal repeats
MIST	metabolites in safety testing
MM	mineral salt minimal medium
MS	mass spectrometry
mü (μ)	growth rate [s^{-1}]
mutein	wildtype protein with one or several mutations
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NCR	NADH / NADPH cytochrome c reductase

NIT	nitrilase
NL	norm liter
OD_{600}	optical density measured at 600 nm
ONC	over night culture
P. pastoris	*Pichia pastoris*
PAMO	phenylacetone monooxygenase
PCR	polymerase chain reaction
PDB	protein data base
PEG	polyethylene glycol
PMSF	phenyl methyl sulfonyl fluoride
pO_2	partial pressure of oxygen
R2	coefficient of determination
rDNA	ribosomal DNA
RNA	ribonucleic acid
RP	reversed phase
RT	room temperature
RT-QPCR	real time quantitative polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SEM	scanning electron microscope
SOC	super optimal broth with catabolite repression, nutrient-rich bacterial growth medium
Str	streptomycine
Suc	sucrose
TEM	transmission electron microscopy
Temp	temperature
Tet	tetracycline
Tgl	triglyceride
TY3	sub-class of LTR retrotransposons (Metaviridae)
Ura	urea
WT	wildtype
Y. lipolytica	*Yarrowia lipolytica*
*Yl*CPR	*Yarrowia lipolytica* cytochrome P450 reductase
Ylt1	*Yarrowia lipolytica* retrotransposon
YNB	yeast nitrogen based with out amino acids, minimal mineral salt media
YPD	yeast extract peptone dextrose, complete medium for yeast growth
Y_{SX}	biomass production per carbon source consumed [g/g]

2. Abstract

Often cytochrome P450 metabolites present the active form or toxic side product of pharmaceuticals. Therefore there is a vast interest in such metabolites, which often can only be prepared selectively using biotransformations. Two-liquid biphasic systems present a valuable biotechnological tool for biotransformation of hydrophobic substrates, since most typical cytochrome P450 substrates, e.g. steroids and other drugs, are mere immiscible in water. The alkane utilizing, non-conventional yeast *Y. lipolytica* has been shown to be able to grow in biphasic environments and efficiently utilize hydrophobic substrates. Furthermore, *Y. lipolytica* has been identified as an attractive host for heterologous protein production and therefore efficient genetic tools are available. The present work shows an evaluation of functional expression of the human liver cytochrome P450 system consisting of human CPR in combination with either CYP2D6 or CYP3A4 and possible advantages of such recombinant *Y.lipolytica* srains for the oxidation of hydrophobic steroids in two-liquid biphasic systems. Employing the organic solvent ethyl oleate, which can be also utilized as a carbon source by Yarrowia, it was possible to increase the conversion rate by two-fold and prolong the bioconversion for several days to an overall product concentration of at least 5 times higher than a biotransformation in aqueous system.

Additionally, in the course of this work several HPLC-MS methods including a high-throughput HPLC-MS method for the analysis of steroids (e.g. testosterone and its metabolites) were developed.

Furthermore, the MuteinDB, a database for storing high quality data about muteins and their kinetic data towards different substrates, has been improved and further developed. After the data format and general rules for data collection were determined, several comprehensive data sets were collected and implemented (e.g. CYP3A4, CYP2D6, HRP, NIT, BM3 (CYP102A1), thereby providing a solid basis for future database supported protein engineering concepts.

3. Zusammenfassung

Sehr oft sind die von cytochrome P450 metabolisierten pharmazeutischen Substanzen die eigentliche aktive bzw. auch toxische Form. Aus diesem Grund besteht ein großes Interesse an solchen Metaboliten, die häufig nur mit Hilfe von selektiven Biotransformationen dargestellt werden können. Zweiphasige Systeme bestehend aus einer wässrigen und einer organischen Phase stellen ein wertvolles Werkzeug für die Biotransformation von hydrophoben Substraten dar. Typische Substrate von cytochrome P450 Enzymen wie beispielsweise Medikamente und Steroide, sind meist schwer wasserlöslich.

Es wurde gezeigt, dass die alkan-verwertende, unkonventionelle Hefe *Y. lipolytica* in solchen zweiphasigen Milieus sehr gut wachsen und hydrophobe Substrate sehr gut verwerten kann. Desweiteren wurde gezeigt, dass die Hefe *Y. lipolytica* als sehr attraktiver Hostorganismus für die heterologe Expression von Proteinen ist was auch dadurch unterstützt wird, dass geeignete genetische Werkzeuge vorhanden sind. In dieser Arbeit wird die funktionelle Expression von dem cytochrome P450 System der menschlichen Leber, welches aus der menschlichen cytochrome P450 Reduktase in Verbindung mit entweder dem CYP2D6 oder dem CYP3A4 besteht, evaluiert. Desweiteren wird die Verwendung von zweiphasigen Systemen für die Oxidation von hydrophoben Stereoiden mit Hilfe der Hefe *Y. lipolytica* als Ganzzellbiokatalysator gezeigt. Durch die Verwendung des verwertbaren organischen Lösungsmittels Ölsäureethylester als organische Phase, welche durch *Y. lipolytica* verwertet werden kann, war es möglich die Umsetzungsrate um den Faktor zwei zu erhöhen, zusätzlich den Biokonversionzeitraum um mehrere Tage zu verlängern und eine um mindestens fünffach höhere Produktkonzentration im Vergleich zum wässrigen System zu erreichen.

Zusätzlich wurden, während dieser Arbeit mehrere HPLC-MS Methoden inklusive einer hoch-druchsatz HPLC-MS Methode für die Analyse von Steroiden, beispielsweise Testosteron und deren Metaboliten entwickelt.

Desweiteren wurde die MuteinDB, eine Datenbank für das Sammeln und Speichern von hoch qualitativen Daten über Muteine und deren kinetischen Informationen von verschiedenen Substraten, verbessert und weiter entwickelt. Nach festlegen des Datenformats und allgemeiner Regeln für das Datensammeln wurden mehrere umfangreiche Datensätze für CYP3A4, CYP2D6, HRP, NIT, BM3 auch bekannt als CYP102A1 und PAMO gesammelt und in die Datenbank aufgenommen. Die Datenbank bietet nun eine solide Grundlage für zukünftige „protein engineering“ Projekte.

4. Introduction

4.1. Whole cell biotransformation in two-liquid phase system

Yeasts and other microorganisms have been used for centuries; simple products like beer, wine and cheese feeding mankind since thousands of years belong to the oldest forms of biotechnology. In the past 100 years, more and more organisms were used as whole cell biocatalysts, to perform chemical transformations on natural or non-natural organic compounds. By the development of recombinant protein technologies, producing larger amounts of heterologous proteins with desired catalytic activities became possible and the number of available technical enzymes and whole cell catalysts increased significantly. Recombinant enzymes expressed in various hosts, have either been expressed and purified from the expression host with more or less expensive and labour/cost-intensive down-stream processing, or have been used for biotransformation by exposing a whole cell lysate or the recombinant strain directly to the substrate mix. However, whole cell catalysis shows advantages as well as disadvantages compared to isolated enzymes.

On the one hand, the encapsulation by the supramolecular structure of cell membranes acts as a protective shield against environmental influences like, for example, sheer forces or denaturing agents. Moreover, some enzymatic systems, i.e. cytochrome P450s, are multicomponent, membrane-bound enzyme systems, which are difficult to isolate and in addition their catalytic reactions depend on expensive and labile redox cofactors (NADH, NADPH). Due to economic reasons, viable whole cells are therefore often preferred over isolated enzymes, as they provide cofactor regeneration during biotransformation using cheap carbon soures as an energy supply. On the other hand, substrates have to pass the outer cell membranes in order to be transformed; furthermore, endogenous proteins and the cell's own metabolism can catalyze unwanted side reactions (Nikolova et al., 1993).

Two-liquid phase biotransformation systems, consisting of an organic water-immiscible solvent and an aqueous solution, are a particular example of whole cell biocatalysis, and a valuable biotechnological tool for biotransformation of hydrophobic substrates (Fig 1). These systems have been intensively studied as a viable alternative to tackle the problem of the poor solubility in aqueous media of many organic compounds of commercial interest and the discovery of microorganisms able to grow in presence of organic solvents has further increased the research efforts in this field (Bühler et al., 2004; León, 1998). In such systems, cells are either grown or incubated in the presence of 10-50% organic phase, where the substrate of interest is dissolved in. Carbon for cell growth is mainly provided by water soluble substrates such as sugar, glycerol or fatty acids. The biomass is then used for

biocatalysis either in the form of resting cells or as growing culture. Growing cells are considered more favorable than resting cells when expressing a protein with low stability, since they enable a constant protein expression during biotransformation. However, resting cells have the advantage that the desired reaction can be investigated independently from growth. Additionally, this can minimize side reactions and allows the identification of potential limitations (Becker et al., 2007). Also resting cells are usually supplemented with some carbon source, i.e. glucose, to maintain energy metabolism for cofactor regeneration (Siedler et al., 2011).

Biocatalysis in two-phase systems shows several key advantages; the presence of organic solvent can increase the overall concentration of poorly water-soluble substrates and products and at the same time reduce toxic and inhibitory effects by regulation of their concentration in the aqueous phase. Additionally hydrolysis of substrates and products, and mass-transfer limitations may be reduced. Furthermore, the stereoselectivity of a biotransformation may be improved and in situ product recovery, favors the bioconversion by shifting the thermodynamic equilibrium, while an easier recovery of both product and biocatalyst can be achieved.

Nevertheless, the system or reactors are not the most crucial steps in developing a biphasic two-liquid phase system, but rather the selection of the right organic solvent. Several parameters have to be considered. First of all, many solvents are cytotoxic or show inhibitory effects. Furthermore many of the non-toxic organic solvents are highly apolar and therefore have rather restricted solvent capabilities and as such are of limited use as a solvent for substrates and products of interest. Additionally, different cell types, lines, or indeed individual strains may vary considerably in their response to a given solvent, even under the same physiological conditions: it has been reported that conditions which give high initial substrate conversion activities show poor stability of whole cells. Different solvent parameters have been introduced to correlate observed effects. The most widely used parameter is log P, definded as the partition coefficient in a standard ocotanol: water mixture. Even though solvents with high log P have been observed to promote the retention of biocatalytic activity, no simple relation exists between these parameters and the different solvent classes. Therefore the toxicity and inactivation of cells can only be addressed by selection of appropriate solvents and studying the damaging effect on cells (Salter et al., 1995). Furthermore, when aiming for large-scale processes challenges for subsequent downstream processing, environmental implications and safety issues have to be considered (Schmid et al., 1998).

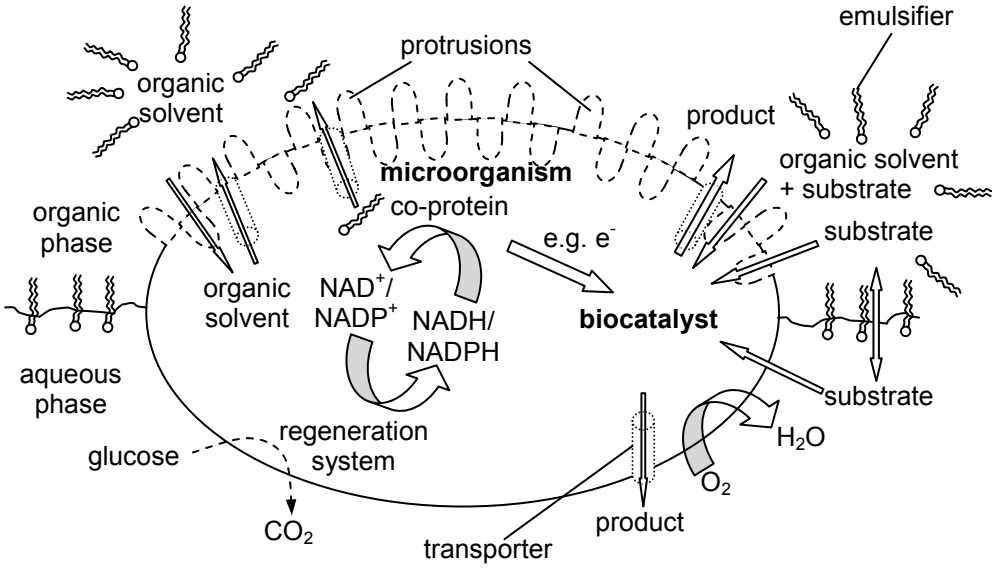


Fig 1: Schematic representation of whole cell biotransformation.

As mentioned before, whole cell biocatalysis is especially suitable for membrane bound, multi compound enzyme systems such as the cytochrome P450 systems. Additionally, typical P450 substrates for example steroids are very hydrophobic, which would promote the use of a two-liquid phase system for the bioconversion of steroids with whole cells harboring heterologously expressed P450 system.

4.2. Cytochrome P450 family

Cytochrome P450 (CYP) enzymes comprise a large and functionally diverse superfamily of heme-thiolate monooxygenases, where the heme domain is bound via a conserved cysteine thiolate group forming the fifth ligand of the heme iron. CYP enzymes have been identified in all domains of life, from prokaryotic organisms like bacteria and archaea, to simple eukaryotes like yeasts and fungi, to mammalians. Even some viruses have been identified to carry genes coding for P450 enzymes (Nelson et al., 1993). More than 6000 members of this CYP superfamily have been identified so far (http://drnelson.uthsc.edu/cytochromeP450.html). Prokaryotic CYPs are typically involved in biosynthesis of antibiotics and catalyze important reactions for the degradation of a variety of hydrocarbons. In fungi, CYPs are often required for the synthesis of steroids and catalyze the detoxification of defense chemicals of target host plants therefore enabling pathogenesis. In plants, many secondary metabolites require CYPs e.g. to synthesize phytohormone, pigment and other compounds serving a wide range of functions. One central and well known role of CYPs in mammalian cells is the degradation of foreign chemicals by catalyzing the first oxidative step (phase I) of the detoxification process in liver. Mammalian CYPs are furthermore involved in natural metabolic pathways, such as steroid biosynthesis and metabolism of fatty acids. CYP-catalyzed reactions can be broadly classified into four

categories. The most common one is the hydroxylation reaction where a hydroxyl group is incorporated replacing a hydrogen atom (Fig 2). The second one is the epoxidation reaction where oxygen is introduced into a carbon-carbon bond. Furthermore, CYPs can catalyse the oxidation of heteroatoms where oxygen is added to nitrogen, sulfur or other heteroatom. Additionally, they are able to catalyze the reduction of oxygen and xenobiotics, which can take place under conditions of limited oxygen (Danielson, 2002).

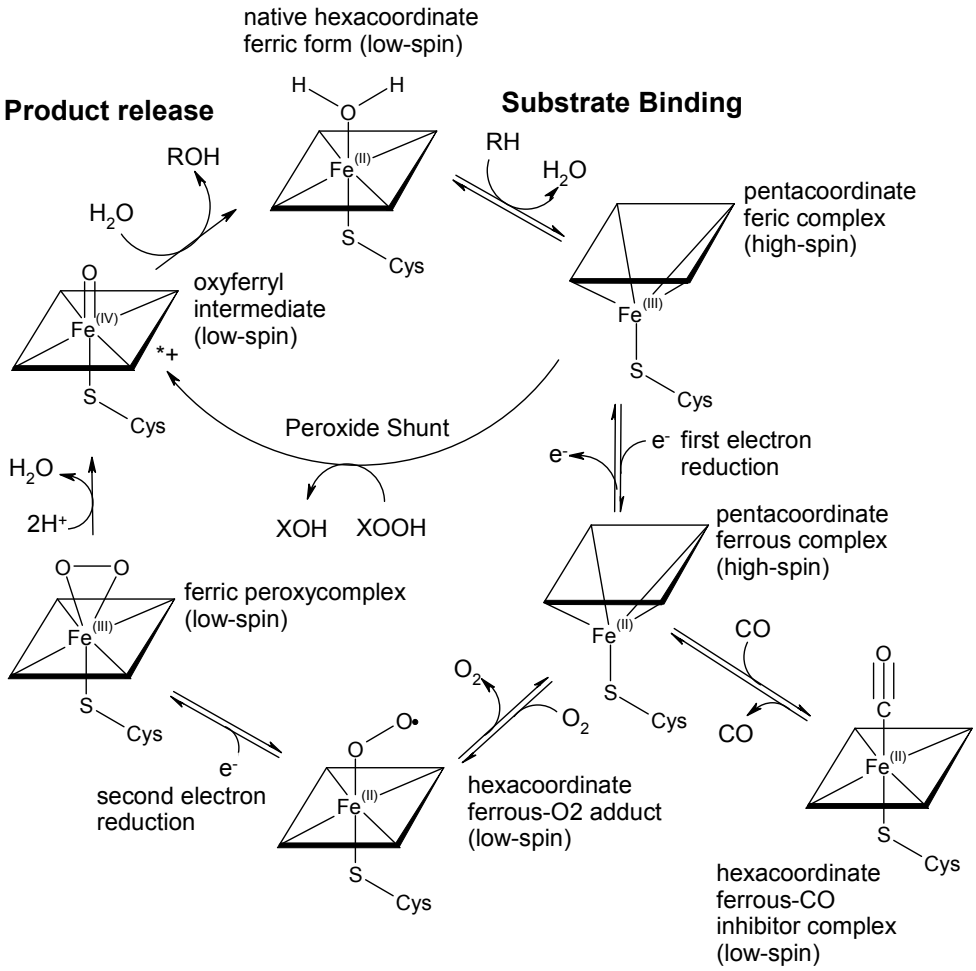


Fig 2: The proposed catalytic cycle of cytochrome P450 for hydroxylation reactions.
Upon substrate binding, the heme iron shifts to a high spin state. Substrate binding is accompanied by an increased redox potential which allows reduction of the iron to form a pentacoordinate ferrous complex that binds molecular oxygen to yield a semistable, low-spin, hexacoordinate ferrous-O2 adduct. Reduction by a second electron is thought to produce a low-spin, ferric peroxycomplex and oxyferryl (Fe+4=O) intermediates. It is hypothesized that transfer of an oxygen atom from this intermediate to the substrate via an oxygen rebound mechanism yields the oxidized product and regenerates the native hexacoordinate state of the P450. The so called "peroxide shunt" pathway allows in the presence of external oxygenation agents, e.g. peracids, to directly form the oxyferryl species from pentacoordinate ferrous. By binding of CO to the pentacoordinated ferrous complex a hexacoordinated ferous-CO complex is formed showing a distinct absorption at 450 nm, giving this enzyme class its name. Picture redrown based on picture from literature (Danielson, 2002)

Many of the members of the P450 family catalyze multiple reactions; therefore the usual method of naming enzymes is inadequate for this group of proteins. Based on structural homology, a systematic nomenclature has been derived. P450 genes are identified by the abbreviation CYP (cytochrome P450) followed by a number, indicating the gene family

(proteins with more than 40% sequence identity), a capital letter specifying the subfamily (more than 55% identity) and a final number for the individual gene within the subfamily, for example CYP2D6 (Nelson et al., 1993). Despite the low sequence identity among cytochrome P450s, the overall fold topography is highly conserved. All members of the CYP protein family consist of carboxy-terminal part, having a relatively high abundance in alpha helix and an amino-terminal domain with a relatively high abundance in beta sheet, which build up a common globular-to-triangular structural framework.

Depending on their subcellular localization, cytochrome P450s can be additionally categorized into three broad groups. While all prokaryotic CYPs belong to the soluble cytosolic form, almost all eukaryotic CYPs are embedded in the membrane of either the mitochondria (mitochondrial-type) or the endoplasmic reticulum (microsomal-type).
Membrane bound CYPs are tethered to membranes by an amino-terminal signal-anchor sequence composed of a string of hydrophobic residues.

The vast majority of cytochrome P450s needs helper proteins to form an active cytochrome P450s system. This systems are composed of the cytochrome P450 itself and one or two additional proteins (i.e. FAD-containing reductase + ferredoxin, FAD/FMN-containing reductase) constituting an electron transfer chain (ET) that shuttles reducing equivalents from a cofactor (i.e. NADH or NADPH) to the cytochrome P450. There are already more then 10 electron transfer chains described in literature (Hannemann et al., 2007). However, the most common monooxygenase system in eukaryotes is the class II (Fig 3). In the simplest form, they contain two integral membrane proteins: the cytochrome P450 and the flavin containing cytochrome P450 reductase (CPR), which transfers both required electrons from NADPH to one of the many cytochrome P450 isozymes. Therefore, the activity of CYPs is not only determined by its abundance, but also by the abundance of the electron transport partners (Crespi et al., 1999). Recent studies showed that the redox partners do not only provide the necessary reduction equivalents, but they are also important for the regulation of the catalytic cycle, where the CPR binding acts as an internal timer of the reaction (Fishelovitch et al., 2010).

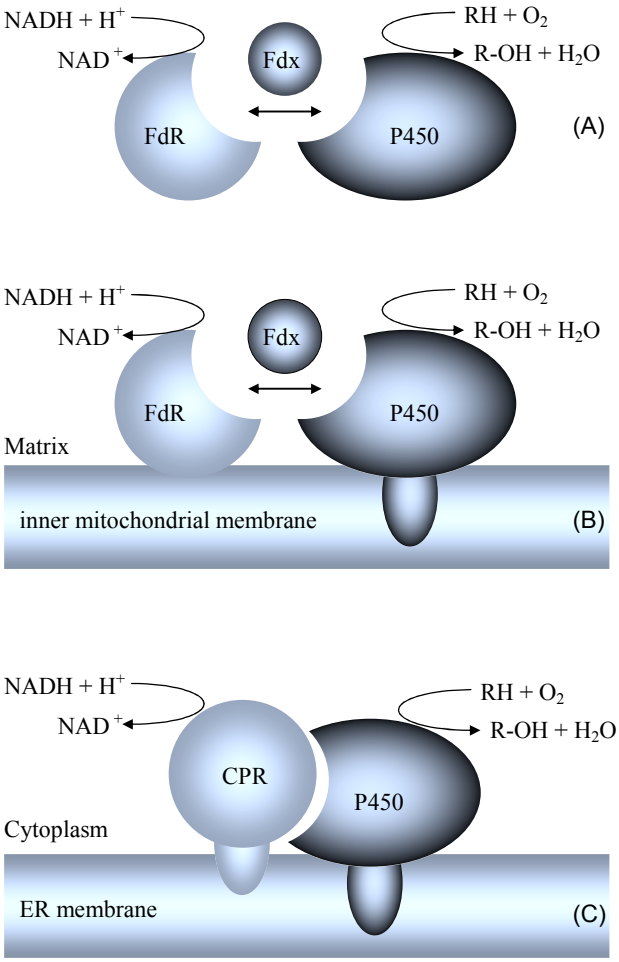


Fig 3: Schematic organization of of the most comone cytochrome P450 systems.
(A) Class I, bacterial system; (B) class I, mitochondiral system; (C) class II microsomal system. Picture is based on picture from literature (Hannemann et al., 2007)

As mentioned before, P450 enzymes play a vital role in metabolism of many drugs and xenobiotics and carcinogens, and are therefore of particularly scientific interest. In this work, a particular focus has been given on human CYP2D6 and CYP3A4.

4.3. Human CYP2D6 and CYP3A4

Human P450s are membrane bound proteins and are expressed in many tissues. So far, 57 cytochrome P450 enzymes and 58 pseudogenes have been identified in the human genome (Zöllner et al., 2010). The highest levels are found in the liver, where they have the principal function to introduce an oxygen atom into hydrophobic substrates. The increased hydrophilicity of the product facilitates its elimination from the human body. Several xenobiotic-metabolizing CYPs are expressed in human liver, among which CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 appear to be most commonly responsible for the metabolism of many structurally diverse drugs and chemicals. Furthermore, many of the liver P450s are involved in endogenous metabolism pathways; i.e. of fatty acids and steroids (Spatzenegger et al., 1995). Many of these drug-metabolizing CYPs are subject to

polymorphism: these sequence variants are associated with modified levels of expression or catalytic activities. Physicians recognized that patients responded differently to applied drugs, according to which allelic variant their genomes were carrying (Shastry, 2006); interestingly, very often, when the influence of polymorphism on pharmacokinetic is mentioned, the redox partners of CYPs are ignored, even though they show a high level of polymorphism as well (Miller et al., 2010).

CYP2D6 is a highly polymorphous, microsomal cytochrome P450 found mainly in the liver and to some extend in duodenum and brain as well. It accounts only for 2% of the expressed hepatic CYPs. Nevertheless, this enzyme is responsible for the metabolism of roughly 25% of available pharmaceuticals. These compounds vary in chemical structure, although they all contain a basic nitrogen and a planar aromatic ring as common feature (Strobl et al., 1993; Unwalla et al., 2010). In 2006, the structure of CYP2D6 was resolved and deposited in the protein data base (PDB: 2F9Q) (Fig 4). The 3D structure was made possible truncating the membrane-bound N-terminal domain and introducing two mutations at position L230 and L231 that helped to solubilize the protein (Rowland et al., 2006).

CYP3A4 is a polymorphous, microsomal cytochrome P450. It is the most abundant one and found mainly in the liver and in the human intestine. Up to 60% of the total hepatic cytochrome P450 is comprised by CYP3A4. It has been shown that a huge variety of pharmaceuticals and drugs are metabolized by CYP3A4, which is therefore responsible for the metabolization of roughly 60% of available pharmaceuticals (McKinnon et al., 1995; Shimada et al., 1994). In 2004, the structure of CYP3A4 was resolved and deposited in the protein data base (PDB: 1W0F) (Fig 4). Likewise for CYP2D6, the protein was truncated to facilitate solubility and crystallization (Williams et al., 2004).

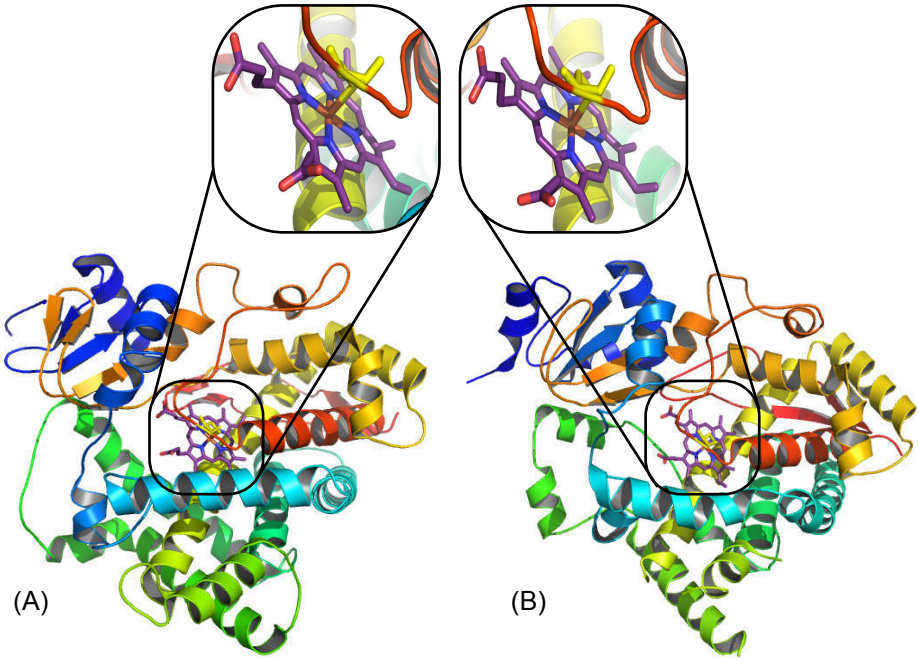


Fig 4: CYP2D6 and CYP3A4 protein structure
Overall fold of (A) human cytochrome P450 2D6 and (B) human cytochrome P450 3A4, colored from blue at the N-terminus to green to yellow to orange to red at the C terminus. The heme is depicted as a stick model in the center of the molecule. The cysteine residue forming the thiolate bridge is shown in form of a yellow stick model.

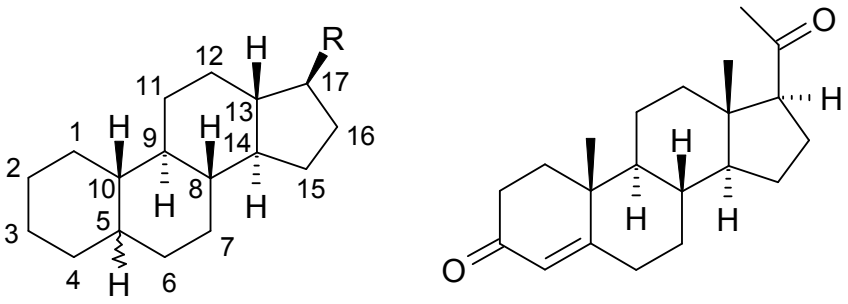
P450 enzymes show rising interest in the scientific community for offering specific reactions to otherwise inaccessible sites required to produce functionalized compounds. The wide use and the complex structures of steroid molecules make the use of P450s as biocatalysts particularly interesting (Fernandes et al., 2003). The next paragraph gives a short introduction into the complex world of steroids.

4.4. Steroids

A large number of lipid compounds belong to the steroid subclass. They show a basic gonane structure, which is composed of three cyclohexane rings and one cyclopentane ring joined to each other in a characteristic arrangement (Fig 5). The steroids vary by the functional group attached to the gonane structure and the oxidation state of the rings (Moss, 1989). Several hundreds distinct steroids are found in plants (phytosterols), vertebrates (steroid hormones) and fungi (ergosterols) (Bun-Ichi, 1973; Fryberg et al., 1972; Heftmann, 1975).
In humans, sterols have several important roles. Cholesterol modulates membrane fluidity and is required to build and maintain membranes. Other steroids bind to receptors and act as sex hormones (e.g. testosterone, etc.). The corticosteroids regulate immune functions, blood volume and electrolyte levels, while anabolic steroids regulate muscle and bone synthesis (Gilliver, 2010; Morfin, 2002; Vandenput et al., 2010). Many naturally occurring and

synthetic steroids are abused by athletes for faster muscle buildup and are also routinely used as therapeutics in medicine (Fragkaki et al., 2009).

Efficient production methods of steroid metabolites are of a general industrial interest. In mammalian cells, cytochrome P450 enzymes are mainly responsible for sterol oxidative metabolism. In particular, progesterone and testosterone belong to the steroid hormones and were used in this work. Testosterone shows anabolic (promotes protein synthesis) and androgenic (stimulation and control of male characteristics development) effects; progesterone belongs to the progestogene hormone family, which has the main function of maintaining pregnancy. Furthermore, progestogenes serve as precursors to all other steroids and therefore play a vital role in steroidogenesis (Bun-Ichi, 1973).



(A) (B)

Fig 5: Structure of steroids
(A) basic skeleton of steroids, (B) human hormone progesterone

4.5. Heterologous expression of P450 enzymes

In the past 20 years, several recombinant expression systems have been investigated. From the more complicated and expensive mammalian (Langenbach et al., 1992) and baculovirus-mediated insect cell system (Asseffa et al., 1989), often exhibiting low levels of functional enzyme, to the "simpler" expression host *E. coli*. Besides the cheap and ease handling, *E. coli* has the additionally advantage of lacking native P450s. However, for high level expression N-terminal truncation or modifications are needed in *E. coli*: changing its native form can lead to a change in the product spectrum of the enzyme (Kim et al., 2008).

In recent years the focus shifted more and more to yeast systems, which combine the ease of handling of prokaryotic systems (cheap media, single cell organism, high cell density) with the features of a eukaryotic system (an endoplasmic reticulum (ER) membrane environment, protein expression and modification processes that resemble those of higher eukaryotes). The endogenous CPR homologues of the different yeasts are able to interact to some extent with heterologously expressed CYPs, although the co-expression of the heterologous P450 redox partner in general leads to an increased CYP activity (Zöllner et al., 2010). A couple of mammalian microsomal CYPs have been functionally expressed so far in *S. cerevisiae*

(Kalsotra et al., 2004; Krynetski et al., 1995; Rao et al., 2010; Renaud et al., 1990; Urban et al., 1990), *P. pastoris* (Dietrich et al., 2005; Kolar et al., 2007) and *S. pombe* (Bureik et al., 2002; Drăgan et al., 2011; Zehentgruber et al., 2010) and the non-conventional yeasts *Y. lipolytica* (Juretzek et al., 2000; Novikova et al., 2009; Nthangeni et al., 2004).

4.6. The non conventional yeast *Yarrowia lipolytica*

In early years of its discovery, *Yarrowia lipolytica* was classified as *Candida lipolytica*. After observation of ascospores, the fungus was reclassified and finally named *Yarrowia lipolytica* (van der Walt et al., 1980). The name "lipolytica" refers to the ability of extensively degrading n-parafines and oils. *Y. lipolytica* can also grow on glucose, alcohols and acetate but not on sucrose. The fungi *Y. lipolytica* is often isolated from biphasic environments like dairy products rich in lipids (Sinigaglia et al., 1994) and oil-polluted soil and water (Kim, Tae Hyun; Lee, Jung-Hyun; Oh, Young Sook; Bae, Kyung Sook; Kim, 1999; Schmitz et al., 2000). For efficient uptake and utilization, *Y. lipolytica* needs to get in direct contact with the hydrophobic substrates: *Y. lipolytica* produces and secretes surfactants to emulsify hydrophobic substrates, therefore increasing the surface area by lowering the interfacial tension. The cell surface is modified to increase hydrophobicity: it has been observed that during cultivation on hydrophobic substrates, the cell surface undergoes structural changes resulting in the formation of protrusions which increases the hydrophobicity of the cell surface and thus decreases mass transfer limitations. Additionally, these structures probably resemble channels that connect the cell wall to the interior of the cell enabling direct contact with and up-take of hydrophobic substrates (Fig 1). These protrusions can be visualized by using electro microscopy (SEM and/or TEM). Using a simple light microscopy, the adhesion to droplets of hydrophobic substrates to *Y. lipolytica* cells can be observed. These observations have led to the hypothesis that hydrophobic substrates can migrate through channels via the plasma membrane to the ER (Aguedo et al., 2003; Fickers et al., 2005; Mlícková et al., 2004). Several studies show the application of *Y. lipolytica's* ability to efficiently utilize hydrophobic substrates. *Y. lipolytica* has been grown in organic/aqueous systems, to bioremediate diesel-contaminated soils (Margesin et al., 1997) and olive-mill waste water (Scioli et al., 1997), for protein production on alkanes (Barth et al., 1996) and aroma compound formation from fatty acid derivates (Waché et al., 2003).

Y. lipolytica grows exclusively (or strictly) under aerobic conditions and is considered as nonpathogenic (optimal growth temperature is below 32-34 °C). Furthermore, this fungus was classified as GRAS (generally regarded as safe) by the FDA (Food and Drug Administration). It is a natural dimorphic fungus and, depending on growth conditions and media composition,

forms yeast cells, pseudohyphae or septate hyphae (Barth et al., 1997) (Fig 6). The complete genome sequence has been made publicly available in 2004 (Dujon et al., 2004). Genome analysis revealed that the organism contains 6 chromosomes that show a high frequency of introns and a rather high G+C content. The organism is distantly related to *Saccharomyces cerevisiae*, and displays an expansion of protein families and genes involved in hydrophobic substrate utilization (Dujon et al., 2004; Thevenieau et al., 2009). The ability to grow on n-paraffins as sole carbon source and the capability to secrete large amounts of protein aroused interest and in the mid 80s efficient genetic tools for gene integration became available. *Y. lipolytica* shows a natural resistance to most commonly used antibiotics. Still, auxotrophy markers are generally used to date as selection method for transformants (Madzak et al., 2004). No natural replicative vectors exist; however, an artificial replicative vector containing both replicative and centromeric functions has been developed, stably maintained as on to three copies per cell (Madzak et al., 2000; Vernis et al., 2001). Integrative vectors are mainly used for heterologous protein production. To increase copy numbers of the integrated expression cassettes, vectors containing defective ura3d4 alleles as selection marker have been used. The defective allele harbors a degenerated ura3d4 promoter, which gives only sufficient amounts of uracyl when several copies of the vector are integrated into the genome, allowing the selection for clones where multicopy integrations or gene multiplications have occurred (Le Dall et al., 1994). As integration target for multicopy integration, rDNA or LTR sites of a *Y. lipolytica* retrotransposon (Ylt1), belonging to the TY3 family, have been used. This retrotransposone is only found in strains isolated from America (Schmid-Berger et al., 1994). For protein expression, several promoters, including non-natural synthetic ones, secretion signals and terminators are available and have been used successfully (Madzak et al., 2005). In a comprehensive study, several yeasts (*S. cerevisiae*, *H. polymorpha*, *K. lactis*, *S. pombe*, and *Y. lipolytica*) have been compared for their efficiency as hosts for heterologous protein production (Müller et al., 1998). In the mentioned study, *Y. lipolytica* was identified as one of the most attractive hosts for heterologous protein production.

Furthermore, new and efficient tools for recombinant protein expression and high-throughput screening with *Y. lipolytica* have been developed (Bordes et al., 2007; Emond et al., 2010). This makes *Y. lipolytica* a potent and desirable alternative to other expression systems.

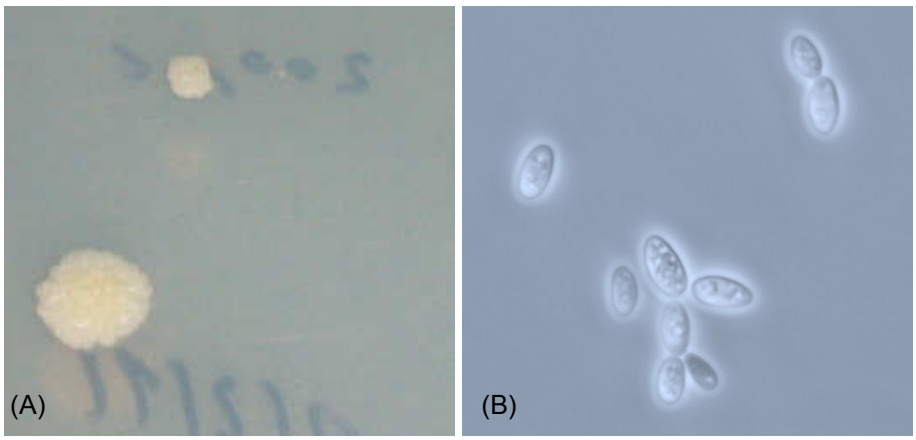


Fig 6: *Y. lipolytica* strain H222-S4 (Dresden, Germany)
(A) morphology when grown on minimal YNBG media and (B) under the microscope.

4.7. Protein engineering and comprehensive mutein data base

As mentioned in paragraph 1.1, the ability of modifying amino acid sequences by recombinant DNA technologies led to the development of widely used protein engineering tools. With these tools, new enzymes with desired traits, i.e. new and/or increased activity, improved stability, etc. have been designed and constructed. Protein engineering can be classified into two categories: rationally designed where detailed knowledge about the structure and function is needed and an evolutionary approach known as directed evolution, where random mutagenesis and screening methods are applied to generate and select muteins with desired properties. These techniques are often combined to screening of sequence space for improved traits. The selected mutated versions with improved properties are used as starting material to create the next mutein library for continued iteration.

Efficient engineering and cloning tools enable the fast construction of vast numbers of muteins to be screened. For high-throughput screening, the detection and identification of clones showing altered properties and/or activity represents always an analytical challenge (Brannigan et al., 2002; Lutz et al., 2008). This is especially the case when no easy detectable color or fluorescence assay is available, i.e. most cytochrome P450 substrates have to be analyzed by separation via HPLC and detected by mass spectroscopy (MS).

Large numbers of muteins have been created by man and nature (e.g. polymorphism of mammalian P450 enzymes). Information about certain proteins and their muteins are widely spread in literature. Even after thorough literature search, there is still the problem of assembling and presenting the data in an easy and comprehensive way. Many studies only describe single mutations and their effects, but do not compare them to already known muteins; therefore, possible additive effects of single amino acid changes and further interesting information may be overlooked. A comprehensive database of laboratory-derived and natural occurring muteins and their catalytic properties would help scientists from

medicine and pharmacokinetics, to structural biology or applied biocatalysis. So far, only a few databases are addressing this complex issue (Fischer et al., 2007; Kawabata et al., 1999; Preissner et al., 2010; Schomburg et al., 2004); however, none of these databases gives detailed information about kinetic characteristics of muteins allowing, at the same time, a fast, systematic and easy way to search for known mutations and catalyzed reactions of interest resulting in a comparative output of muteins in respect to their catalytic properties. Retrieved informations can give potential starting points for directed evolution experiments. The MuteinDB (http://www.muteindb.org) is meant as a platform to collect experimentally derived data about muteins from publicly available sources. Additionally it allows easy access by flexible searches options.

4.8. Aim of the work

As mentioned before, most of the typical substrates for cytochrome P450 enzymes are very hydrophobic. One of the biggest obstacles in performing bioconversions with hydrophobic substrates is the very low solubility in the aqueous phase, which limits cell uptake and represent a frequent bottleneck to the overall biotransformation performance. The unique properties of alkane-utilizing yeast *Y. lipolytica* to efficiently utilize and grow on hydrophobic substrates have been shown in several examples. However, so far its potential for the biotransformation of hydrophobic substrates in a two-liquid biphasic setup with heterologous expressed proteins has not been studied. Furthermore, *Y. lipolytica* has been identified as a potential host for the heterologous production of cytochrome P450 enzymes, but only a few examples exist so far.

The main topic of this work was to use and improve the available genetic tools for this species to functionally express the human liver CYP system CYP2D6 and CYP3A4 in combination with either functionally expressed human cytochrome P450 oxidoreductase and/or the endogenous cytochrome P450 oxidoreductase in *Y. lipolytica*.

The next step after the successful expression of the human liver CYP system was the assessment of *Y. lipolytica* as a whole cell biocatalyst in biphasic two-liquid phase systems for hydroxylation of hydrophobic substrates. In this work I focused on the bioconversion of the very hydrophobic substance class of steroids, namely testosterone, 17alpha-methyltestosterone and progesterone. For the analysis of steroids and their bioconversion products several HPLC-MS methods have been developed. Additionally, a HPLC-MS high-throughput screening methods for the screening of P450 mutants showing changed steroid metabolite production has been developed.

The second part of this work focused on the improvement and further development of the muteinDB. As mentioned before, the MuteinDB (http://www.muteindb.org) provides a platform for not only collecting but also for searching for in-depth information of experimentally derived kinetic properties of muteins. This comprehensive database will help researchers from the field of applied biocatalysis to identify potential starting points for further enzyme engineering. As an additional use, medical scientists can also get information about the influence of mutations on drug metabolization and in vivo activation. The new implemented structure search will allow the prediction of structure scaffolds that are accepted by muteins, which might provide helpful information for the development of new biocatalysts and most probably will facilitate drug metabolite prediction in pharmaceutical research and development.

To accomplish this goal, the data collection was standardized and general rules for data collections were written down. The informatics part was done by and in close cooperation with Bettina Halwachs and Gerhard G. Thallinger (Institute for Genomics and Bioinformatics, TU Graz). Several exemplary datasets of different enzyme classes were collected to demonstrate the general applicability of the database. Information about CYP3A4, CYP2D6, HRP, NIT were collected at the TU Graz by myself (Andreas Braun, IMBT) and co-workers (Katrin Weinhandl, Martina Geier, IMBT). Data about the BM3 also known as CYP102A1 were collected by cooperation partners at the RWTH Aachen University (Jan Marienhagen, Anna Joelle Ruff, Department of Biotechnology), and cooperation partners from the University of Groningen helped with the data collection about PAMO (Daniel E. Torres Pazmino, Groningen Biomolecular Sciences and Biotechnology Institute). All data sets were curated at the TU Graz (Andreas Braun, Bettina Halwachs) before implementation to the database.

5. Materials and Methods

More detailed informations about reagents, media and used devices are given in the appendix.

5.1. Growth media

All following media were prepared and sterilized by either autoclaving (20 min at 121 °C) or sterile filtration (0.22 µm filter, Roth, Germany). Solid form was obtained by setting up a 15 g/L agar concentration.

5.1.1. media for Escherichia coli (E. coli)

- LB medium (Luria-Bertani)
  When needed, ampicillin was added to a final concentration of 100 µg/ml.
- SOC medium
  SOC is a suitable medium for use in the final step of cell transformation to obtain maximum transformation efficiency of *E. coli* (Hanahan, 1983).

5.1.2. Growth media for Yarrowia lipolytica (Y. lipolytica)

- YPD
  Complex standard medium often used for growing yeast.
- YNB (Yeast nitrogen based with out amino acids)
  Standard media for classification of yeasts based on amino acid and carbohydrate requirements. D-glucose was added as carbon source to a final concentration of 0.5 – 1 %. When needed, amino acids and bases were added (Uracile, Leucine) to a final concentration of 20 – 50 mg/L
- MM (mineral salt minimal medium) (Mauersberger et al., 1996)
  More or less the same composition as YNB w/o amino acids, but salts were adopted from the Reader-Medium (Reader, 1929). D-glucose was added as carbon source to a final concentration of 0.5 – 1 %.

5.2. Strains

Strains used in this work are summarized in Table 1.

5.3. Plasmids

p64ICL1, p65ICL1, p67RYI were provided by Dr. Mauersberger (TU Dresden, Germany) Plasmids used in this work are summarized in Table 2.

5.4. Oligonucleotides

All primers used during this work were obtained from the company IDT (Integrated DNA Technologies, USA). The sequences as well as the purpose of each oligonucleotide used in this work are given in the appendix section.

5.5. Methods

Unless stated otherwise, all genetic methods applied during this work were carried out according to the standard methods as described previously (Sambrook et al., 2001).

5.5.1. DNA electrophoresis and manipulation

Products from PCR and endonuclease reactions were analysed by agarose gel electrophoresis using agarose concentration of 1 % (w/v) in TBE-buffer. The GeneRulerTM 1kb plus DNA Ladder and MassRulerTM DNA Ladder (Fermentas, Germany) have been used as reference DNA. The ethidium bromide stained DNA fragment of interest was isolated from gel and purified using a commercially available kit (Promega, USA), according to the manufacturer's instructions. DNA concentration was determined either by comparing to MassRuler DNA ladder (Fermentas, Germany) or spectroscopically at 260 nm using nanodrop (Thermo Scientific, Germany).

5.5.2. DNA restriction and ligation

All used restriction endonucleases were from Fermentas (Fermentas, Germany). All restriction reactions were performed according to the manufacturer's instructions in the recommended buffers. Double restrictions were simultaneously performed in the most appropriate buffers. Ligation of DNA fragments were performed using the commercially available T4 DNA ligase™ from Fermentas (Fermentas, Germany). The ligation reactions were performed according to the manufacturer's instructions, at different molar ratios of plasmid backbone to insert.

Table 1: Strains used (in this work)

***E. coli* strains**	**Genotype**	**Phenotype**	**Source**
Top10F'	*F'{lacIq Tn10 (TetR)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG*	Tet+, Str+	Invitrogen, UK
Top10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*	Tet+, Str+	Invitrogen, UK
***P. pastoris* strains**			**Reference**
CBS7435	wild type strain		(Küberl et al., 2011)
***Y. lipolytica* strains**	**Genotype**	**Phenotype**	**Reference**
H222-S4	*MATA, ura3-302*[a]	Ura$^-$, Alk$^+$, Tgl$^+$, Lip$^+$, Eth$^+$, Glu$^+$,Suc$^+$	(Mauersberger et al., 2001)
Production strains	**Description**	**Plasmid target / Auxotrophy**	**Reference**
YL23	H222-S4 transformed with *Sac*II – linearized p65D-linker plasmid, negative control	rDNA, none	this work
YL21	H222-S4 transformed with *Sac*II - linearized p64D-HCPR-WT-2D6syn plasmid	rDNA, none	this work
YL13	H222-S4 transformed with *Sac*II - linearized p64D-HCPR-WT-2D6WT plasmid		
YL10	H222-S4 transformed with *Sac*II - linearized p64D-HCPRsyn-2D6syn plasmid		
YL22	H222-S4 transformed with *Sac*II - linearized p64D-HCPR-WT-3A4syn plasmid		
YL11	H222-S4 transformed with *Sac*II - linearized p64D-*Yl*CPR-WT-2D6syn plasmid		
YL18	H222-S4 transformed with *Sac*II - linearized p64D-*Yl*CPR-WT-3A4syn plasmid		
YL15	H222-S4 transformed with *Sac*II - linearized p65D-HCPR-WT-2D6syn plasmid	rDNA, none	this work
YL20	H222-S4 transformed with *Sac*II - linearized p65D-HCPR-WT-3A4syn plasmid		
YL12	H222-S4 transformed with *Sac*II - linearized p65D-*Yl*CPR-WT-2D6syn plasmid		
YL19	H222-S4 transformed with *Sac*II - linearized p65D-*Yl*CPR-WT-3A4syn plasmid		
YL24	H222-S4 transformed with *Sac*II - linearized p64D-HCPR-WT-2D6syn-F120A plasmid	rDNA, none	this work
YL25	H222-S4 transformed with *Sac*II - linearized p64D-HCPR-WT-2D6syn-E216A/F483G		
YL28	plasmid		
YL29	H222-S4 transformed with *Sac*II - linearized p64D-HCPR-WT-2D6syn-F483G plasmid		
	H222-S4 transformed with *Sac*II - linearized p64D-HCPR-WT-2D6syn-E216M/F483G plasmid		
YL26	H222-S4 transformed with *Sac*II - linearized p64DAS1-2D6syn-HCPR-WT plasmid	rDNA, none	this work
YL27	H222-S4 transformed with *Sac*II - linearized p64DAS2-2D6syn-HCPR-WT plasmid		

[a] *ura3-302*: *URA3* disrupted by a construct pXPR2 SUC2 from *S. cerevisiae*. This allele confers the ability to grow on sucrose or mollasses (Mauersberger et al., 2001).

Table 2: Plasmids used (in this work)

Plasmid	Description	Marker gene (selection in yeast)	Reference
p64ICL1	parental vector	*ura3d4* (mc)[a]	(Förster et al., 2007)
p65ICL1	parental vector	*ura3d1* (sc)[a]	
p67RYL	source for *Y. lipolytica* cytochrome P450 reductase (*Yl*CPR-WT)	*ura3d4* (mc)[a]	(Förster, 2001; Nthangeni et al., 2004)
pNMTS-CYP-OR-spe	source for codon optimized human cytochrome P450 3A4 (3A4syn)		Weis et al., unpublished
pBdpTrcRed-3A4(192v)wtA305S	source for codon optimized human cytochrome P450 reductase (HCPRsyn)		
ID30915411	cDNA wild-type human cytochrome P450 2D6 (2D6-WT)		BioCat GmbH, Germany
ID7262313	cDNA wild-type human cytochrome P450 3A4 (3A4-WT)		
ID7262313	cDNA wild-type human cytochrome P450 reductase (HCPR-WT)		
Cloning vectors for integration into Y. lipolytica:			
pJet1.2	cloning vector		Fermentas
p64D-linker	cloning and expression vector	*ura3d4* (mc) [a]	this work
p65D-linker	cloning, expression vector and negative control	*ura3d1* (sc) [a]	
p64DAS1-linker	cloning and expression vector	*ura3d4* (mc) [a]	this work
p65DAS2-linker	cloning, expression vector and negative control	*ura3d1* (sc) [a]	
Integrative vectors for CPR and P450 production in Y. lipolytica:			
p64D-HCPR-WT-2D6syn **p64D-HCPR-WT-2D6WT** **p64D-HCPRsyn-2D6syn** **p64D-HCPR-WT-3A4syn** **p64D-HCPR-WT-3A4WT** **p64D-*Yl*CPR-WT-2D6syn** **p64D-*Yl*CPR-WT-2D6WT** **p64D-*Yl*CPR-WT-3A4syn** **p64D-*Yl*CPR-WT-3A4WT**	multi copy co-expression of human wild-type CPR or *Y. lipolytica* own wild-type CPR in combination with wild-type or codon optimized CYP2D6 or wild-type or codon optimized CYP3A4, respectively	*ura3d4* (mc) [a]	this work
p65D-HCPR-WT-2D6syn **p65D-HCPR-WT-2D6WT** **p65D-HCPR-WT-3A4syn** **p65D-HCPR-WT-3A4WT** **p65D-*Yl*CPR-WT-2D6syn** **p65D-*Yl*CPR-WT-2D6WT** **p65D-*Yl*CPR-WT-3A4syn** **p65D-*Yl*CPR-WT-3A4WT**	single copy co-expression of human wild type CPR, codon optimized CPR or *Y. lipolytica* own wild type CPR in combination with wild type / codon optimized CYP2D6 or wild type/ codon optimized CYP3A4, respectively	*ura3d1* (sc) [a]	this work
Integrative vectors for CPR and CYP2D6 mutant production in Y. lipolytica:			
p64D-HCPR-WT-2D6syn-F120A **p64D-HCPR-WT-2D6syn-E216A/F483G** **p64D-HCPR-WT-2D6syn-F483G** **p64D-HCPR-WT-2D6syn-E216M/F483G**	multi copy co-expression of human wild-type CPR in combination with codon optimized CYP2D6 mutants	*ura3d4* (mc) [a]	this work
Integrative vectors for CPR and CYP2D6 mutant production in Y. lipolytica:			

p64DAS1-2D6syn-HCPR-WT **p64DAS2-2D6syn-HCPR-WT**	multi copy co-expression of codon optimized CYP2D6 in combination with human wild-type CPR where 2D6syn is under the control of the DAS1 or DAS2 promoter from *P. pastoris*	*ura3d4* (mc) [a]	this work

[a] *URA3* marker genes for single copy (sc, *ura3d1*) or multicopy (mc, *ura3d4*) selection with different promoter lengths; Abbreviations: HCPR-WT, wild type human cytochrome P450 reductase; *Yl*CPR-WT, wild type Y. lipolytica cytochrome P450 reductase; 2D6syn, codon optimized human cytochrome 2D6; 2D6WT, wild type human cytochrome 2D6; 3A4-sp-opt, codon optimized human cytochrome 3A4; 3A4WT, wild type human cytochrome 3A4

5.5.3. DNA purification and DNA sequencing

Plasmid purification was performed using a commercially available kit (Fermentas, Germany) according to the manufacturer's instructions. After isolation, the plasmid concentration was determined either by comparing to MassRuler DNA ladder (Fermentas, Germany) or spectroscopically at 260 nm using nanodrop (Thermo Scientific, Germany).
Correctness of all DNA inserts was verified by sequencing (LGC Genomics, Germany)

5.5.4. Vector construction

The multicopy vectors p64D-linker and single copy vector p65D-linker were obtained by replacing the ORF and intron of the ICL1 gene with a linker, containing cutting site *Spe*I and *Asc*I, in parental vectors p64ICL1 and p65ICL1 (Förster et al., 2007), respectively (Fig 7). Both vectors contain the URA3 as selection marker. The p64 vector variant contains a degenerated ura3d4 promoter which gives only sufficient amounts of urea when several copies of the vector are integrated into the genome. This allows the selection for clones where multicopy integrations or gene multiplications have occurred. The p65 vector variant contains the normal ura3d1 promoter for single copy selection. Multicopy Vectors containing DAS1 or DAS2 promoter from *P. pastoris* instead of the ICL1 promoter were obtained by replacing the *Sph*I and *Spe*I flanking fragment of the p64D-linker plasmid with DAS1 or DAS2 isolated from wild-type *P. pastoris* strain CBS7435 (Küberl et al., 2011) respectively.
Genes of interest were amplified from plasmid- or genomic DNA using PCR (Table 3). p67RYL was used to isolate wild-type *Y. lipolytica* CPR. pNMTS-CYP-OR-spe and pBdpTrcRed-3A4(192v)wtA305S were used to isolate codon optimized human CYP3A4syn and codon optimized human CPRsyn respectively. Both genes were optimized for yeast expression (hybrid optimized for *P. pastoris*, *S. pombe* and *S. cerevisiae*). They were already available in our laboratory from previous studies (Weis et al., unpublished).
Wild-type human CYP2D6 (ID30915411), wild-type human CYP3A4 (ID7262313) and wild-type human CPR (ID3882411) were isolated from cDNA clones. (BioCat GmbH, Germany),
For the codon optimization of CYP2D6, the free software "Gene Designer V1.1.4.1" (DNA 2.0, USA) was used to create an optimized gene for yeast expression (hybride optimized for *Pichia pastoris* and *Y. lipolytica*). The codon optimized gene 2D6syn was synthesized by GenScript Corporation (USA).

Table 3: PCR reaction setup for DNA amplification - example
***Yarrowia lipolytica* CPR WT**

1 µL temp (p67RYI ~100ng)	98°C 30''
5 µL Primer *Yl*CPR_WT_fw (5µM)	(98°C 5'' – 58°C 20'' – 72°C 35'') x 30
5 µL Primer *Yl*CPR_WT_rv (5µM)	72°C 5'
5 µL dNTPmix (2mM each)	4°C ∞
10 µL Phusion Buffer HF 5x	
24,5 µL deion. H2O	
0,5 µL Phusion Polymerase	
50µL	

Co-expression single and multicopy vectors with combinations of different CPR's and cytochrome P450's were obtained by cloning overlap PCR inserts at the corresponding site into the linker region (Fig 7). To have each gene under the control of their own promoter, the inserts were constructed by overlap extension PCR. Shortly: Each part (CPR, isocitrate lyase (ICL1) promoter and terminator and CYP) was amplified via PCR using Primer with overhangs containing homologues regions with the adjusting parts. The second PCR was done in two steps (Table 4). The first step is the actual overlap PCR. All fragments were added to the PCR reaction mix and the PCR was run for 20 cycles using Phusion Polymerase (FINNZYMES, Finland). After the first step flanking primers were added to the reaction and the PCR was run for another 35 cycles to amplify the overlap construct. This PCR construct were then cloned into pJet1.2 according to manufacturer'S instructions using the CloneJET™ PCR Cloning Kit (Fermentas, Germany) and sequences verified by sequencing. The inserts were than isolated using the corresponding restriction enzymes.

Table 4: overlap extension PCR reaction setup - example
2 HCPR_T_pD_2D6syn (6005bp)

1 µL ol-temp HCPR _ol_T (preOL 1)	98°C 30''
1 µL ol-temp ol_HCPR _T_ol_pD (preOL 2)	(98°C 5'' – 69°C 20''– 72°C **3'**) x 20
1 µL ol-temp ol_T _pD_ ol_2D6syn (preOL 7)	72°C 5'
1 µL ol-temp ol_p_2D6syn (preOL 6)	4°C ∞
5 µL dNTPmix (2mM each)	
10 µL Phusion Buffer HF 5x	
30,5 µL deion. H2O	
0,5 µL Phusion Polymerase	
50µL	
	98°C 30''
+ (after 20 cycles)	(98°C 5'' – 69°C 20'' – 72°C **3'**) x 35
5 µL Primer HCPR_pp_opt_fw (5µM)	72°C 5'
5 µL Primer 2D6_syn_rv (5µM)	4°C ∞
4 µL Phusion Buffer HF 5x	
2 µL dNTPmix (2mM each)	
3,5 µL deion. H2O	
0,5 µL Phusion Polymerase	
70µL	

Multicopy co-expression vectors with combinations of human CPR and different CYP2D6 mutants were obtained by applying the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, USA) according to the supplier's instructions. pJet1.2 vectors containing the

HCPR-WT-CYP2D6syn insert were used as template and sequences were verivied by sequencing. Subsequently, the appropriate DNA fragment of the overlap PCR insert was cloned into the corresponding site of the p64D-linker multicopy vector.

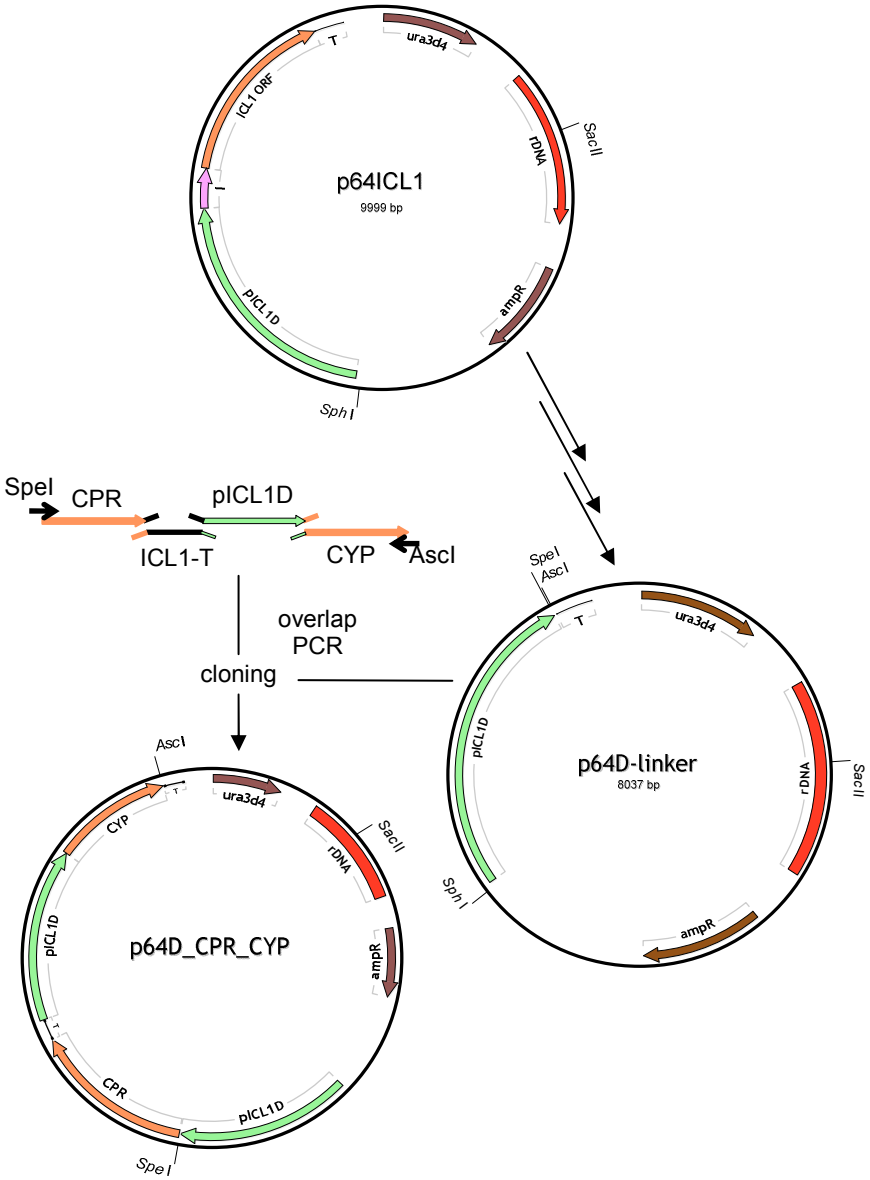


Fig 7: Schematic representation of multi copy co-expression vector concstruction
Construction of multi copy co-expression vectors by inserting overlap extention PCR fragments into the multi copy integrative vector p64D-linker (constructed by removing the open reading frame (ORF) as well as the ICL1 Intron) containing the selective multi copy ura3d4 marker. Green arrows, ICL1 promoter; light violet ICL1 intron; stroked goalpost, ICL1 terminator; red box, rDNA integration region; brown arrows, selection marker for E. coli or Y. lipolytica respectively; gene localization and orientation are indicated by orange arrows

5.5.5. E. coli cultivation and transformation

Unless otherwise noted, *E. coli* cells were grown in LB medium (100 µg/mL ampicillin) and incubated at 37°C and 180 rpm.

Transformation of 80 µL competent E. coli cells after addition of approximately 100 ng plasmid-DNA was performed via electroporation. Mix was transferred into prechilled electroporation cuvettes. The cuvettes were placed into the electroporator (Bio-rad, Germany) and transformation was done with 1.25 kV/mm charging voltage, 200 Ω resistance and 25 µF capacitance. Afterwards, 920 µL SOC-Media were added and the suspension was transferred to Eppendorf tubes. For regeneration, tubes were shaken at 37°C, 650 rpm for 1 h in a thermomixer (Eppendorf, Germany). Aliquots were plated on LB-agar plates (100 µg/mL ampicillin)

5.5.6. Preparation of electro-competent E. coli cells

For overnight culture (ONC), 50 mL LB media in 100 mL culture flasks were inoculated with a single colony of *E. coli* Top10F', picked from a freshly grown LB-Agar plate. The ONCs were incubated at 37°C overnight while shaking.

The next day, 500 mL LB media in 2 L baffled flasks were inoculated with 5 mL overnight cultures. The main cultures were incubated at 37°C while shaking, until they reached an OD_{600} between 0.7 and 0.8 (2-3 h). Then they were chilled on ice for approximately 30 min.

For cell harvest, cultures were transferred to prechilled 500 mL centrifuge bottles and centrifuged at 2,000 g for 15 min at 4°C. The supernatants were discarded and the pellets were twice resuspended in prechilled 500 mL ddH_2O, centrifuged at 2,000 g for 15 min and the supernatants discarded. Then, the cell pellets were resuspended in 35 mL prechilled, sterile 10% glycerol and centrifuged at 4,000 g for 15 min and the supernatants were discarded. Each pellet was resuspended in 1 mL cold, sterile 10% glycerole.

The pooled cell suspension was then aliquoted (80 µL) into Eppendorf tubes and frozen in liquid N_2 or stored on ice to be used freshly respectively.

5.5.7. Transformation and preparation of chemical competent Y. lipolytica cells

Procedures were performed according to literature (Barth et al., 1996). Short: 5 mL of YPD (pH 4.0) was inoculate in the morning with a loop of cells and incubated in 100 mL flasks at 28°C while shaking. In the evening 10 mL of YPD (pH 4.0) were inoculated to an OD600 of 0.001, 0.004, 0.008 and 0.016 and incubated in 100 ml flasks at 28°C overnight while shaking. The culture showing the optimal OD_{600} between 5 and 6 was chosen and cells harvested by centrifugation for 5 min at 2000 g and 28°C. Cells were washed twice with TE-

buffer (prewarmed at 28°C). Cells were resuspended in 0.1 M lithium acetate (pH 6.0) (prewarmed at 28°C) and incubated for one hour at 28°C while gently shaking (60-100 rpm). Afterwards, cells were centrifuged at 28°C and 1000 g for 5 min and resuspended in one-tenth of the volume in lithium acetate buffer (pH 6.0). These competent cells were directly used for transformation as follows.

For transformation all operations were performed on ice, if not stated otherwise. 0.5-1 µg of transforming DNA (linearized Vector) were placed at the bottom of a 2 ml Eppendorf tube, heatshocked at 95°C for 5min and place immediately on ice. Denatured carrier DNA (5µL per trafo, heatshocked for 5min at 95°C) was added to the transforming DNA. DNA mix was overlaid with 100 µl of competent cells (the cells are very sensitive, the tips of the pipetting tips were cut off). Cells and DNA were gently mixed and incubate at 28 °C for 15 min without shaking. Afterwards, 0.7 ml 40 % PEG 4000 solved in lithium acetate buffer (pH 6.0, 0.1 M) was added and suspensions gently mixed and incubate for 1 h at 28 °C, while shaking. Subsequently the mix was heatshocked at 39 °C for 10 min in a water bath and 1.2 ml lithium acetate buffer (pH 6.0, 0.1 M) added. Transformation suspension was gently plated in aliquots of 200 µL on selective YNB medium. Single copy tranformants appeard after 1-2 days, multicopy transformants took up to 3-4 weeks. Positive transformants were transferred to fresh YNB.

5.5.8. Transformation and preparation of electro-competent Y. lipolytica cells

Procedures were performed according to condensed *Pichia pastoris* transformation protocol from literature (Lin-Cereghino et al., 2005). Short: 5-10 ml of YPD media (pH 4.0) was incubated in 300 mL buffled flask overnight at 28°C while shaking. Next day, 50 mL YPD media (pH 4.0) were inoculated to an OD600 of 0.15 – 0.20 in a 300 mL buffled flask. Cells were grown to an OD600 of 0.8 – 1.0 at 28°C while shaking. Cells were harvested by centrifugation at room temperature (RT) and 500g for 5 min. Afterwards, cells were resuspended in 9 mL of ice-cold BEDS solution supplemented with 1 mL DTT (1 M) and incubated for 5 min at 100 rpm and 28 °C. Subsequently cells were centrifuged again at RT and 500g for 5 min. Finally, the cells were resuspend in 1 mL (0.02 volumes) of BEDS solution without DTT. The competent cells were directly used for transformation as follows.

Linearized plasmid DNA (0.5-1 µg for single copy, 4-8 µg for multicopy) was transferred to an electroporation cuvette and overlaid with 80 - 100 µL of competent cells. The cell and DNA mix was incubate for 2 min on ice. Electroporation was performed with Gene Pulser™ (BIO-RAD, USA) (conditions: cuvette gap, 2.0 mm; charging voltage, 1.5 (single copy) – 2.0 (multicopy) kV; resistance, 200 Ω; capacitance, 25 µF). Immediately after electroporation 1

mL cold sorbitol (1.0 M) was added and gently mixed. Aliquots were plate on selective YNBG media.

5.5.9. Cultivation of Y. lipolytica cells and induction

Yeast clones were incubated in YNBG (1% D-glucose) overnight at 28°C while shaking. Exponentially growing cells were taken to inoculate the YNBG (0.6% D-glucose) main culture to a starting OD600 of ~ 0.5. The main culture was incubated overnight at 28°C while shaking. After roughly 17- 20 hours D-glucose was depleted. After additional 1-2 h the expression of CPR and CYP under the control of the ICL1 promoter was induced by adding ethanol to a final concentration of 1%. Additional 1% ethanol was added after ~ 8 h and ~ 20 h. After 24h the cells were harvested and further used for either whole cell conversions or microsome isolation (Fig 8).

$$C_{Ethanol}[g/L] = \frac{V * MW * \Delta A}{\varepsilon * d * v * 1000}$$

$\Delta A = (A_2 - df * A_1)_{sample} - (A_2 - df * A_1)_{SB} - (A_2 - df * A_1)_{RB}$
df = (sample volume + R1)/sample volume + R1 + R2)
V (total volume)
MW (molecular weight)
d (optical path)
v (sample volume)
ε (extinction coefficient NADH)
340 nM = 6.3, 334 nm = 6.18, 365 nm = 3.4 L/mmol/cm

Fig 8: Formula for determination of ethanol

5.5.10. Ethanol determination during induction

Samples were taken shortly before and shortly after addition of ethanol. The ethanol concentration was determined with ENZYTEC™ fluid ethanol test kit (scil diagnostics GmbH, Germany) according to manufacturer's instructions. Short: cell suspension was centrifuged at 4°C and 16100g for 10 min. pH was adjusted to 8-9 by adding a potassium hydroxide solution (2 M) and incubated for 15 min on ice. ddH_2O was used to determine reagent blank (RB, no sample) and sample blank (SB, no reagent 2). Reagent 1 and diluted sample were incubated for 3 min at RT. Reagent 2 was added, mixed and further incubated at R for 15 min. Absorption was measured at 340, 334 and 365 nm using UV/Vis DU 800 spectrophotometer (Beckman Coulter, USA). Ethanol concentration was calculated as shown in Fig 9.

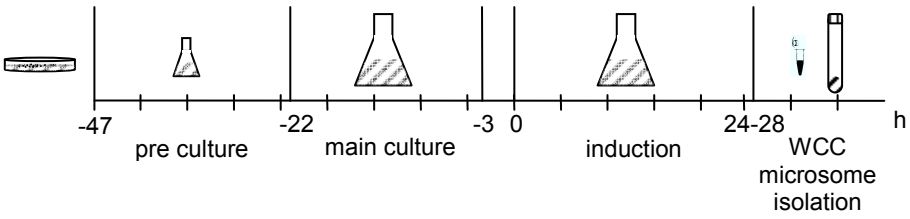


Fig 9: Principle scheme of the *Y. lipolytica* cultivation, heterologous protein production in shake flasks

5.5.11. Microsome preparation

Yeast cells were harvested by centrifugation at 2000g for 10 min. The cell pellet was washed twice with water. 4-6 g cells were resuspended in ~ 20 ml buffer A. Crude enzyme lysate was obtained by "Merkenschlager homogenization" (Merkenschlager et al., 1957). Homogenization bottles were filled to 1/3 with glass beats (0.25-0.5 µm) and mixed with cell suspension. After homogenization for 3 min at 2-8°C with Merkenschlager homogenizer, cell debris and glass beats were removed by centrifugation at 10000 g and 4°C for 10 min. To pellet the microsomes the supernatant was centrifuged at 100000 g and 4°C for 1 hour. The brownish pellet was resuspended in buffer A (~ 1 mg microsome pellet per mL buffer) using potter homogenizer. The microsomes were stored at -20°C till use.

5.5.12. SDS (sodium dodecylsulfate) polyacrylamid gel electrophoresis

Total protein concentrations of microsomal preparations were determined according to the manufacturer's instructions (Bio-Rad, Germany). 20 µg total proteins were added to 15 µl of NuPage loading buffer. Samples mix were incubated at 40°C for 10 min and loaded onto NuPAGE Novex Bis-Tris-Gel (Separation Gel: 4-12%, Stacking Gel: 4%, 1.0 mm thick).
SDS-PAGE was run according to manufacturer's instructions (Invitrogen, UK).

5.5.13. Immunologic detection of proteins

Western blotting was done according to protocol provided with the MAB-2D6 and WB-3A4 kit (BD Gentest™). Blotting was performed for 1 h with a current of 400 mA as the limiting variable. Proteins were transferred electrophoretically from the polyacrylamide gel onto a nitrocellulose membrane (GE Healthcare Europe GmbH) in a wet blotting system.
The Western blotting sandwich was built up as follows:
positive electrode (white) // sponge // filter // membrane // gel // filter // sponge // negative electrode (black) (Fig 10)

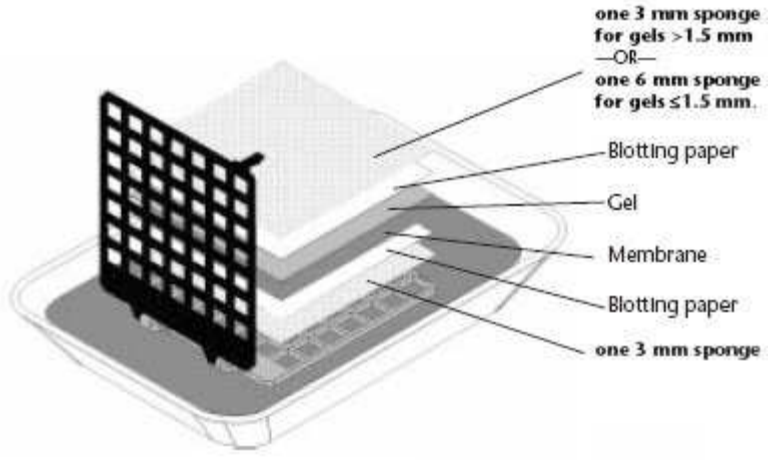


Fig 10: Western blot sandwich (picture copied from manufacturer manual)

The membranes were stained with Ponceau S to detect proteins. Upon taking an image, the membranes were washed with ddH_2O and blocked with TBST-milk for 1 h at 37°C or overnight at 4°C respectively. This step was followed by applying the primary antibody (MAB-2D6, WB-3A4, BD Gentest, USA) for 1 h at 37°C with moderate shaking. Subsequently, the membranes were washed 5 times with TBST for 5 min each. Then the secondary antibody (antibody conjugated with alkaline phosphatase) was applied for 1 h at 37°C with moderate shaking, which was followed by washing 5 times with TBST for 5 min each. Finally, detection was done by placing 2-3 mL BCIP/NBT substrate onto the membranes for 2-3 min. The reaction was stopped by washing the membrane with water.

5.5.14. Genomic DNA isolation

Genomic DNA was isolated from *Y. lipolytica* tranformants grown at 28°C overnight in minimal YNB media supplemented with 1% D-glucose (YNBG) essentially as described in literature (Hoffman et al., 1987). Short: the equivalent volume of 100 OD units (100= OD_{FINAL} x Xml) of a 50 ml overnight yeast culture (OD_{600} 4-6) was transferred into a 50ml tube and centrifuged for 5 min at 500 g. Cell pellet was resuspended in 0.5 mL sterile distilled water and transferred to 1.5 mL tube and centrifuged for 5sec at 16100 g. Supernatant was discarded and resuspended in residual water. In the following order, 200 µL Yeast lysis buffer, 200 µL Roti®-Phenol, and 0.3 g of acid-washed glass beads were added to the cell suspension. Cells were homogenized by shaking (Vortex) for 3-4 min. 200 µL TE buffer were added and gently mixed. After centrifugation for 5 min at 161000 g the aqueous phase was transferred to a new tube. Genomic DNA was precipitated by adding 1 mL 100% EtOH and gently mixing by inversion. After centrifugation for 1min at 161000 g the supernatant was removed by aspiration. The remaining pellet was resuspended in 400 µL TE-buffer and 5 µL RNAse A (10mg/ml) and incubate at 37°C for roughly 2 hours. Purified genomic DNA was precipitated by adding 10 µL ammonium acetate (4M) and 1 mL 100% EtOH. After gently

mixing by inversion, genomic DNA was centrifuged for 1 min at 16100 g and supernatant was discarded. DNA was washed with 1 mL 70% EtOH. The remaining pellet was air-dried and resuspended in 50 µL ddH_20. DNA concentration was determined spectroscopically at 260 nm using nanodrop (Thermo Scientific, Germany).

5.5.15. Southern blot

Southern blotting was used to confirm that *Y. lipolytica* strain YL23 contains only one copy of Amp target sequence. DIG DNA labeling and detection was performed according to the manufacturer's instructions (Roche, Germany). Short: using PCR a 540 bp long part of the AmpR ORF was amplified and used as template for the generation of the DIG labled probe according to the method of random primed labeling (Höltke et al., 1995). Labeling efficiency was estimated by serial dilutions and comparing to DIG-labeled control DNA (Roche, Germany). 5 µg genomic DNA of *Y. lipolytica* strains or 1 µg of control plasmid were digested overnight with *Spe*I or *Asc*I or with both and fragments separated with agarose gel electrophoresis. DNA was depurinated by soaking the agarose gel in HCl (250 mM) and denatured by submerging the gel in denaturing buffer. DNA was transferred to a nylon membrane (Biodyne A, Pall, USA) by capillary transfer with transfer buffer (10x SSC) (Fig 11)

DNA was fixed by baking the air-dried membrane at 120°C for 30 min. Denatured DIG labeled DNA probe in pre-heated hybridization buffer was added to the, in hybridization buffer, prehybridized nylon membrane and incubated at 68°C overnight while shaking in a hybridization oven. Nylon membrane was washed two times for 5 min with each washing buffer. For the immunological detection an anti-digoxigenin-AP conjugate antibody was used. After 30 min blocking the membrane was incubated for 30 min with antibody followed by several washing and equilibration steps. Detection was accomplished by adding colorsubstrate solution and reaction stopped by washing with TE-buffer.

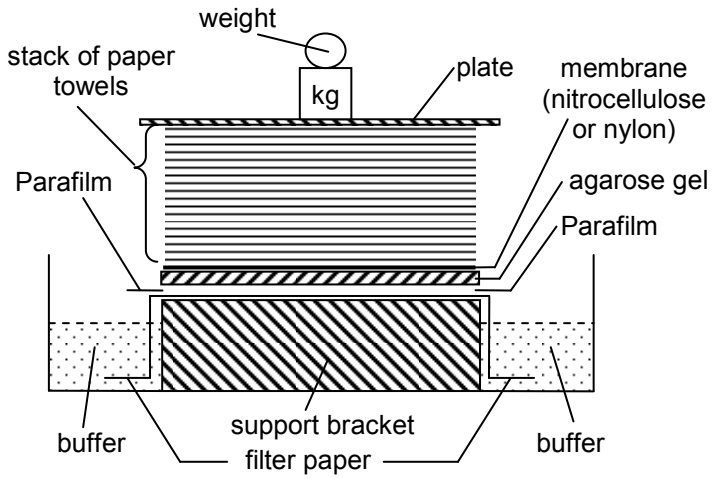


Fig 11: Principle scheme of the DNA transfer assembly.
Sheet of nitrocellulose membrane was laid over the agarose gel, and the separated DNA fragments were transferred to the sheet by blotting. The agarose gel was placed on a support bracket on top of a filter paper of which the ends are dipping in a bath of alkali buffer solution.

5.5.16. Real-time QPCR

Real-time QPCR (RT-QPCR) was used to estimate the copy number of the integrated expression cassette. The ICL1 set of primers, ICL1-fw and ICL1-rv, anneal to the single-copy of the endogenous *Y. lipolytica* ICL1 gene within the chromosome. The Amp primers, Amp-fw and Amp-rv, target the Amp marker gene present on the integration cassette. *Y. lipolytica* YL23 was used as a control organism with a single-copy of both the ICL1 and Amp target sequences. Reaction mixes (18 µl) consisted of 100 pg template DNA, Power SYBR Green Master Mix (Applied Biosystems, CA, USA), and 250 nM of each primer. Each reaction was run in duplicate in an ABI PRISM 7300 Real Time PCR machine (Applied Biosystems, USA). The profile used was: 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 60 s. Data collection was done after each 60°C step. A melting curve analysis was conducted after the amplification from 45°C to 95°C. Analysis was done using "Sequence Detection Software" SDS (Applied Biosystems, version 1.2, CA, USA). Average Ct values of the 2 profiles (ICL1 and Amp) were used to estimate the relative copy number for the selected transformant (Livak et al., 2001).

5.5.17. CO difference spectrum

P450 concentrations in the isolated microsomes were determined by reduced carbon monoxide spectra (Omura et al., 1964). 0.4 mL microsome fraction (15–30 mg protein per mL) was added to sodium phosphate buffer (0.1 M, pH 7.4) containing glycerol (20%) to the end volume of 2 mL. 100 µL KCN (200 mM, pH 7.7) were added to mask the negative peak of cytochrome oxidases at 445 nm (Förster, 2001). Some sodium dithionite was added to reduce the CYP. The mixture was split into a reference and a measuring sample and transferred into separate polystyrol/-styren cuvettes (Sarstedt, Germany) The reference

spectrum was recorded from 400 to 500 nm with the two-beam Specord 205 UV/Visible spectrophotometer (Analytik Jena, Germany). The measuring cuvette was then slowly aerated with carbon monoxide for 30-60 s, and the spectrum was measured again.

The cytochrome P450 concentration was calculated with an extinction coefficient of ε_{450nm} = 91 /mM/cm (pmol/µL cytochrome P450 = $\Delta E_{450-490nm}$ x dilution factor x 1 cm / ε)

5.5.18. NADPH cytochrome c reductase (NCR) activity

The reductase-catalyzed reduction of bovine heart cytochrome c at 550 nm was measured essentially as described (Phillips et al., 1962). Cytochrome c solution (300 µM) in Tris-HCl buffer (50 mM, pH 7.5) was mixed with diluted microsome fraction (1:50 – 1:400) and made up with Tris-HCl buffer to 650 µL. 50 µL KCN solution (50mM, pH 7.7) was added to mask the cytochrome c oxidase activity. Reaction was started by adding 50 µL aqueous NADPH (1.5 mM). Activities were determined by measuring the increase of absorption at 550 nm using UV/Vis DU 800 spectrophotometer (Beckman Coulter, USA) and calculated by using an extinction coefficient of ε = 21/mM/cm.

5.5.19. Hydroxylation assay / CYP activity of microsomes

Microsomal substrate conversion was performed essentially as described (Phillips et al., 2006). Short: Substrate (bufuralol solved in H_2O → 25 µM end concentration, steroids solved in DMSO → 2 mM end concentration) and NADPH (1mM end concentration) were added to potassium phosphate buffer (100 mM, pH 7.4) and pre-incubated for few minutes at 37°C. The reaction was started by adding 20 µL microsomal preparation to give the total volume of 200 µL. The reaction mixture was incubated at 37°C for 20 min (bufuralol) or 1 h (progesterone) and stopped by adding 20 µL perchloric acid (70% v/v). After 20 min incubation on ice IS (prednisolone) was added and the mixture centrifuged for 10 min at 161000g. 100 µL supernatant were transferred to a fresh microtiterplate and stored at -20°C till analysis by HPLC-MS.

Alternatively a regeneration system for cofactor regeneration was added (Weckbecker et al., 2005). Procedure was performed as described above with the addition of glucose dehydrogenase (50 U/mL) and glucose (50 mM ~ 1%) to the reaction mixture.

5.5.20. Hydroxylation assay / aqueous whole cell bioconversion

Yeast cultures were centrifuged at 2000g for 15 min and resuspended in same growth media (supplemented with 1% D-glucose) or in potassium phosphate buffer (100 mM, pH 7.4, supplemented with 1% D-glucose) to an OD of ~ 40 (biomass concentration ~ 10 g/L CDW). Stock solutions of substrate were added to 200 (Eppendorf tubes) or 1000 µL (PYREX tubes)

of cell suspension. Bufuralol was solved in water (1 mM) and added to a final concentration of 25 µM, steroids were solved in DMSO (80-100 mM) and added to a final concentration of 2 mM. The whole cell conversion was done at 30°C for 20 – 60 min with bufuralol or several hours for progesterone. After the desired time internal standard (prednisolone or boldenone) was added and the reaction stopped by centrifugation at 16100 g for 10 min. Supernatant was transferred to a fresh Eppendorf tube.

5.5.21. Hydroxylation assay / two-liquid biphasic whole cell bioconversion

Yeast cultures were centrifuged at 2000g for 15 min and resuspended in potassium phosphate buffer (100 mM, pH 7.4, 1% glucose) to an OD of ~ 40 (biomass concentration ~ 10 g/L CDW). 1000 µL of organic solvent, containing steroid (20 mM), were added to 1000 µL cell suspension. The biphasic whole cell conversion was done in 10 mL PYREX tubes at 30°C while shaking at high speed for several hours. After the desired time the reaction was stopped and the two phases separated by centrifugation at 16100 g for 10 min. 500 µL aqueous phase was transferred to a fresh Eppendorf tube. Internal standard (prednisolone) was added and samples were stored at -20°C. 500 µL organic phase was transferred to a fresh GC-Vial. 500 µL isopropanol and internal standard (prednisolone) was added and samples were stored at -20°C.

5.5.22. Y. lipolytica Fed-batch culture in bioreactor

Cultivation process was performed in a 5 l stirred tank bioreactor (Biostat C, Sartorius). The initial batch working volume was 3.5 l and increased to total of 4 l at the time of harvesting. The set-points of all control variables were maintained the same during the entire process duration and, thus, the cultivations were accomplished under the conditions of constant temperature 28 C, 10 L/min airflow (i.e. without any oxygen enrichment), 1500 r.p.m. agitation, pH 5.5. Automated control of the pH was achieved by using 25% ammonia solution and 25% phosphoric acid. The process consisted of the biomass growth phase (i.e. in both batch and exponentially fed fed-batch cultures) and the expression phase with linear feed inducer. The cultivation began at time 0 h with a batch, during which the pO_2 value continuously decreased and the base consumption increased (i.e. no control of the pO_2 set-point applied). After 14–16 h, the pO_2 rose again rapidly and the base consumption stopped. At this point the exponential addition of D-glucose “feed solution 1” was started and continued over 10 h in accordance with the function $f(t)=1.05*e^{0.18*t}$ (in gram of glucose per hour). During the subsequent production phase, ethanol was added during the time period of 34-36 h in accordance with the function $f(t)=1.05*e^{0.002*t}$ (in gram of ethanol per hour) Biomass concentration was determined by measuring cell dry weight (CDW) or by OD

measurement and calculating cell dry weight (using a factor determined by correlating OD to CDW). Cells were harvested by centrifugation at RT, 2000 g for 15 min and resuspended in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 0.5% glucose to a desired OD of 100 - 120 (biomass concentration ~ 25 g/L CDW).

5.5.23. Bioreactor aqueous and two-liquid biphasic whole cell bioconverison

Biotransformations were performed in a 1.5 L stirred tank bioreactor (DASGIP Parallel Bioreactor Systems, DASGIP BioTools). The batch working volume was 600 mL for aqueous phase system and 450 mL + 150 mL for biphasic system. The set-points of all control variables were maintained the same during the entire biotransformation temperature 30°C, 1 NL/min airflow (i.e. without any oxygen enrichment), 1000 r.p.m. agitation, pH 7.4 .

The transformation began at time 0 h by adding progesterone (100 mM in DMSO) to an end concentration of 2 mM or 150 mL water immiscible organic solvent as 2nd phase containing 20mM progesterone. Glucose was provided at a low constant rate of 0.17 g of glucose h-1. Samples were taken at different time points and the reaction was stopped removing the cells by centrifugation at 16100 g for 10 min. 500 µL aqueous phase was transferred to a fresh Eppendorf tube. Internal standard (prednisolone) was added and samples were stored at -20°C. 500 µL organic phase was transferred to a fresh GC-Vial. 500 µL isopropanol and internal standard (prednisolone) were added and samples were stored at -20°C.

5.5.24. HPLC-MS analysis methods

Bufuralol metabolites in the supernatant were separated by HPLC with a XDB-C18, 1.8µm, 4.6x50mm column (Agilent technologies) at 50°C using following gradient: 0-1.6 min/20% B; 1.61-3 min/40% B; 3.01-4/20% B, with a flow rate of 0.9 mL/min. Solvent A was aqueous ammonium acetate (10 mM, pH 5.5) and solvent B was acetonitrile.

Steroids metabolites in aqueous and organic supernatant were separated by HPLC system (1200 series, Agilent Technologies) with a Chromolith RP-C18e, 5µm, 4.6x100mm (MERCK KGaA, Germany) at 26°C using following gradient: 0-3 min/25% B; 3-7 min/75% B; 7-9 min/75% B; 9.01-9.5 min/25% B; 9.5-10 min/25% for aqueous samples and 0-3 min/25% B; 3-7 min/75% B; 7-11.3 min/75% B; 11.31-13 min/25% B for organic samples, with a flow rate of 1.0 mL/min. Solvent A was ddH_2O acidulated (0.1% acetic acid) solvent B was acetonitrile acidulated (0.1% acetic acid). Metabolites were detected using MSD SL detector in positive mode equipped with an electron spray ionization (ESI) unit (Table 5). Yields of the hydroxylated products were calculated by using an external calibration of a reference metabolite or metabolite derivate.

Table 5: m/z values for MSD detection

substance	m/z	substance	m/z
17alpha-methyltestosterone	303	hydroxyl-17alpha-methyltestosterone	319
testosterone	289	hydroxytestosterone	305
		androstenedione	287
		hydroxyandrostenedione	303
progesterone	315	hydroxyprogesterone	331
bufuralol	262	hydroxybufuralol	278
boldenone (IS)	287		
prednisolone (IS)	361		

6. Publications

6.1. Manuscript 1 (muteinDB – published in database; doi:10.1093/database/bas028)

MuteinDB: The mutein database linking substrates, products and enzymatic reactions directly with genetic variants of enzymes

→ *see supplementary data 1*

6.2. Manuscript 2 (*Y. lipolytica – accepted for publication in Microbial cell factories*)

Expression of human liver cytochrome P450s using the non conventional yeast Yarrowia lipolytica for steroid hydroxylation in biphasic systems

→ *see supplementary data 2*

7. Standard Operating Procedure (SOP) –

7.1. SOP I (HPLC-MS)

MT-HPCL-MS whole cell screening system for P450 mediated steroid hydroxylation

→ *see supplementary data 3*

7.2. SOP II (Y. lipolytica)

Constructing a Y. lipolytica P450 expression strain

→ *see supplementary data 4*

8. Additional results and discussion:

8.1. HPLC-MS method development

8.1.1. Bufuralol measurement

Bufuralol is a well-known standard substrate for CYP2D6 and was used to verify the functional expression of CYP2D6. Therefore, a HPLC-MS method was developed (Fig 12). The method to measure bufuralol and its hydroxylated products was optimized based on a method from literature (Paine et al., 2003).

With this optimized method a bufuralol metabolite analysis takes only 4 minutes.

Method Name: AGB-Bufuralol-ESI.M

Column: XDB-C18, 1.8µm, 4.6x50mm Agilent, in-line filter unit (0.2 µm filter)

Column Temp: 50°C

Flow = 0.9 mL/min

Solvent A: 10mM Ammonium acetate + AA till pH 5.0

Solvent B: Acetonitril

Gradient: 0-1.6/ 20% B, 1.6-1.61/40% B, 1.61-3/40% B, 3-3.01/20% B, 3.01-4/20% B

StopTime: 4.0 min

Injection: 5 µL Sample was injected (100µL per Well in a 96 Well Plate)

Overlapped Injection, after 1.0 min

Detector: MS-ESI, positive, (3000V, drying gas flow 12.0 L/min, nebulizer pressure 410 kPa, drying gas temp. 350 °C), delayed MS start time (1.0 min), VWD

- *MSD1 m/z = 278 → hydroxybufuralol*
- *MSD2 m/z = 262 → bufuralol*
- *MSD3 m/z = 361 → IS (Prednisolone)*
- *VWD1 = 252 nm*

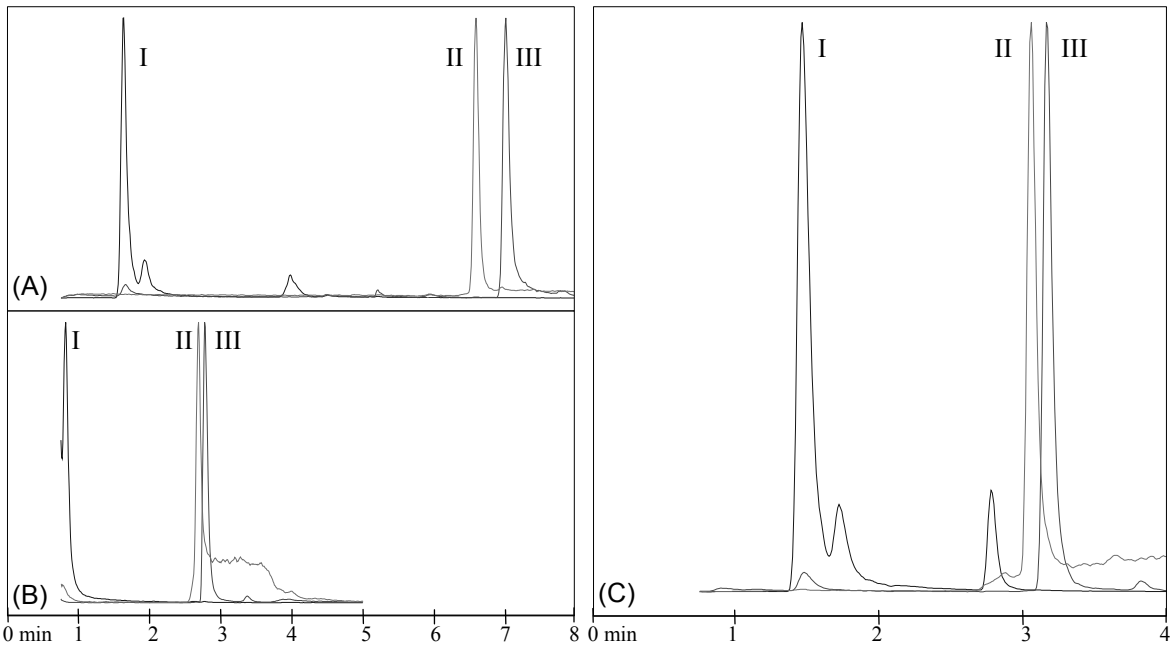


Fig 12: Method development for HPLC-MS analysis of bufuralol and its metabolites.
Mobile phase: ammonium acetate (solvent A, pH 5, 100 mM); acetonitril (solvent B). (A) isocratic 30% B, flow = 1 mL/min; (B) 0-1.6/20% B, 1.6-1.61/40% B, 1.61-3/40% B, 3-3.01/20% B, 3.01-5/20% B, flow = 1 mL/min; 0-(C) 1.6/20% B, 1.6-1.61/40% B, 1.61-3/40% B, 3-3.01/20% B, 3.01-5/20% B, flow = 0.9 mL/min, ammonium acetate (solvent A, pH 5, 10 mM). Peak designation: I, 1'-hydroxybufuralol; II, prednisolone (IS); III, bufuralol.

8.1.2. Steroid measurement

Steroids are a class of organic compounds synthesized from isopentenyl building blocks. They belong to the family of lipids and are very hydrophobic. They occur in mammalian plants and fungi where they have diverse functions from vitamins and sex hormones to toxins. Many hormone derivates are used as anti-inflammatory drug or to increase muscle and bone synthesis. There is vast interest in steroid metabolites which are generated mainly by cytochrome P450 enzymes. Therefore steroids were chosen as hydrophobic substrates.

To be able to separate steroid substrate and hydroxylated products as well as organic solvent of aqueous or organic phase samples, the Chromolith RP-c18e (5 mM, 4.6x100 mm, MERCK) was used. The method was optimized based on a method from literature (Paine et al., 2003). As mobile phase ddH2O acidulated with 0.1% formic acid (solvent A" and acetonitril acidulated with 0.1% formic acid (solvent B)) were chosen. With the optimized method, the favored separation could be achieved (Fig 13).

Method name: AGB-Testo-CL-ESI-DBP.M

Column: Chromolith RP-C18e, 5µm, 4.6x100mm Merk, in-line filter unit (0.2 µm filter)

Column Temp: 26°C

Flow:1.0 mL/min

Solvent A: ddH_2O + 0.1% formic acid

Solvent B: Acetonitril + 0.1% formic acid

Gradient: 0-3/ 25% B, 3-7/75% B, 7-11.3/75% B, 11.3-11.31/25% B, 11.31-13/25% B

StopTime: 13.0 min

Injection: 5 µL Sample was injected (~100µL per Well in a 96 Well Plate)

Overlapped Injection: after 6.0 min

Detector: MS-ESI, positive, (3000V, drying gas flow 12.0 L/min, nebulizer pressure 410 kPa, drying gas temp. 325 °C), delayed MS start time (2.5 min), VWD

- *MSD1 m/z = 289 → Testosterone*
- *MSD2 m/z = 305 → Hydroxytestosterone*
- *MSD3 m/z = 287 → Boldenone, Androstendione*
- *VWD1 = 240 nm*

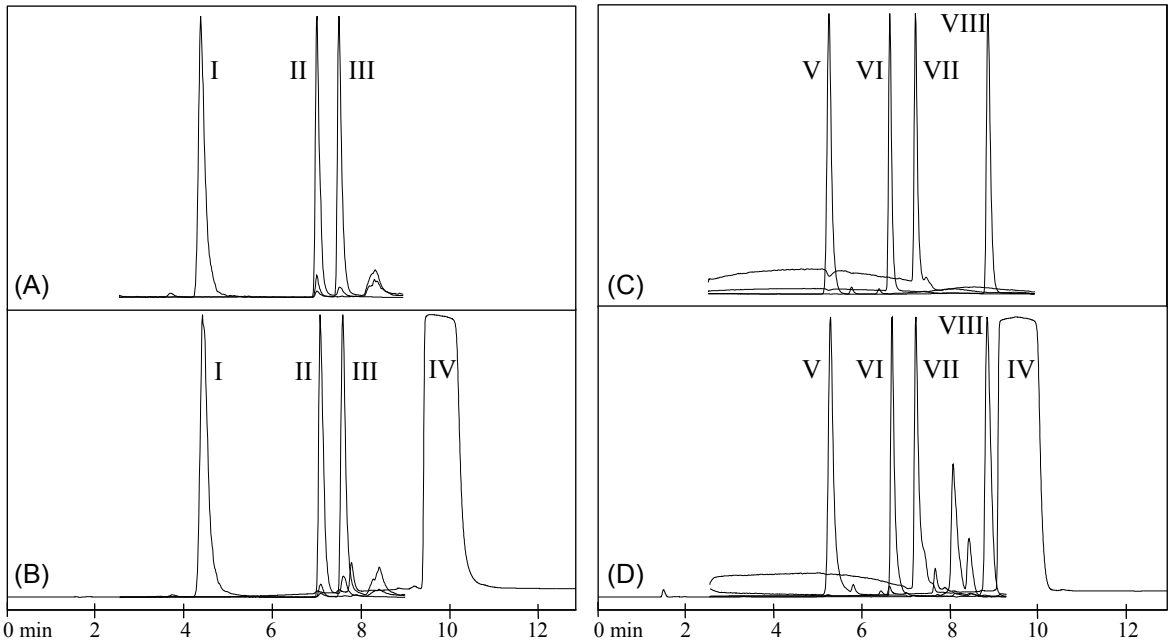


Fig 13: Method development for HPLC-MS analysis of steroids (A/B, testosterone; C/D, progesterone) and their metabolites.
Mobile phase: ddH2O acidulated with 0.1% formic acid (solvent A); acetonitril acidulated with 0.1% formic acid (solvent B). (A/C) aqueous phase 0-3/ 25% B, 3-7/75% B, 7-8/75% B, 8-8.01/25% B, 8.01-.8.5/25%. (B/D) organic phase 0-3/ 25% B, 3-7/75% B, 7-11.3/75% B, 11.3-11.31/25% B, 11.31-13/25% B. Peak designation: I, 6beta-hydroxytestosterone; II, boldenone (IS); III, testosterone; IV, dibutylphthalate (DBP, organic solvent); V, prednisolone (IS); VI, 6beta-hydroxyprogesterone; VII, 16alpha-hydroxyprogesterone; VIII, progesterone.

8.1.3. High-throughput HPLC screening of testosterone metabolites

For managing many hundred samples in a reasonable time, a short analysis time per sample is needed. This can be achieved by using short columns with small particle size packaging that can withstand high pressures and optimization of the used mobile phases (Fig 14). With the new established method one sample takes only 1.8 minutes to be analyzed, while at the same time the different metabolites are still separated. With this method and the automated well plate handler is it possible to analyze up to 700 samples per day.

Method name: AM-Testosterone-fast_ESI.M

Column: XDB-C18, 1.8µm, 4.6x50mm Agilent, in-line filter unit (0.2 µm filter)

Column Temp: 60°C/25°C

Flow = 1.5 mL/min

Solvent A: ddH_2O + 0.1% formic acid

Solvent B: Acetonitril + 0.1% formic acid

Gradient: 0-0.5/40% B, 0.5-0.75/85% B, 0.75-1.2/75% B, 1.2-1.21/40% B, 1.21-1.6/40% B

StopTime: 1.6 min

Injection: 5 µL Sample was injected (~100µL per Well in a 96 Well Plate)

Overlapped injection: after 1.5 min

Detector: MS-ESI, positive, (2850V, drying gas flow 12.5 L/min, nebulizer pressure 410 kPa, drying gas temp. 350 °C), delayed MS start time (0.4 min), VWD

- *MSD1 (80%) m/z = 305 → Hydroxytestosterone*
- *MSD2 (10%) m/z = 289 → Testosterone*
- *MSD3 (10%) m/z = 287 → Boldenone (IS), Androstendione*
- *VWD1 = 240 nm*

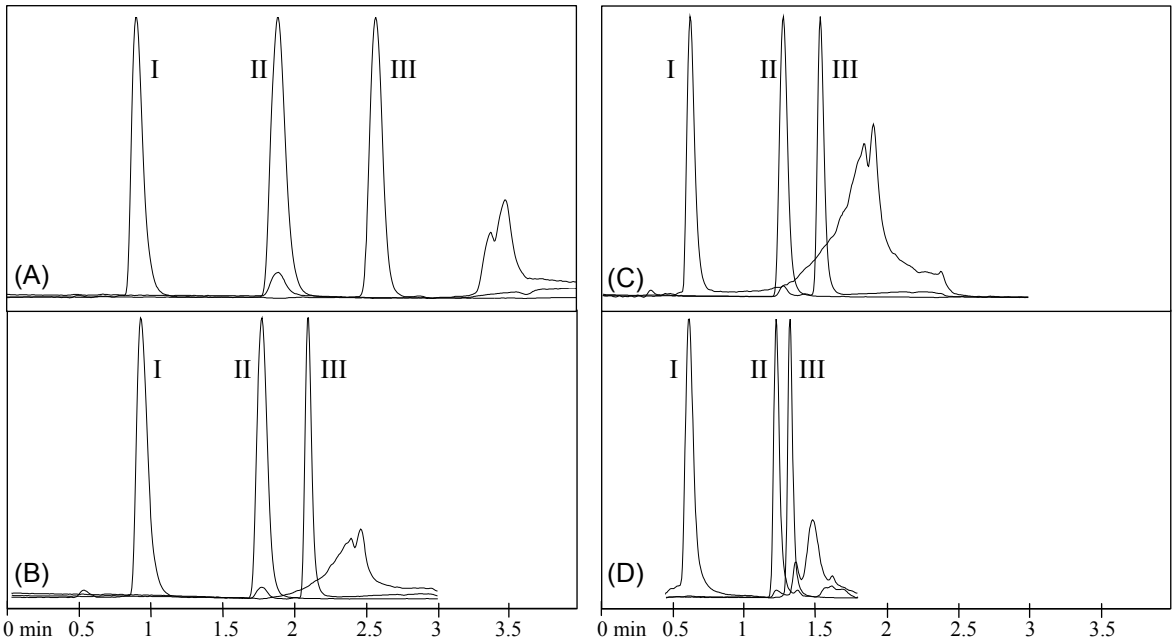


Fig 14: Method development for high-throughput HPLC-MS analysis of testosterone and their metabolites.

Mobile phase: ddH2O acidulated with 0.1% formic acid (solvent A); acetonitril acidulated with 0.1% formic acid (solvent B). (A) 0-2/40% B, 2-3/70% B, 3-3.3/70% B, 3.3-3.31/40% B, 3.31-4.0/40%B, flow = 1.0 mL/min (B) 0-1/40% B, 1-2/70% B, 2-2.3/70% B, 2.3-2.31/40% B, 2.31-3/40% B, flow = 1.0 mL/min (C) 0-1/40% B, 1-2/70% B, 2-2.3/70% B, 2.3-2.31/40% B, 2.31-3/40% B, flow = 1.5 mL/min (D) 0-0.5/40% B, 0.5-0.75/85% B, 0.75-1.2/85% B, 1.2-1.21/40% B, 1.21-1.8/40% B, flow = 1.5 mL/min. Peak designation: I, 6beta-hydroxytestosterone; II, boldenone (IS); III, testosterone

8.2. Proof of principle solubility of hydroxysteroid in aqueous phase

All experiments with aqueous systems were done with steroid concentrations way above their solubility limits in water. However, in none of the experiments with aqueous systems do we reach the solubility limit of hydroxy-steroids. Nevertheless, to exclude the influence of the buffer (supplemented with D-glucose) and high concentration of steroids, calibrations have

been made with 6beta-, and 16alpha-hydroxyprogesterone under reaction conditions. Hydroxy-progesterone show according to SciFinder (Ridley, 2009) the lowest solubility limits of the steroids chosen for this study. The results show, that for both hydroxyl-progesterone the solubility is not influenced by the presence of any compound of the reaction buffer (Fig 15). Therefore, the hydroxy-steroids were directly measured in the aqueous phase and not subject to any extraction procedure.

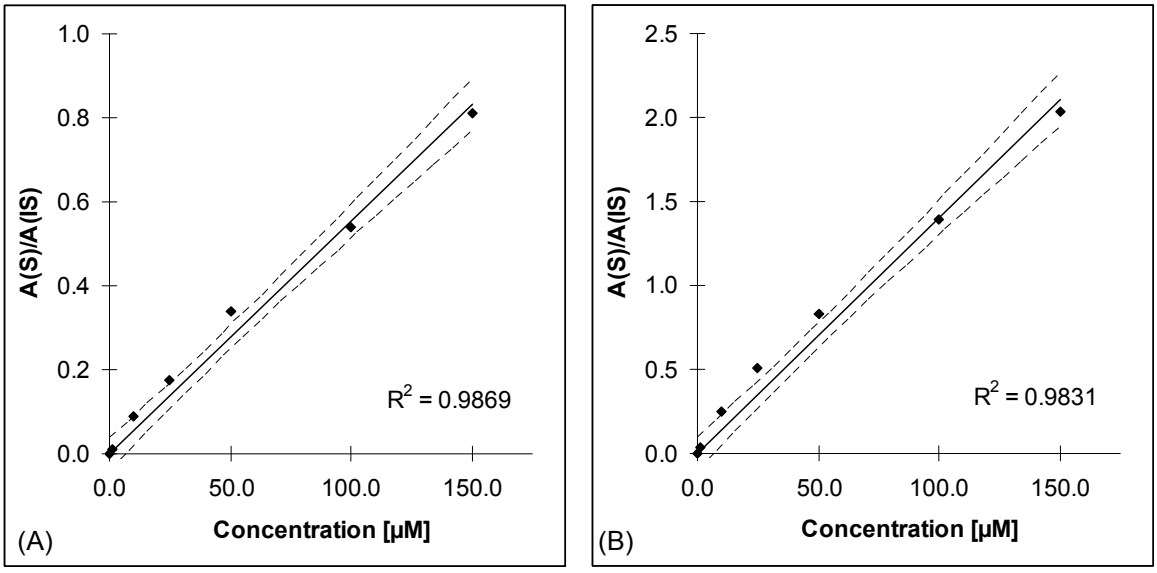


Fig 15: Calibration curves of (A) 6beta-hydroxyprogesterone and (B) 16alpha-hydroxyprogesterone with potassium phosphate buffer (100 mM, pH 7.4, supplemented with 1% D-glucose) saturated with progesterone as solvent. dashed lines, uncertainty trumpets

8.3. CDW vs. OD

It is common practice to measure the biomass using OD_{600} and calculated the corresponding cell dry weight (CDW) using a predetermined conversion factor. Cell dry weight (CDW) and OD_{600} measurements showed a nice correlation with an R^2 of 0.998 (Fig 16). For calculating cell dry weight from OD measurements, the slope = 0.237 ± 0.003 and axis intercept = 0.147 were used through out this study.

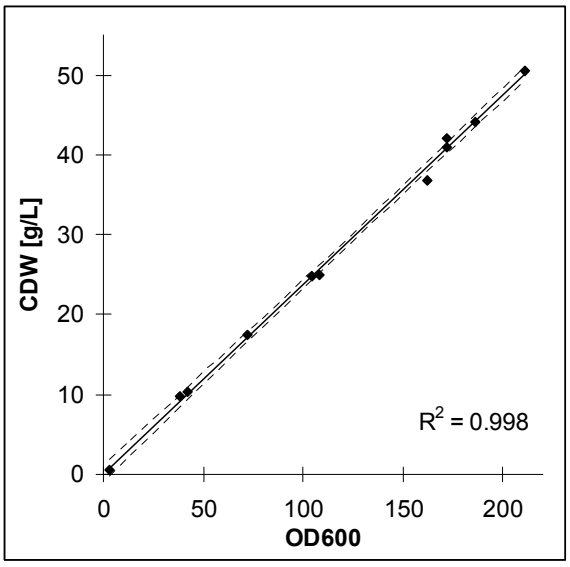


Fig 16: Correlation between cell dry weight (CDW) and OD_{600} of *Y. lipolytica* cells.
Samples taken during biomass production on in minim media (MM) supplemented with D-glucose in 5 L stirred tank reactor. dashed line, uncertainty trumpet

8.4. Southern blot

With Southern transfer and the associated hybridization techniques for DNA it is possible to obtain information about the physical organization of single and multicopy sequences in genomes. For obvious reasons the non-radioactive labeling digoxigenin-antidigoxigenin detection systems was used. Part of the ampicillin resistance maker was used as target DNA to create the DIG labeled probe. The ampicillin resistence marker does not naturally occur in the *Y. lipolytica* genome but is present on both, single and multicopy shuttle vector plasmids (p65D/p64D).

The DIG labeling of AmpR showed a high labeling efficiency. A concentration of 500 ng/µL DIG labeled target DNA was achieved (Fig 17).

Looking at the agarose gel after electrophoresis did indicate that digestion with *Asc*I did not work as well as hoped for. Nevertheless, after DNA transfer to nylonmembrane and detection with alkaline phosphatase substrate BCIP/NBT, showed that all digestions worked well enough including the digestion with *Asc*I, except for the single copy YL23 strain (Fig 18).

Depending on used restriction enzymes and integration events one would expect different bands visible (Fig 19). The southern blot shows a mixture of head to tail, tail to tail and head to head integration and duplication events that have occurred in strains harboring multiple copies of the integration cassette (Fig 18). Unfortunately, it seems that some unspecific binding of the AmpR probe did occur, as some small bands one would not expect were visible. However, single copy transformants showed on specific band, as expected.

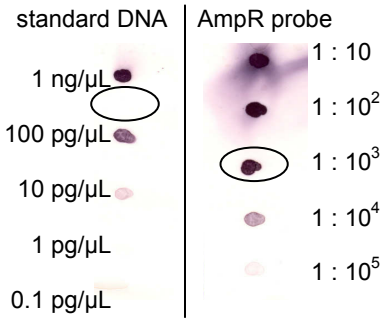
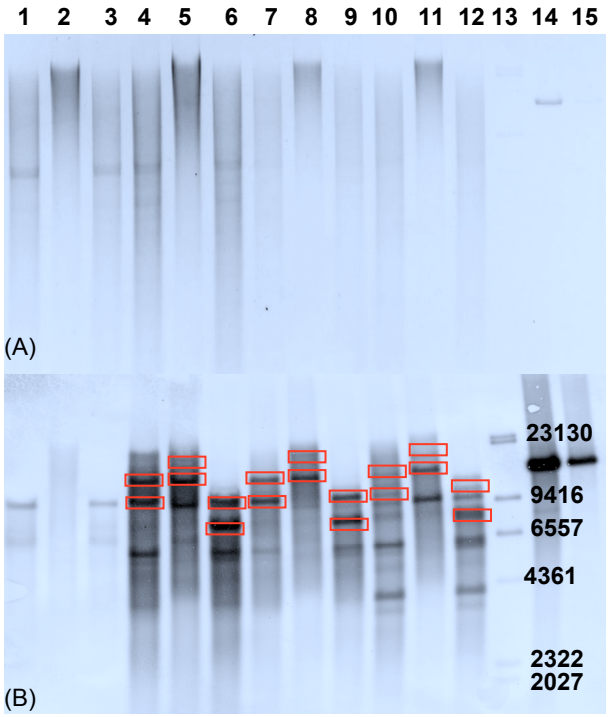


Fig 17: Dot blot, determine the DIG labeling efficiency of AmpR target probes



lane	DNA	enzyme
1	YL23 (sc)	SpeI
2	YL23 (sc)	AscI
3	YL23 (sc)	SpeI/AscI
4	YL17 (mc)	SpeI
5	YL17 (mc)	AscI
6	YL17 (mc)	SpeI/AscI
7	YL21 (mc)	SpeI
8	YL21 (mc)	AscI
9	YL21 (mc)	SpeI/AscI
10	YL22 (mc)	SpeI
11	YL22 (mc)	AscI
12	YL22 (mc)	SpeI/AscI
13	DNA marker	5 µL
14	10 ng control DNA	SpeI/AscI
15	1 ng control DNA	SpeI/AscI

Fig 18: Southern blot
(A) Agarose gel of digested genomic and control DNA. (B) Nylone membrane after southern bloting and detection with alkaline phosphatase substrate BCIP/NBT.

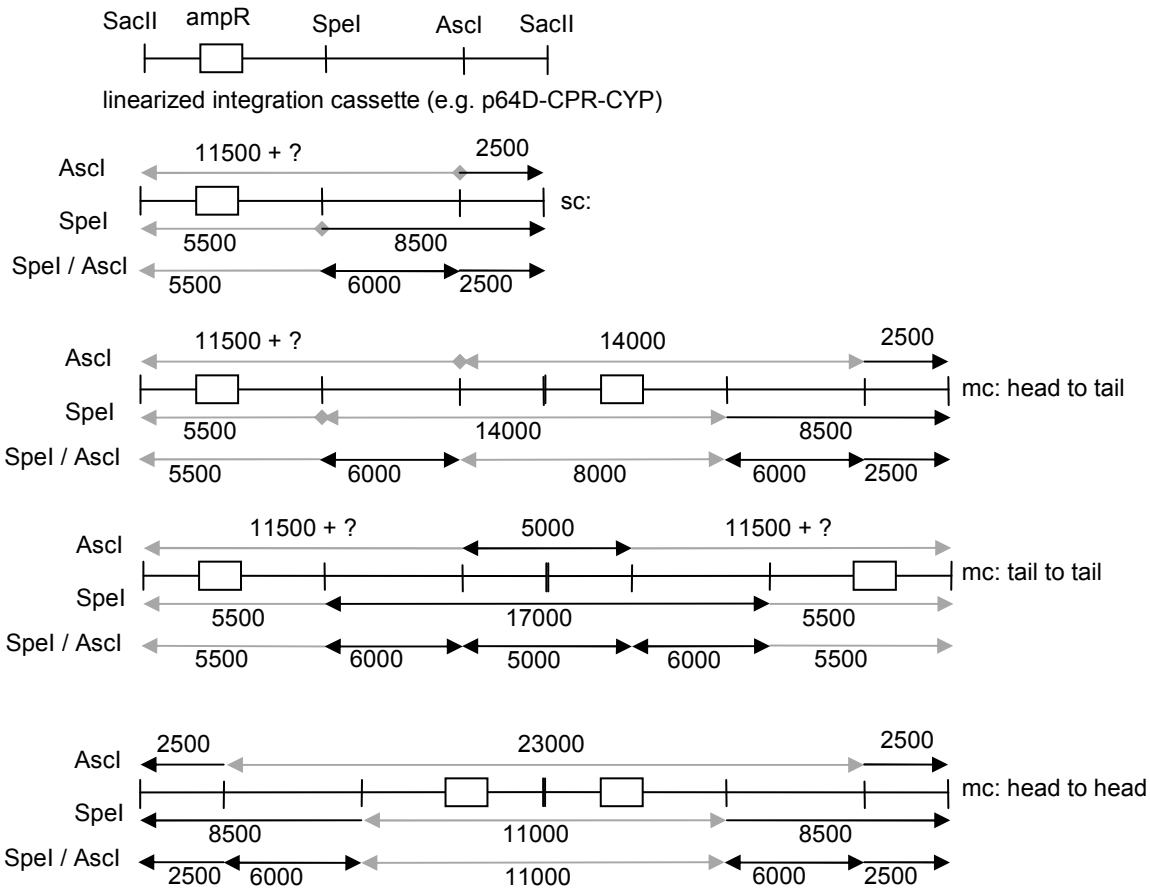


Fig 19: Expected bands of multicopy strains after southern blotting.
Bands are depending on digestion setup and integrated orientation. grey arrows, DNA fragments that contain the sequence recognized by the probe

8.5. Ethanol consumption

Y. lipolytica can not produce but can utilize ethanol as sole carbon source. However, concentrations above 3% are toxic (Barth et al., 1997). Therefore it is important to add the right amount of ethanol for the induction of genes under the ICL1 promoter. The apparent ethanol consumption is made up of two parts. The part that is utilized by the growing cells and the part that is lost due to evaporation. During the induction phase of shake flask cultures, ethanol is added at different time points leading to spikes in ethanol concentration (Fig 20). In shake flask culture we observe a growth rate on ethanol of $\mu = 0.035\ s^{-1}$ and an apparent biomass production of $Y_{SX} = 1.16$ g per g ethanol consumed.

Under more controlled conditions in a bioreactor, the values differ significantly from that of the shake flask culture. Ethanol is continuously fed to the cell suspension (Fig 20). There we observe a growth rate on ethanol of $\mu = 0.002\ s^{-1}$ and an apparent biomass production of $Y_{SX} = 0.06$ g per g ethanol consumed.

The bioreactor results were used to calculate an optimal exponential feed of ethanol during continuous induction, with the function $f(t)=1.05*e^{0.002*t}$ (in gram ethanol per hour). The results show that the measured ethanol levels are well below toxic levels.

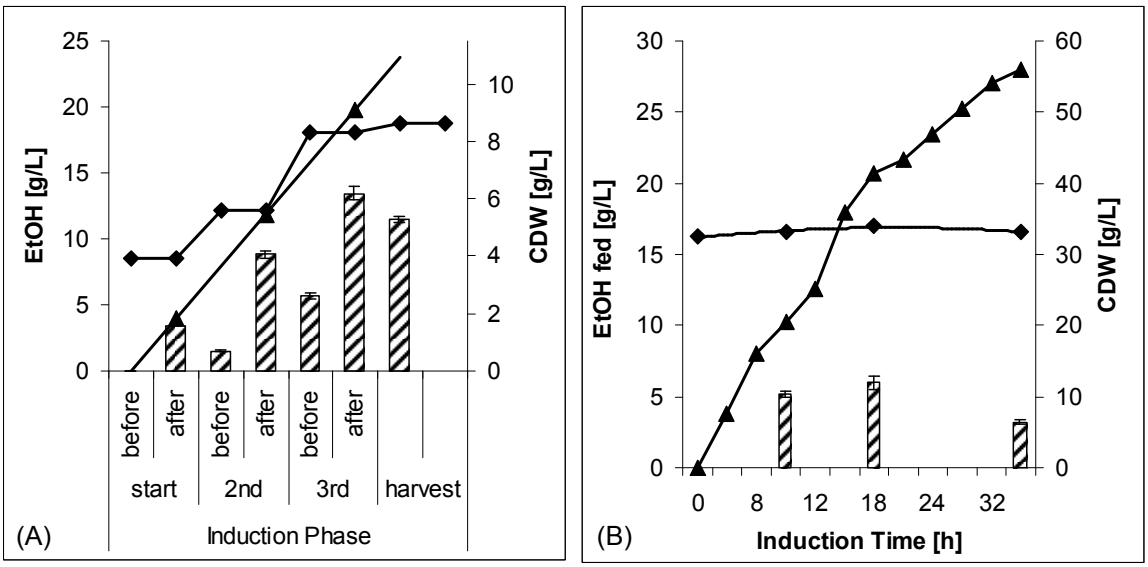


Fig 20: Ethanol concentration during induction of Y. lipolytica.
Strain expressing CYP3A4 and HCPR in (A) shake flask culture with spiked inducton and (B) in bioreactor with continuous ethanol feed. Samples were taken and cells removed by centrifugation. Ethanol concentration was determined with ENZYTECTM fluid ethanol test kit (scil diagnostics GmbH, Germany) according to manufacturer's instructions. angular striped bars, measured ethanol; diamonds, biomass; triangles, total fed ethanol (calculated)

8.6. Microsomal conversion with GDH regeneration system

Cofactors are usually very expensive. This can be circumvented to some extent by using regeneration systems for the regeneration of the cofactors. Several such regeneration systems are known and have been used (Woodyer et al., 2004; Zhang et al., 2009). The glucose dehydrogenase system was chosen as it was already available at our lab. Adding another enzyme to an already complex system makes the system even more complex. A conversion experiment, run for several hours, showed that the hydroxylation of progesterone more or less stops after 30 – 60 minutes (Fig 21). This can be due to several reasons. Product or substrates could show inhibitory effects on the heterologous expressed human CYP system. One of the enzymes of the human CYP system, the CYP itself or the reductase is degenerated at this conditions, which is unlikely as the optimal temperature for these enzymes is 37°C. Furthermore, the glucose dehydrogenase is already degenerated and therefore the regeneration of cofactors is failing. However, this results show that using regeneration systems at lab scale is already problematic let alone for medium and large scale production of metabolites (Zöllner et al., 2010).

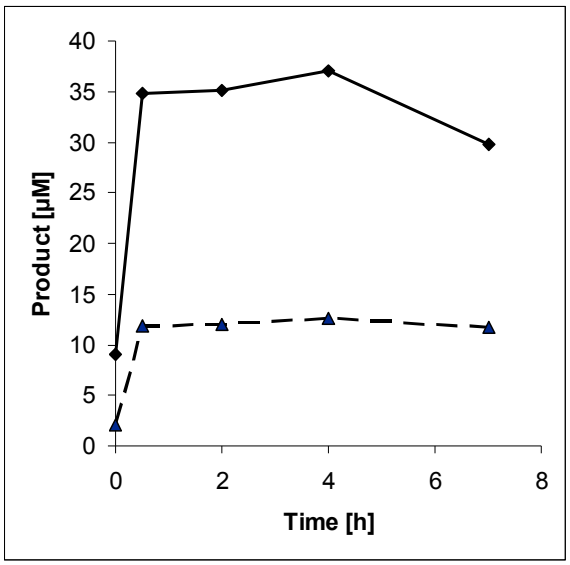


Fig 21: Product formation during microsomal conversion of progesterone with glucose dehydrogenase as regeneration system.
Y. lipolytica microsomes harboring human CYP3A4 and human CPR were used. solid line, 6beta-hydroxyprogesterone; dashed line, 16alpha-hydroxyprogesterone

8.7. Optimized conditions for *Y. lipolytica* whole cell conversion of steroids

The activity of biocatalysts and whole cell biocatalysts are typically influenced by pH and temperature of the reaction system. In order to determine the optimum pH, the hydroxylation of progesterone was performed under different pH values at 30°C (Fig 22). The results indicate an optimal pH of 6.0 closely followed by pH 7.5. As pH 7.4 is the standard conditions for hydroxylation experiments with cytochrome P450 enzymes (Phillips et al., 2006), this pH value was used throughout this study. It is well-known that pH values have a significant influence on the protonation sate of substrates, which can lead to decreased or increased uptake rates. Additionally, pH values have also a big influence on the outer cell wall leading also to an increased or decreased uptake rate.

With the increase of temperature from 20 to 40°C, hydroxylation rate of progesterone by *Y. lipolytica* cells harboring CYP3A4 and HCPR increased as well, with extensive increase at 40°C. However, further increase in temperature led to a significant decrease in hydroxylation rate. One reason for this increased activity at 40°C is probably due to the fact that most of the mammalian CYPs have their optimum temperature at 37°C. Furthermore, elevated temperatures increase the water solubility of very hydrophobic compounds such as progesterone. However, even though elevated temperatures have an effect of permeabilization of the cell wall, exceeding with the temperature leads to a point where the integrity of the cell is compromised. Most of the *Y. lipolytica* species have their optimal growth and viability around 30°C (Barth et al., 1996): although 40°C showed the highest whole cell hydroxylation rates, 30°C was chosen as a compromise between long-time viability and whole cell hydroxylation activity.

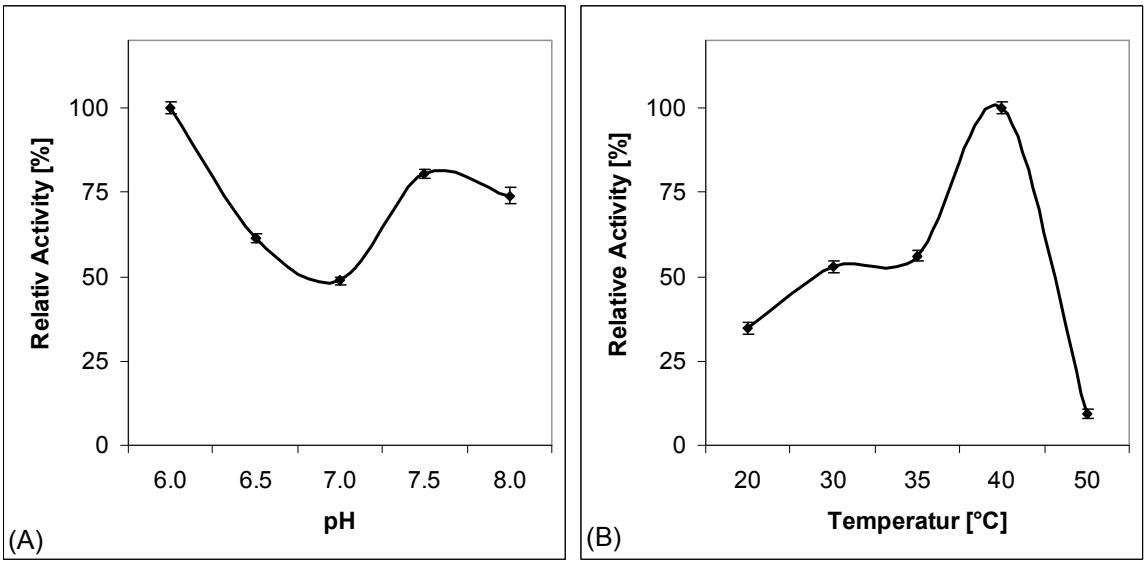


Fig 22: Influence of (A) pH and (B) temperature on whole cell conversion
Conversion was done using *Y. lipolytica* cells harboring CYP3A4 and HCPR; reaction conditions: ~ 12 g CDW/L whole cells harboring CYP3A4 and HCPR, 2 mM progesterone, 30°C, 3 h. potassium phosphate buffer solutions were used: 100mM supplemented with 1% glucose. (B) Influence of temperature; reaction conditions: ~ 12 g CDW/L whole cells harboring CYP3A4 and HCPR, 2 mM progesterone, pH 7.4 (100mM potassium phosphate supplemented with 1% glucose), 3 h. Highest observed activities have been set to 100%.

8.8. Fermentation and biotransformation procedure in bioreactors

Based on information from literature (Biryukova et al., 2009; Hartner et al., 2008; Nicaud et al., 2002), colleagues (Andrea Mellitzer, IMBT, TU Graz; Thomas Züllig and Shaikh Rafeek, LSFM, ZHAW-Wädenswil) and own experience the fermentation procedure for *Y. lipolytica* under more controlled conditions in bioreactors for the heterologous co-expression of human CYPs and human CPR and subsequent biotransformation of steroids was established. The procedure in short: an 50 mL pre-seed culture is inoculated with some *Y. lipolytica* cells form a freshly grown plate. After 23 hours shaking at 28°C, the 200 mL seed culture is inoculated to an OD600 of 1 and incubated at 28°C for 10 hours while shaking. Afterwards the seed culture is added to 3300 MM-media (1.5 % D-glucose) in a 5 L stirred tank reactor (Biostat, Sartorius, Germany). Following bioreactor parameters are kept constant during biomass production and protein expression: pH at 5.5 (25%, NH_4OH, 25% H_3PO_4), airflow at 10 NL/min, stirring at 1500 rpm, temperature at 28°C. Batch culture is grown till complete depletion of glucose. Subsequently the fed-batch is started. A constant growth rate of $\mu = 0.18$ s^{-1} is achieved by exponential feeding of 0.3 g feed solution 1 (50% glucose supplemented with trace elements and thiamin) per g CDW per hour. After 10 hours of fed-batch the induction is started by constantly adding ethanol at a low feed of 0.033 g ethanol per g CDW per hour, so that the ethanol concentration after 36 hour of induction does not reach toxic concentrations. *Y. lipolytica* cells are harvested by centrifugation (28°C, 10 min, 2000g) and resuspended to an OD of roughly 100 (corresponding to roughly 24 g/L CDW) with

potassium phosphate buffer (pH 7.4, 200 mM) supplemented with 0.5% D-glucose. For whole cell bioconversion, 450 to 600 mL *Y. lipolytica* cell suspensions are transferred to 1.5 L stirred tank reactor (DasGip, Germany). Following parameters are monitored and kept constant: pH, airflow at 1 NL/min, stirring at 1000 rpm, temperature at 30°C. To accommodate for the need of a carbon source for cell maintenance and cofactor regeneration a low feed of 0.01 g D-glucose per g CDW per hour is provided. After 90 – 160 hours cells are removed by centrifugation for 10 min at 10000g (Fig 23).

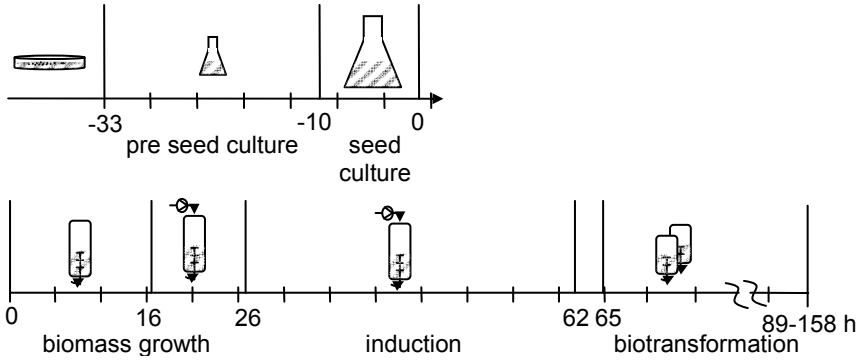


Fig 23: Principle scheme of the *Y. lipolytica* cultivation, heterologous protein production and bioconversion in bioreactors

Results show, that the measured biomass formation is very similar to the calculated one. The highest expression level was reached after 24 h of induction and stayed constant till the end of the induction phase. Furthermore, the ethanol concentrations during the induction phase never reach toxic levels (Fig 24). Hence, that the established bioreactor parameters and procedure are suitable for the cultivation of *Y. lipolytica* and the heterologous protein production with this host organism.

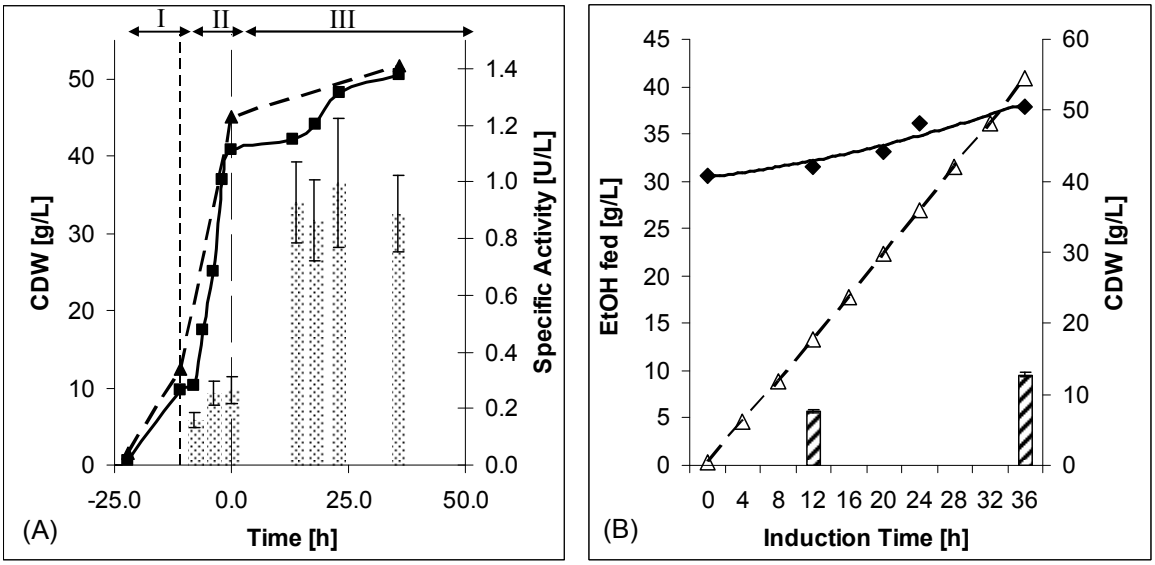


Fig 24: Fermentation and biotransformation of *Y. lipolytica* in bioreactor
(A) Biomass production and CYP3A4 activity towards progesterone of Y. lipolytica strain YL22, during fed-batch and induction phase under controlled conditions in bioreactor. Whole cell activity measurements were done as described in materials and methods. (B) Ethanol concentration during induction with continuous ethanol feed. Samples were taken and cells removed by centrifugation. Ethanol concentration was determined with ENZYTECTM fluid ethanol test kit (scil diagnostics GmbH, Germany) according to manufacturer's instructions. The Roman numerals show the following process phases: I, batch growth with glucose; II, fed-batch exponential growth on D-glucose; III, induction phase (36 h). closed triangles, calculated biomass concentrations; open triangles, fed ethanol (calculated); squares, biomass concentrations determined as cell dry weight; filled bars, CYP expression levels measured as activity per volume of cell suspension; angular striped bars, measured ethanol

As mentioned before one disadvantage of using yeast systems can be the formation of unwanted side products by endogenous P450 enzymes. However, not only endogenous P450 can present a problem. More or less any endogenous protein can show unwanted side reactions when using whole cells, independent of the used host organism. When using testosterone as substrate for the whole cell bioconversion with *Y. lipolytica* cells. Testosterone is very efficiently converted to androstenedione (Fig 25). Apparently, this reaction is also known in other yeasts (Mauersberger S., Geier M., personal communication). From the beginning on is testosterone converted to androstenedione. After roughly 40 hours most of the testosterone is gone. During the first 24 hours of the whole cell conversion, some hydroxytestosterone is produced, after this time the hydroxytestosterone concentration is rapidly reduced. From the start on the formation of hydroxyandrostenedione is observed. One possibility is that androstenedione is recognized as substrate by the CYP3A4 and converted to hydroxyandrostenedione, furthermore, hydroxytestosterone can by converted to hydroxyandrostenedione by *Y. lipolytica*, which would be indicated by the steep reduction of the hydroxytestosterone concentration after 24 hours. This side product formation was also observed when using microsomes for *Y. lipolytica* cells harboring CYP3A4 for the conversion of testosterone, however with much lower rate.

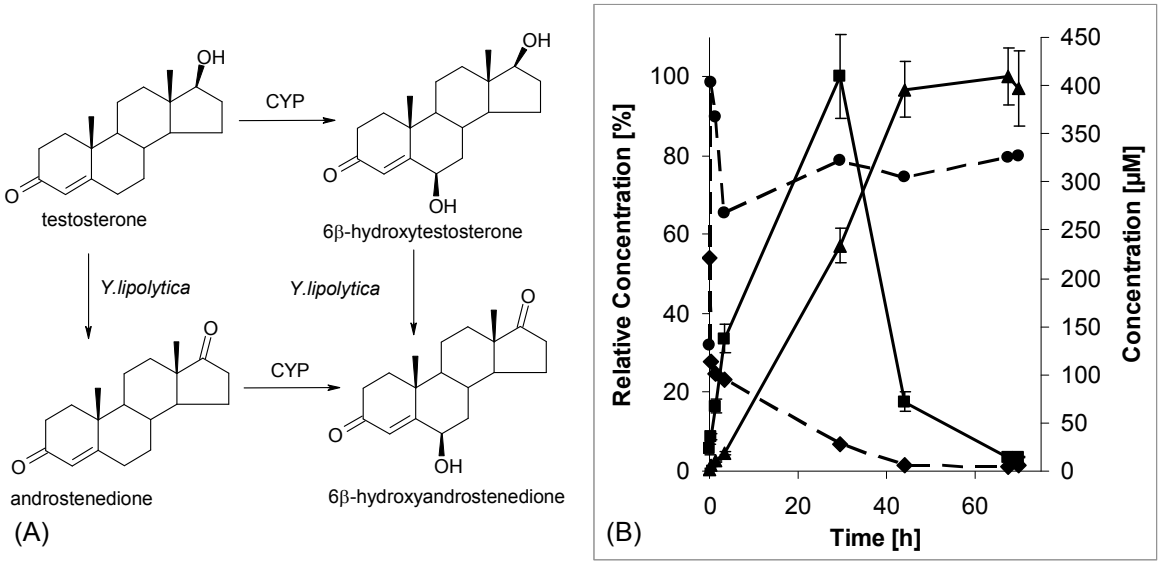


Fig 25: Side product formation during whole cell conversion of testosterone
(A) Schematic representation of whole cell bioconversion of testosterone; (B) time course of products formation and substrate depletion during whole cell conversion of testosterone by Y. lipolytica cells harboring human CYP3A4 and human, under controlled conditions in 1.5 L stirred tank reactor (DasGip). closed squares, relative 6beta-hydroxytestosterone concentration; closed triangles, relative hydroxyandrostenedione concentration; closed diamond, testosterone concentration; closed circles, androstenedione concentration

There are two possible enzyme classes that could be responsible for this side reaction. On the one hand an alcoholoxidase (AOX) which is cofactor independent, on the other hand a cofactor (NAD+) dependent alcoholdehydrogenase (ADH). This presents a simple test to distinguish which enzyme class is responsible for this side reaction. *Y. lipolytica* microsomes harboring no CYP were used to convert testosterone in the presence and without the presence of cofactors NAD^+, NADH, $NADP^+$ and NADPH. Results show (Fig 26) that providing the cofactors NAD^+/NADH during the microsomal transformation lead to roughly a 5 fold increase in androstene formation rate. This indicates that most probably a NAD^+/NADH dependent alcoholedehydrogenase is responsible for the observed side reaction – in addition alcohol oxidases accepting secondary alcohols are rare.

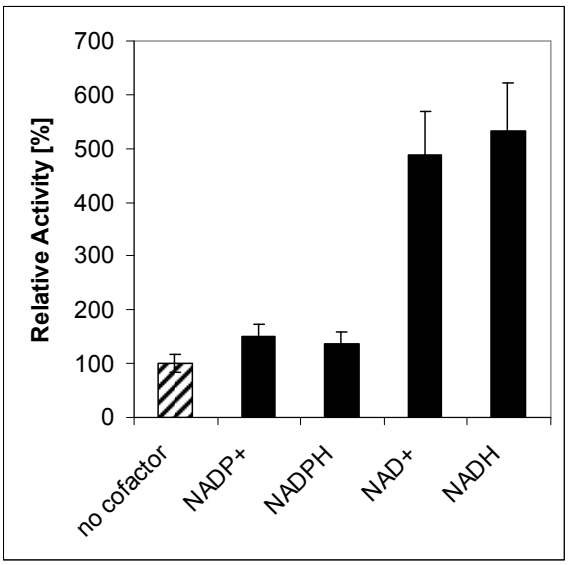


Fig 26: Cofactor dependent conversion of testosterone to androstenedione.
Conversion rate of testosterone to androstenedione by *Y. lipolytica* microsomes harboring no heterologous CYPs in the presence or absence of cofactors. angular striped bar, conversion without any additional cofactor; full bars, conversion with addition of cofactors

Due to the side product formation when using testosterone, progesterone was choosen as testsubstrate. Protesterone is a known standard substrate for CYP3A4 and no side product formation could be observed during whole cell conversion. Additionally the structure is very similar to that of testosterone, and several hydroxyprogesterone standards are commercially available.

8.9. Influence of organic solvents on the biphasic whole cell conversion

Adding small amounts of water-immiscible organic solvents during induction should help the cells to adjust themselves to the organic solvent before using them in the biphasic setup.
To determine if pre-adjusting the cells to an inert, not utilizable organic liquid would improve the subsequent whole cell conversion in a biphasic setup with that inert organic solvent, dibuthylphtalat (DBP) was chosen due to availability and convenient handling. It was hypothesized that cells already adjusted to a hydrophobic environment would subsequently give higher conversion rates during whole cell conversion in aqueous as well as biphasic systems: surprisingly, this is not the case for biphasic whole cell conversion with inter organic solvent dibuthylphtalat. Conversion rates of adjusted cells turned out to be considerable lower in the aqueous system (Fig 27).

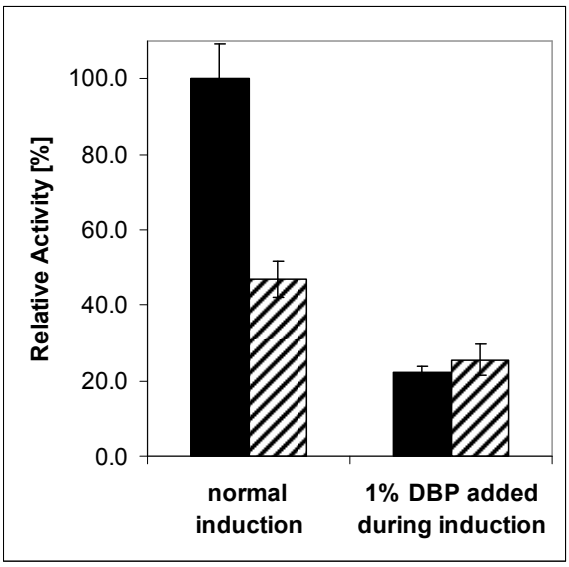


Fig 27: Influence of inert dibutylphathalat (DBP) when added during induction.
Whole cell conversion (total hydroxyprogesterone) after 26 h of ethanol induction, of resting *Y. lipolytica* cells harboring CYP3A4 and HCPR. Filled bar, in aqueous system; angular striped bar, in biphasic system with dibutylphthalate as org phase. All activities are shown in percent relative to normal aqueous system.

Furthermore, the already significantly lowered hydroxylation rate was further lowered when using adjusted cells for the biphasic whole cell conversion of progesterone with dibuthylphtalat (DBP) as 2nd phase (Fig 27).

Additionally, every time *Y. lipolytica* cells interacted with the inert, non utilizable organic solvent dibuthylphtalate (DBP) the hydroxylation pattern of the whole cell progesterone bioconversion was changed (Fig 28). This change was also observed in aqueous phase bioconversion with cells where dibuthylphtalat was added during induction. However, no change was observed when using the degradable organic solvent ethyl oleate as 2nd phase.

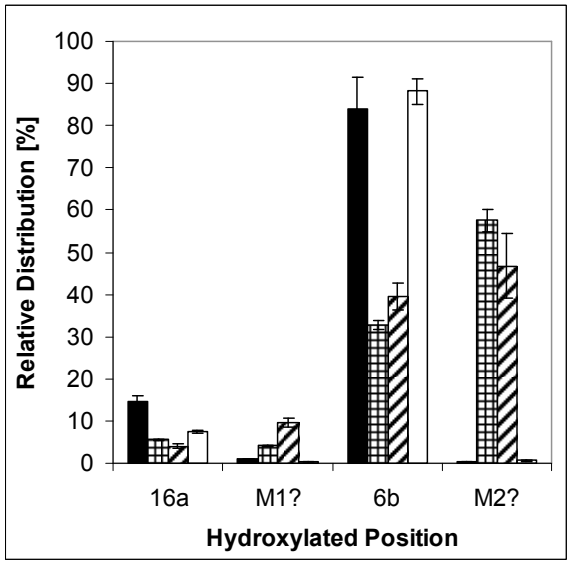


Fig 28: Progesteronehydroxylation pattern under different whole cell conversion conditions
Conversion was done with Y. lipolytica cells harboring CYP3A4 and HCPR after 26 h of ethanol induction. Filled bars, aqueous system; squared bars, aqueous system with cells induced in the presence of 1% dibutylphthalate; striped bars, biphasic system with dibutylphthalat as organic phase; blank bars, biphasic system with ethyl oleate as organic phase.

In regard to the hydroxylation pattern, even after 2 hour there is already a discrepancy in the hydroxylation pattern of dibutylphthalate biphasic system, compared to the aqueous and the ethyl oleate biphasic system. The longer bioconversion takes place, the bigger is the discrepancy. At the beginning, the 6beta-hydroxylase activity is dominant. However, the longer the *Y. lipolytica* cells adjust to the dibutylphthalate environment, the more the hydroxylase specificity is shifted to an uncharacterized unknown modification. After roughly 4 hours, the hydroxylase rate towards the unknown position exceeds the rate towards the 6beta-position. At the end of the two-liquid phase bioreactor experiment the hydroxylation pattern is considerable shifted (Fig 29), indicating that the changes of the cell surface, due to the presence of the inert dibuthylphtalat, not only influence the uptake of progesterone, but also its conversion.

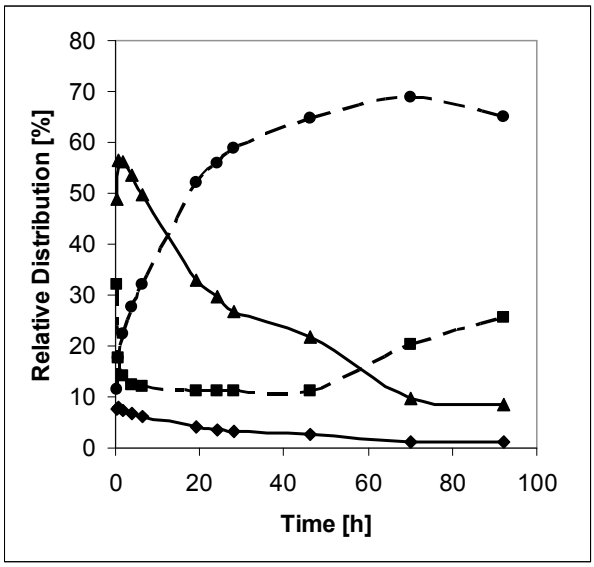


Fig 29: Progesteronehydroxylation trend during biphasic DBP whole cell conversion in bioreactor
Conversion was done with resting *Y. lipolytica* cells harboring CYP3A4 and HCPR after 37 h of ethanol induction. Dashed line, unknown hydroxyprogesterone product; diamonds, 16alpha-hydroxyprogesterone; squares, unknown hydroxyprogesterone product M1; triangles, 6beta-hydroxyprogesterone; circles, unknown hydroxyprogesterone product M2.

8.10. Biphasic whole cell conversion of steroids by *P. pastoris* harboring human CYP2D6

Fresh cultivated *P. pastoris* cells harboring human CYP2D6 and human CPR were provided by Martina Geier (PhD Thesis, Geier, 2012). Biphasic whole cell conversion was performed as described for *Y. lipolytica*. Compared to *Y. lipolytica*, P. pastoris showed a total different behavior in the presence of inert organic solvents. There were no obvious physiological changes or any interaction with the organic solvents e.g. cell aggregation, etc. visible. However, the found conversion rates were very similar between *P. pastoris* and *Y. lipolytica*. Both yeast systems showed at least 70 % reduced conversion rates during biphasic whole cell conversion compared to the conversion in aqueous system (Fig 30).

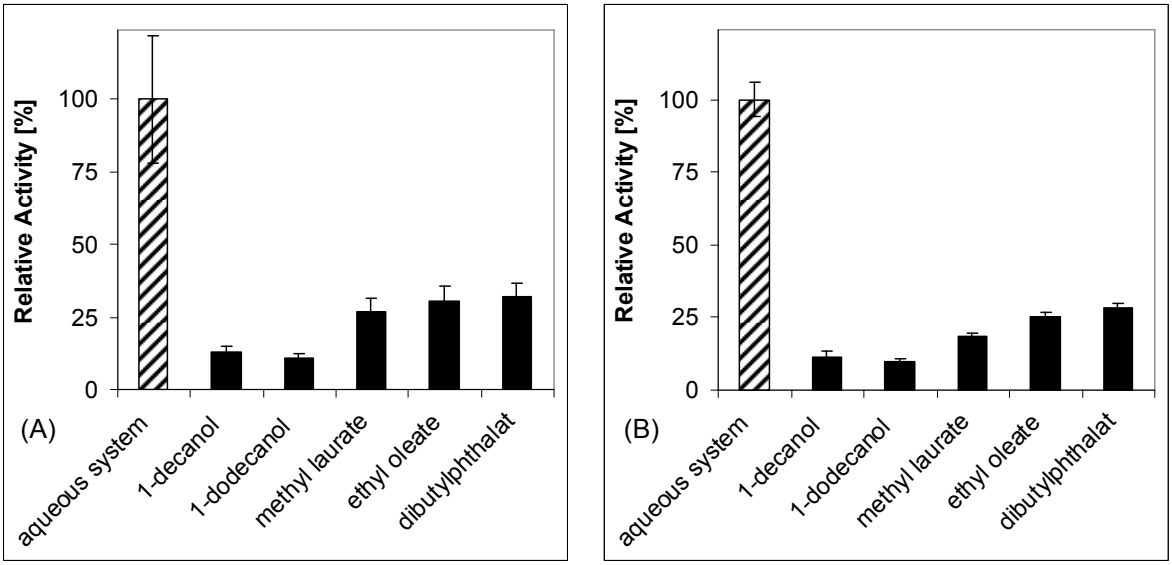


Fig 30: Hydroxylation rates of 17alpha-methyltestosterone with *P. pastoris* under different conditions
Aqueous and biphasic conversions of 17alpha-methyltestosterone was done with resting cells (A) *P. pastoris* harboring CYP2D6 and HCPR after 72 h of methanol induction and (B) *Y. lipolytica* harboring CYP2D6 and HCPR after 26 h of ethanol induction.

As *P. pastoris* was not the scope of this thesis, these certainly curious results were not further pursued.

8.11. CO spectrum

Measuring a CO difference spectrum may sound trivial, but its not. The measurement of isolated soluble P450 proteins is quite simple. However, measurement of membrane bound P450 in microsomes or even whole cells is much trickier especially when working with eukaryotes. Most yeasts contain some cytochrome oxidase that can bind CO showing a negative peak at 445 nm. This interferes with the CYP measurements which show, upon reduction and binding of CO, a peak at 450 nm. Potassium cyanide (KCN) has to be added to

mask the negative cytochrome oxidase peak (Förster, 2001; Phillips et al., 2006). It was not possible to measure the CO-spectrum of all expression strains. Only the once with the highest copy numbers gave a CO-spectrum (Fig 31). The highest expression levels for human liver cytochrome P450 2D6 was 92.34 ± 9.19 [pmol/mg total protein] corresponding to 140.1 ± 14.8 [nmol/g CDW]) and for human liver cytochrome P450 3A4 60.93 ± 5.86 [pmol/mg total protein] corresponding to 55.2 ± 5.3 [nmol/g CDW].

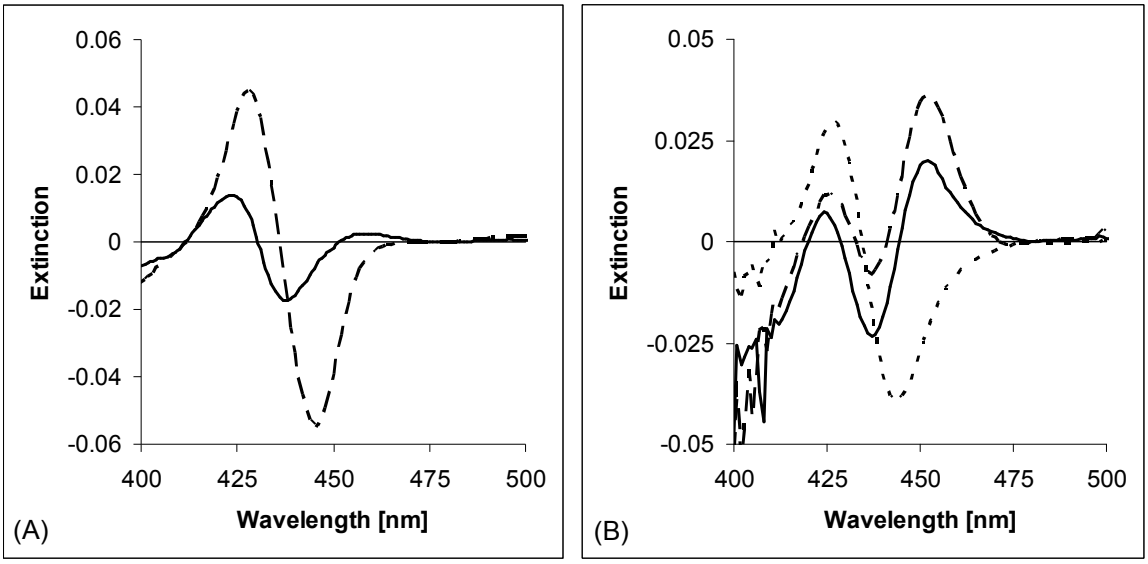


Fig 31: CO spectra of *Y. lipolytica* microsomes harboring human CYPs
(A) Influence of KCN on the CO difference spectrum. dashed line, no KCN added; solid line, KCN added (B) CO difference spectrum of human liver CYPs measured in microsomes isolated from Y. lipolytica after 24 h of ethanol induction. doted black line, control (no P450); dashed black line, strain YL21 (HCPR-WT, 2D6syn) ; solid black line, Yl22 (HCPR-WT, 3A4syn)

8.12. Heterologous promoters from *P. pastoris*

Promoters play a vital role in heterologous protein expression. They are responsible for efficient transcription of heterologous genes. There is a large variety of heterologous and homologous promoters available for yeast systems. Although, homologous promoters are preferred, heterologous promoters can some times have advantageous properties (Porro et al., 2005).

Several promoters of *Y. lipolytica* have been described which are either constitutive (pTEF, pRPS7), growth-phase-dependent (hp4d) or inducible (ICL1, XPR2, POX1,…). However, the inducible promoters need undesirable conditions for effective induction and are not tightly regulated. Therefore the "perfect" inducible promoter for *Y. lipolytica* remains to be found (Juretzek et al., 2000; Madzak et al., 2004).

In this study we tested the heterologous promoters of DAS1 (GenBank: FJ752551) and DAS2 (GenBank: FJ752552) gene from the methylotrophic yeast *P. pastoris* for the expression of

human liver cytochrome P450 while the human cytochrome P450 reductase was kept under the control of the endogenous promoter of the ICL1 gene.

During induction, *Y. lipolytica* cells were able to grow on ethanol as well as on ethanol/methanol (1:1) mixture. However, they did not grow very well on methanol (Fig 32).

The results of the whole cell activity towards bufuralol show that the cells show no activity at all when the CYP2D6 is under the control of the *P. pastoris* DAS1 promoter independent of the used inducer. When using the *P. pastoris* DAS2 promoter for CYP2D6 expression we reach roughly 10-20 percent activity compared to the standard system (both CYP2D6 and HCPR are under the control of the ICL1 promoter). Interestingly, the standard system where the expression of CYP2D6 and HCPR are both controlled by the ICL1 promoter is independent on the used inducer. Even though cells were not able to grow on methanol, cells showed more or less the same specific hydroxylation activity as cells induced by ethanol or a mixture of ethanol and methanol (1:1) (Fig 32). It has been shown that dissecting promoters into its elements (cis-elements, UAS-elements) and creating new synthetic and also hybrid promoters can significantly improve its properties for protein expression (Hartner et al., 2008; Madzak et al., 2000). Recently a group from India showed that there are potential binding sites for the Mxr1p transcription factor in the promoter regions of the DAS genes (Kranthi et al., 2010). This could be a starting point to further identify important regions. Dissecting the DAS2 promoter in its elements and combining it with promoter elements of one of the natural promoters could give a new synthetic hybrid promoter with improved properties for heterologous protein production.

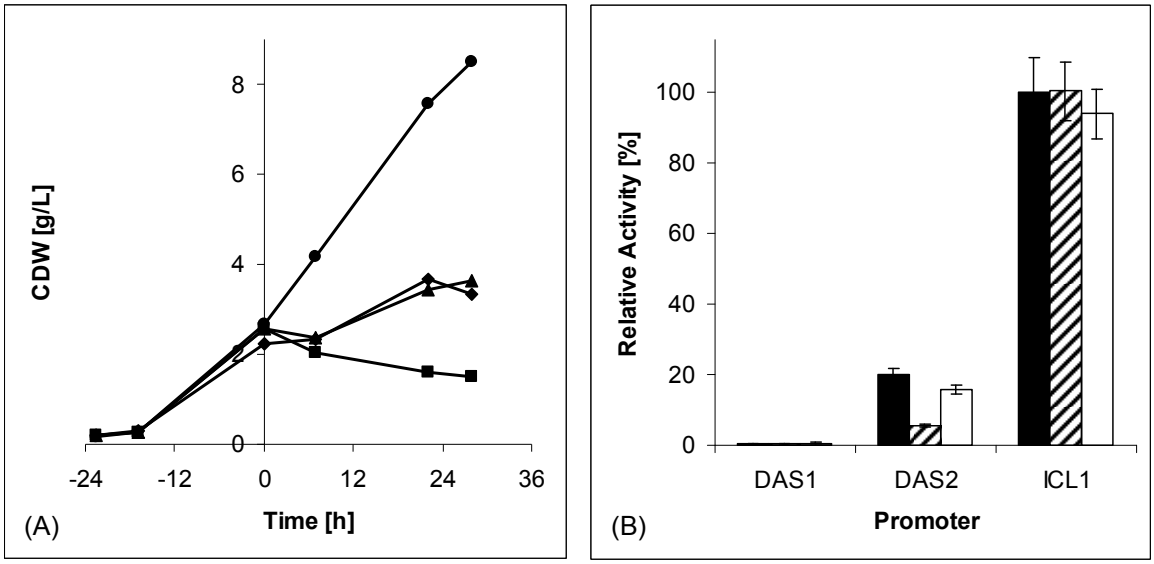


Fig 32: Expression of CYP2D6 in *Y. lipolytica* with heterologous promoters from *P. pastoris*
(A) Growth of Y. lipolytica cells on glucose and on different carbon sources during induction. (B) Whole cell hydroxylation activity towards bufuralol of Y. lipolytica cells after 22 h of induction. Cells were harboring CYP2D6 under the control of different promoters and HCPR under the control of ICL1 promoter. Carbon source added during induction: circles, glucose; diamonds, ethanol; squares, methanol; triangles, methanol/ethanol (1:1); filled bar, ethanol; angular stripped bar, methanol; open bar, methanol/ethanol (1:1)

8.13. CYP2D6 mutants

Based on the information from the MuteinDB, site saturation mutagenesis on positions E216 and F483 of the cytochrome P450 2D6 have been performed (Geier et al. unpublished results). These positions have been described to have an effect in substrate specificity (Paine et al., 2003; Smith et al., 1998).

Selected mutants were created by site-directed mutagenesis (QuikChange®, Stratagene, USA) and expressed in *Y. lipolytica* (Table 1). All selected mutants showed changed testosterone hydroxylation patterns (Geier et al. unpublished results). Expressed CYP2D6 muteins showed the expected changed testosterone hydroxylation patterns (Geier et al. unpublished results). The observed hydroxylation pattern was different from CYP3A4 as well and CYP3A4 does not show any formation of the new hydroxytestosterone (Fig 33).

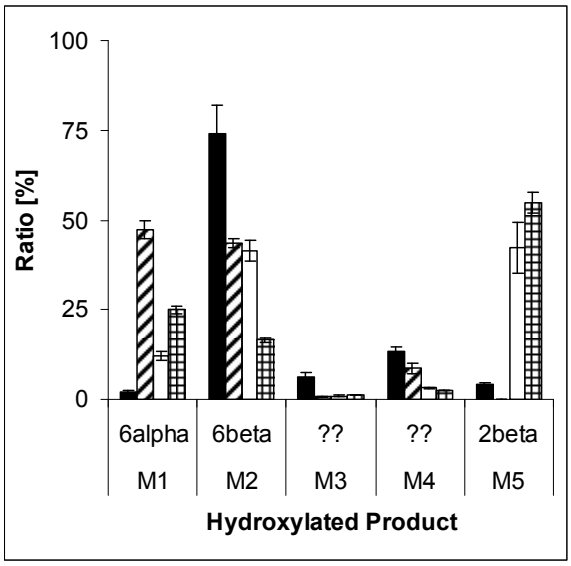


Fig 33: Testosterone hydroxylation pattern of CYP2D6 muteins and CYP3A4, expressed in *Y. lipolytica*.
Y. lipolytica cells harboring native and mutated CYPs. filled bars, CYP2D6; angular striped bars, CYP3A4; open bars, CYP2D6-E216M/F483G; checked bars, CYP2D6-E216G/F483G

9. Conclusion and Outlook

In 2008 the US FDA published a guidance for metabolism studies and MIST (Metabolites In Safety Testing). Many of the metabolites can not be synthesized by chemical means, generating a need for efficient metabolite production using recombinant microorganisms (Zöllner et al., 2010). As mentioned before, most of the substrates for P450 enzymes are very hydrophobic, hence their involvement in the so called “Phase I: functionalization” of the detoxification metabolism pathway. Many studies showed the potential usage of two-liquid phase systems for the bioconversion of hydrophobic substrates employing whole cells as catalysts (Bosetti et al., 1992; Bühler et al., 2004; Desmet et al., 1983; León, 1998; Liu et al., 1996; Salter et al., 1995; Schmid et al., 1998).

Organisms isolated from environments rich in organic solvents and able to utilize them are thought to be particularly suitable for such two-phase systems. The alkane utilizing yeast *Y. lipolytica* is often isolated from biphasic environments and many studies show its application in such systems (Kim, Tae Hyun; Lee, Jung-Hyun; Oh, Young Sook; Bae, Kyung Sook; Kim, 1999; Margesin et al., 1997; Schmitz et al., 2000; Waché et al., 2003). Additionally *Y. lipolytica* as an expression system has been shown to be particularly interesting as a host for the production of complex proteins (Madzak et al., 2004). By adaptation of the available genetic tools for *Y. lipolytica*, it was possible to functionally express the complex membrane bound human liver cytochrome P450 system consisting of human cytochrome P450 reductase and either human P450 2D6 or human P450 3A4. Both expressed CYPs are responsible for the metabolization of a significant part of the known pharmaceuticals including steroids (Spatzenegger et al., 1995). The functionally expression was confirmed by western blot analysis and hydroxylation of standard substrates. When performing whole cell conversions of different steroids, the formation of androstenedione from testosterone was observed, witch is most probably due to a NAD^+/NADH dependent alcoholdehydrogenase. Several ADHs have been identified in *Y. lipolytica* (Barth et al., 1979) of which the ADH1 was reported to be NAD+/NADH dependent. By knocking out this particularly ADH, the range of possible steroids used for whole cell hydroxylation with *Y. lipolytica* cells harboring human CYPs could be increased.

Different organic solvents from toxic to non-toxic inert and utilizable organic solvents have been used to evaluate the potential of *Y. lipolytica* for the two-liquid biphasic biotransformation of hydrophobic substrates solved in organic solvent. The observed conversion rates were considerable dependent on the type of organic solvent used as second phase. The non-toxic, utilizable organic solvent ethyl oleate showed a significant 2 fold improved conversion rate compared to the aqueous phase system. Furthermore, a hydroxylation pattern dependency of the used organic solvent was observed. The progesterone hydroxylation pattern was significantly shifted when using dibuthylphthalat as organic phase compared to ethyl oleate as organic phase as well as to the normal aqueous system. Choosing the right organic solvent depends on different parameters; i.e. toxicity towards the organism as well as solubility of the hydrophobic substrate (Bühler et al., 2004). However, as the organic solvents influence the outer membranes as well as the sub cellular arrangements of *Y. lipolytica* (Mlícková et al., 2004). Its influence on the heterologous expressed membrane bound proteins stereo specificity has to be considered and investigated as well. In this work we could show that *Y. lipolytica* is suitable for the whole cell bioconversion of hydrophobic substrate progesterone in two-liquid phase systems. However,

each substrate has to be individually reviewed to find the optimized organic solvent. Now that a procedure is available for analyzing two-liquid biphasic systems it would be of most interest to compare other non-alkane utilizing yeasts with *Y. lipolytica* in such systems. Additionally, it would be very interesting and most probably would give a deeper understanding to have electro microscopy pictures of *Y. lipolytica* with different organic solvents. First "cryo TEM" experiments have been done in cooperation with Nadejda Matsko (Institute for Electron Microscopy and Fine Structure Research, FELMI, TU Graz). However, it seems that freezing the samples for "cryo TEM" is more challenging than anticipated. So far it was not possible to get usable pictures. To improve the possibility of getting meaningful pictures, people from the Laboratoire de Microbiologie et Ge´ne´tique Mole´culaire (INRA INA-PG, France) who were able to get nice TEM pictures before could be contacted for assistance (Mlícková et al., 2004).

It is well-known that comprehensive databases compiled of well revised and standardized data sets can improve scientific research significantly. However, not only the quality of the data, furthermore functionality of the database is important for a good workflow. Reviewing and optimizing a database and its structure for a desired purpose is only possible when both sides, the informatic and the scientific, work close and interactively together.
To standardize the data format and to constitute general rules for data collection made it possible so that different scientists were able to collect data of the same quality. Several exemplary datasets have been collected; CYP3A4, CYP2D6, HRP, NIT (Andreas Braun, Katrin Weinhandl, Martina Geier, Institute of molecular biotechnology, TU Graz), BM3 also known as CYP102A1 (Jan Marienhagen, Anna Joelle Ruff, Department of Biotechnology, RWTH Aachen University), and PAMO (Daniel E. Torres Pazmino, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen). Searching the database revealed, that besides CYP3A4 and some CYP102A1 muteins, CYP2D6 as well is able to hydroxylate steroids. Two amino acid positions of CYP2D6 were identified to significantly influence the activity and the hydroxylation pattern of CYP2D6 towards steroids. Generating site saturation libraries at these two positions, separately and combined revealed new hydroxylation patterns, as well as improved variants showing mainly one distinct hydroxylated product (Geier et al. unpublished results). Selected muteins were expressed in *Y. lipolytica* which showed the same changed hydroxylation patterns as reported before (Geier et al. unpublished results). These examples show the usability of the MuteinDB as a fast way to get important information about muteins and the influence of their mutations on substrate specificity and kinetic data. To further improve the applicability and usability of the MuteinDB, the database has to be populated with more datasets. Collecting datasets with this

high quality is very time consuming. Therefore, this can only be done as a joint effort of a working group and with help of the scientific community. Additionally, improving and revising the database as well as the guidelines for data collection should be continued to be able to adjust to new challenges in the future.

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11. Appendix

11.1. Index of Figures

11.2. Index of Tables

11.3. Materials and Methods

11.3.1. Reagents, Media, Buffer

Table 6: Reagents used (in this work)

Name	description
1'-hydroxybufuralol	BD Gentest, USA
11alpha-hydroxyprogesterone	gift from TU Vienna
16alpha-hydroxyprogesterone	Steraloids, Inc., USA
17alpha-hydroxyprogesterone	Sigma-Aldrich, Germany
17alpha-methyltestosterone	Sigma-Aldrich, Germany
1-decanol	SAFC, USA
1-dodecanol	Sigma-Aldrich, Germany
1-octanol	Sigma-Aldrich, Germany
6beta-hydroxyprogesterone	Steraloids, Inc., USA
6beta-hydroxytestosterone	Sigma-Aldrich, Germany
acetic acid	Roth GmbH, Germany
acetic acid (CH3COOH)	Roth GmbH, Germany
	J.T. Baker, Netherlands
acetonitrile	Roth GmbH, Germany
	VWR, Belgium
Agar Agar	Roth GmbH, Germany
agarose LE	Biozyme, Germany
ammonium acetate	Sigma-Aldrich, Germany
ammonium sulfate ((NH4)2SO4)	Roth GmbH, Germany
Ampicillin (Amp)	Sigma-Aldrich, Germany
androstendione	Sigma-Aldrich, Germany
anti-digoxigenin antibody conjugated with alkaline phosphatase (AP) (southern blot)	Roche, Germany
BCIP/NBT	Calbiochem, USA
bicin	Roth GmbH, Germany
bisethylhexylphthalate (BEHP)	Merck, Germany
blocking reagent	Roche, Germany
boldenone	Sigma-Aldrich, Germany
boric acid (H3BO3)	Roth GmbH, Germany
bovine serum albumin (BSA)	Roth GmbH, Germany
bufuralol	BD Gentest, USA
calcium chloride dehydrate (CaCl2 * 2H2O)	Roth GmbH, Germany
carrier DNA (fisch sperm, MB grade)	Roche, Germany
citric acid monohydrate	Roth GmbH, Germany
copper sulfate pentahydrate (CuSO4 * 5H2O)	Merck, Germany
cytochrome c	Sigma-Aldrich, Germany
decane	Sigma-Aldrich, Germany
D-glucose monohydrate (C6H12O6 * H2O = dextrose)	Roth GmbH, Germany
dibutylphthalate (DBP)	Merck, Germany
DIG labeled dNTP Mix (southern blot)	Roche, Germany
dimethylsulfoxid (DMSO)	Roth GmbH, Germany
dithiothreitol (DTT)	Roth GmbH, Germany
DNA ladder (MassRulerTM DNA ladder mix, GeneRulerTM 1 kb plus DNA Ladder)	Fermentas, Germany
dodecane	Merck, Germany
dry whey (low fat) and protein powder	bought in a local supermarket
EDTA disodium dihydrate	Roth GmbH, Germany
ethanol (C2H5OH)	J.T. Baker, Netherlands
ethyl oleate	Sigma-Aldrich, Germany
ethylene glycol	Sigma-Aldrich, Germany
glycerol	Roth GmbH, Germany
glycin (C2H5NO2)	Roth GmbH, Germany
goat anti-rabbit IgG antibody conjugated with alkaline phosphatase	BD Gentest, USA

heptan	Sigma-Aldrich, Germany
hexanucleotide Mix (southern blot)	Roche, Germany
hydrochloric acid (HCl)	Roth GmbH, Germany
iron chloride (FeCl3 * 6H2O)	J.T. Baker, Netherlands
isopropanol	Roth GmbH, Germany
Klenow enzyme (southern blot)	Roche, Germany
lithium acetate	Sigma-Aldrich, Germany
MAB-2D6 mouse monoclonal antibody against human CYP2D6	BD Gentest, USA
magnesium chloride (MgCl2)	Roth GmbH, Germany
magnesium sulfate (MgSO4)	Roth GmbH, Germany
magnesium sulfate heptahydrate (MgSO4 * 7H2O)	Roth GmbH, Germany
maleic acid	Roth GmbH, Germany
manganese sulfate tetrahydrate (MnSO4 * 4H2O)	Roth GmbH, Germany
methyl laurate	SAFC, USA
NADPH-Na4	Roche, Germany
n-lauroylsarcosine	Sigma-Aldrich, Germany
NuPAGE LDS sample buffer (4x)	Invitrogen, UK
NuPAGE SDS PAGE gel	Invitrogen, UK
NuPAGE® SDS Running Buffer (20x, MOPS)	Invitrogen, UK
PEG 4000	Roth GmbH, Germany
perchloric acid 70%	Fluka, Germany
phenylmethylsulfonylfluoride (PMSF)	Fluka, Germany
phosphoric acid (H3PO4, 98%)	Roth GmbH, Germany
Ponceau S (C22H16N4O13S4)	Sigma-Aldrich, Germany
potassium chloride (KCl)	Roth GmbH, Germany
potassium cyanide	Roth GmbH, Germany
potassium dihydrogene-phosphate (KH2PO4)	Roth GmbH, Germany
potassium hydrogene-phosphate (K2HPO4)	Roth GmbH, Germany
potassium hydroxide (KOH)	Roth GmbH, Germany
potassium iodide (KI)	Roth GmbH, Germany
Power sYBR Green MM	Applied Biosystems, UK
prednisolone	Sigma-Aldrich, Germany
progesterone	Sigma-Aldrich, Germany
protein ladder (PageRulerTM prestained)	Fermentas, Germany
rabbit anti-alpha-mouseto mouse IgG antibody conjugated with alkaline phosphatase	BD Gentest, USA
restriction enzymes (used in this work)	Fermentas, Germany
Roti®-Phenol	Roth GmbH, Germany
sodium acetate	Roth GmbH, Germany
sodium chloride (NaCl)	Roth GmbH, Germany
sodium dithionite	Sigma-Aldrich, Germany
sodium dodecyl sulfoxid (SDS)	Roth GmbH, Germany
sodium hydroxide (NaOH)	Roth GmbH, Germany
sodium molybdate dehydrate (Na2MoO4 * 2H2O)	Roth GmbH, Germany
sodium nitrate (NaNO3)	Merck, Germany
sorbitol (sorbit)	Roth GmbH, Germany
testosterone	Sigma-Aldrich, Germany
thiamin-hydrochlorid	Roth GmbH, Germany
toluol	Sigma-Aldrich, Germany
Tris	Roth GmbH, Germany
tri-sodium citrate dehydrate	Roth GmbH, Germany
Triton X 100	Roth GmbH, Germany
	Roth GmbH, Germany
Trypton	Oxoid, USA Bacto Laboratories Pty Ltd, Australia
WB-3A4 rabbit antibody against human CYP3A4	BD Gentest, USA
Yeast extract	Roth GmbH, Germany

	Oxoid, USA Bacto Laboratories Pty Ltd, Australia
zinc sulfate heptahydrate (ZnSO4 * 7H2O)	Sigma-Aldrich, Germany

Table 7: Media / Buffer used (in this work)

Name	description
ammonium acetate (pH 5.0, 10 mM)	0.7708 g ammonium acetate, acetic acid till pH 5.0, to 1 L with ddH2O
ampicillin Stock (Amp)	100 mg/mL dissolved in H2O → 100 µg/mL final concentration
assay buffer (pH 7.4, 100 mM)	microsomal: 100 mL KPi-buffer (pH 7.4, 1 M), to 1 L with ddH2O whole cell conversion (WCC): 100 mL KPi-buffer (pH 7.4, 1 M), 50 mL D-glucose (20%), to 1 L with ddH2O
BEDS solution	1.63 g bicin (→ 10 mM), NaOH till pH 7.3, 192 g sorbitol (→ 1 M), 30 mL ethylene glycol, 50 mL DMSO, to 1 L with ddH2O
blocking solution (southern blot)	5 mL blocking stock solution to 50 mL with maleic acid buffer
blocking stock solution (10x, southern blot)	5 g blocking reagent to 100 mL with maleic acid buffer
buffer A (Merckenschlager)	50 mL KPi-buffer (pH 7.9, 1 M), 50mL glycerol, 5 mL PMSF (200 mM), 1 mL EDTA (1 M), 1 mL dithiothreitol (2 M), to 1 L with ddH2O
bufuralol (1 mM)	1 mg bufuralol, to 3.3 mL with assay buffer
color-substrate solution (southern blot)	200 µL BCIP/NBT, to 10 mL with detection buffer
CO-spectra buffer (pH 7.4, 100 mM)	20 g glycerol, 10 g KPi-buffer (pH 7.4, 1 M), to 100 g with ddH2O
cyt c assay buffer (pH 7.5)	605.7 mg Tris (→ 50 mM), 37.2 mg EDTA disodium dehydrate (→ 1 mM), HCl till pH 7.5, to 100 mL with ddH2O
cytochrome c (300 µM)	18 mg cytochrome c, to 5 mL with cyt c assay buffer
denaturing solution (southern blot)	10.0 g NaOH (→ 0.5 M), 43.83 g NaCl (→ 1.5 M) to 500 mL with ddH2O
detection buffer (pH 9.5, southern blot)	1.21 g Tris (→ 0.1 M), 0.58 g NaCl (→ 0.1 M), HCl till pH 8.0, to 200 mL with ddH2O
D-glucose solution	50%: 550 g D-glucose monohydrate, to 1 L with ddH2O 20%: 220 g D-glucose monohydrate, to 1 L with ddH2O
dithiothreitol (DTT)	1 M: 1.542 g dithiothreitol, to 10 mL with ddH2O 2 M: 3.08 g dithiothreitol, to 10 mL with ddH2O
EDTA disodium dihydrate (pH 8.0)	1 M: 3.72 g EDTA disodium dihydrate, NaOH till pH 8.0, to 10 mL with ddH2O 0.2 M: 3.72 g EDTA disodium dihydrate, NaOH till pH 8.0, to 50 mL with ddH2O
equilibration buffer (10x, southern blot)	250 mL SSC (20x, transfer buffer) to 500 mL with ddH2O
ethanol (75%)	30 mL ethanol, to 40 mL with ddH2O
feed solution 1	550 g D-glucose monohydrate, 3 mL trace element solution (PTM), 3 mL thiamin hydrochloride and 1 mL iron chloride, to 1 L with ddH2O
glycerol (10%)	25g glycerol, to 250 g with ddH2O
HCl (250 mM)	10.4 mL conc. HCl (37%) to 500 mL with ddH2O
hybridization solution (southern blot)	50 mL SSC (20x → 5x), 20 mL blocking stock solution (→ 1%), 2 mL N-laurylsarcosine (10% → 0.1%), 0.4 mL SDS (10% → 0.02%), to 500 mL with ddH2O
interner standard (IS)	10 mg boldenone, to 34.915 with mL DMSO 10 mg prednisolone, to 27.743 mL with H2O/DMSO (1:1)
iron chloride solution (5000x)	3 g FeCl3 x 6 H2O, to 100 mL with ethanol
KCN (pH 7.7)	50 mM: 16.3 mg KCN, verd. HCl till pH 7.7, to 5 mL with cyt c assay buffer 200 mM: 65.1 mg KCN, verd. HCl till pH 7.7, to 5 mL with CO-spectra buffer
LB medium (Luria-Bertani)	10 g tryptone, 5 g yeast extract, 5 g NaCl, to 1 L with ddH2O solid form by adding 15 g/L agar
lithium acetate (pH 6.0, 100 mM)	10.2 g lithium acetate dehydrate, acetic acid till pH 6.0, to 1 L with ddH2O
Loading Buffer (NuPAGE® LDS Sample	250 µL NuPAGE LDS sample buffer (4x), to 1000 µL with ddH2O

Buffer)	
lysis buffer	4 mL triton X 100, 2 g SDS, 1.17g NaCl, 0.07 g EDTA disodium dehydrate, 0.24 g Tris, HCl or NaOH till pH 8.0, to 200 mL with ddH2O
maleic acid buffer (pH 7.5)	11.6 g maleic acid (→ 0.1 M), 8.77 g NaCl (→ 0.15 M), NaOH till pH 7.5, to 1 L with ddH2O
mineral salt solution (5x)	30 g (NH4)2SO4, 10 g KH2PO4, 1.2 g K2HPO4, 7 gMgSO4 x 7 H2O , 3 g NaCl, 2.9 g NaNO3, 1.9 g CaCl2, to 1 L with ddH2O
minimal media (self made)	400 mL mineral salt solution, 1 mL trace element solution (PTM) 0.2 mL iron chloride, 1 mL Thiamine hydrochloride, 30 mL dextrose solution (50%), to 1 L with ddH2O
MOPS (Nu Page® SDS Running Buffer)	50 mL NuPAGE® SDS Running Buffer (20x, MOPS), to 1000 mL with ddH2O
NADPH-Na4	20mM: 7 mg NADPH-Na4, to 420 µL with assay buffer 1.5 mM: 1.3 mg NADPH-Na4, to 1.04 mL with cyt c assay buffer
neutralization solution (southern blot, pH 7.4)	121.14 g Tris (→ 1 M), 87.66 g NaCl (→ 1.5 M), HCL till pH 7.4, to 1 L with ddH2O
N-lauroylsarcosine sodium salt (10%)	5.7 mL N-lauroylsarcosin (35%), to 20 mL with ddH2O
PEG4000 (40%)	4 g PEG4000, to 10 mL with lithium acetate (100 mM)
phenylmethylsulfonyl fluoride (PMSF, 200 mM)	1.74 g PMSF, 10 mL ddH2O, to 50 mL with ethanol
phosphoric acid (25%)	294 g phosphoric acid (98%), to 1000 g with ddH2O
Ponceau S solution	0.1% Ponceau S in 5% acetic acid
potassium hydroxide solution (2 M)	4 g potassium hydroxide, to 50 mL with ddH2O
potassium phosphate buffer (KPi-buffer, different pH)	pH 6.5, 1 M: 57.39 g K2HPO4, 90.57 g KH2PO4, to 1 L with ddH2O pH 7.4, 1M: 139.69 g K2HPO4, 26.95 g KH2PO4, to 1 L with ddH2O pH 7.9, 1M: 160.94 g K2HPO4, 10.34 g KH2PO4, to 1 L with ddH2O + KOH till desired pH
primary antibody	5 µL primary antibody (MAB-2D6, or WB-3A4) in 30 mL TBST-Milk
Roti®-Phenol (composition)	phenol : chloroform : isoamyl alcohol (25:24:1)
running solution (western blot)	50 mL 20x Transfer buffer, 100 mL methanol, to 1L with ddH2O
SDS PAGE (NuPAGE ® gel)	Bis-Tris Gel (separation gel 4-12%, stacking gel 4%, 1.0 mm thick)
SDS solution (10%)	2 g SDS, to 20 mL with ddH2O
secondary antibody	5 µL secondary antibody (MAB-2D6, or WB-3A4) in 30 mL TBST-Milk
SOC-Media	20 g bacto tryptone, 0.58 g NaCl, 5 g bacto yeast extract, 2 g MgCl2, 0.16 g KCl, 2.46 g MgSO4, 3.46 g glucose, to 1 L with ddH2O
sodium acetate (3 M)	4.92 g sodim acetate, to 20 mL with ddH2O
sodium citrate buffer (pH 4.0, 500 mM)	52.54 g citric acid monohydrate, NaOH till pH 4.0, to 1L with ddH2O
sorbitol (1 M)	18.2 g sorbitol, to 100 mL with ddH2O
SSC (20x, transfer buffer, southern blot	350.64 g NaCl (→ 3 M), 176.46 g tri-sodium citrate dehydrate (→ 0.3 M), citric acid till pH 7.0, to 2L with ddH2O
steroid (80 mM)	10 mg testosterone, to 0.867 mL with DMSO 10 mg 17alpha-methyltestosterone, to 0.827 mL DMSO 10 mg progesterone, to 0.795 with DMSO
TBS-buffer (10x)	30.3 g Tris (→ 0.25 M), 87.6 g NaCl (→ 1.5 M), diluted HCl till pH 7.5, to 1 L with ddH2O
TBST-buffer (washing)	100 mL TBS-buffer (10x), 0.5 mL TritonX 100, to 1 L with ddH2O
TBST-milk (blocking solution)	4 g dry whey powder, 1 g protein powder (90% protein), to 100 mL with TBST-buffer
TBST-milk (for antibodies)	0.4 g dry whey powder, 0.11 g protein powder (90% protein), to 100 mL with TBST-buffer
TE-Buffer (pH 8.0)	1.21 g tris (→ 10 mM), 0.37 g EDTA disodium dehydrate (→ 1 mM), HCl or NaOH till pH 8.0, to 1 L with ddH2O
thiamine hydrochloride solution (1000x)	400 mg thiamine-hydrochloride, to 100 mL with ddH2O
trace element solution (PTM, 1000x)	50 mg H3BO3, 4 mg CuSO4 x 5 H2O, 10 mg KI, 30 mg MnSO4 x 1 H2O, 20 mg Na2MoO4 x 2 H2O, 40 mg ZnSO4 x 7 H2O, to 100 mL with ddH2O
transfer buffer (20x,	14.5 g Tris (→ 24M), 72 g Glycin (→ 1920mM), to 500mL ddH2O

western blot)	
Tris-HCl (pH 7.0, 100 mM)	12.1 g Tris diluted, to 1 L with ddH2O pH adjusted with 1 M HCl
washing buffer 3 (southern blot)	500 mL maleic acid buffer, 1.5 mL tritonX-100
washing solution 1 (southern blot)	50 mL SSC (20x → 2x), 5 mL SDS (10% → 0.1%), to 500 mL with ddH2O
washing solution 2 (southern blot)	2.5 mL SSC (20x → 0.1x), 5 mL SDS (10% → 0.1%), to 500 mL with ddH2O
YNB Difco (yeast nitrogen base , composition)	5 g ammonium sulfate, 2 µg biotin, 0.4 mg calcium pantothenate, 2 µg folic acid, 2 mg inositol, 0,4 mg niacin, 0.2 mg p-aminobenzoic acid, 0.4 mg pyridoxine hydrochloride, 0.2 mg riboflavin, 0.4 thiamine hydrochloride, 0.5 mg boric acid, 40 µg copper sulfate, 0.1 mg potassium iodide, 0.2 mg iron chloride, 0.4 mg manganese sulfate, 0.2 mg sodium molybdate, 0.4 mg zinc sulfate, 1 g potassium dihydrogene-phosphate, 0.5 g magnesium sulfate, 0.1 g sodium chloride, 0.1 g calcium chloride, to 1 L with H2O
YNB solution (10x)	67 g YNB Difco, to 1 L with ddH2O
YNBG (different glucose concentrations)	1%: 100 mL YNB (10x), 50 mL D-glucose (20%) and 200 mLKPi-Buffer (1M, pH 6.5), to 1 L with ddH2O 0.6%: 100 mL YNB (10x), 30 mL D-glucose (20%) and 200 mL KPi-Buffer (1M, pH 6.5), to 1 L with ddH2O solid form by adding 15 g/L agar
YPD	20 g tryptone, 10 g yeast extract, 100 mL D-glucose (20%), optional: 100 mL sodium citrate buffer (pH 4.0, 500 mM) to 1 L with ddH2O solid form by adding 15 g/L agar

11.3.2. Instruments/Devices used

Table 8: Instruments/Devices used (in this work)

used for……	description
absorption measurement	DU® 800 Spectrophotometer, Beckman CoulterTM, USA
(cyt c activity assay)	Cuvettes, same as for OD measurement
bioreactor for cell culture	Biostat (5 L), Sartorius, Germany
bioreactor for whole cell conversion (different setups)	DasGip (1.5 L), DasGip, Germany
centrifuge for cell harvesting	AvantiTM Centrifuge J-20XP + JA-14 rotor and JA-25,50 rotors, Beckman CoulterTM, USA
CloneJETTM PCR Clining Kit	Fermentas, Germany
CO differential spectra	SPECORD 205, analytikjena, Germany
electro transformation	Gene PulserTM, BIO-RAD, USA Electroporation Cuvettes (2mm gap) cellprojects Lt., UK
electrophoresis	PowerPacTM Basic + Sub-Cell GT, Biorad, USA
GeneJETTM Plasmid Miniprep Kit	Fermentas, Germany
mixing of small volumes	Vortex-Genie 2/Scientific Industries Inc, USA
OD measurement	BioPhotometer, Eppendorf, Germany Cuvettes (10x4x45mm), Sarstedt, Germany
PCR reaction	GeneAmp®PCR System 2700, Applied Biosystems, USA
PfuUltra ® II Fusion HS DNA Polymerase	Stratagene, USA
Phusion High-Fidelity DNA Polymerase	FINNZYMES, Finland
SDS Page	XCellSurelock, Novex MiniCell, Invitrogen, UK
shaker for small volumes	Thermomixer comfort, Eppendorf, Germany
shakers for cell culture	AG20 and HT MiltronII, Infors AG, Swiss
table centrifuge	Centrifuge 5810, Eppendorf, Germany
(also for cell harvest)	Centrifuge 5415R + A-4-62 and F34-6-38 rotor, Eppendorf, Germany
ultracentrifuge for microsome isolation	Optima LE-80K ultracentrifuge + TI 70 rotor, Beckman CoulterTM, USA Merkenschlager homogeniser, B.Braun, Germany

western Plot	TE 22 Mighty Small Transphor Tank Transfer Unit, Amersham Bioscience, USA Nitrocellulose membrane, Hybond-ECLTM, Amersham Biosciences, Sweden
Wizard ® SV Gel and PCR Clean-Up System	Promega, USA

11.3.3. oligonucleotides

Table 9: Primers for amplification

name	sequence	information
ICL-T_PCR_rv	AAA**GGATCC**GTAAAGTCACGATAG	*Bam*HI-part of promoter-terminator-*Bam*HI
ICL-T_PCR_fw	A**GGATCC**CCAGTAGACTGACCAAGCATACAAAAA**ACTAGT**AAAA **GGCGCGCC**GCAGTTTGTTTAGCAAAATATATTTAAC	
ICL-T_PCR2_fw	AAAA**GGATCC**CCAGTAGACTGACC	
ICLA_PCR_fw	AAAA**GTCGAC**GATCAGGACAAACAG	*Sal*I-part of promoterA-*Bam*HI
ICLA_PCR_rv	AAAA**GGATCC**CCAGATCTATATATACTGC	
*Yl*CPR_WT_fw	AAAA**ACTAGTGCCACC**ATGCCTCTACTCGACTCTCTCG	*Yarrowia lipolytica* CPR
*Yl*CPR_WT_rv	TTTT**GGCGCGCC**CTACCACACATCTTCCTGGTAGAC	
2D6_syn_fw	AAAA**ACTAGTGCCACC**ATGGGTTTGGAGGCTTTGG	CYP2D6 synthetisch
2D6_syn_rv	TTTT**GGCGCGCC**TTATCTTGGGACGGCACACAG	
2D6_WT_fw	AAAA**ACTAGTGCCACC**ATGGGGCTAGAAGCACTGG	CYP2D6 short and long wild type
2D6_WT_rv	TTTT**GGCGCGCC**CTAGCGGGGCACAGCAC	
HCPR_pp_opt_fw	AAAA**ACTAGTGCCACC**ATGGGTGACTCTCACGTTGAC	human P450 reductase HCPRsyn codon optimized
HCPR_pp_opt_rv	TTTT**GGCGCGCC**TTAGGACCAAACGTCCAAAGAG	
ICLA_PCR2_rv	TTTT**ACTAGT**TTTTTGTATGCTTGGTCAGTCTACTGG	*Sal*I-promoterA-*Spe*I
ICLA_PCR2_fw	AAAA**GTCGAC**GATCAGGACAAACAGAGGCC	
HCPR-WT_SpeI_fw	AAAA**ACTAGTGCCACC**ATGGGAGACTCCCACGTGG	Human CPR WT
HCPR-WT_AscI_rv	TTTT**GGCGCGCC**CTAGCTCCACACGTCCAGGG	
rDNA_SacII_fw	AAAA**CCGCGG**CAGACACTGC	rDNA *Y. lipolytica*
3A4-sp-opt_fw	AAAA**ACTAGTGCCACC**ATGGCTTTGATTCCTGATTTG	3A4syn codon optimized
3A4-sp-opt_rv	TTTT**GGCGCGCC**TTAAGCACCGGAAACAGTACC	
3A4WT_fw	AAAA**ACTAGTGCCACC**ATGGCTCTCATCCCAGACTTG	3A4WT
3A4WT_rv	TTTT**GGCGCGCC**TCAGGCTCCACTTACGGTG	
Pro-Pp-DAS1-fw	TTT**GTCGAC**TTTTGATGTTTGATAGTTTGATAAGAGTG	*P. pastoris* DAS1 promoter
Pro-Pp-DAS1-rv	CCC**ACTAGT**TTTGTTCGATTATTCTCCAGATAAAATC	
Pro-Pp-DAS2-fw	CTC**GTCGAC**TTTGTTCGATTATTCTCCAGATAAAATC	*P. pastoris* DAS2 promoter
Pro-Pp-DAS2-rv	CGT**ACTAGT**TTTTGATGTTTGATAGTTTGATAAGAGTG	

Table 10: Primers for overlap extension PCR

name	SEQUENCE	info
HCPR_ol_T_rv	**GCTAAACAAACTGC**TTAGGACCAAACGTCCAAAGAGTAAC	Human CPR overlap with ICL1-Terminator
T_ol_HCPR_vw	**CTTTGGACGTTTGGTCCTAA**GCAGTTTGTTTAGCAAAATATATTTAACG	
T_ol_pD_rv	**CAAGTATGTCCATCTCGA**GGATCCGTAAAGTCACGATAGCTTAAC	ICL1-Terminator overlap with ICL1D-Promoter variant
pD_ol_T_fw	**CTATCGTGACTTTACGGATCC**TCGAGATGGACATACTTGTATCGTC	
p_ol_2D6WT_rv	**GTGCTTCTAGCCCCAT**TTTTTGTATGCTTGGTCAGTCTACTGG	all ICL1-Promoter variants overlap with 2D6WT
2D6WT_ol_p_fw	**CTGACCAAGCATACAAAAA**ATGGGGCTAGAAGCACTGGTG	
*Yl*CPR_ol_T_rv	**GCTAAACAAACTGC**CTACCACACATCTTCCTGGTAGACG	*Yarrowia lipolytica* CPR overlap with ICL1-Terminator
T_ol_*Yl*CPR_vw	**CCAGGAAGATGTGTGGTAG**GCAGTTTGTTTAGCAAAATATATTTAACG	
p_ol_2D6syn_rv	**CAAAGCCTCCAAACCCAT**TTTTTGTATGCTTGGTCAGTCTACTGG	all ICL1-Promoter variants overlap with 2D6syn
2D6syn_ol_p_fw	**CTGACCAAGCATACAAAAA**ATGGGTTTGGAGGCTTTGGTTC	
T_ol_pC_rv	**GCTTCTACAGCGTCGAC**GGATCCGTAAAGTCACGATAGCTTAAC	ICL1-Terminator overlap with ICL1C-Promoter
pC_ol_T_fw	**CTATCGTGACTTTACGGATCC**GTCGACGCTGTAGAAGCACATG	
T_ol_pA_rv	**CTCTGTTTGTCCTGATC**GGATCCGTAAAGTCACGATAGCTTAAC	ICL1-Terminator overlap with ICL1A-Promoter
pA_ol_T_fw	**CTATCGTGACTTTACGGATCC**GATCAGGACAAACAGAGGCCAC	
HCPR-WT_ol_T_rv	**GCTAAACAAACTGC**CTAGCTCCACACGTCCAGGG	WT Human CPR overlap with ICL1-Terminator
T_ol_HCPR-WT_vw	**GGACGTGTGGAGCTAG**GCAGTTTGTTTAGCAAAATATATTTAACG	
p_ol_3A4-sp_rv	**CAGGAATCAAAGCCAT**TTTTTGTATGCTTGGTCAGTCTACTGG	all ICL1-Promoter variants overlap with 3A4syn
3A4-sp_ol_p_fw	**CTGACCAAGCATACAAAAA**ATGGCTTTGATTCCTGATTTGG	
p_ol_3A4WT_rv	**CTGGGATGAGAGCCAT**TTTTTGTATGCTTGGTCAGTCTACTGG	all ICL1-Promoter variants overlap with 3A4WT
3A4WT_ol_p_fw	**CTGACCAAGCATACAAAAA**ATGGCTCTCATCCCAGACTTGG	
2D6syn_ol_T_rv	**GCTAAACAAACTGC**TTATCTTGGGACGGCACACAG	Human 2D6syn overlap with ICL1-Terminator
T_ol_2D6syn_fw	**GTGCCGTCCCAAGATAA**GCAGTTTGTTTAGCAAAATATATTTAACG	
P_ol_HCPR-WT_rv	**CGTGGGAGTCTCCCAT**TTTTTGTATGCTTGGTCAGTCTACTGG	all ICL1-Promoter variants overlap with HCPR-WT
HCPR-WT_ol_P_fw	**CTGACCAAGCATACAAAAA**ATGGGAGACTCCCACGTGG	

Table 11: Primers for site-directed mutagenesis

name	sequence	information
2D6syn_F120A_fw	CTCGTTCTCAGGGTGTC**GCT**CTGGCTAGATACGGTC	F120A mutation
2D6syn_F120A_rv	GACCGTATCTAGCCAG**AGC**GACACCCTGAGAACGAG	
2D6syn_E216G_fw	CAAGAGGGTCTGAAGGAA**GGC**TCTGGATTCTTGAGAG	E216G mutation
2D6syn_E216G_rv	CTCTCAAGAATCCAGA**GCC**TTCCTTCAGACCCTCTTG	
2D6syn_F483G_fw	CCATGGAGTCTTTGCT**GGC**TTGGTTTCTCCATCCC	F483G mutation
2D6syn_F483G_rv	GGGATGGAGAAACCAA**GCC**AGCAAAGACTCCATGG	
2D6syn_E216F_fw	CAAGAGGGTCTGAAGGAA**TTC**TCTGGATTCTTGAGAG	E216F mutation
2D6syn_E216F_rv	CTCTCAAGAATCCAGA**GAA**TTCCTTCAGACCCTCTTG	
2D6syn_E216M_fw	CAAGAGGGTCTGAAGGAA**ATG**TCTGGATTCTTGAGAG	E216M mutation
2D6syn_E216M_rv	CTCTCAAGAATCCAGA**CAT**TTCCTTCAGACCCTCTTG	

Table 12: Primers for sequencing

name	sequence	information
rDNAko_seq_rv	CCTTAGGATCGACTTACCCGTG	rDNA integration
seq_homo_fw	GCTCTGTACACCGAGAAACAGG	
seq_rDNA_fw	GAGTTTGAACCTAGAGGTGCCAG	
Seq_CPRYL_1_rv	CCTCGCCGTAGTCACCAATTC	Yarrowia lipolytica cytochrome P450 reductase
Seq_CPRYL_2_rv	CTTGTCGGAACCAAGTCGCAG	
seq_CPRYL_3_fw	GATCCCGCCTACAAGCCCTC	
seq_CPRYL_4_fw	GAAAGCGGCACCGACGTG	
seq_URA3_rv2	CTCGGTTCTGGCCGTACAGAC	ura3
seq_URA3_rv1	GGTGGTGGTAACATCCAGAGAAGC	
seq_T_rv	GTCCAGCGCCTCTATCAAACTC	ICL1 terminator
ICL-T_seq_fw	CCTTTTCCCGTCGCAAAAC	
seq_T_fw	GCTGGACTACATCATTACTGAATCAC	
seq_T_rv2	GTAAAGTCACGATAGCTTAACTATGAGC	
seq_AmpR_fw1	CATCGAACTGGATCTCAACAGC	AmpR gene
seq_AmpR_fw2	GCCCTCCCGTATCGTAGTTATC	
seq_AmpR_rv	GTATGCGGCGACCGAGTTG	
seq_ori_fw1	GGTTGGACTCAAGACGATAGTTACC	E.coli origin of replication
seq_ori_fw2	CGCAATTAATGTGAGTTAGCTCAC	
seq_ori_fw3	CGACACCCGCCAACACC	
seq_ori_fw4	CAGTCGCTTCACGTTCGCTC	
seq_ori_fw5	GCCTGCTTCTCGCCGAAAC	
seq_pD_fw1	CGTGACTATCTGCTCCGTTCCTC	ICL1 promoter
seq_pD_fw2	GTGTCATAATCTCTGAACGTGAAGG	
seq_pD_fw3	GTTTGTTGGGTGTCAAATCACG	
seq_pD_rv1	CCGCATGAGTCACGCTACG	
seq_HCPR-WT_rv1	AACTTGACCCCAGAGAGATCCAC	human cytochrome P450 reductase
seq_HCPR-WT_fw1	ACCCTGAGGAGTATGACCTGGC	
seq_HCPR-WT_fw2	CCTGAACAACCTGGATGAGGAG	
seq_HCPR-WT_fw3	CAGTTCCACAGGGACGGTG	
seq_3A4WT_rv1	TACTGGGCAATGATAGGGACC	wild type CYP3A4
seq_3A4WT_rv2	GGGAATAATCTGAGCGTTTCATTC	
seq_3A4WT_fw1	CCTGATGTCCAGCAGAAACTGC	
seq_3A4WT_fw2	CACGAGCAGTGTTCTCTCCTTC	
seq_3A4-sp_rv1	CACCGAAAGAAGTAGAGGTAATGACG	codon optimized CYP3A4syn
seq_3A4-sp_fw1	TTCGTAACCTTAGACGTGAAGCTG	
seq_3A4-sp_fw2	TGAGATCAACGGTATGTTCATTCC	

seq_3A4-sp_rv2	AAGGAAATCGAAACGCAAAAGC	
2D6seq_syn_rv	GCAAACGCAGAAATCTAGGGTC	codon optimized CYP2D6syn
2D6seq_syn_fw1	AGGAAGCTGCCTGTTTGTGC	
2D6seq_syn_fw2	GACATCGAGGTTCAAGGTTTCC	
2D6seq_fw	TCTCTTGGACAAAGCCGTGAG	wild type CYP2D6
2D6seq_rv	GACTCCTCCTTCAGTCCCTCC	
2D6Seq_fw1	AACACTTCCTGGATGCCCAG	
seq_Pro-Pp-DAS1_fw1	CGATGGTGTTGCTCAACAAAGG	P. pastoris DAS 1+2 promoter
seq_Pro-Pp-DAS1_fw2	GCTGGAACGCAGGTCCTTATG	
seq_Pro-Pp-DAS1_fw3	CATTCTGTCGAAAATGGAAGCG	
seq_Pro-Pp-DAS1_rv	CCGCCCAAACGAACAGATAATAG	

Table 13: Primers for RT-QPCR

name	sequence	binding region
RT-ampR-fw	GCTATGTGGCGCGGTATTATC	AmpR gene
RT-ampR-rv	GTATGCGGCGACCGAGTT	
RT-ICL1-fw	CCAGCAGCCCGAGATTGA	ICL1 promoter
RT-ICL1-rv	ACTCAGCACCGGACCACTTC	

Table 14: Probe-Primers for southern blot

name	sequence	binding region
Splot-ampR-fw	cgcggtattatcccgtattg	AmpR gene
Splot-ampR-rv	atacgggagggcttaccatc	

12. Supplementary Data

Supplementary data 1

Manuscript I
(muteinDB - published in database; doi:10.1093/database/bas028)

MuteinDB: The mutein database linking substrates, products and enzymatic reactions directly with genetic variants of enzymes

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Original article

MuteinDB: the mutein database linking substrates, products and enzymatic reactions directly with genetic variants of enzymes

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Mutational events as well as the selection of the optimal variant are essential steps in the evolution of living organisms. The same principle is used in laboratory to extend the natural biodiversity to obtain better catalysts for applications in biomanufacturing or for improved biopharmaceuticals. Furthermore, single mutation in genes of drug-metabolizing enzymes can also result in dramatic changes in pharmacokinetics. These changes are a major cause of patient-specific drug responses and are, therefore, the molecular basis for personalized medicine. MuteinDB systematically links laboratory-generated enzyme variants (muteins) and natural isoforms with their biochemical properties including kinetic data of catalyzed reactions. Detailed information about kinetic characteristics of muteins is available in a systematic way and searchable for known mutations and catalyzed reactions as well as their substrates and known products. MuteinDB is broadly applicable to any known protein and their variants and makes mutagenesis and biochemical data searchable and comparable in a simple and easy-to-use manner. For the import of new mutein data, a simple, standardized, spreadsheet-based data format has been defined. To demonstrate the broad applicability of the MuteinDB, first data sets have been incorporated for selected cytochrome P450 enzymes as well as for nitrilases and peroxidases.

Database URL: http://www.MuteinDB.org

Introduction

One of nature's fundamental mechanisms to create genetic diversity in living organisms is the creation of mutants, which, in turn, leads to evolution. Mutational events and selection of the optimal variant are essential to obtain a better catalyst. In human medicine, enzyme polymorphisms arising from evolutionary events have been identified since the 1960s (1). Physicians recognized that patients with the same disease responded differently to drugs, according to which allelic variant their genomes were carrying. This opened the road to what is nowadays called 'personalized medicine' (2). Additionally, industry desires to artificially improve enzymes through mutation and selection. To this end, efficient protein engineering tools to create tailor-made enzyme variants, named 'muteins', have been developed over the past decades (3). Muteins generated either by rational design or by directed or designed evolution were adapted to the needs of industrial processes or for completely new applications.

Increasing interest in personalized medicine and in tailor-made enzymes in the fast-growing biocatalysis industry has led to an exponential increase of literature about muteins and their influence on enzymes' kinetic properties.

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In a plethora of examples, the artificial substitution of one or more amino acids in a polypeptide resulted in a significant increase or decrease of stability, turnover rate or substrate specificity for the enzyme (4). Even new reactions or activities on molecules that were not substrates for the natural parental enzyme can be caused by just a few or every single mutation. For example, esterases could be changed to hydroxynitrile lyases and epoxide hydrolases (5,6). Papain, a protease, was modified to an enzyme with efficient nitrile hydratase activity (7). Furthermore, the fatty acid hydroxylase CYP102A1 and the camphor hydroxylase CYP101 were redesigned to efficient alkane hydroxylases (8–10). By a single mutation, the broadly applied lipase CalB was modified to perform aldol additions and epoxidations (11). More recently, a transaminase showing almost no activity for a commercially interesting substrate was mutated to a highly active and selective catalyst enabling a new efficient industrial process for sitagliptin production (12).

Information about specific proteins and their muteins are widely spread in the literature. Many studies only describe single mutation and its effects without comparison to already known muteins. Possible additive effects of single amino acid changes are scarcely described or used. Even after a thorough and time-consuming literature search, researchers face the problem of assembling and presenting the data in an easy understandable and comprehensive way. Essential information may be lost such as details about potentially cooperative mutations or reactions one would not expect in certain protein families. Therefore, a web-accessible database combining available knowledge about a specific enzyme and its muteins in a single place are highly desirable. Such a database would allow researchers to access relevant information about their protein of interest in a fast and easy way and accelerate the engineering of new and improved variants.

Existing, comprehensive enzyme engineering databases such as CYPED are mainly focused on enzyme sequences and their structures (13). Only a few databases go beyond that and contain, to some extent, information about muteins and their properties. The most recently published database introducing mutein information is SuperCYP (14), which exclusively addresses human cytochrome P450s. Another example is SPROUTS (15), which provides details on the influence of point mutations on protein stability. The Protherm database (16) contains experimental thermodynamic data, and BRENDA (17), a well-known enzyme databases, includes only a small section about muteins. Finally, the Protein Mutant Database (18) includes references to mutant proteins from the literature. However, none of these databases provides kinetic characteristics of muteins and allows a fast, systematic and user-friendly way to search for known mutations and catalyzed reactions of interest. All these databases focus on enzymes and provide information about their variants from the view of the protein. Additionally, none of the existing databases is searchable by substrate or product molecule structures allowing comparison of muteins with respect to their catalytic properties.

In this article, we present the novel database MuteinDB (http://www.MuteinDB.org). It is a user-friendly graphically appealing database devoted to provide easy access to detailed information on naturally occurring and laboratory-evolved muteins as well as on the influence of mutations on kinetics of catalyzed reactions, including inhibition. It allows to search for the best biocatalyst for a given substrate, reaction or product simply by substrate name, Chemical Abstracts Service (CAS) number or molecule structure. In addition, a structure search tool offers the possibility to predict muteins which most likely accept a new substrate, if no enzyme/substrate properties were described so far.

MuteinDB overview

The MuteinDB is a platform to collect, catalog, and store experimentally derived data about muteins from publicly available sources as well as data directly submitted by the scientists. Additionally, it allows flexible searches by reaction type, molecular (sub) structures, substrate, product or mutein name. MuteinDB provides details on catalyzed reactions, kinetic data (activity, kinetic resolution) and experimental conditions used for data generation as well as for possible substrates or products consumed or produced by a reaction of choice including relevant scientific publication or patent information. Furthermore, it is possible to screen all enzyme variants for known interactions with specific inhibitors. Substrate, product and inhibitor data are linked to CAS and/or CID number (PubChem) as a unique identifier for unambiguous reference. The use of these distinct identification numbers allows even to extract information about (comparative) stereo- or enantioselectivity of individual muteins. Furthermore, once the user has identified a mutein of interest, information about its sequence, the employed expression hosts, cofactors, cosubstrates, and coproteins can be directly shown. In the sequence view, all mutations of a specific mutein are highlighted and liked to other muteins with known mutations of the same position. Additionally, the wild type sequence including all known amino acid exchanges which are again linked to muteins containing the modified position is illustrated for all muteins.

For first-time users, a comprehensive frequently asked question (FAQ) section and in-depth tutorial movies are provided. The key features are described in more detail below.

Search options

In contrast to other databases, MuteinDB allows users not only to query for muteins and mutations but also for substrates, products, or inhibitors, using the corresponding name or CAS number as well as catalyzed reaction types. The database also provides means to easily and efficiently search the data (e.g. by allowing to enter wildcards in the values) and display it in a clear, tabular form.

Structural search

Another important difference to other existing databases is the fully integrated (sub) structure search tool. It allows searching for substances with a similar structure by drawing an arbitrary chemical structure in the JME Molecule Editor (19). The database will provide all possible hits related to the drawn structure, and the user can navigate amongst them to refine the search. Based on knowledge about mutein/substrate combinations and their specific products, this for the first time also allows predictions of other possible substrates and products for known muteins which were not experimentally evaluated so far.

Individual features of MuteinDB

MuteinDB uses a mutein-based classification. A unique ID is assigned to each mutein and is linked to the reference source, the catalyzed reaction and the corresponding wild type protein. This mutein-centric approach allows more flexible and specific searches compared to the publication-based classification of the Protein Mutant Database (18) or the reaction based classification of BRENDA (17). Each reaction and publication reference can be independently surveyed, which is especially important when amino acid changes result in new functionalities. An example for such a case is the lipase CALB that was modified to a C–C bond forming enzyme for aldol additions (11).

Basic information about underlying wild type protein sequences, structures and source organism as well as compound structures, their respective references and reactions are retrieved from the public databases GenBank, PDB, UniProt, PubChem, PubMed, CrossRef, and KEGG (20–24). Wherever third party data is presented, it is linked to the corresponding database entry.

Standardized format for data collection and import

The recent introduction of experimental high-throughput techniques required the development of standardized formats for data from biological experiments. They facilitate exchange of data, their storage in publicly accessible repositories, increase experimental transparency and allow reproduction of bioinformatic analyses from publications. For example, such formats are available for DNA-microarray experiments (25), proteomics studies (26), and data deriving from qPCR experiments (27). Most of them are XML based, which can be difficult to create and manipulate. Therefore, simpler, spreadsheet-based formats have been introduced which are more accessible for the individual researcher. A prominent representative is the MAGE-TAB format for 45 DNA-microarray experiments (28).

Here, we propose a standardized spreadsheet-based data exchange format for muteins and related experimental kinetic data. The MuteinDB import spreadsheet comprises seven sections for each entry: (i) basic data; (ii) signal sequences; (iii) pH conditions; (iv) temperature conditions; (v) storage stability; (vi) reaction data and (vii) activity data. The basic data section includes the enzyme's name, the GenBank protein ID and the PDB ID (if available). Additionally, the corresponding wild-type name and the sequence mutations are illustrated for muteins. The reaction section contains the substrate and the product of the reaction (both with CAS number and name), the enzyme classification (EC) number of the reaction and the reaction type. The activity section can cover one of following types: conversion activity, enatiomeric excess or inhibition. All three types are followed by the corresponding kinetic values and the experimental conditions. The provided standards for kinetic data necessitate a minimum quality of biochemical protein data (e.g enzyme activity provided in μmol product made by μmol enzyme per minute).

A detailed description of the fields along with guidelines for data collection and a template spreadsheet are available on the MuteinDB homepage. Standardized entry of data into the spreadsheet is ensured by drop-down lists for fields with a defined value set. Drop-down lists can be extended if new values for a field are required.

For data import, the files are checked for data consistency according to the guidelines and compared with the already existing mutein data to prevent duplicate entries (Figure 1). A detailed report on the import is provided, allowing focused modification of the data to adjust it conforming to the guidelines. Upon successful import, the data is reviewed by an expert team at Graz University of Technology and feedback is provided to the submitter. After all inconsistencies are resolved, the new content is publicly released. New data can be submitted any time and is made available immediately after the review.

MuteinDB structure and implementation

The MuteinDB is implemented using Java, an object-oriented and platform-independent programming language. The application is based on a 3-tier architecture with an Oracle database as the persistence tier, an

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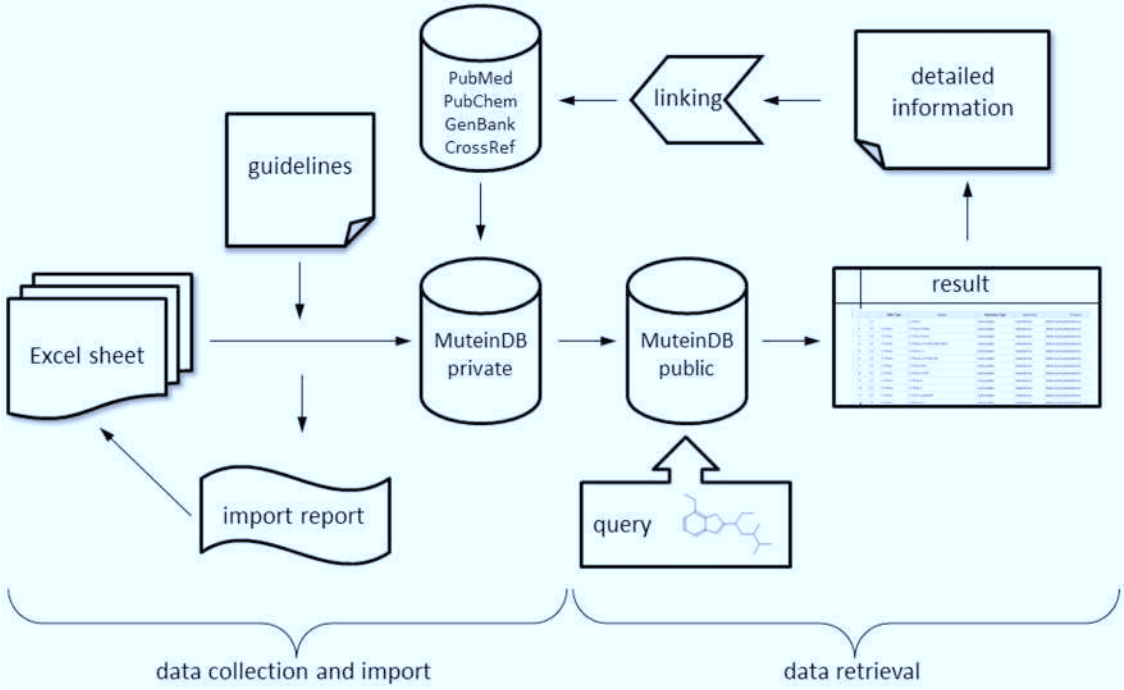

Figure 1. Schematic diagram of database structure. MuteinDB structure can be divided into two major parts. Firstly, the data collection and import structure within MuteinDB, illustrated on the left. Detailed guidelines structure and specify the correct and unified data collection as well as the data import. The standardized excel data import template guarantees data quality and consistency. During the automated data import from the data import excel sheet, metadata from third party databases such as PubMed, PubChem, GenBank and CrossRef are retrieved and added. The data import procedure ends either with a summary including imported muteins, molecules, reactions, activities or with a detailed error report. Secondly, stored public mutein data can be easily retrieved via various search mechanisms. For example, chemical structures can be used for identifying molecules of interest and their catalyzed reactions. Results are presented in tabular listings with links to third party databases or to detailed information contained in MuteinDB.

application server (JBoss) as the middle tier and a WEB interface as the client tier. Business logic is implemented using Enterprise JavaBeans 3. The web interface depends on JavaServer Faces 2, Asynchronous JavaScript and XML and JBoss Seam. The relational database schema has been designed to accommodate controlled vocabularies in form of a data dictionary. Attributes with a defined value set are linked to data dictionary entries to facilitate standardized content in the database.

For substructure search (5), the JME Molecule Editor (19) and the Chemistry Development Kid (CDK)—an open-source Java library—are used.

Use of MuteinDB

The MuteinDB was developed as a user-friendly and intuitive resource of mutein-related properties for scientists in the fields of biology, biotechnology, organic chemistry and pharmaceutical sciences. The top information bar offers 'FAQs' where users will find helpful information. Furthermore, first-time users will find tutorial movies explaining the database usage and the different MuteinDB sections.

The simplest search option 'Search by Substrate' is directly accessible via the home screen. The left side navigation bar gives access to further querying options.

Search options

(i) Substrate: enables the user to search for muteins that convert a certain substrate of interest.
(ii) Reaction: enables the user to search for specific reactions by entering a molecule name or a CAS number for the substrate and/or the product (including single enantiomers)
(iii) Structure: enables the user to draw chemical structures to search for similar or exact (sub) structure matches in either one or all of the molecule categories (substrate, product and/or inhibitor).
(iv) Inhibitor: enables the user to search for inhibitors of muteins and wild-type enzymes by entering a molecule name or a CAS number.

(v) Mutation: enables the user to search for muteins containing mutations at a certain position.
(vi) Wild type: enables the user to browse all muteins and their reactions for a defined wild-type enzyme.
(vii) Mutein: enables the user to search for all relevant reactions for a defined mutein name.

To keep the additional querying options simple and flexible, further refinements of the query can, but do not have to, be specified or selected. For example, the search can be restricted amongst others to the reaction type, the underlying wild type protein, or to a specific organism.

All text fields are equipped with 'suggest input'. While typing a box will appear and provide suggestions one can choose from. Furthermore, selected fields allow 'wildcard search' with '*' as a placeholder.

Example workflow

The ability to search for exact or similar structures is one of the unique main features of the MuteinDB. Therefore, we will describe this search type in more detail and use it as example to demonstrate the ability of MuteinDB for valuable data retrieval (Figure 2).

Selecting 'Search by Structure' will open the JME Molecule Editor Applet (19) and allows the user to draw an arbitrary chemical structure (Figure 2A). After submitting the search, results will be presented as a table listing all molecules containing the drawn structure (Figure 2B). The 'structure result' page proposes several related substrates, products and inhibitors with similar structures to the drawn molecule structure. In all result views, moving the cursor over a molecule name will show its chemical structure. Additionally, each molecule name is linked to PubChem (22). This also facilitates the search if the CAS number or the exact molecule name is unknown or if different trivial names of the molecule are commonly used.

One or several molecules can be selected via checkboxes and can be used for a subsequent search by substrate/product or inhibitor. The results are shown again in tabular form listing all muteins that convert the selected substrates or produce the selected products or are inhibited by the chosen inhibitors.

Selecting 'testosterone' from the list for a subsequent search reveals several muteins that are able to convert this steroid (Figure 2C). This supports predictions about possible transformations of testosterone derivatives where no experimental data is available so far. Hits from such searches are preferred muteins for experimental evaluation.

Information about the catalyzed reactions such as substrate, product and reaction type are presented in the 'substrate view' (Figure 2D). A link to KEGG reaction (29) is provided when a corresponding entry exists. As the kinetic data are one of the most important pieces of information stored in the database, kinetic parameters such as K_m and k_{cat} are given. Furthermore, enantiomeric access and E-values are provided if available. The view can be customized using 'edit display settings'.

As multiple publications may have reported the same reaction for a given mutein, the one stating the highest activity is shown in the main result screen. By using the expand button the data from the other reports are also shown. Clicking on the mutein name will bring up the 'mutein view' where detailed information about the mutein and the reaction are provided.

In the 'substrate section', several mouse-over buttons (Figure 2D) give further information about the catalyzed reaction. 'C' shows comments on the reaction, 'W' gives activity data of the underlying wild-type reference, 'R' shows information about reaction conditions and analysis and 'L' provides detailed information about the corresponding literature. The PubMed ID or the digital object identifier (DOI, http://crossref.org) of the publications are given and directly linked to PubMed or to the webpage associated with the digital object identifier, respectively. Additionally, the EC number is provided and linked to the comprehensive enzyme database BRENDA (Figure 2F–H).

The 'sequence section' shows the mutein sequence aligned with its corresponding wild-type sequence (Figure 2E). In the mutein sequence, the mutations are highlighted in violet. The sequence can be downloaded as FASTA format. The amino acids of the wild-type sequence highlighted in blue mark the positions of known mutations. These positions are linked to the 'enzyme mutation view'. In this view, all muteins that contain a mutation at this position are listed. Via the mutein name it is possible to navigate to the mutation view of the corresponding mutein.

Another highlight of the MuteinDB is the ability to select two or more muteins, which convert the substrate of interest or form the product of interest, for comparison in side by side view. In the 'compare view' the kinetic data of the catalyzed reaction as well as information about the mutations, expression system and involved cofactors and coproteins are displayed.

Inhibitors have a special status and may have been reported in the 'structure result' page for the inhibitor search. The results are shown in tabular form (Figure 3) listing muteins that are inhibited by the chemical compound. Instead of kinetic data, the inhibitor constant K_i or the IC_{50} value are provided. Additionally, the underlying reaction used to determine the inhibitor constant is shown.

As the same inhibitor measurements can be found in different publications, only the one with the highest inhibition constant is shown as the main result. Via the expand button, the data of the other literature sources is shown. The mutein name is again linked to the 'mutein view', where detailed information on the mutein and the inhibition reaction are provided.

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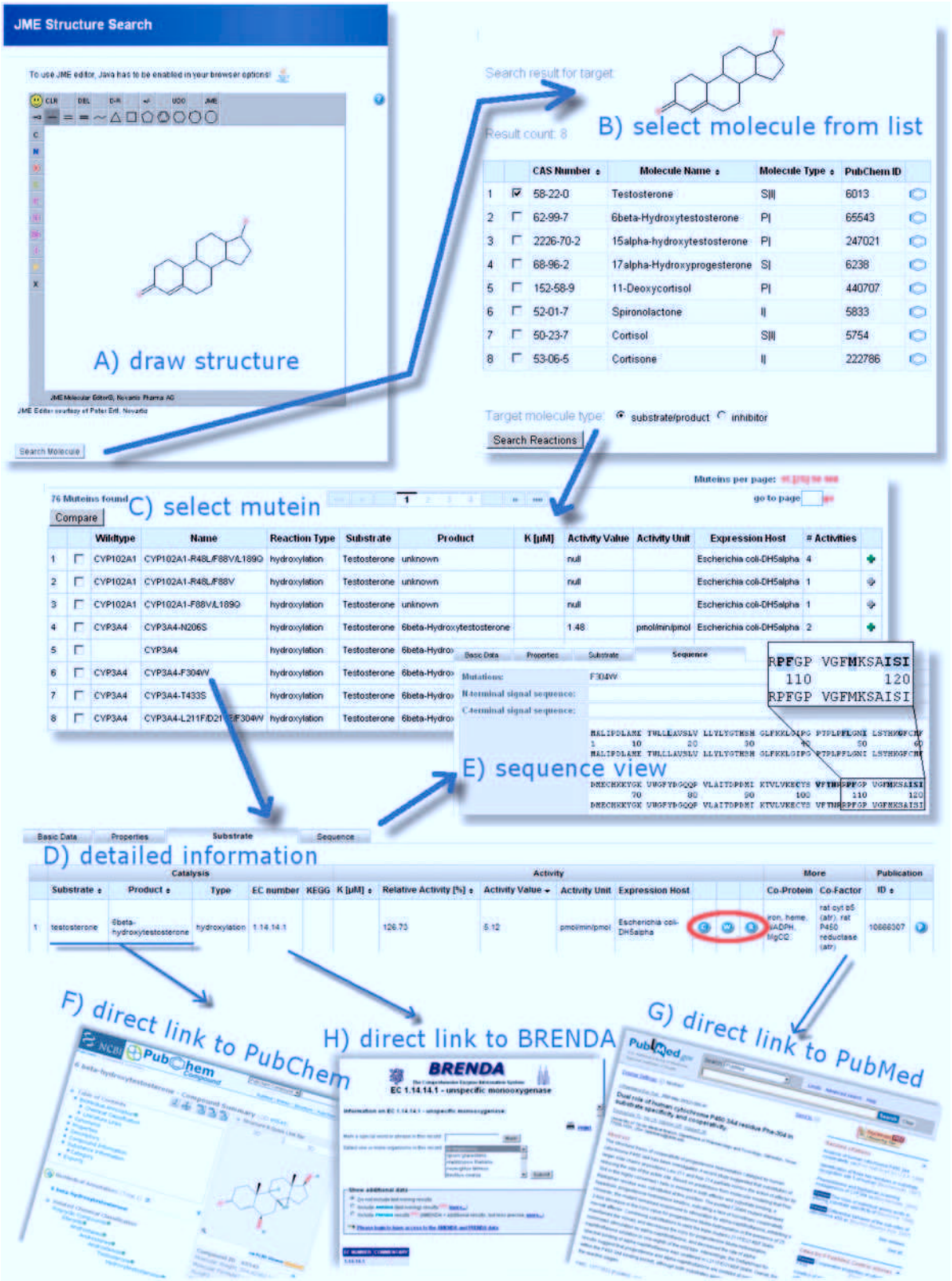

Figure 2. MuteinDB structure search, its results and the capabilities of the MuteinDB webinterface. (**A**) The MuteinDB (sub) structure search uses the JME editor, which allows users to draw arbitrary molecular structures. (**B**) The user-drawn structure is used as seed for the following database search and shown on top of the structure search result table. In this table all molecules,

(continued)

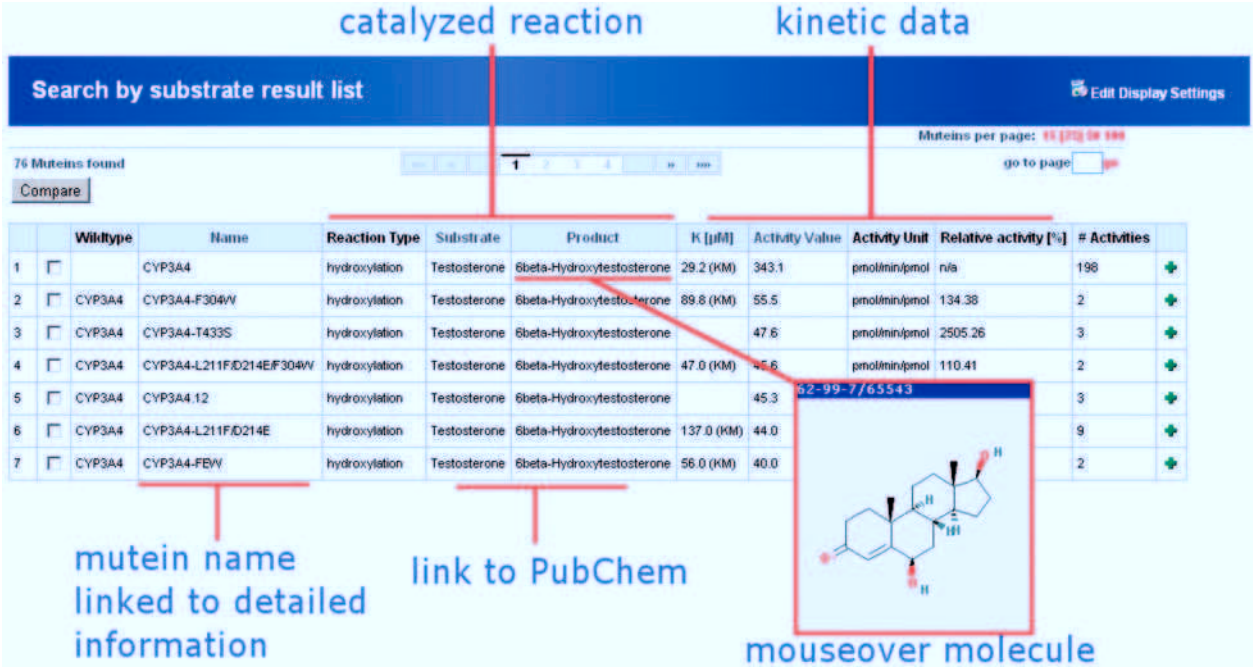

Figure 3. Result display of the MuteinDB web-interface for testosterone as a substrate. Information within the result listing for each mutein is by default grouped into catalyzed reaction and kinetic data. Reaction information comprises the reaction type as well as the catalyzed substrate and product. Molecules are directly linked to their corresponding PubChem entry. Additionally, the molecule structure can be displayed by moving over the compound's name. Important kinetic parameters such as K value, activity value including its unit as well as the relative activity in (%) are directly available in the result view. All presented information and further links for each mutein or wild type is directly linked by its name.

Results and conclusions

MuteinDB is a comprehensive and carefully curated database for specific muteins and their kinetic data of catalyzed reactions including inhibition. It provides in-depth information on mutein properties combined with flexible search capabilities. The MuteinDB has been designed to be broadly applicable to proteins and their muteins from any enzyme class including those with no known catalytic function. We demonstrated this by entering data sets of several enzymes and their variants of different enzyme classes.

Presently, the understanding of the structure–function relationship of proteins is still limited. Scientists are trying to tackle the problem from different perspectives (from medicine and pharmacokinetics, to structural biology or applied biocatalysis) and are, therefore, interested in how mutations can influence catalytic properties.

By means of MuteinDB a user can find enzymes that catalyze a particular reaction not only in expected enzyme classes but also in others [e.g. a C–C bond forming mutein derived from a hydrolase (30)]. This feature helps to identify potential starting points for further enzyme engineering. Moreover, medical scientists can get information about the influence of mutations on the drug metabolism and the *in vivo* activation. This helps to predict a patient's personal response to certain administered drugs. In addition, the implemented structure search for substrates, products and inhibitors allows the prediction of structure scaffolds that could be accepted by muteins. This might provide helpful information for the development of new biocatalysts and, most probably, will facilitate drug metabolite prediction in pharmaceutical research and development.

At present MuteinDB contains several thousand reactions (Table 1) for muteins of different enzyme

Figure 2. Continued
substrates, products or inhibitors which contain the query structure are presented. A selection of these molecules can be used for a subsequent 'Search by Reaction'. (**C**) All wild type enzymes and muteins which catalyze the selected molecules are shown. (**D**) For each row of the tabular result, further information can be obtained via the mutein or wild type name. The detailed information is organized in four main categories: (i) basic data; (ii) properties; (iii) substrate and (iv) sequence. (**E**) The 'Sequence' tab of the selected mutein allows to explore the sequence of the mutein as well as the wild type sequence. Known mutations are highlighted and linked to the corresponding entries of MuteinDB. (**F**) Information in the 'Substrate' tab is linked to third party databases. For example, (**F**) molecules are linked to PubChem, (**H**) EC-Numbers to Brenda and (**G**) literature to PubMed or to its DOI location. For muteins, experimental settings and wild type activity values are available from the 'Substrate' tab.

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Table 1. MuteinDB data overview

Wild-type Name	Muteins	Reactions	Activities	Publications
CYP102A1	168	909	995	42
CYP102A2	0	4	4	1
CYP2D6	98	648	1259	213
CYP3A4	124	825	1908	220
HAPMO	6	106	114	5
HRP C1	17	32	45	8
Nitrilases	8	26	26	3
NITAf	11	42	42	2
P3H	0	1	12	1
P3H type1	0	21	31	3
P3H type2	0	16	23	2
P4H	0	21	53	4
PAMO	31	309	385	10
Total: 11	444	2892	4829	422

classes. It is the largest collection of kinetic data of muteins compiled in a single database. To demonstrate the general applicability of the database, different types of enzymes from different origins have been searched in literature and imported into MuteinDB. Data were collected by searching SciFinder (www.cas.org) and PubMed (21) abstracts for specific keywords. Detailed data from texts, tables and figures were manually extracted from the matching full-text publications and were curated by a team of scientists, who enriched the published information with first-hand kinetic data wherever possible.

CYP2D6 and CYP3A4 are human liver enzymes and known to be involved in drug metabolism. Both enzymes have been chosen as primary data sets due to their pronounced polymorphism and high importance for human drug and xenobiotic metabolism. We selected CYP102A1 (BM-3) from *Bacillus megaterium* as a prokaryotic representative. This protein is one of the most mutated and investigated proteins known.

To import the data, we used the standardized spreadsheet-based import file format described previously. It contains all attributes necessary to describe a mutein and its properties.

In order to augment the database content, data collection is on-going. To make the database as comprehensive and up-to-date as possible, we are addressing the research community with a request to aid us in the collection of kinetic data sets for enzymes of different type and origin. We appreciate any contribution to the database both updates to existing data and new kinetic data sets.

Future directions

In the course of integrating new data sets, the MuteinDB will be adapted, and the guidelines for data collection will be adjusted. Feedback from end users and data collectors will ensure a continued focus on a user-friendly development.

The collection of data sets was carried out as part of the OXYGREEN (www.oxygreen.org) project, a research collaboration funded by the European Commission Seventh Framework Programme (EU FP7), and will be continued to do so. MuteinDB will be used and extended in the context of BIONEXGEN, a recently funded EU project. To ensure continuation of data collection and curation of the database, MuteinDB will be integrated into future projects.

A downloadable version of the MuteinDB is in preparation. It will be provided for companies or universities that would like to store their own data in-house. The data can be integrated into the public online database on request. The download will be available in exchange for new mutein data sets or for a fee for database curation and data collection.

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Conflict of interest. None declared.

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Supplementary data 2

Manuscript II
(*Y. lipolytica* – accepted for publication in Microbial cell factories)

Expression of human liver cytochrome P450s using the non conventional yeast *Yarrowia lipolytica* for steroid hydroxylation in biphasic systems

Steroid biotransformations in biphasic systems with *Yarrowia lipolytica* expressing human liver cytochrome P450 genes.

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Abstract

Background

Yarrowia lipolytica efficiently metabolizes and assimilates hydrophobic compounds such as n-alkanes and fatty acids. Efficient substrate uptake is enabled by naturally secreted emulsifiers and a modified cell surface hydrophobicity and protrusions formed by this yeast. We were examining the potential of recombinant Y. lipolytica as a biocatalyst for the oxidation of hardly soluble hydrophobic steroids. Furthermore, two-liquid biphasic culture systems were evaluated to increase substrate availability. While cells, together with water soluble nutrients, are maintained in the aqueous phase, substrates and most of the products are contained in a second water-immiscible organic solvent phase.

Results

For the first time we have co-expressed the human cytochromes P450 2D6 and 3A4 genes in Y. lipolytica together with human cytochrome P450 reductase (hCPR) or Y. lipolytica cytochrome P450 reductase (YlCPR). These whole-cell biocatalysts were used for the conversion of poorly soluble steroids in biphasic systems.

Employing a biphasic system with the organic solvent and Y. lipolytica carbon source ethyl oleate for the whole-cell bioconversion of progesterone, the initial specific hydroxylation rate in a 1.5 L stirred tank bioreactor was further increased 2-fold. Furthermore, the product formation was significantly prolonged as compared to the aqueous system.

Co-expression of the human CPR gene lead to a 4-10-fold higher activity specific activity, compared to the co-overexpression of the native Y. lipolytica CPR gene. Multicopy transformants showed a 50-70-fold increase of activity as compared to single copy strains.

Conclusions

Alkane-assimilating yeast Yarrowia lipolytica, coupled with the described expression strategies, demonstrated its high potential for biotransformations of hydrophobic substrates in two-liquid biphasic systems. Especially organic solvents which can be efficiently taken up and/or metabolized by the cell might enable more efficient bioconversion as compared to aqueous systems and even enable simple, continuous or at least high yield long time processes.

Keywords

Yarrowia lipolytica, biphasic sytem, cytochrome P450, steroid, whole-cell, bioconversion

Introduction

Cytochrome P450s (CYPs) are a large, ubiquitous family of heme-containing monooxygenases that are responsible for the oxidative metabolism of a wide variety of drugs, environmental chemicals and endogenous compounds, such as steroids, prostaglandins and fatty acids [1].

Most cytochrome P450 systems are composed of a monooxygenase and one or two additional proteins, constituting an electron transfer chain. Genes encoding these components are either expressed individually or linked resulting in self sufficient CYPs. To some extent, the natural electron transport chain from NAD(P)H to the heme containing cytochrome P450 can be replaced by either homologues or different proteins with similar function e.g. flavodoxin and flavodoxin reductase to support catalytic activity [2]. Therefore, the activity of CYPs is not only determined by its abundance, but also by the abundance of the electron transport partners [3] and possibly by their molar ratio.

Eukaryotic CYPs are membrane associated and many of those are located on the cytosolic side of the endoplasmic reticulum membrane. However, several important CYPs such as the vitamin D3 25-hydroxylase CYP27B1 are also associated to mitochondrial membrane [4]. In mammalian cells, expression takes place in different tissues, but the highest levels are found in the liver, where CYPs have the principal function to introduce an oxygen atom into hydrophobic substrates. The increased hydrophilicity of the product facilitates its elimination from the mammalian body.

Several genes of xenobiotic-metabolizing CYPs are expressed in human liver, among which CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 appear to be most commonly responsible for drug metabolism [5]. The relative importance for drug metabolism is reflected in the abundance of these enzymes, e.g., CYP3A4 being most abundant with ~ 30% of total CYP in liver cells, and the preference to bind and/or metabolize chemicals with structures

commonly found in drugs, e.g., CYP2D6 preferentially binds widely used drugs with basic amine functions [3].

Many of these drug-metabolizing CYPs are subject to polymorphisms, duplications, and differential expression levels, which gives rise to wide variation in pharmacokinetics profiles. This makes CYPs highly important to the pharmaceutical industry, where human drug metabolites are essential for drug development and analysis.

In order to provide sufficient amounts of human CYPs, several recombinant expression systems have been investigated in the past 20 years, from the more complicated and expensive mammalian [6] and Baculovirus-infected insect cell system [7] to the "simpler" expression host Escherichia coli. Since the first functional expression of mammalian CYPs in bakers's yeast Saccharomyces cerevisiae was demonstrated in the mid 80s [8, 9], in recent years, the focus shifted more and more to yeast systems, which combine the ease of handling of prokaryotic systems with the sub-cellular structure of eukaryotic systems reassembling a more natural environment. Mammalian CYP genes have been expressed, e.g., in the yeasts S. cerevisiae [8–14], P. pastoris [15, 16], S. pombe [17–19], and Y. lipolytica [20–22]. Frequently, whole-cells were used for drug metabolite synthesis to deal with inherent stability problems of human CYP enzymes and regeneration of NADPH.

Most of the typical substrates for CYPs are very hydrophobic and most probably enter the enzyme's active site via biological membranes. This also explains why specific activities of truncated and soluble human CYPs are lower than those of their native membrane bound counterparts [23]. Another obstacle in performing bioconversions with hydrophobic substrates is their very low solubility in the aqueous phase, which limits cellular uptake and thus overall biotransformation performance. Investigations showed that the substrates have to be added in concentrations above the solubility limit to achieve efficient biotransformation. To ensure a homogenous suspension, the substrates have to be dissolved at high concentration in an organic solvent and quickly added to the aqueous solution [24].

However, there are several approaches to increase substrate availability. The addition of water-miscible organic solvents or detergents has been tested [17], which may results in a drastic increase of substrate solubility. Some solvents and most of the detergents, however, have a strong impact on cell membranes, compromising cell integrity and viability, and thus biocatalyst functionality.

In a more sophisticated approach, water-soluble cyclodextrins, toroid-shaped cyclic oligosaccharids, have been used to capture hydrophobic substances in their cavities, thereby increasing their apparent solubility. This approach has been successfully used with Mycobacterium sp. [25], but seems to have toxic effects on yeast cells as well [17].

Biphasic systems consisting of an organic and an aqueous phase, represent a valuable tool for the biotransformation of hydrophobic substrates [26–32]. The organic phase regulates the substrate and product concentration in the aqueous phase, allowing high overall concentrations of otherwise toxic hydrophobic substrates. Furthermore, such a system can be used to avoid inhibition effects by substrate and/ or product and can help to guide equilibrium reactions into the desired direction enhancing stereoselectivity. A crucial step is the choice of the organic phase depending on different parameters, including toxic or inhibitory effects of the solvent on the cells as well as substrate and product solubility [31, 32].

Our studies have been focused on Y. lipolytica which is naturally adapted to such two-phase systems. Y. lipolytica is often isolated from biphasic environments like dairy products rich in lipids [33] or oil-polluted soil and water [34, 35] . Applications of Y. lipolytica include bioremediation of diesel-contaminated soils [36] and olive-mill waste water [37], protein production on alkanes [38], and aroma compound formation from fatty acid derivatives [39]. When Y. lipolytica is grown on hydrophobic substrates, the cell surface is in direct contact with substrate droplets and several modifications in cell structure occur, which are probably related to hydrophobic substrate transport. These observations have led to the hypothesis that

hydrophobic substrates can migrate through channels via the plasma membrane to the ER [40, 41].

These unique properties of the alkane utilizing yeast Y. lipolytica together with the availability of efficient genetic tools for this species underscore its potential for biotransformations in biphasic systems. The yeast Yarrowia lipolytica has been revealed as one of the most suitable host for heterologous protein production [42] and several CYP genes of mammalian e.g. from Bos taurus and H. sapiens s. [20–22], plant e.g. green bell pepper [43], and fungal origin e.g. Candida maltosa and Rhodotorula minuta [44, 45], have been expressed in this yeast so far.

In this study, we report the first example of functional co-expression of genes encoding the important human liver CYPs CYP2D6 or CYP3A4 together with hCPR and intrinsic YlCPR in Y. lipolytica and their use in biphasic whole-cell bioconversions aming at steroid oxidation and as a model for the hydroxylation of other poorly soluble substrates.

Results and discussion

Cloning and expression of genes for human liver cytochrome P450s and CPRs

One of the most frequently studied bottlenecks in heterologous gene expression is the variable codon-usage and -bias of different organisms. By exchanging rare codons with more frequently used ones of the host, the expression of heterologous genes can be significantly improved in some cases [46]. In some cases, however native genes provided better results than codon optimized variants. In this study, we compared expression of wild-type and codon optimized genes for CYP2D6, CYP3A4 and human CPR in Y. lipolytica.

Using Y. lipolytica, it is possible to create multicopy integration clones employing the auxotrophic marker ura3 linked to a largely truncated and deficient promoter [47, 48]. This approach previously allowed the human CYP1A1, YlCPR and bovine CYP17 gene expression to be increased several fold [22, 49, 50].

In a similar approach, the genes encoding CYP2D6, CYP3A4, human cytochrome P450 reductase (hCPR) and Y. lipolytica cytochrome P450 reductase (YlCPR) were cloned and ligated into (i) the integrative plasmid vector p64D-linker for selection of multicopy transformants, as well as into (ii) p65D-linker promoting single copy integration. The resulting series of expression vectors contained each of the two expression cassettes under the control of the ICL1 promoter (pICL1D) and terminator (ICL1t), one for CYP and one for CPR. Y. lipolytica H222-S4 was transformed with the resulting SacII (Fig. 1) linearized plasmids.

Copy numbers of selected clones were verified by RT-PCR. Supposed single copy transformants indeed showed one integrated copy, while multicopy transformants showed between 10 and more than 40 integrated copies (Table 2) as determined by quantitative PCR, which is in good accordance with previous results [20, 22, 47, 48, 51].

It is common knowledge that separating biomass production and the expression phase of microbiologic cell cultures can be beneficial for heterologous protein production, both in terms of a reduced metabolic burden and limitation of a potentially harmful toxic activity. Therefore, Y. lipolytica was first grown in YNB medium with glucose as a sole carbon source (YNBG). Upon depletion of glucose and a short starvation phase, heterologous protein production in shake flasks was initiated by induction of the ICL1 promoter with addition of ethanol. After a short lag phase, while switching from glucose to ethanol, a further increase in biomass was observed (Fig. 2). Bufuralol and progesterone were used to assess CYP2D6 and CYP3A4 activity, respectively. Already after 4 to 8 h of induction, Y. lipolytica cells harboring CYP genes showed activity towards bufuralol or progesterone, the specific activity did not further increase and remained constant for 70 h (Fig. 2). For practical reasons all further experiments were carried out with cells harvested after 26 h of induction.

To confirm expression of heterologous cytochrome P450s, microsomal protein of each clone sampled after 26 h of ethanol induction were analyzed by Western blotting. Bands of CYP2D6 (~ 55 kDa) and CYP3A4 (~ 57 kDa) were detected at the correct sizes. No Western blots were performed for CPR, since strong protein bands with the correct sizes for hCPR (~ 75 kDa) and YlCPR (~ 85 kDa) were already detectable by Ponceau-staining of the blots for multicopy transformants, indicating the high expression levels of CPR. (Fig. 3).

Human liver cytochromes P450 2D6 and 3A4 and human cytochrome P450 reductase (hCPR) as well as Y. lipolytica cytochrome P450 reductase (YlCPR) were functionally expressed. The codon optimized versions of either CYPs showed best activities in combination with wild-type human CPR and wild-type Y. lipolytica CPR (data not shown). Therefore, these combinations were used for Western blot analysis and further used throughout this study.

For both CYP2D6 and CYP3A4, the use of the p64D-derived multicopy integration vectors led to a significant increase of expression (Fig. 3) and whole-cell CYP activity compared to single copy integrants. CYP2D6 multicopy gene integration gave ~ 70 fold increase of activity towards bufuralol, and multiple copies of the CYP3A4 coding sequence resulted in a ~ 50 fold increased activity towards progesterone (Fig. 4). These increase was similar to those observed by overexpressing a CYP1A1 gene in the Y. lipolytica strain PO1d [22]. The highest expression levels assessed by CO-spectra for human liver cytochrome P450 2D6 and 3A4 were 92.3 ± 9.2 and 60.9 ± 5.9 pmol per mg microsomal protein, respectively. No results from CO spectra have been reported for CYP1A1 expression in Y. lipolytica. Expression levels in the range of 90 and 51-250 pmol per mg microsomal protein have been reported for CYP2D6 and CYP3A4 in bakers yeast, respectively [12, 52, 53].

Coexpression of CYPs and CPRs

It has been shown that the coexpression of a functional CPR is important for optimal CYP activity [54, 55]. In some cases the proteins of the native electron transfer chain can be replaced by other proteins. For example, the adrenodoxin homologue of Schizosaccharomyces pombe etp1fd was shown to be able to replace mammalian adrenodoxin and transfer electrons from adrenodoxin reductase to CYP11B1 and CYP11B2 [56]. However, in most cases the mammalian CPRs are more effective in supplying electrons to mammalian CYPs than other CPRs [57]. This suggests that the intrinsic yeast CPRs may be a limiting factor in the heterologous expression and application of mammalian CYPs in yeast. Therefore, we coexpressed not only the endogenous Y. lipolytica CPR but also the human CPR.

P450 reductase activity measured as NADPH cytochrome c reductase activity was detected in all clones expressing either human CPR or Y. lipolytica CPR, while a control strain transformed with the empty vector showed only low endogenous CPR activity. The multicopy effect described for the hydroxylation activity was also observed for the CPR activity towards cytochrome c (Fig. 4).

Multicopy transformants YL11 and YL18 over-expressing the endogenous Y. lipolytica CPR showed 7-fold or 40-fold higher activity towards cytochrome c compared to multicopy transformants Yl21 and Yl22 over-expressing the human CPR, respectively. This may be due to a higher affinity of the Y. lipolytica CPR to cytochrome c as compared to the human CPR. However, the more likely reason might be the higher YlCPR expression level, as indicated by the more intense band on the Western blot when compared to the human CPR (Fig. 3). Despite the higher activity of over-expressed YlCPR towards cytochrome c, YlCPR coexpression did yield much lower CYP activities than achieved with clones coexpressing the human CPR (Fig. 4). This indicates that coupling of the human CYPs with Y. lipolytica CPR, although possible, is inferior to coupling with human CPR. This is in accordance with earlier

results suggesting that mammalian CPR is more effective in transporting electrons to mammalian CYPs as compared to alternative CPRs [3, 56, 58].

Determination of operation parameters

Resting and growing whole-cells can be employed as catalysts for biotransformations. Growing cells are considered more favorable than resting-cells when expressing a protein with low stability, since they permit sustained protein expression during biotransformation. However, resting-cells have the advantage that the desired reaction can be investigated independently of growth phenomena and at higher cell densities. Furthermore, biotransformation conditions can be chosen independently from growth conditions minimizing side reactions and allowing identification of potential limitations [59].

Differences between growing and resting cells on bufuralol and protesterone hydroxylation rates were investigated for Y. lipolytica harboring hCPR combined with CYP2D6 and CYP3A4, respectively. The hydroxylation rates were determined as product formation rates measuring the concentrations of hydroxybufuralol or hydroxyprogesterone, respectively. Resting-cells showed significantly higher hydroxylation rates than growing cells (data shown in Additional File 1). Therefore, resting-cells were used further in this study.

Both CYP2D6 and CYP3A4 have also been tested for their ability to hydroxylate testosterone, 17-alpha-methyltestosterone and progesterone. CYP2D6 usually prefers substrates containing a basic amine function [3]. Nevertheless, some studies have shown that CYP2D6 is also involved in steroid hydroxylation [60, 61]. Both recombinant human liver cytochrome P450 whole-cell Yarrowia catalysts hydroxylated testosterone, 17alpha-methyltestosterone and progesterone (Fig. 5). Although expression levels have been lower for CYP3A4 compared to CYP2D6, Y. lipolytica cells harboring CYP3A4 and hCPR in all cases showed the expected several-fold higher activity towards steroids, with a preference towards testosterone. The untransformed control strain YL23 showed no formation of any

hydroxylated steroid. However, Y. lipolytica is oxidizing the hydroxy function of testosterone at position 17 to the corresponding keto-function giving androstenedione [62, 63]. Such undesired side reactions may hamper the reliability of activity measurements. Therefore, testosterone was excluded and progesterone, another known standard substrate for CYP3A4, was used for bioconversion studies. Furthermore, progesterone was used to investigate the potential of two-liquid biphasic systems and Y. lipolytica catalysts for more efficient whole-cell bioconversions.

Whole-cell conversion in biphasic systems

Most of the typical human liver CYP substrates are very hydrophobic. Hence, one main function of CYPs is to incorporate a hydroxyl function to render the compound more water soluble and to activate it for further metabolization. Alkane-utilizing yeasts, such as Y. lipolytica, can excrete surfactants and adjust their cell surface and cell wall to directly interact with water immiscible organic substrates, like alkanes, long-chain fatty acids and triglycerides, facilitating their uptake [40, 41, 64–67]. These properties as well as metabolic adaptations and the subcellular organization of the alkane-utilizing yeast cell are obviously supporting the high in vivo turnover numbers observed for the host-own P450s of the CYP52 family involved in primary alkane and fatty acid oxidation [40, 41, 67–69].

In this study, we investigated whether the unique properties of Y. lipolytica will support also the function of heterologously expressed CYPs in this yeast and will give this organism an advantage in CYP-catalyzed bioconversion of hydrophobic substrates dissolved in organic solvents. A spectrum of 10 water immiscible organic solvents ranging from (i) solvents with known good solvent properties (toluene, 1-octanol), (ii) inert fluids (bis-ethyl hexyl phthalate, dibutyl phthalate) to (iii) potential carbon sources (1-decanol, 1-dodecanol, methyl laureate, ethyl oleate, n-decane, n-dodecane) [70, 71] were chosen. The solvents toluene and 1-octanol, which are known to be toxic for microorganisms, enabled the highest solubility of steroids. Indeed, they showed the expected toxic effect on Y. lipolytica cells, too. Two solvents, known

to be utilized as a carbon source by Y. lipolytica, n-decane and n-dodecane, conferred very low solubility of progesterone and were therefore excluded from further analyses. 1-decanol, 1-dodecanol, methyl laureate, ethyl oleate, bisethylhexylphthalate (BEHP) and dibutyl phthalate (DBP) had no obvious toxic effects and enabled reasonable solubility of steroids (Additional File 2).

As mentioned before, exposing Y. lipolytica cells to water immiscible organic solvents triggers changes in cell structure resulting in increased hydrophobicity [66]. Even when the organic solvent is not utilized by Y. lipolytica, morphological changes of the cells immediately become obvious. The cell suspensions become less homogenous and cell agglomerates form. It has been reported that significant changes to the cell surface (increased cell wall hydrophobicity, occurrence of protrusions and special channel like structures) are important for the cells to attach to the hydrophobic substrates as well as for the uptake of these substrates [40, 41, 66, 72, 73]. Y. lipolytica was observed to adsorb to the organic solvent droplets of DBP and ethyl oleate, similarly to the previously observed adsorption to hexadecane when cells are grown in media containing that alkane as sole carbon source [40, 41].

Based on the experiences with aqueous whole-cell bioconversions, biotransformations in biphasic systems also were performed with resting-cells. Cells were taken after 26 h of ethanol induction and resuspended in 100 mM potassium phosphate, pH 7.4, containing 1% w/v glucose to a biomass concentration of ~ 12 g/L CDW. The cell suspension was then mixed with an equal volume of the organic phase containing 20 mM of progesterone. Conversions were performed at 30°C over night while shaking. All data were calculated with respect to the aqueous phase volume. Based on other reported experiences [24] for conversion in aqueous systems steroids were added above their solubility limit as DMSO stock solution resulting in precipitation of most of the hydrophobic substrate. Surprisingly, most of the

tested water immiscible organic solvents led to a decrease in conversion rate of at least 50% compared to the pure aqueous system where just the organic solvent of the pre-solubilized substrate was added. Only ethyl oleate enabled a slightly increased conversion rate (Fig. 6).

Thus, ethyl oleate was identified as the most promising water immiscible organic solvent for the biphasic conversion of progesterone in shake flask cultures. Comparing one utilizable and one non degradable organic solvent, ethyl oleate and dibutyl phthalate [Mauersberger et al. unpublished results][74], respectively, were chosen to perform bioconversions under more controlled conditions in bioreactors.

Two-liquid phase bioreactor process

Biphasic and aqueous bioconversions of progesterone with recombinant Y. lipolytica catalysts harboring CYP3A4 and hCPR have been performed in a stirred tank bioreactor. For biomass production, a standard batch was run for 16 h (phase I) followed by a fed-batch run for 10 h at a constant growth rate of $\mu = 0.18$ /h (phase II). Subsequently CYP3A4 and hCPR production was induced by a low, linearly increasing feed of ethanol over 36 h (phase III). Samples were taken at different time points and specific 6-beta-hydroxyprogesterone formation was determined in test tubes. Interestingly, a low CYP activity was already observed during fed-batch (phase II) on glucose. This reflects a derepression of the pICL1 promoter under conditions of glucose limited growth or glucose depletion. The highest specific 6-beta-hydroxyprogesterone activity of 22 mU/g CDW was reached after roughly 12 h of ethanol-induction and remained constant during the induction time. However, the highest volumetric progesterone hydroxylation rate of 1 U/L (calculated from the specific activity) was reached after roughly 24 h of induction (Fig. 7). Cells were harvested after 36 h of induction and resuspended in glucose containing potassium phosphate buffer to a biomass concentration of ~ 25 g/L CDW. Aqueous and biphasic bioconversions in 1.5 L stirred tank reactors used the same cell suspension as starting material. Glucose at a low constant feed of 0.007 g/h/g CDW

compared to exponential feed of 1.05 g/h/g CDW during fed batch, was provided as energy source for cell maintenance and regeneration of cofactors.

While initially, i.e. during the first hour, both biphasic systems formed less hydroxyprogesterone than the aqueous system, as of 2 h of conversion the ethyl oleate based biphasic system provided the highest conversion rates (Fig. 8). However, after 6 h the product formation rate of all systems started to drop significantly. While the product formation of the aqueous system came even nearly to a halt after 6 h. The product formation of both biphasic systems continued at a reduced rate throughout the whole biotransformation experiment (Fig. 8).

In the beginning of the bioconversion of the different systems the substrate is probably taken up directly from the aqueous phase. The discrepancy in the hydroxylation rate of the different bioconversion systems in this early phase of conversion, is likely due to the difference in availability of progesterone in the aqueous phase. Indeed, 8 times and 42 times higher concentrations of ethyl oleate and dibutyl phthalate, respectively were measured in the aqueous system compared to the aqueous phases of the respective biphasic system. The available substrate concentrations in the aqueous phases stayed more or less constant during the whole bioconversion process excluding the influence of partitioning of substrate on observed variations of bioconversion rates during the conversion process. Y. lipolytica cells most probably need some time to adjust to the new hydrophobic environment. This seems to be significantly faster for the naturally occurring utilizable ethyl oleate giving much faster product formation compared to the inert non degradable dibutylphthalate.The generally lower conversion rates in biphasic systems might be explained by lower substrate concentration in the aqueous phase in presence of organic solvents.

Subsequently, the uptake of bioconversion substrate may be achieved in different ways; either Y. lipolytica cells continue to take up the low concentrations of substrate from the aqueous phase, or a direct uptake of substrate from the organic phase takes place. Although, the ethyl oleate might be cleaved by secreted lipases, the most reasonable explanation would be the co-uptake of ethyl oleate and dissolved steroid, similarly to the alkane uptake. In such a case, the conversion rate might be limited by the uptake rate of the organic phase.

Although dibuthylphtalate was shown to be a very well suitable substrate for the conversion of hydrophobic substrates in biphasic system using another alkane utilizing yeast [75], in our study dibutyl phthalate as second phase enabled significantly lower conversion rates compared to ethyl oleate.

After approximately 1.5 and 43 h, the overall product formation of both, the ethyl oleate and DBP biphasic systems, respectively, exceed the aqueous system, with an ongoing catalyst activity within the evaluated period of 100 h of bioconversion. However, it was observed that surprisingly the use of DBP as organic phase also lead to a significant change in the product profile formed from progesterone, while ethyl oleate did not. Already after 2 h there is a discrepancy in the hydroxylation pattern in the dibutyl phthalate biphasic system, compared to the aqueous and the ethyl oleate biphasic system. One might speculate that a different extent of emulsification and transport effects in different solvent systems might contribute to this effect. However no experiments were performed to proof this hypothesis. At the beginning, 6-beta-hydroxylation is dominant. However, the hydroxylase specificity is shifted to the formation of an unknown hydroxy product. After roughly 4 hours, the hydroxylase rate towards the unknown position exceeds the rate towards the 6-beta-position. At the end of the two-liquid phase bioreactor experiment the hydroxylation pattern is considerable shifted. The unknown product makes up 65% of the total product compared to only traces in aqueous system. By comparing the unknown product to in-house available hydroxyprogesterone standards 6-beta, 11-alpha, 16-alpha and 17-alpha hydroxyprogesterone, we were not able to

identify the new product. Unfortunately the concentration was not high enough to identify the product by other means e.g. NMR.

Oleate esters naturally occur in the membranes of Y. lipolytica. They are incorporated into lipids or degraded as energy source and might show less influence on cellular membranes or membrane bound proteins than dibutyl phthalate which is not degraded by Y. lipolytica. Furthermore, DBP is more similar to steroids and may enter the binding pocket of CYP3A4 which might explain the distorted hydroxylation patterns.

Conclusion

The Y. lipolytica strain H222-S4 was engineered to express the human liver CYP2D6 and CYP3A4. Co-expressing human cytochrome P450 reductase as well as increasing the copy numbers of both genes improved the catalytic performance of these whole-cell biocatalysts. However the human CYP reductase was the more efficient redox partner. Our results support previous findings, stating that not only human CYP3A4 but to some extent also CYP2D6 is able to hydroxylate steroid molecules such as testosterone, 17α-methyltestosterone and progesterone which are non-typical CYP2D6 substrates.

By using bio-degradable ethyl oleate in a two-liquid biphasic setup instead of aqueous systems, we could show that Y. lipolytica cells harboring human CYP3A4 and human CPR can be applied as a whole-cell system with increased biocatalytic stability. This might be caused by different cellular adaptation mechanisms to the organic compounds such as cell wall adaptation or by different transport mechanisms. A systems biology approach might help to clarify these issues, which was out of scope of this study. As expected the alkane-assimilating yeast Yarrowia lipolytica, coupled with above described expression strategies, demonstrated to be promising tools for biotransformations of hydrophobic substrates in two-liquid biphasic systems. Especially organic solvents which can be efficiently taken up and/or metabolized by the cell might enable more efficient bioconversion as compared to aqueous

systems and even enable simple, more stable, continuous or at least high yield long time processes.

Materials and Methods

Chemicals and reagents

Unless stated otherwise, all chemicals were purchased from BD Bioscience (USA), Sigma–Aldrich (Germany), Carl Roth GmbH (Germany), J.T. Baker (Netherlands) and Roche Diagnostics (Germany) at the highest purity available. Oligonucleotides were purchased from IDT (Belgium).

Strains, media and culture conditions

E. coli Top10 or E. coli Top10F' (Invitrogen Corp.) strains were used as host for recombinant plasmid propagation. Cells were grown on Luria-Bertani Agar (Carl Roth GmbH) supplemented with 100 µg/mL ampicillin at 37°C. DNA isolation and purification was done using Fermentas Miniprep kit or Promega Wizard SV Gel isolation and PCR cleanup kit.

In this study, the Y. lipolytica strain H222-S4 (MATA ura3-302) [76] was used as recipient strain for integration of plasmids described in Table 1. The Y. lipolytica strains were grown at 28°C on solid complete medium YPD or on minimal medium YNB (Difco, BD Biosciences, USA) supplemented with 2% w/v glucose (YNBD) and 15 g/L agar [48]. Transformants were selected on solid YNBD (15 g/l agar). Cultivation in liquid media was performed with 200 or 250 mL of buffered minimal YNB medium supplemented with 0.6% w/v glucose and 200 mM potassium phosphate buffer, pH 6.5, in 1 L Erlenmeyer flasks at 28°C.

All seed and inoculum cultures were prepared using buffered minimal medium YNB (Difco) supplemented with 1% w/v glucose and 200 mM potassium phosphate, pH 6.5. The defined mineral medium used for the main fed-batch cultures contained per liter

6.0 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.24 g K_2HPO_4, 1.4 g $MgSO_4$ x 7 H_2O, 0.6 g NaCl, 0.58 g $NaNO_3$, 0.38 g $CaCl_2$, 1 mL PTM trace element solution , i.e. 0.5 g/L H_3BO_3, 0.04 g/L $CuSO_4$

x 5 H_2O, 0.1 g/L KI, 0.303 g/L $MnSO_4$ x 1 H_2O, 0.2 g/L Na_2MoO_4 x 2 H_2O, 0.4 g/L $ZnSO_4$ x 7 H_2O, 0.2 mL of ethanolic iron chloride solution i.e. 30 g/L $FeCl_3$ x 6 H_2O, 1 mL thiamin hydrochloride solution i.e. 4 g /L and 16.5 g glucose monohydrate.

The feed solution contained 550 g glucose monohydrate per liter, 3 mL PTM trace element solution, 0.2 mL ethanolic iron chloride solution and 3 mL thiamin hydrochloride solution.

For induction of the pICL1-controlled CYP expression, pure ethanol was used as feed solution.

Genes and vectors

The integrative multicopy and single copy Y. lipolytica vectors p64ICL1, p65ICL1 [51, 77, 78] were adapted by replacing the ICL1 intron and gene with a small linker containing SpeI and AscI restriction sites and used for cloning and co-expressing CYPs and CPRs. The vector p67RYL was used to isolate the Y. lipolytica CPR gene which is essentially the wild-type gene but alanine at position 2 was chanced to proline [48, 79, 80]. pNMTS-CYP-OR-spe and pBdpTrcRed-3A4(192v)wtA305S were used to isolate codon optimized human CYP3A4 and codon optimized human CPR gene, respectively. Both were optimized for expression in yeast, i.e. hybrid optimized for P. pastoris , S. pombe and S. cerevisiae (Weis et al., unpublished).

Wild-type human CYP2D6 (ID30915411), wild-type human CYP3A4 (ID7262313) and wild-type human CPR (ID3882411) genes were isolated from cDNA clones (BioCat GmbH, Germany).

For the codon optimization of the CYP2D6 gene, the free software “Gene Designer V1.1.4.1” (DNA 2.0, USA) was used to design a gene optimized for expression in yeast, i.e. hybrid optimized for P. pastoris and Y. lipolytica, and then synthesized.

Vectors constructions

For vector construction, standard molecular biology procedures were performed [81]. The multicopy vector p64D-linker and single copy vector p65D-linker were obtained by replacing

the isocitric lyase 1 (ICL1) gene and intron with a linker containing SpeI and AscI site, in the parental vectors p64ICL1 and p65ICL1, respectively. Both vectors contain the URA3 selection marker. The p64-vector variants contain a deficient, truncated ura3d4 promoter as multicopy selection marker, which gives only sufficient amounts of gene product when several copies (at least 8-10) of the vector are integrated into the genome. This allows for selection of clones with multicopy integrations or gene multiplications. The p65-vector variants contain the ura3d1 promoter sufficient for single copy selection [47, 48].

Co-expression of single and multicopy vectors with combinations of different CPRs and cytochrome P450s were obtained by cloning overlap extension PCR products into the SpeI and AscI sites (Fig. 1). Each gene was placed under the control of the ICL1 promoter [82]. Shortly; each fragment, i.e. CPR, ICL1 promoter and terminator, CYP was amplified via PCR using primers with overhangs homologous to adjoining parts. Overlap extension PCR was done in two steps. All fragments were added to the first reaction mix PCR was run for 20 cycles using Phusion Polymerase (NEB). Then flanking primers were added to the reaction which was run for another 25 cycles to amplify the overlap construct. The fuel-length PCR construct was then cloned into pJet1.2 using the CloneJET™ PCR Cloning Kit (Fermentas) and verified by sequencing (LGC genomics, Germany). Confirmed inserts were cloned with SpeI/AscI into p64D-linker or p65D-linker, respectively, using T4 DNA ligase (fermentas) according to standard procedure.

Transformation into Y. lipolytica

Transformation of Y. lipolytica H222-S4 was performed by the lithium acetate method [38] or by electroporation according to a condensed protocol [83]. All vectors contained the rDNA (ribosomal DNA fragment) of Y. lipolytica as integration site and were digested by SacII before transformation. The transformants were selected for Ura^+ phenotype on minimal YNBD medium (2% w/v glucose). Colonies appearing after 2-3 days (single copy) and 2-3 weeks (multicopy) were transferred onto fresh plates and sub-cultured. The resulting

prototrophic recombinant Y. lipolytica strains used for CYP and CPR expression and steroid biotransformation studies and their estimated integrated vector copies are given in Table 2.

Real-time PCR (RT-PCR) was used to estimate the copy number of the integrated expression cassettes. Genomic DNA was isolated from Y. lipolytica transformants grown overnight in minimal YNB medium supplemented with 1% w/v glucose (YNBD) [84]. The ICL1 set of primers, ICL1-fw (5′-CCA GCA GCC CGA GAT TGA-3′) and ICL1-rv (5′-ACT CAG CAC CGG ACC ACT TC-3′), anneal to the single copy of the endogenous Y. lipolytica ICL1 gene within the chromosome. The Amp primers, Amp-fw (5′-GCT ATG TGG CGC GGT ATT ATC-3′) and Amp-rv (5′-GTA TGC GGC GAC CGA GTT-3′), target the amp^R marker gene present on the integrated vectors. Y. lipolytica YL23 was used as a control organism with a single copy of both the ICL1 and amp^R target sequences. Reaction mixes of 18 µl consisted of 100 pg template DNA, Power SYBR Green Master Mix (Applied Biosystems, CA, USA), and 250 nM of each primer. Each reaction was run in duplicate in an ABI PRISM 7300 Real Time PCR machine (Applied Biosystems, CA, USA). The profile used was, 95°C for 10 min, fallowed by 40 cycles of (95°C for 15 s, 60°C for 60 s). Data collection was done after each 60°C step. A melting curve analysis was conducted after the amplification, heating from 45°C to 95°C. Analysis was done using Sequence Detection Software SDS (Applied Biosystems, version 1.2). Average Ct values of the 2 profiles (ICL1 and Amp) were used to estimate the relative copy number for the selected transformant [85].

CPR and CYP induction

Y. lipolytica clones were grown in YNBG (1% w/v glucose) overnight at 28°C and 220 rpm. Exponentially growing cells were taken to inoculate the YNBG (0.6% w/v glucose) main culture to a starting OD_{600} of ~ 0.5. The main culture was grown at 28°C and 220 rpm for 17 – 20 h until glucose was fully consumed. After additional 2-4 h, the expression of CPR and CYP under the control of the ICL1 promoter was induced by adding ethanol to a final concentration of 1% v/v. Additional 1% v/v ethanol was added after ~ 8 h and ~ 20 h. After 24 h the cells were harvested by centrifugation and further used for either whole-cell biotransformation assays or microsome isolation.

Isolation of microsomes

Yeast cells were harvested by centrifugation at 2 000 g for 10 min and washed twice with water. 4-6 g of cells were resuspended in ~ 20 ml disruption buffer (50 mM potassium phosphate, pH 7.9, containing protease inhibitor 1 mM PMSF, 5% w/w glycerol, 1mM EDTA and 2 mM DTT). A crude cell lysate was obtained by mechanical cell disruption using "Merkenschlager homogenization" [86]. After cell disruption cell debris was removed by centrifugation at 10 000 g and 4°C for 10 min. To pellet the microsomal fraction, the supernatant was centrifuged at 100 000 g and 4°C for 1 h. The membrane pellet was resuspended in disruption buffer to ~ 1 mg pellet per mL using a Dounce homogenizer and pestle.

SDS-PAGE and Western blot analyses

Total protein content of the microsomal preparation was determined by Bradford using the biorad protein assay kit (Bio-Rad, Germany) before separating the proteins by using the NuPAGE® electrophoresis system (Invitrogen Ltd). Samples containing ~ 20 µg total protein in 15- 25 µL loading buffer without reducing agent were incubated at RT for at least 10 min. For separation, a NuPAGE Novex 4-12% Bis-Tris-Gel and MOPS buffer were used. The

PageRuler Prestained Protein Ladder (Fermentas) was used as molecular mass calibration standard.

Western blotting was done according to the protocol provided with the MAB-2D6 and WB-3A4 kits (BD Gentest™). On completion of PAGE, the proteins were transferred electrophoretically onto nitrocellulose membrane (GE Healthcare Europe GmbH) in a wet blotting system. Then the membranes were blocked at room temperature overnight with whey powder. The blot was developed by incubation with NBT/BCIP at RT for 5 min.

NADPH cytochrome c reductase (NCR) activity

The cytochrome P450 reductase-catalysed reduction of bovine heart cytochrome c was measured at 550 nm essentially as described [70]. A 300 µM cytochrome c solution in 50 mM Tris-HCl buffer, pH 7.5, was mixed with 2 to 80 µg of microsomal protein and made up with Tris-HCl to 650 µL. Fifty µL of 50 mM KCN solution, pH 7.7, were added to mask cytochrome c oxidase activity. Reaction was started by adding 50 µL of 1.5 mM NADPH. Activities were measured on a UV/Vis DU 800 spectrophotometer (Beckman Coulter, USA) and calculated by using ε_{550} = 21 mM^{-1} cm^{-1} as molar extinction coefficient of cytochrome c.

Quantification of cytochrome P450

CYP concentrations in the isolated microsomes were determined by reduced carbon monoxide spectra [87]. Six to 12 mg of microsomal protein were added to 100 mM sodium phospate buffer, pH 7.4, containing 20% w/w glycerol to an end volume of 2 mL. One hundred µL 200 mM KCN, pH 7.7, were added to mask the spectral interference of cytochrome oxidase (negative absorption at 445 nm) with the CYP peak at 450 nm in the CO difference spectrum [49]. A few grains sodium dithionite were added to reduce the CYP. The mixture was transferred into polystyrene cuvettes (Sarstedt, Germany) and a reference spectrum was recorded from 400 to 500 nm (Specord 205 UV/Visible spectrophotometer,

Analytik Jena, Germany). The mixture was then bubbled with carbon monoxide for 60 s, and the spectrum was measured repeatedly several times.

The CYP concentration was calculated using a molar extinction coefficient of $\varepsilon_{450nm} = 91\ mM^{-1}\ cm^{-1}$.

CYP activity of microsomes and whole-cells

In vitro substrate conversion with microsomes was performed essentially as described in [88]. Twenty five µM bufuralol or 2 mM steroid (in DMSO) e.g. progesterone, 17alpha-methyltestosterone or testosterone, and 1 mM NADPH were added to 100 mM possium phosphate buffer, pH 7.4, and pre-incubated for 2-3 min at 37°C. The reaction was started by adding 20 µL microsomal preparation (0.6 to 0.8 mg total protein) to give a total volume of 200 µL. The reaction mixture was incubated at 37°C for 20 min (bufuralol) or 1 h (steroid) while shaking and stopped by adding 20 µL 70% perchloric acid. After 20 min of incubation on ice, prednisolone (in DMSO) was added to a final concentration of 50 µM as internal standard and the mixture centrifuged for 10 min at 16 100 g. One hundred µL supernatant were transferred to a fresh microtiter plate and stored at -20°C.

For whole-cell conversions, yeast cultures were spun at 2000 g for 15 min and the cell pellets were resuspended in 100 mM potassium phosphate buffer, pH 7.4, containing 1% w/v glucose to a biomass concentration of ~ 10 g/L CDW. Stock solutions of substrate were added to 200 (eppendorf tube) or 1000 µL (PYREX tubes) of cell suspension. Bufuralol was added as 1 mM stock solution to cell suspension yielding a final concentration of 25 µM. Steroid e.g. progesterone, 17alpha-methyltestosterone or testosterone was added as 100 mM stock solution in DMSO to cell suspension yielding a final concentration of 2 mM. Whole-cell conversion of bufuralol and progesterone was performed at 30°C for 20 – 60 min or several hours while shaking, respectively. Then prednisolone (in DMSO) was added to a final concentration of 50 µM as internal and the reaction was stopped by centrifugation at 16 100 g for 10 min. Supernatants were transferred to fresh reaction tubes and stored at -20°C.

CYP activity in whole-cell biphasic systems

Twenty to fifty mL yeast cultures were centrifuged at 2 000 g for 15 min and the pellet was resuspended either in induction media supplemented with 1% v/v ethanol or in 100 mM potassium phosphate buffer, pH 7.4, containing 1% w/v glucose to a biomass concentration of ~ 10 g/L CDW. An equal volume of organic solvent containing 20 mM progesterone, was added to 1000 µL of cell suspension. The biphasic whole-cell conversions were performed in 10 mL PYREX tubes at 30°C while shaking at 320 rpm for several hours. Reactions were stopped at desired time points and the two phases were separated by centrifugation at 16 100 g for 10 min. Two to five hundred µL aqueous and organic phase were transferred to fresh reaction tube or GC-vial, respectively. Prednisolone (in DMSO) was added to a final concentration of 50 µM as internal standard. An equal volume of isopropanol was added to the organic phase, and samples stored at -20°C.

Fed-batch cultivation in bioreactors

Cultivation process was performed in a 5 L stirred tank bioreactor (Biostat C, Sartorius, Germany) The initial batch working volume was 3.5 L (minimal mineral salt medium M with initial 1.5% w/v glucose) and was increased to 4 L at the time of harvesting. The set-points of all control variables were maintained during the entire process, thus, the cultivations were accomplished under the conditions of constant temperature of 28°C, 10 L/min air flow (i.e. without any oxygen enrichment), 1500 rpm agitation, pH 5.5. Automated control of the pH was achieved by using 25% ammonia and 25% phosphoric acid solutions. The process consisted of the biomass growth phase on glucose, i.e. batch and exponential fed-batch cultures, and the expression phase on ethanol with linear feed addition. The cultivation started at time 0 h with a batch (1.5% w/v glucose) during which the pO_2 value continuously decreased and the base consumption increased, i.e. no control of the pO_2 set-point was applied. After 14–16 h, the pO_2 increased rapidly and base consumption stopped due to glucose depletion. At this point the exponential addition of glucose feed solution was started

and continued over 10 h according to the function $f(t)=1.05*e^{0.18*t}$ in grams of glucose per hour. During the subsequent production phase, 0.5% v/v ethanol was maintained for 34-36 h in accordance with the function $f(t)=1.05*e^{0.002*t}$ in grams of ethanol per hour. Biomass concentration was determined by measuring cell dry weight (CDW) e.g. 2 mL cell suspension was centrifuged at 16 000 g for 10 min. Supernatant was discarded and cell pellet dried at 100 °C till constant weight. Cells where harvested by centrifugation at RT and 2 000 g for 15 min and cell pellets were resuspended in 100 mM potassium phosphate buffer, pH 7.4, supplemented with 0.5% w/v glucose as energy source to a desired biomass concentration of ~ 25 g/L CDW.

Resting-cell biotransformation in bioreactor

Biotransformations were performed in a 1.5 L stirred tank bioreactor (DASGIP Parallel Bioreactor Systems, DASGIP BioTools, Germany). The batch working volume was 600 mL (phosphate buffer with glucose and ethanol-induced Y. lipolytica cells as indicated above) for aqueous phase system and 450 mL aqueous phase plus 150 mL organic phase for biphasic systems. The set-points of all control variables were maintained during the entire biotransformation at 30°C, 1 NL/min airflow (i.e. without any oxygen enrichment), 500 rpm agitation and pH 7.4.

The transformations began at 0 h by adding 100 mM progesterone stock solution (in DMSO) to a final concentration of 2 mM or 150 mL water immiscible organic solvent containing 20 mM progesterone. Glucose was provided at a low constant rate of 0.17 g h^{-1} over the entire biotransformation time of 90 hour. Samples were taken at different time points and the reaction was stopped removing the cells by centrifugation at 16 100 g for 10 min. Aqueous and organic phase samples were withdrawn as described above.

Analysis by HPLC-MS

Bufuralol and metabolites were separated by HPLC (1200 series, Agilent technologies, USA) with a XDB-C18, 1.8 µm, 4.6 x 50 mm column (Agilent technologies, USA) using a gradient based on 10 mM ammonium acetate, pH 5.5, and acetonitrile at a flow rate of 0.9 mL/min. Metabolites were detected using MSD SL detector equipped with an electron spray ionization (ESI) unit (Agilent technologies, USA).

Hydroxylated products were quantified by external calibration using reference metabolites or metabolite derivates.

Steroid metabolites were separated by HPLC (1200 series, Agilent technologies, USA) with a Chromolith RP-C18e, 5 µm, 4.6 x 100 mm column (MERCK KGaA, Germany) using a gradient based on water and acetonitrile, both acidified with 0.1% v/v acetic acid, at a flow rate of 1 mL/min.

List of abbreviations:

Yl, Yarrowia lipolytica; H, human; CPR, cytochrome P450 reductase; CYP, cytochrome P450; DBP, dibuthylphtalat; EO, ethyl oleate; CDW, cell dry weight; NCR, NADPH cytochrome c reductase; ICL1, isocitric lyase 1; sc, single copy; mc, multicopy.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

AG, SM, and AG drafted the outline of the expression experiments. BB, AS and AG drafted the outline of the two-phase system and fermentation experiments. SM helped with the selection of expression vectors, strains and cultivation conditions. AB and GM carried out the experiments and analyzed the data. AB wrote the paper which was later revised and corrected by AG and BB. All authors read and approved the final manuscript.

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Figures

Fig. 1: Schematic representation of construction of the co-expression vector

Construction of multicopy coexpression vectors by inserting overlap extension PCR fragments into the multicopy integrative vector p64D-linker containing the multicopy selective *ura3d4* marker.

Fig. 2: CYP activity and *Y. lipolytica* biomass production during induction

CYP2D6 (1'-hydroxybufuralol formation, A)and CYP3A4 (6β-hydroxyprogesterone formation, B) activity during ethanol induction of *Y. lipolytica* strains YL21 and YL22, respectively. The cultures were incubated at 28°C and induced by ethanol after glucose depletion. The Roman numerals indicate the following process phases: I) batch growth with glucose; II) induction phase of about 70 h. Squares highlight biomass concentrations determined as cell dry weight, while bars document CYP expression levels measured as specific whole-cell activity. Experiments were performed in triplicates. Samples were taken after 4,8,21,26,31,45,50,55 and 69 h.

Fig. 3: Western blot analysis

Western blot analysis of microsomes isolated after 26 h of ethanol induction from cultures of *Y. lipolytica* H222-S4 transformed with p64D- or p65D-based integrative vectors, containing different combinations of CYPs and CPRs. Protein transfer was monitored by Ponceau S (*) staining, where putative CPR-protein bands in multicopy transformants were already visible. CYP-protein bands CYP2D6 (A) and CYP3A4 (B) were immunodetected with anti CYP2D6 and CYP3A4 antibodies, respectively.– CYP2D6 was detected in single copy clones (sc) YL12 and YL15 expressing YlCPR-WT + 2D6syn (1), hCPR-WT + 2D6syn (2) respectively and multicopy clones (mc) YL11, YL10 and YL21 expressing YlCPR-WT + 2D6syn (5), hCPRsyn + 2D6syn (6), hCPR-WT + 2D6syn (7) respectively. CYP3A4 was detected in single copy clones

(sc) YL19 and YL20 expressing YlCPR-WT + 3A4syn (1), hCPR-WT + 3A4syn (2) respectively and multicopy clones (mc) YL18 and YL22 expressing YlCPR-WT + 3A4syn (5), hCPR-WT + 3A4syn (6) respectively. YL23 clone harboring empty p65D-linker vector (3+4 in both panels) was used as negative control. PageRuler Prestained Protein Ladder (M) was used for monitoring protein separation and transfer efficiency.

Fig. 4: Influence of copy number and type of reductase on CYP activity.

CYP2D6 (A) and CYP3A4 (B) activity of and CPR activities of *Y. lipolytica single-copy and multicopy* transformants. Strains coexpressed under pICL1-control the codon-optimized P450 2D6syn (A) or P450 3A4syn (B) in combination with the wild-type human or *Y. lipolytica* NADPH-P450 reductases (hCPRwt, YlCPR) from integrated single-copies or multiple vector copies as indicated, except the control strain YL23 on the left in A and B, containing only one endogenous chromosomal YlCPR copy, which is present as background in all strains. Cultivation at 28°C in YNBG on glucose, induction by 1% ethanol, microsomes isolation, whole-cell CYP activity and cytochrome P450 reductase (CPR) activity (determined as NADPH-cytochrome c reductase, NCR) measurements were performed as described in Materials and Methods. Filled bars, CPR activity; hatched bars, CYP activities; -, no genes; + genes in single copy; ++ genes in multicopy

Fig. 5: Whole-cell hydroxylation of different steroids

Whole-cell hydroxylation of different steroids in aqueous phase system, employing *Y. lipolytica* YL21 and YL22 cells harboring CYP2D6 (filled bars) or CYP3A4 (striped bars), respectively. Data is shown as activity relative to the highest measured apparent hydroxylation rate of testosterone (3.9 ± 0.1 mU per g CDW). Compounds were converted by cells equivalent to ~ 10 g/L CDW for 16 hours. Activity was

determined in test tubes as product formation rate after adding steroids dissolved in DMSO to a final concentration of 2 mM.

Fig. 6: Whole-cell conversions of *Y. lipolytica* in biphasic conditions.

Comparison of whole-cell conversion with resting *Y. lipolytica* YL22 cells harboring CYP3A4 and hCPR in aqueous (striped bar) and biphasic systems (filled bars) using different organic solvents as 2nd phase. Activity was determined as product formation rate.

Fig. 7: Biomass and CYP3A4 activity during cultivation of *Y. lipolytica* YL22 in bioreactor.

Time course of biomass concentration and volumetric whole-cell CYP3A4 activity determined during fed-batch cultivation and ethanol induction. Open symbols, biomass concentration determined as cell dry weight; closed symbol, CYP3A4 expression levels measured as volumetric whole-cell activity of 6beta-hydroxyprogesterone formation determined by separate activity assays in test tubes. The Roman numerals show the following process phases: I) batch growth with glucose; II) fed-batch growth over 10 h with exponential addition of glucose at an exponentially increasing feed rate; III) induction phase of about 36 h with a low linear addition of ethanol.

Fig. 8: Hydroxyprogesterone formation during whole-cell conversion in bioreactor.

Time course of hydroxyprogesterone formation during whole-cell conversion of progesterone by *Y. lipolytica* YL22 cells harboring CYP3A4 and hCPR. Closed triangles: aqueous phase system; open circles: with dibutylphthalate (DBP) as second organic phase; open squares: with ethyl oleate as second organic phase.

Tables

Table 1: Plasmids used

Plasmid	Description	Marker gene (selection in yeast)	Reference
p64ICL1	Parental vector for multicopy integration in Y. lipolytica	ura3d4 (mc)[a]	[51]
p65ICL1	Parental vector for single copy integration in Y. lipolytica	ura3d1 (sc) [a]	
p67RYL	Source for Y. lipolytica cytochrome P450 reductase (YlCPRwt)	ura3d4 (mc)[a]	[22, 49]
pNMTS-CYP-OR-spe	Source for codon optimized human cytochrome P450 3A4 (3A4syn)		Weis et al.,
pBdpTrcRed-3A4(192v)wtA305S	Source for codon optimized human cytochrome P450 reductase (hCPRsyn)		unpublished (IMBT, TU Graz)
ID7262313	Source for cDNA wild-type human cytochrome P450 reductase (hCPRwt)		BioCat, Germany
Cloning vectors:			
pJet1.2	E. coli cloning vector		Fermentas, Germany
p64D-linker	Cloning and basic integrative multicopy vector	ura3d4 (mc) [a]	This work
p65D-linker	Cloning and basic integrative single copy vector, negative control	ura3d1 (sc) [a]	
Integrative vectors for CPR and CYP expression in Y. lipolytica:			
p64D-hCPRwt-2D6syn p64D-hCPRsyn-2D6syn p64D-hCPRwt-3A4syn p64D-YlCPR-2D6syn p64D-YlCPR-3A4syn	Multicopy coexpression of human wild-type CPR (hCPRwt), codon optimized CPR (hCPRsyn) or Y. lipolytica-own CPR (YlCPR) in combination with codon optimized CYP2D6 or codon optimized CYP3A4, respectively.	ura3d4 (mc) [a]	This work
p65D-hCPRwt-2D6syn p65D-hCPRwt-3A4syn p65D-YlCPR-2D6syn p65D-YlCPR-3A4syn	Single copy coexpression of human wild-type CPR (hCPRwt) or Y. lipolytica-onw CPR (YlCPR) in combination with codon optimized CYP2D6 or codon optimized CYP3A4, respectively.	ura3d4 (mc) [a]	This work

[a] URA3 marker genes for single copy (sc, ura3d1) or multicopy (mc, ura3d4) selection with different promoter lengths

Table 2: Used Strains and their expression plasmid copy number determined by RT-PCR

Strains	Description	Copy number	Reference
H222-S4	MATA ura3-302[a] (Ura$^-$, Alk$^+$, Tgl$^+$, Lip$^+$, Eth$^+$, Glu$^+$,Suc$^+$)		[76]
YL23	H222-S4 transformed with SacII – linearized p65D-linker plasmid, negative control		This work
YL21	H222-S4 transformed with SacII - linearized p64D-hCPRwt-2D6syn plasmid	46 ± 4	This work
YL10	H222-S4 transformed with SacII - linearized p64D-hCPRsyn-2D6syn plasmid	30 ± 3	
YL22	H222-S4 transformed with SacII - linearized p64D-hCPRwt-3A4syn plasmid	23 ± 2	
YL11	H222-S4 transformed with SacII - linearized p64D-YlCPR-2D6syn plasmid	26 ± 2	
YL18	H222-S4 transformed with SacII - linearized p64D-YlCPR-3A4syn plasmid	10 ± 1 20 ± 3	
YL15	H222-S4 transformed with SacII - linearized p65D-hCPRwt-2D6syn plasmid	0.9 ± 0.1	This work
YL20	H222-S4 transformed with SacII - linearized p65D-hCPRwt-3A4syn plasmid	1.1 ± 0.1	
YL12	H222-S4 transformed with SacII - linearized p65D-YlCPR-2D6syn plasmid	1.1 ± 0.1	
YL19	H222-S4 transformed with SacII - linearized p65D-YlCPR-3A4syn plasmid	1.1 ± 0.1	

[a] ura3-302: URA3 disrupted by a construct pXPR2 SUC2 (invertase gene from S. cerevisiae). This allele confers the ability to grow on sucrose or mollasses [76, 77]

supplementary data

File name: Additional file 1

File format: pdf

Title: Influence of growth phase on whole-cell conversion rates

Description: Comparing Diagrams of whole-cell conversions in aqueous systems by growing and resting cells of Y. lipolytica harboring CYP2D6 or CYP3A4, respectively

File name: Additional file 2

File format: pdf

Title: Solubility of progesterone in organic solvents.

Description: Solubility of progesterone in the different organic solvents shown as a diagram

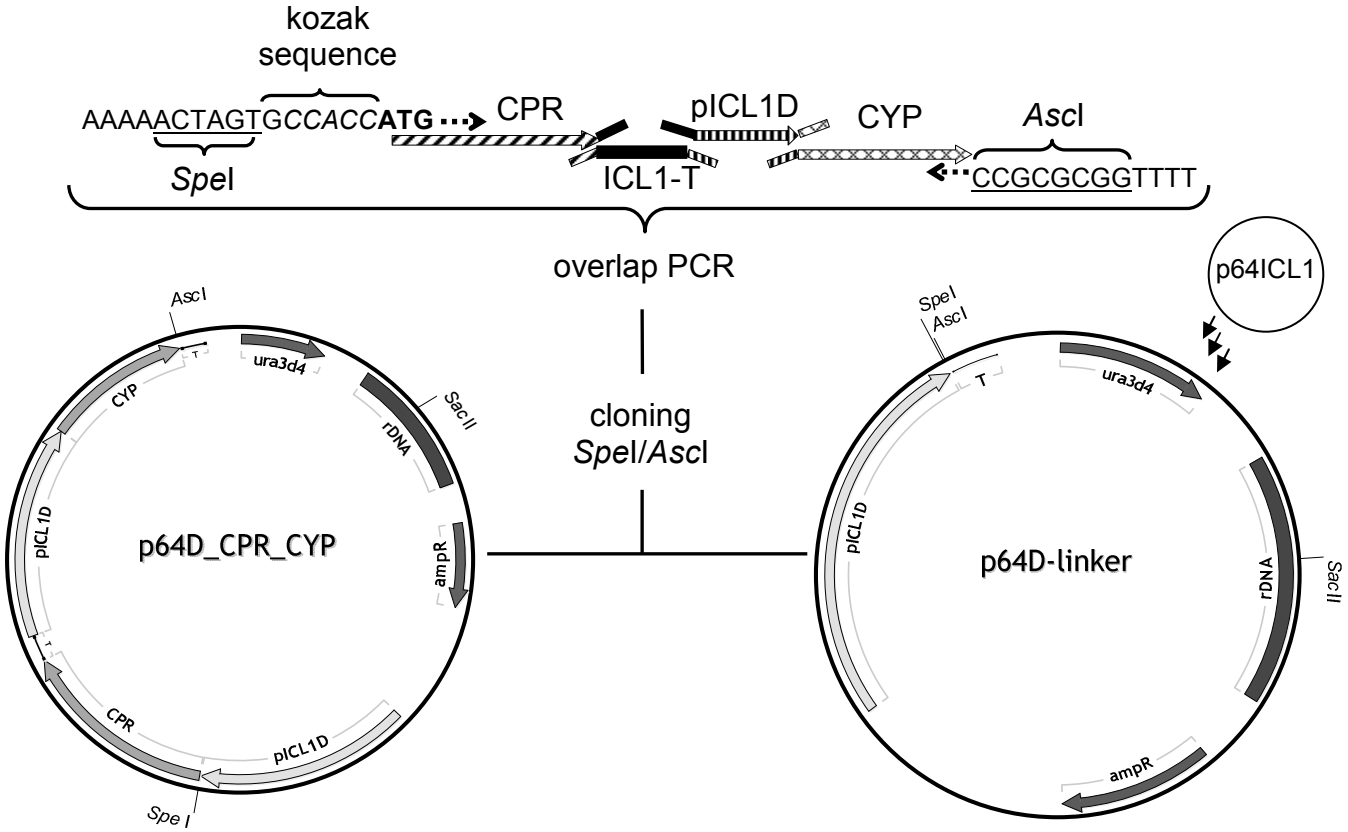

kozak
sequence
AAAAACTAGTGCCACCATG
SpeI
CPR
pICL1D
ICL1-T
CYP
AscI
CCGCGCGGTTTT
overlap PCR
p64ICL1
cloning
SpeI/AscI
p64D_CPR_CYP
p64D-linker
ura3d4
rDNA
SacII
ampR
pICL1D
CYP
CPR
T

Figure 1

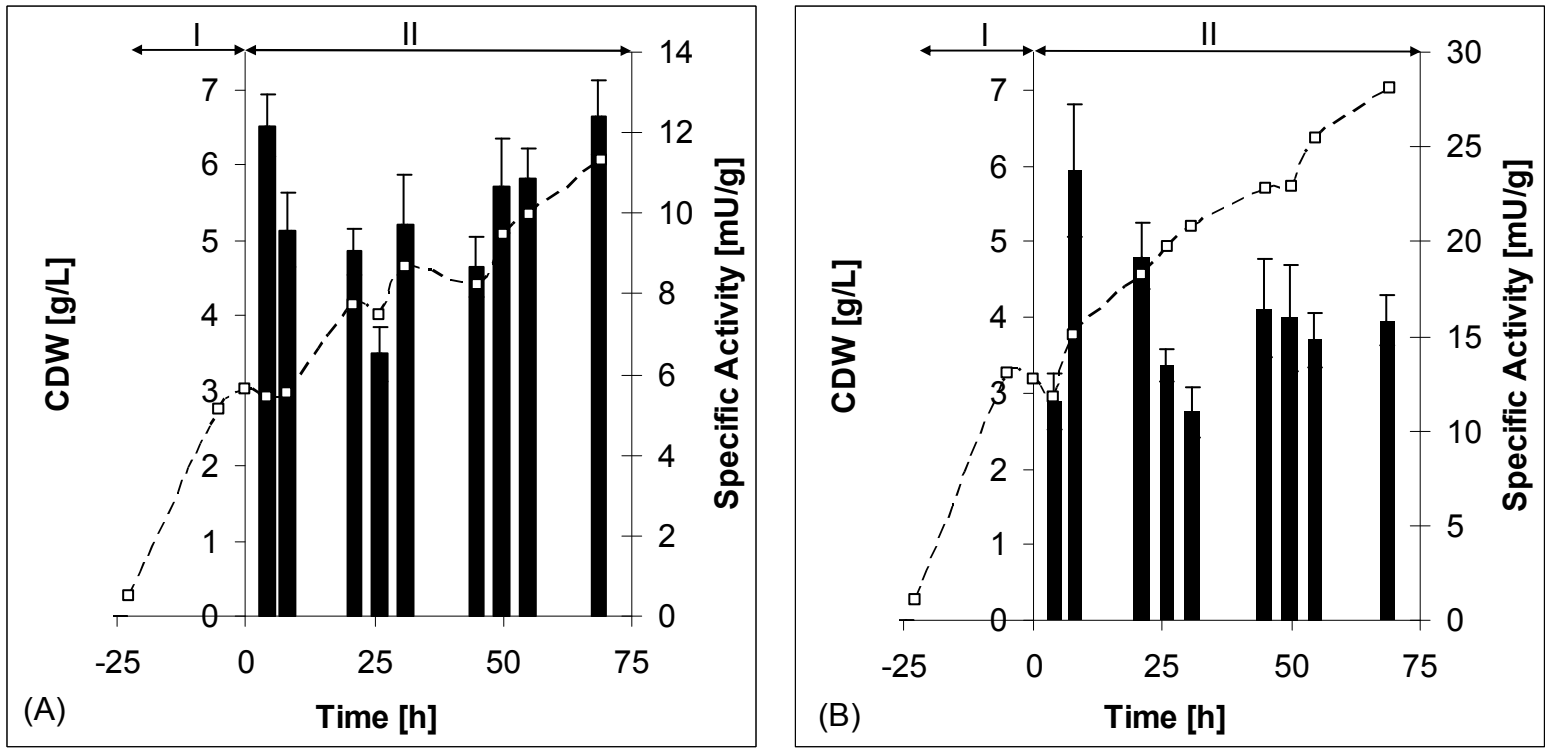


Figure 2

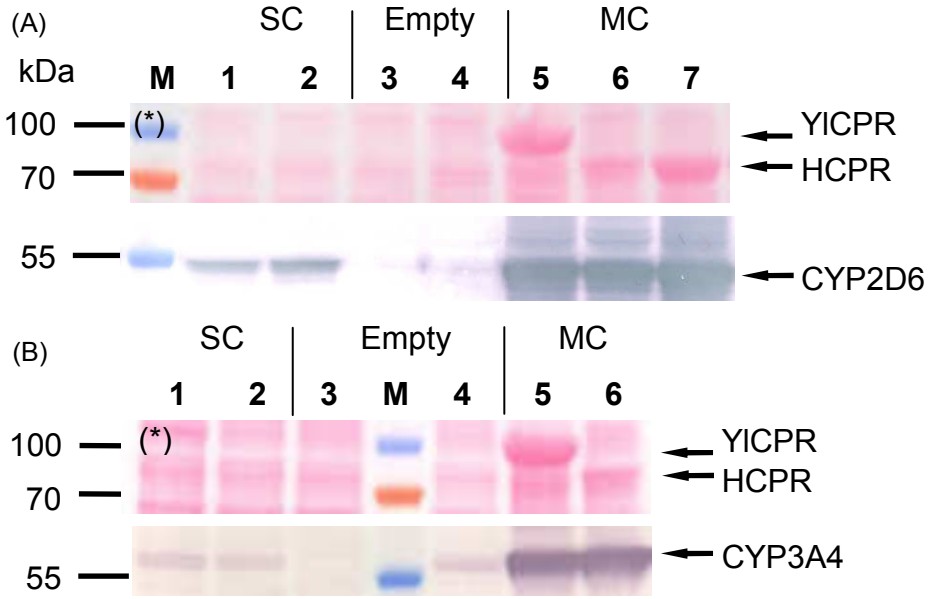

(A)
SC
Empty
MC
kDa
M
1
2
3
4
5
6
7
100
70
55
(*)
YICPR
HCPR
CYP2D6
(B)
SC
Empty
MC
1
2
3
M
4
5
6
100
70
55
(*)
YICPR
HCPR
CYP3A4

Figure 3

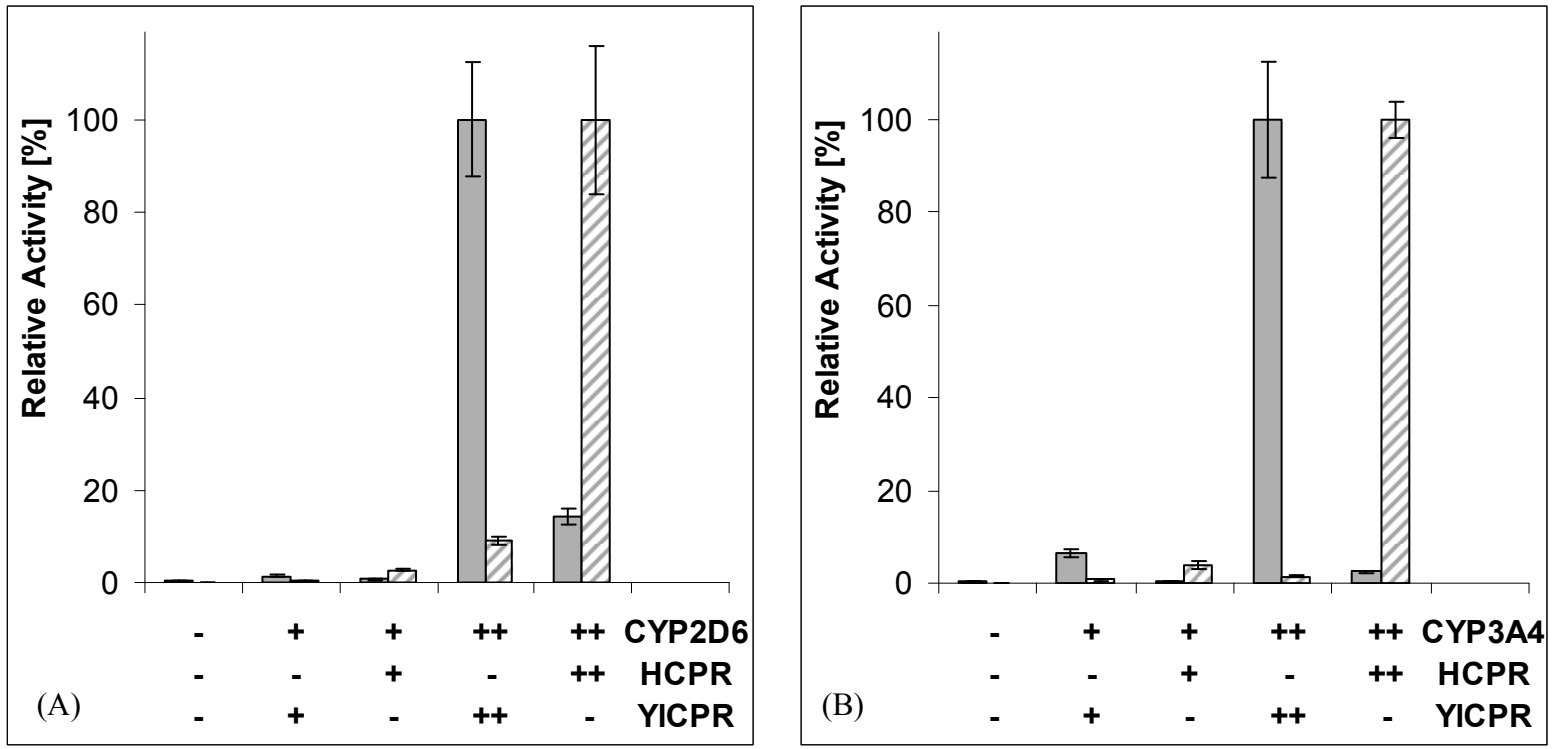

Relative Activity [%]
100
80
60
40
20
0
- + + ++ ++ CYP2D6
- - + - ++ HCPR
- + - ++ - YICPR
(A)
Relative Activity [%]
100
80
60
40
20
0
- + + ++ ++ CYP3A4
- - + - ++ HCPR
- + - ++ - YICPR
(B)

Figure 4

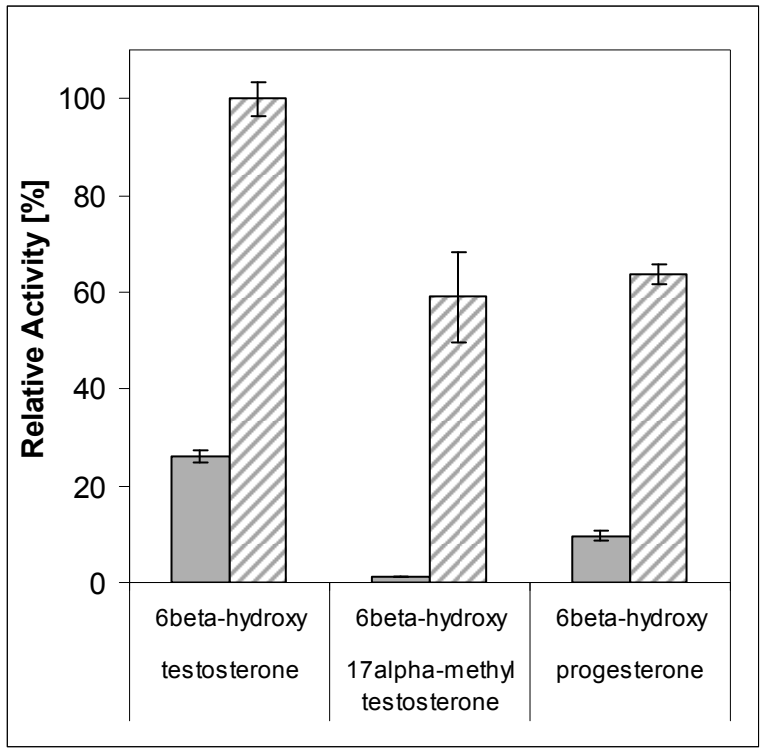

Relative Activity [%]
100
80
60
40
20
0
6beta-hydroxy testosterone
6beta-hydroxy 17alpha-methyl testosterone
6beta-hydroxy progesterone

Figure 5

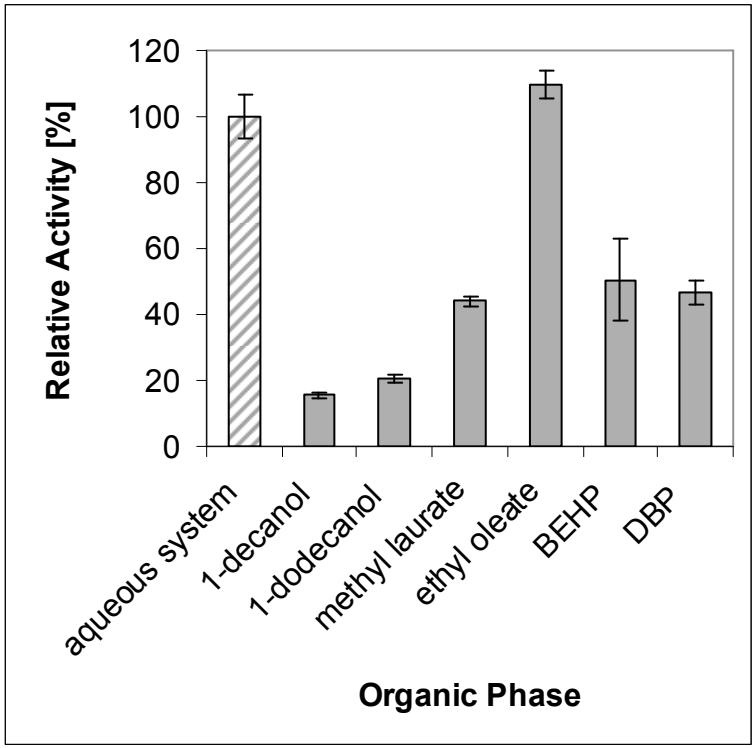

Relative Activity [%]
120
100
80
60
40
20
0
aqueous system
1-decanol
1-dodecanol
methyl laurate
ethyl oleate
BEHP
DBP
Organic Phase

Figure 6

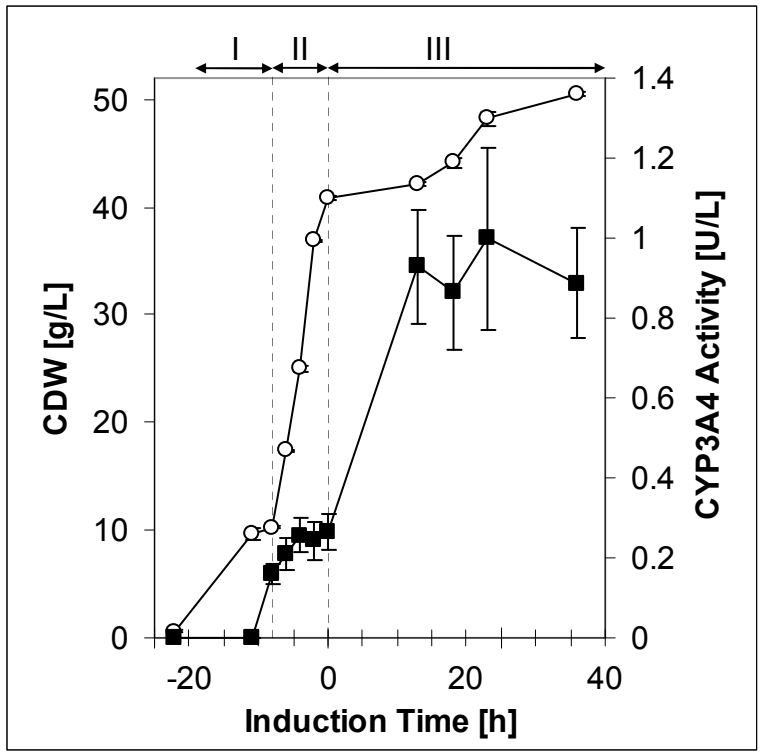

I
II
III
CDW [g/L]
CYP3A4 Activity [U/L]
Induction Time [h]
0
10
20
30
40
50
0
0.2
0.4
0.6
0.8
1
1.2
1.4
-20
0
20
40

Figure 7

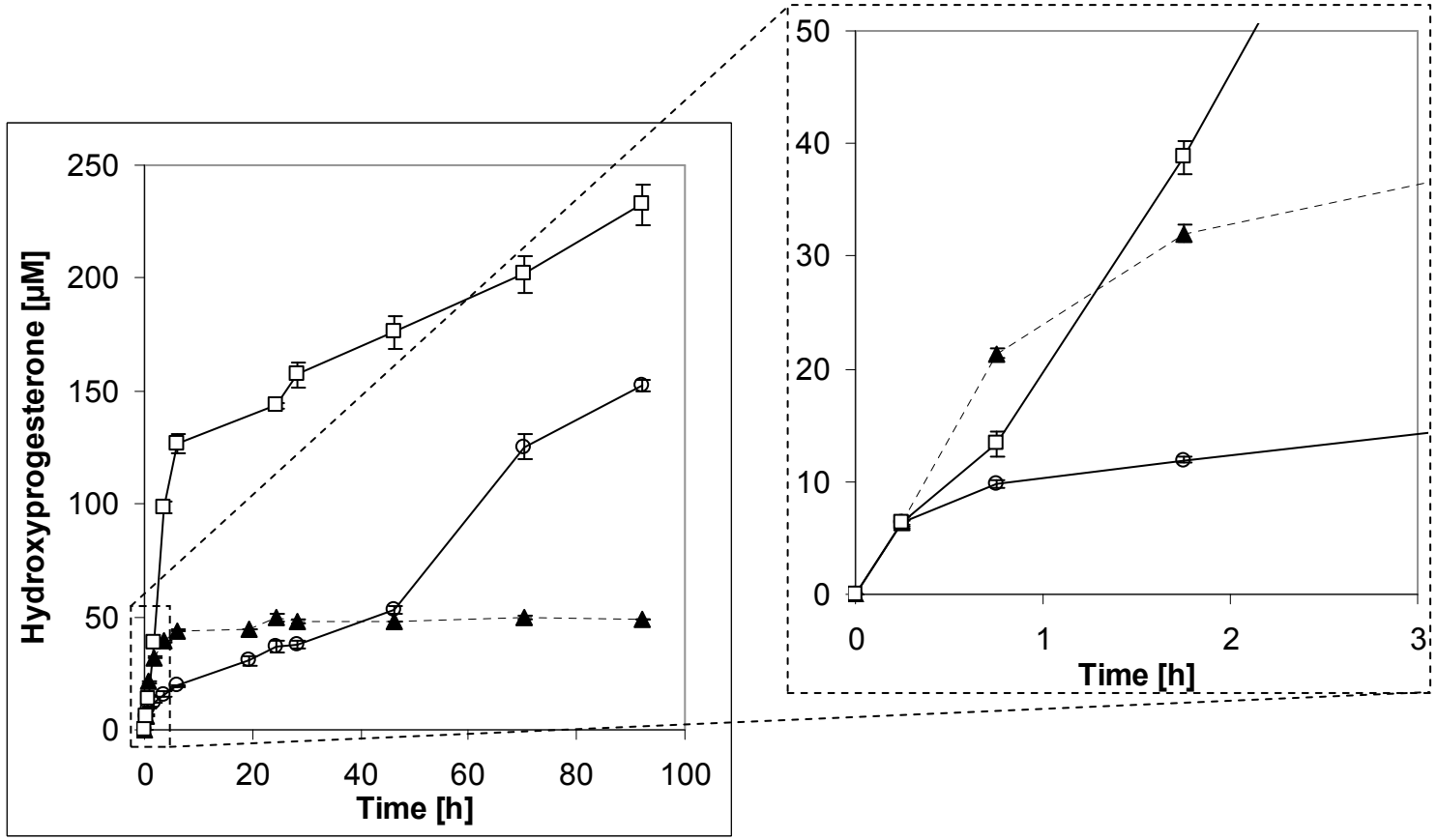

Hydroxyprogesterone [µM]
250
200
150
100
50
0
0
20
40
60
80
100
Time [h]
50
40
30
20
10
0
0
1
2
3
Time [h]

Figure 8

Supplementary data 3

SOP I (HPLC-MS)

MT-HPCL-MS whole cell screening system for P450 mediated steroid hydroxylation

ACIB Protocol

MT-HPCL-MS whole cell screening system for P450 mediated steroid hydroxylation

Author: **Braun Andreas**
andreas.braun@tugraz.at

Creation date: December. 2011 Validity: until revoked/cancelled This ACIB protocol replaces the version from: Developed in project/working group: EU-Project Oxygreen		
Notice of modification:		
Written by: Andreas Braun		
Date/signature:		
Checked: Martina Geier	Yes ☐ No ☐	Date/Signature
Approved by:		

1. Purpose and Field of Application

A protocol for whole cell screening of heterologous expressed P450 mutein libraries for their hydroxylation activity towards steroids and resulting hydroxylation patterns.

This method can be used to screen mutein libraries of membrane bound P450 enzymes expressed in *Pichia pastoris*, which show activities towards testosterone. The substrate conversion is meant to be done with resting, whole cells to circumvent cumbersome cell disruption which often leads to activity loss of the P450 enzyme system due to reduced stability.

This is a medium throughput HPLC-MS screening method and therefore shows reliable but preliminary results. Using this screening system one can determine activity trends and also trends in the hydroxylation pattern.

The method is not limited to *Pichia pastoris*. Any expression host that is able to functionally express membrane bound P450 enzymes and can be cultivated in 96 Deep-well plates can be applied. For other host the cultivation and induction procedure in 96 Deep-well plates have to be adjusted.

Furthermore, besides testosterone this system is also viable for the screening of other steroids. For other steroids the MS signals have to be adjusted. Additionally, in some cases one will have to adjust the mobile phases for optimal separation via HPLC.

2. Principle

A gene library is transformed into the host organism and positive transformants are transferred to 96 Deep-well plates. Each Deep-well plate contains positive and negative controls to which product formation is corresponded to. Cultivation and substrate conversion is performed in the 96 Deep-well plates. Products formed during the whole cell conversion are analyzed by HT-HPLC-MS in the resting cell culture supernatant. The conversion times are adjusted so that the product concentrations are below the solubility limits.

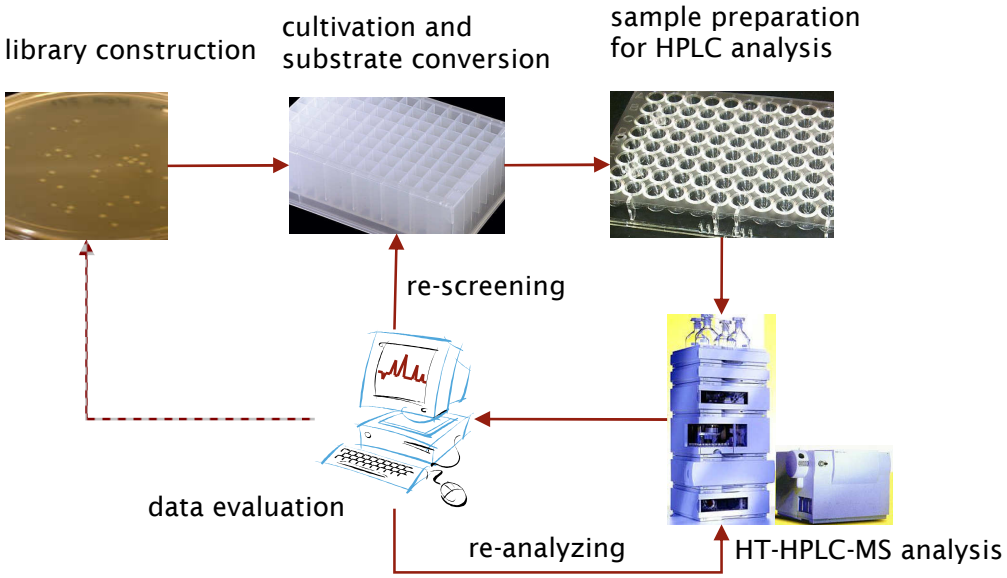


Figure 1: Principle workflow of a HPLC-MS screening system.
P450 mutein library is constructed by using standard genetic methods (Sambrook et al., 2001). Strains are cultivated and muteins are expressed in 96 Deep-well plates (Weis et al., 2004). Cultivation media is replaced by conversion buffer and the conversion started by adding of substrate. After the desired reaction time, internal standard (IS) is added to each well and cells are removed by centrifugation. Supernatant is transferred to 96 microtiter plates and sealed with sealing tape. Samples are analyzed by HT-HPLC-MS. After data evaluation, interesting samples can be re-analyzed. Positive hits showing desired results can be used for a re-screen and also as a staring point for the next round of library construction.

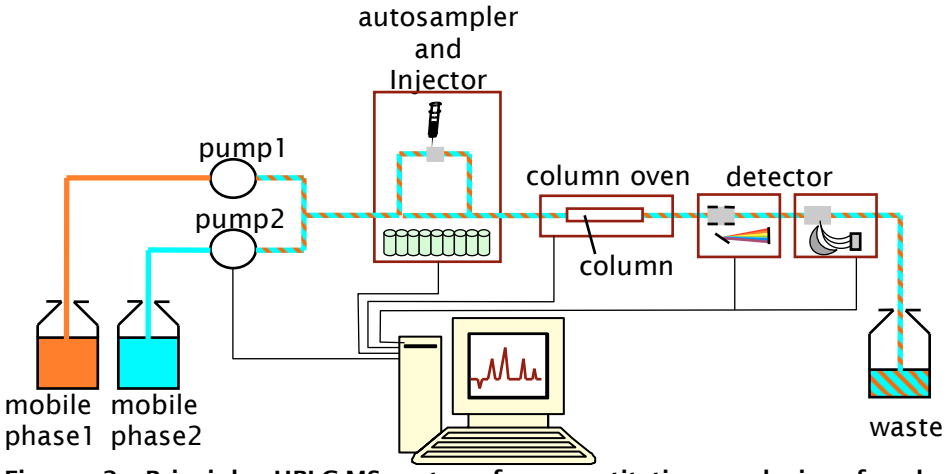


Figure 2: Principle HPLC-MS setup for quantitative analysis of substrate and products. With high pressure a mobile phase is constantly pushed trough a tightly packed column. The analysis sample is injected into the mobile phase stream and substrate and products are separated by interaction with the packing material of the column. After separation, individual chemical compounds are detected by UV/VIS and MS. Detected peaks are used for identification and quantification. The peak area is proportional to the concentration (Dong, 2006).

3. Key Words, Definitions & Abbreviations

Key words: HPLC-MS, hydroxylation pattern, cytochrome P450, mutein, *Pichia pastoris*, whole cell screening, resting cells

Abbreviation	Description
ACN	acetonitril
CDW	cell dry weight
ddH2O	double distilled H2O
ESI	electrospray ionization
FA	formic acid
HPLC	high pressure liquide chromatography
HT	high throughput
MS	mass spectroscopy
MT	medium throughput
OD600	optical density at 600 nm
RT	room temperature
UV	ultra violet
VIS	visibile

4. Methodology

4.1. Reagents

List of the chemicals needed

Name	Formula	MW	Purity	Supplier	Order No.	Comments
6beta-hydroxytestos-terone	C19H28O3	304.42	≥97%	Sigma-Aldrich	H-2898-25MG	
acetonitrile	CH3CN	412.05	≥99.9%	Bartelt GmbH	9017.2500	
biotin, D(+)-	C10H16N2O3S	244.31	≥98.5%	Carl Roth GmbH	3822.1	store at 4°C
boldenone	C19H26O2	286.41	≥97%	Sigma-Aldrich	46431-10MG-R	
D-glucose monohydrate	C6H12O6 * H2O	198.17	≥99.5%	Carl Roth GmbH	6780.2	
dimethylsul-foxid (DMSO)	(CH3)2SO	78.13	≥99.8%	Carl Roth GmbH	4720.4	
formic acid	HCOOH	46.02	≥98%	Carl Roth GmbH	4724.2	
methanol	CH3OH	32.04	≥99.9%	Carl Roth GmbH	4621.2	highly toxic
phosphoric acid	H3PO4	98.00	≥85%	Carl Roth GmbH	6366.1	
potassium di-hydrogene-phosphate	KH2PO4	136.09	≥99%	Carl Roth GmbH	3904.1	
potassium hy-drogene-phosphate	K2HPO4	174.18	≥98%	Carl Roth GmbH	6875.1	
potassium hy-droxide	KOH	56.11	≥85%	Carl Roth GmbH	6751.1	
sodium azide	NaN3	65.01	≥99.5%	Sigma-Aldrich	71289-5G	highly toxic

testosterone	C19H28O2	288.43	≥99%	Sigma-Aldrich	86500-25G

4.2. Solutions

List of the solutions needed to perform the method

Name	Ingredients	Comments
6beta-hydroxytestos-terone stocks	10 mM: 5 mg 6beta-hydroxytestosterone, to 1.64 mL with DMSO 2 mM: 40 µL 10 mM stock solution, to 200 µL with DMSO 0.5 mM: 10 µL 10 mM stock solution, to 200 µL with DMSO	store in the dark
acidified H2O mobile phase 1	1 L H2O + 1 mL formic acid + some grains of sodium azide	
assay buffer (pH 7.4, 100 mM)	100 mL KPi-buffer (pH 7.4, 1 M), to 1 L with ddH2O	
biotin (500x)	10 mg biton, to 50 mL with ddH2O	store at 4°C
BMD1	1%: 100 mL YNB (20x), 50 mL D-glucose (20%) and 200 mL KPi-Buffer (1M, pH 6.0), to 1 L with ddH2O	store in the dark
BMM different methanol con-centrations	BMM2 (1%): 50 mL YNB (20x), 2 mL methanol, 0.4 mL biotin (500x) and 200 mLKPi-Buffer (1M, pH 6.0), to 200 mL with ddH2O BMM10 (5%): 40 mL YNB (20x), 10 mL methanol, 0.4 mL biotin (500x) and 40 mL KPi-Buffer (1M, pH 6.0), to 200 mL with ddH2O	prepare fresh
boldenone	1 mM: 14 mg boldenone, to 50 mL with DMSO	store in the dark
D-glucose solu-tion	20%: 220 g D-glucose monohydrate, to 1 L with ddH2O	
interner stan-dard (IS)	10 mg boldenone, to 34.915 with mL DMSO	store at -20°C
mobile phase 2 acidified ace-tonitrile	1 L acetonitrile + 1 mL formic acid	store in the dark
potassium phosphate buffer (KPi-buffer, different pH)	pH 6.0, 1 M: 22.99 g K2HPO4, 118.13 g KH2PO4, to 1 L with ddH2O pH 7.4, 1M: 139.69 g K2HPO4, 26.95 g KH2PO4, to 1 L with ddH2O + KOH for phosphoric acid till desired pH	
steroid (200 mM)	25 mg testosterone, to 0.867 mL with DMSO	store in the dark
testosterone stock	200 mM: 577 mg testosterone, to 10 mL with DMSO	store in the dark
YNB Difco	5 g ammonium sulfate, 2 µg biotin, 0.4 mg calcium pantothen-	Supplier:

(yeast nitrogen base , composition)	ate, 2 µg folic acid, 2 mg inositol, 0,4 mg niacin, 0.2 mg p-aminobenzoic acid, 0.4 mg pyridoxine hydrochloride, 0.2 mg riboflavin, 0.4 thiamine hydrochloride, 0.5 mg boric acid, 40 µg copper sulfate, 0.1 mg potassium iodide, 0.2 mg iron chloride, 0.4 mg manganese sulfate, 0.2 mg sodium molybdate, 0.4 mg zinc sulfate, 1 g potassium dihydrogene-phosphate, 0.5 g magnesium sulfate, 0.1 g sodium chloride, 0.1 g calcium chloride, to 1 L with H2O	BD Biosciences
YNB solution (20x)	134 g YNB Difco, to 1 L with ddH2O	store in the dark

4.3. Materials

Description of materials needed to perform the method

Name	Supplier	Order No.	Comments
96 Well microplates F-from (25-340 µL volume)	Greiner, Germany	655101	
96-Deep-Well Plate (2 mL volume)	Bel-Art Products, USA	F378600000	
Biohit Tips: 0,5-300 µL 50-1200 µL	Schmiedt Laborgeräte, Austria	 790302 780043	
column: Zorbax Eclipse XDB-C18 TT HAT 600bar	JAS, Austria	927975-902	
column: Chromolith ® RP-18e	Merck, Germany	1.02129.0001	
Filter Disc 0.2x2.1mm (10 St.)	JAS, Austria	5067-1555	
seal foil	Carl Roth GmbH, Germany	EN76.1	
Tips, 10 µL	Biozyme Scientific GmbH, Germany	720031	
Tips, 1000 µL	Greiner, Germany	740290	
Tips, 200 µL	Biozyme Scientific GmbH, Germany	760201	
Toothpicks	PAPSTRAR, Germany	12713	bought at a local vendor

4.4. Apparatus

Description of the instruments needed to perform the test and specifications.

Name	Supplier	Comments
Centrifuge 5415 R + A-4-62 rotor	Eppendorf AG, Germany	
HPLC 1200	Agilent technologies, USA	
HT MiltronII shaker	Infors AG, Swiss	
MS (single quadrupol)	Agilent technologies, USA	
Multichannel pipettes: 5-100 µL, 50-1200 µL	Biohit, Germany	
pipettes: 0.1 – 2,5 µl, 0.5 – 10 µl,	Eppendorf AG, Germany	
pipettes: 2 – 20 µl, 20 – 200 µl, 100 – 1000 µL	Gilson Inc., USA	
Platereader Spectramax Plus 384	Molecular Devices, Germany	
RRLC Inline-Filter, max. 600bar	Agilent technologies, USA	
Splitter 1:2 (Figure 3)	selfmade using **Order number**: 0890-1915 — **Name**: PEEK-Kapillare, 0,13mm ID 5063-6591 — PEEK finger tight 1/16 in, PK 5022-2144 — T-Verbrauchsstück, PEEK 1/16 " , 057µl sewpt vol MB 1650 — Upchurch Tub. PEEK nature, 1/16"x0,064mm ID; 5ft	
Vortex-Genie 2	Scientific Industries Inc, USA	

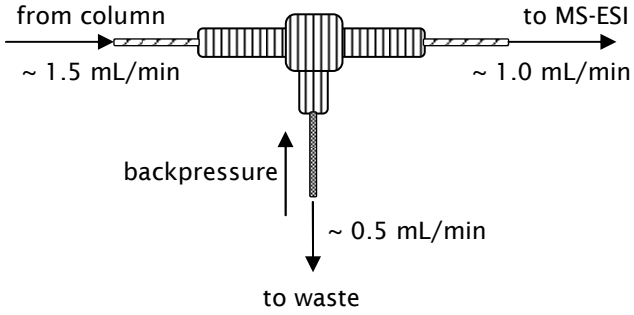


Figure 3: Splitter (1:2)

4.5. Software

List of the software needed to perform the method

Name	Distributor
ChemStation B.04.01. SP1	Agilent technologies, USA
excel macro "Data Import" V2.1	Institute for genomics and bioinformatics

4.6. Procedure

4.6.1. Cultivation and induction

Cultivation and induction is performed as described (Weis et al., 2004). In short: clones are picked and transferred to 96 Deep-well plates filled with 250 µL glucose minimal media BMD1. The cultures are grown from Friday to Monday for 60 h at 28°C, 340 rpm and 80% humidity in HT MiltronII shaker (Infors AG, Swiss). Protein expression is performed from Monday to Thursday for 72 h at the same conditions used for growth. Induction is started by adding 250 µL methanol minimal media BMM2 and subsequent addition of 50 µL methanol minimal medium BMM10 after 10, 24, 34, 48 and 58 h of induction to a total volume of 750 µL.

Important: Each plate should contain negative and positive controls to which results are corresponded to.

4.6.2. Resting, whole cell conversion

After induction, cells are centrifuged at RT and 500 g for 5 min. Cells are washed once with water and re-suspended in 200 µL reaction buffer (100 mM potassium phosphate, pH 7,4). The reaction is started by adding 5 µL testosterone stock solution (200 mM solved in DMSO) to the end concentration of 10 mM. After 8 h of conversion 10 µL IS (1 mM boldenone solved in 1:1 H2O/DMSO) is added to a final concentration of 50 µM. The reaction is stopped by centrifugation at RT and 2000 g for 10 min. 100 µL supernatant are transferred to fresh 96 microtiter plates and sealed with sealing tape. Samples can be stored at -20°C till HPLC-MS analysis.

Important: To account for different growth, OD600 is measured before the reaction is started.

As each plate contains positive and negative controls, only the relative OD600 to each other is important.

4.6.3. OD600 measurement

Using the multichannel pipette 10 µL cell suspension is added to 90 µL H2O provided in a 96 well-plate. The plates are placed on a well-plate shaker at 2000 rpm for 5 min. The OD600 is measured with Spectramax Platereader using the software SoftMax Pro.

SoftMax Pro

After double click on the SoftMax Pro icon on the desktop, go to “file” and “new” in the menu bar. A new “Experiment” will pop up. Clicking on “Setup” in the ”experiment” screen will bring up the “Instrument Settings” dialog box. OD600 is an “Endpoint” measurement. In the “Wavelengths setup” enter the Wavelength 600 nm. “PathCheck” is left at “off”. Go to “Automix&Blanking” and activate “Automixing” for 5 sec by activating the checkbox. Blanking is not necessary as we only measure the relative OD600 of each well. “AutoCalibrate” is left at “on”. Go to “Strips” and check that the whole plate is marked (underlined blue). If this is not the case mark them by clicking the left mouse button at the A1 position, holding the left mouse button and moving the mouse cursor to the A12 position. “AutoRead” is left at “off”. Confirm the settings by clicking “Ok”. After placing the 96 well-plate in the drawer click the “read” button to start the measurement.

To add several measurements (plates) to the same “experiment” click on “experiment” in the menu bar and select new plate or use the key combination “Ctrl+K”. All new added plates will have the same settings as the first plate. The grey underlined “plate bar” marks the selected plate. By clicking with the left mouse button a different “plate bar” can be selected. Clicking “read” again will start the next measurement and the OD600 date will be written in

the new selected “plate bar”. After the measurements, save the data by going to “file” in the “menu bar” and selecting “save as…”.

Data can be exported in two different ways. One way is to go to “file” in the “menu bar” choosing “import/export” and “export”. The data of all plates is saved as a text file and the data can then be imported into excel. The easier way is copy/paste each measurement. Select the measurement you want to export by clicking on the “plate bar”. Press “Ctrl+C” open a excel file click somewhere in the excel sheet and press “Ctrl+V”.

4.6.4. Calibration sample preparation

Prepare several calibration samples in the range from 1 to 200 µM (see table blow). Calibration samples are prepared with the same buffer used for the conversion. To 100 µL of each calibration sample 5 µL boldenone (1mM in DMSO) is added to a final concentration of 50 µM. Each time a fresh internal standard solution is prepared the calibration has to be redone.

List of calibration samples

sample designation	end conc.	stock conc.	stock volume	buffer volume	total volume
	[µM]	mM	[µL]	[µL]	[µL]
1	0	0	0	250	250
2	1	0.5	0.5	249.5	250
3	10	0.5	5	245	250
4	25	0.5	12.5	237.5	250
5	50	2	6.25	243.75	250
6	100	2	12.5	237.5	250
7	200	10	5	245	250

4.6.5. HPLC-MS analysis

The HPLC and the MS are expensive and very sensitive. Only instructed and trained staff are allowed to us it. It does not matter if you used an HPLC-MS before, each machine is different and each lab has own rules. To get a working permission, contact the person responsible for operation for an introduction.

4.6.5.1. Preparations

The HPLC-MS should never be switched off. Only for maintenance, electricity downtime or prolonged holidays the HPLC-MS will be switched off. If this is the case only the person responsible for operation will be able to switch the HPLC-MS back on.

The first step for HPLC-MS analysis is to prepare enough mobile Phase 1 (H2O + 1% FA) and 2 (ACN + 1% FA).

If some old ones are available, check if they are ok (not turbid, etc.). If you prepare them fresh, add some grains of NaN3 as preservative to the aqueous mobile phase.

The HPLC 1200 has two pumps and each pump has two mobile phase channels. Pump A and therefore channel A1 and A2 are always for aqueous mobile phases. Pump B and therefore channel B1 and B2 are always for organic mobile phases. Make sure you put the mobiles phases to the right channels (tubings are marked). Usually the aqueous channels are filled with and kept in 10% isopropanol solution to prevent growth in the tubing. Before putting the tubing into a new mobile phase, dry them slightly with a paper towel.

The second step is to check if the right column is installed. The column oven can hold up to two columns. The Zorbax Eclipse XDB-C18 has to be installed at the column position 2 which is the top position.

The next step is to check if the inline filter is installed (Figure 4). The inline filter assembly holds a frit with 0.2 µm pore size for protection of the column. This precaution significantly increases the column life time.

Figure 4: Inline filter assembly

The last step is to check the ionization chamber of the MS. For this screening method the ESI is used which usually is already installed. If this is not the case install the ESI as instructed by the person responsible for operation.

Open the ionization chamber and clean it with 50% isopropanol and a lint free cotton towel.

Important: Never spray directly onto the acceleration capillary tube opening.

Before samples can be analyzed, the tubing system has to be purged to flush out residual liquids. Purging is achieved by opening the purge valve (Figure 5) and starting the pumps. ***Important:*** Make sure that the right tubes are selected in the pump setup. To reduce the

needed purge time the flow rate can be increased. Prolonged purging time is needed when some air bobble were introduced during change of the mobile phase. Make sure that there are now air bubbles left in any of the used tubings.

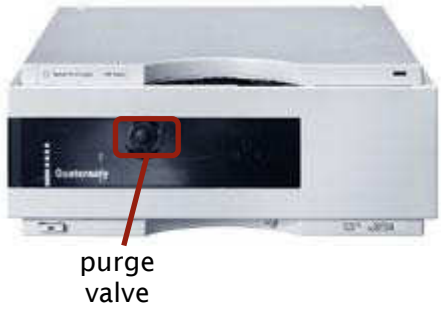


Figure 5: quaternary pump module
Opening the purge valve will direct the pump flow to the waste instead of the injector.

After purging the tubes, the column has to be equilibrated. Stop the pump and open the AM-TESTOSTERONE-FAST-ESI.M method. Due to changes made to the flow rate, a "save current changes?" window will open up.

Important: Do not save any changes.

Close the purge valve and switch on all modules. The whole system is now flushed by the mobile phase and should by equilibrated for at least 20-30 minutes. After equilibration the HPLC-MS system is ready for analysis.

Put the 96 well-plates in the correct order into the corresponding well-plate handler racks.

The analysis is started by selecting "Run Hypersequence" in the "RunControl" section of the menu bar.

How to setup the sequences and hypersequences and to start the HPLC-MS analysis is described in the following sections.

4.6.6. ChemStation software

ChemStation is a very complex software. This SOP will only describe basic procedures for handling existing methods, generating sequences and hypersequences and exporting data for later data analysis. Two instances of the ChemStation can be run at the same time. The Instrument 1 (online) where the HPLC-MS can be controlled and the Instrument 1 (offline) where usually part of the data analysis takes place. By using the offline instance, already measured samples can be viewed while the HPLC-MS system is still analyzing the rest of the samples.

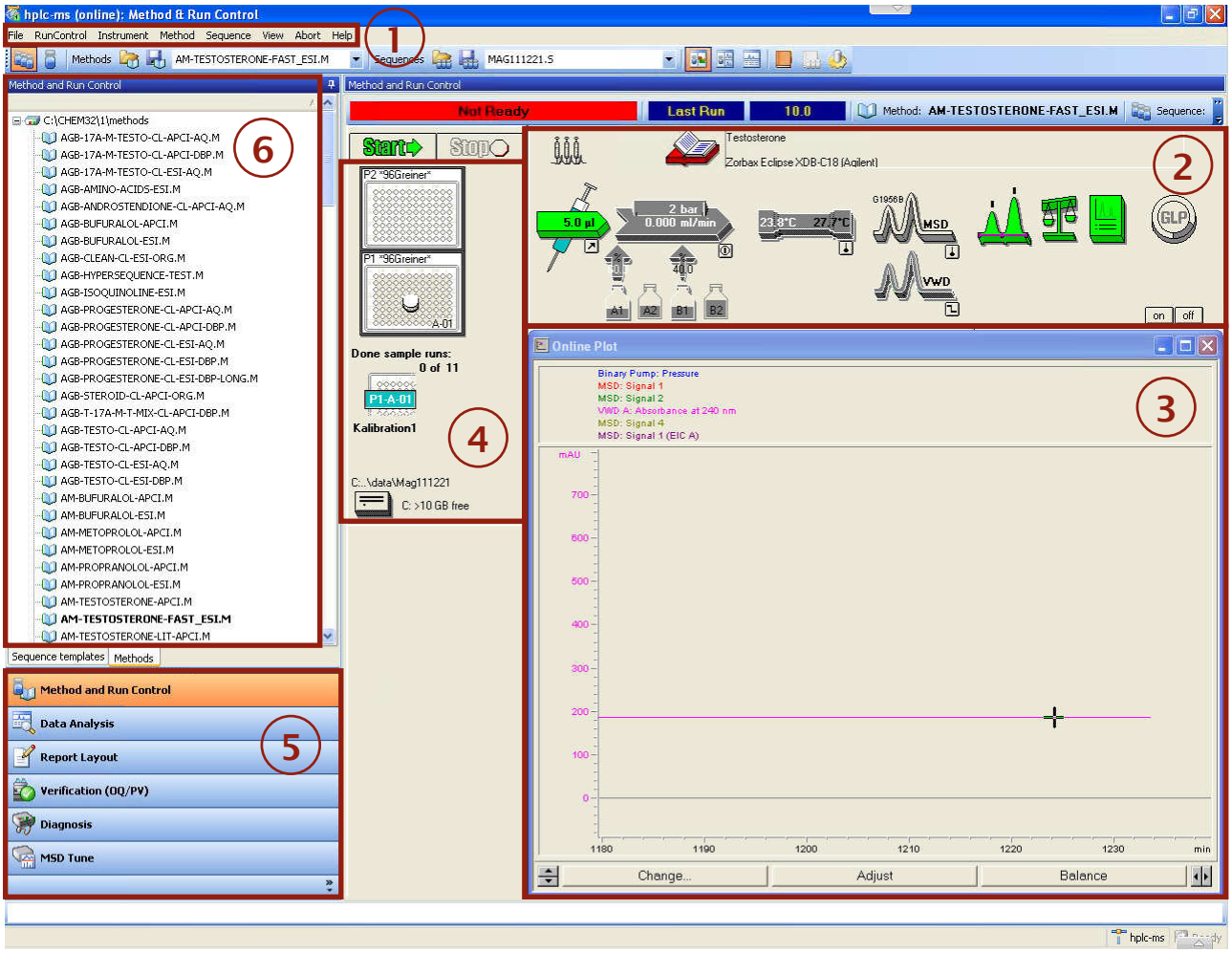


Figure 6: ChemStation: main screen.
menu bar (1) control panel (2), online chromatogram window (3), run and sequence table window (4), task window (5), path window (6)

4.6.7. Software settings for MT-HPLC-MS run

If the software is not already running, open the online instance by clicking the "instrument 1 online" icon on the desktop. Starting the software will take some time. The last known method and sequence will be loaded as well.

Open the method AM-TESTOSTERONE-FAST_ESI.M. either by opening the "Method and Run Control" window by selecting the corresponding bar in the "task window" or by selecting "Load Method" in the "Method" section of the menu bar.

Important: Do not save any changes at this point. Check once again if the mobile phases are connected to the right tubing. Check the bottle fillings and enter the information by left click on the bottle icons and selecting "Solvent bottles fillings…" (Figure 7). With this information the ChemStation will calculate the theoretical bottle fillings during the HPLC run. Therefore, the information is crucial to prevent the HPLC of running dry and pumping air into the system.

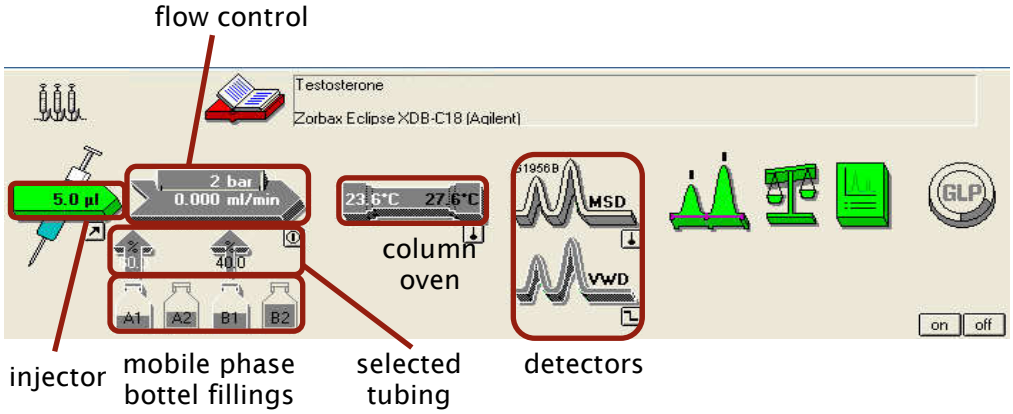


Figure 7: ChemStation: HPLC-MS control pannel.
The on-switch icons are located at the bottom right corner of each module. The "on/off" switch in the right bottom corner controls all modules at once.

Activate all modules, either by clicking each modules on-switch separately or using the "on" button in the right bottom corner of the control panel (Figure 7). All modules should be green. If this is not the case, go to troubleshooting section (4.8) or contact the person responsible for operation.

4.6.8. Sequences and hypersequences setup

Sequence

In the ChemStation, each 96 well-plate is represented by a sequence.

Select "New Sequence Template" in the "Sequence" section of the menu bar. This will discard all previous sequence settings. Do not save changes to a previous sequence.

Select "Sequences Parameters" in the "Sequence" section of the menu bar. Enter your name as "operator". Enter a "subdirectory" where all the data will be saved. The subdirectory should be named as following: your initials, year, month, day. e.g. AGB111227. You will be asked if the subdirectory should be created. Activate the "Post-Sequence Commando/Macro" in the "Shutdown" section. Select "STANDBY" when the run is over night, and the measurement will be finished before you will be able to check on the HPLC-MS run. Otherwise you can select " PUMPALL OFF" which keeps the detectors online but the pump offline. Press "OK" to c onfirm the settings (Figure 8).

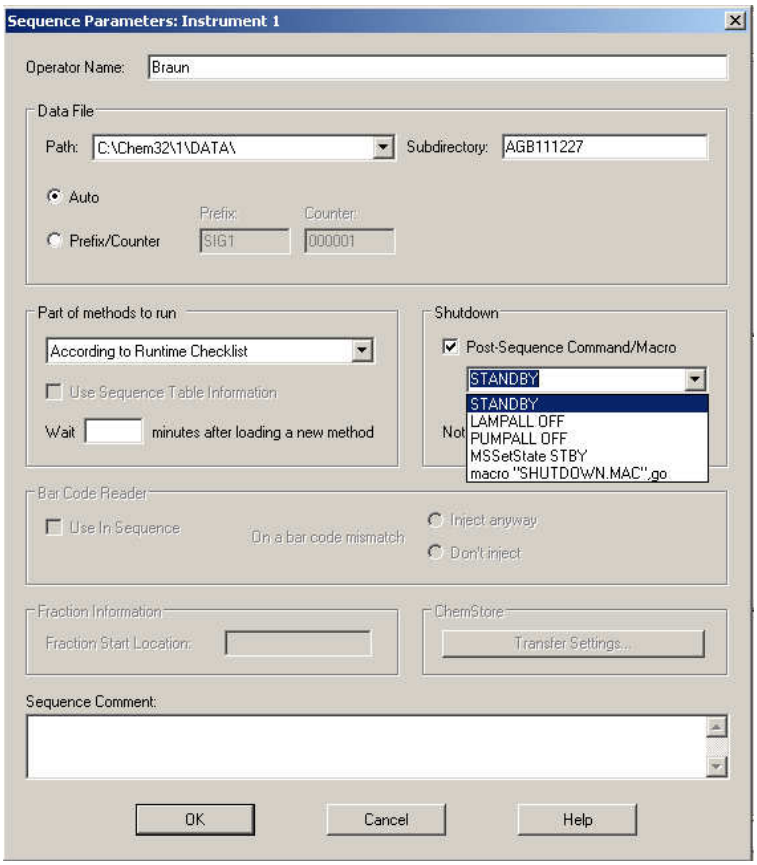


Figure 8: ChemStation: Sequence Parameters section.

Select “Sequences Table” in the “Sequence” section of the menu bar. In this section sample locations and names are entered. Additionally the used method, times of injection sample type and injection volume is entered.

The first row is selected and removed. Select the “Insert/FillDown Wizard” to enter all the necessary informations (Figure 9). Plate locations are entered as “plate position in injector” + position e.g. 2A1.

Important: For a sequence that will be integrated in a hypersequence the “position in injector” is always “2”.

Enter the location range from 2A1 to 2H12. Choose the “AM-Testosterone-Fast_esi” as method. As “Sample name” enter an identifier corresponding to the whole plate. Enter “1” in the “Inj./Location” field. The “Inj. volume” can be left empty. If left empty the 5 µL injection volume set in the chosen method will by used. If for any reason you want to have a different injection volume enter this volume here. Press “OK” to confirm the settings. The Sequence Table will now contain 96 lines. Again press “OK” to confirm the settings. Save the sequence by selecting “Save Sequence Table As …” in the “Sequence” section of the menu bar. Choose a unique identifier that can be easily recognized later on.

Repeat this procedure for each plate.

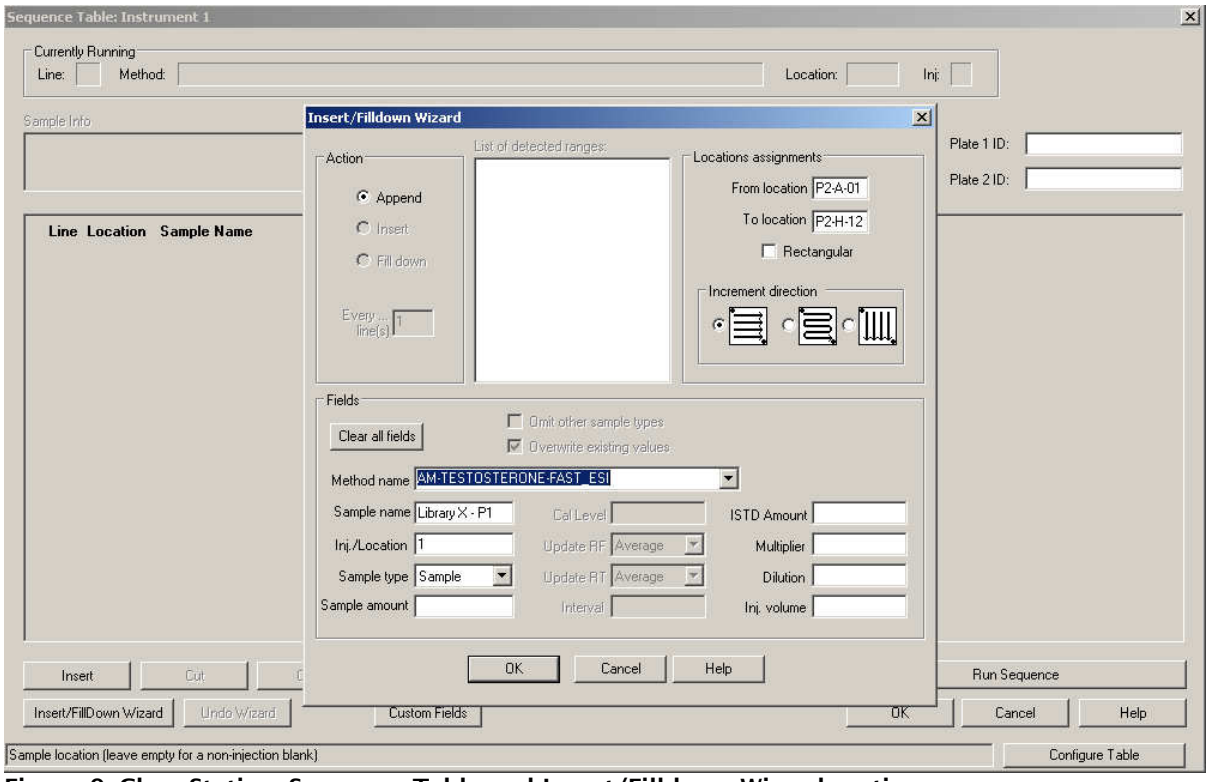


Figure 9: ChemStation: Sequence Table and Insert/Filldown Wizard section.

Hypersequence

Several sequences are combined to a hypersequence. Hypersequences are only available if a well plate handler is installed.

Select “Hypersequence” and “New Hypersequence” in the “Sequence” section of the menu bar. This will discard all previous sequence settings. Do not save changes to a previous sequence.

Select “Hypersequence” and “Hypersequence Parameters” in the “Sequence” section of the menu bar. Activate the “Shutdown command” and “onError command” and put “Standby” in the fields next to them. Select “Input” and “Output” racks. At least one rack must be marked as the Input rack and at least one must be marked as the Output rack. If you mark four input racks and one output rack, the first free input rack becomes an output rack, and so on. Each rack can hold up to 10 “greiner” 96 well-plates. Enter a “Subdirectory” where all the data will be saved. The subdirectory should be named as follows: your initials, year, month, day. e.g. AGB111227 (Figure 10). Press “OK” to confirm the settings.

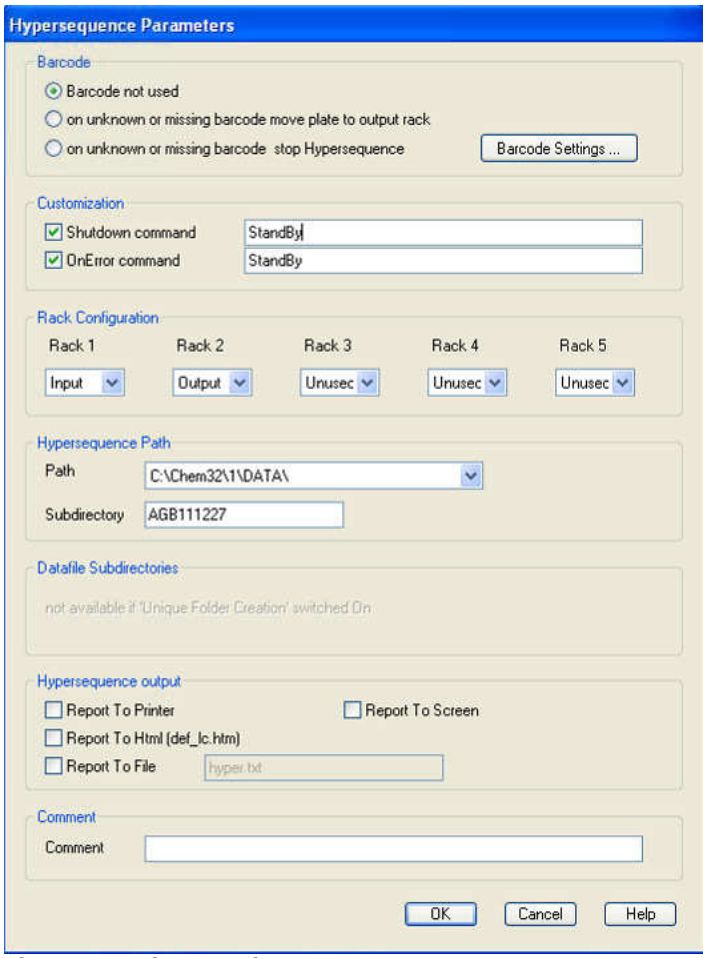


Figure 10: ChemStation: Hypersequence Parameters.

Select “Hypersequence” and “Hypersequence Table” in the “Sequence” section of the menu bar. Delete all existing rows. Select the “Hyperseq. Wizard”. Enter the numbers of plates to be analyzed. Select the sequence corresponding to the first plate to be analyzed and enter its name in the “Sequence name” field. Select AM-Testosterone-Fast_esi as “Method”. Select the “96Greiner” as “Plate Type”. Press “OK” to confirm the settings.

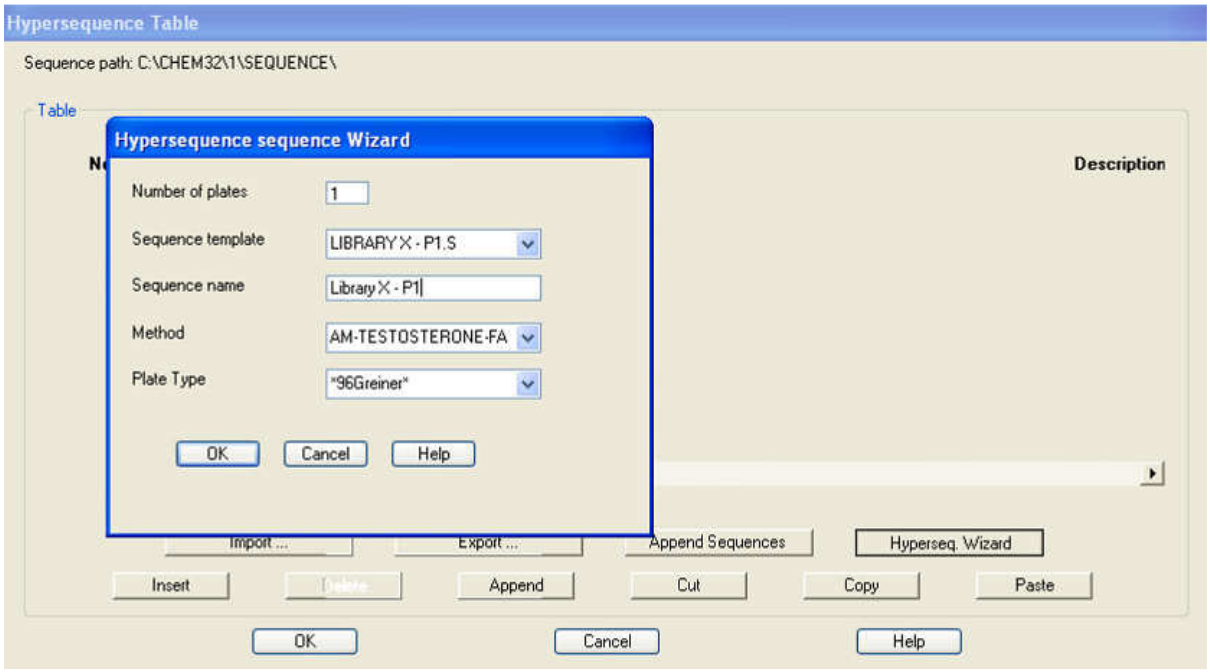


Figure 11: ChemStation: Hypersequence sequence Wizard.

All lines will have the same “Sequence name”. Select the “Sequence name” field and choose the right sequence (Figure 12).Press “OK” to confirm the settings. Save the hypersequence by selecting “Hypersequence” and “Save Hypersequence Table As …” in the “Sequence” section of the menu bar.

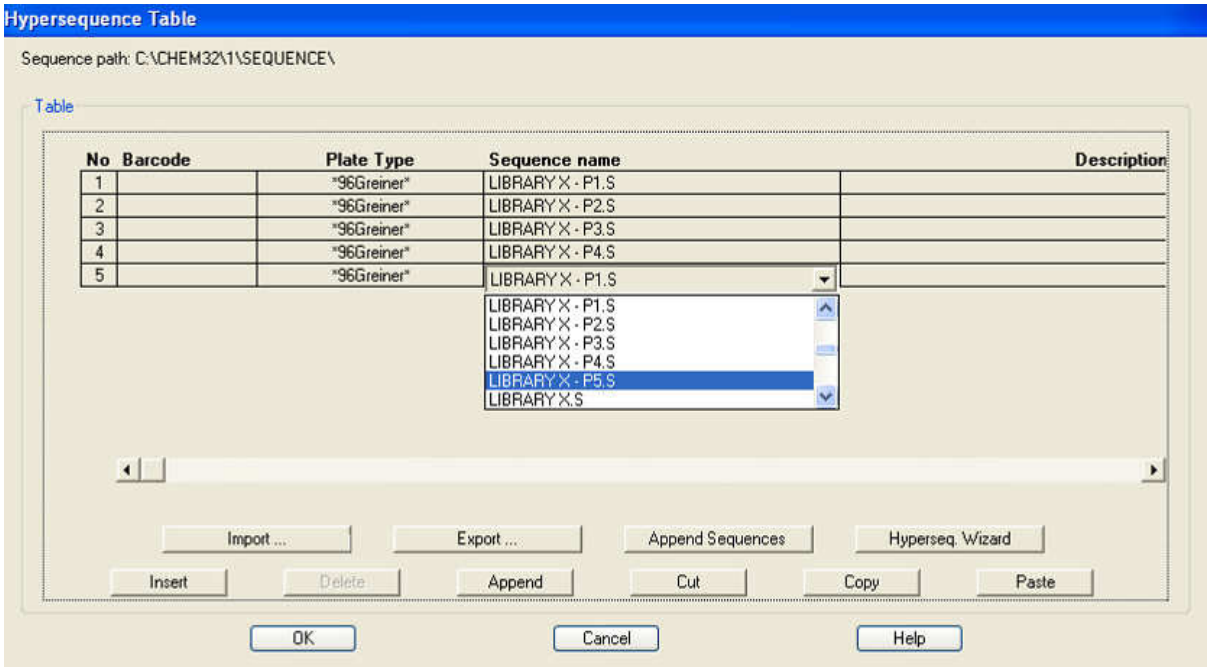


Figure 12: ChemStation: Hypersequence Table.

4.7. Calculations

Calculations are done using Microsoft Excel. For that MS signals have to be integrated and peak areas exported to a text file. An excel macro is then used to import the data to a prepared excel sheet.

4.7.1. Export of the data from ChemStation software

Open the offline instance by clicking the "instrument 1 offline" icon on the desktop. Starting the software will take some time. The offline instance looks the same as the online software (Figure 6). If not already active, select "Data Analysis" in the "task window". Open your "signals data" by clicking on the appropriate file in the "paths window" (Figure 13). Make sure that the method used for integration is set to "Sequence Method". Open the "Preference Dialog"  and select "Sequence Method" in the "Signal/Review Options" and "Method used for Reviews of Sequence Date" section.

Figure 13: ChemStation: "Data Analysis" screen.
sample window (1) signals window (2), task window (3), path window (4)

MSD1 shows the hydroxytestosterone signals, MSD3 shows the internal standard boldenone signal. Write down their retention times for the later on data import.

Select "Sequence Output"  in the "sample window" menu bar. Activate the "Printer Sequence Summary Report". Deselect the "Report to Printer" and instead select the "Report to File" and give a file name. ***Important:*** Filename should end with ".txt". Press "Setup…" to open the "Sequence Summary Parameters" select "6. Analysis reports" and deselect everything else. Press two times "OK" to accept the settings.

Press "Start Sequence reprocessing"  in the "sample window" menu bar. The ChemStation will integrate all signals and print the data to a text file with the entered name. The text file will be located in the sequence data path on the HPLC-MS computer. This is usually "C:\Chem32\1\data".

4.7.2. Adjust integration settings in ChemStation

The integration settings are optimized so that only the needed signal peaks are integrated. For correct integration the retention times of signal peaks should stay constant. Any changes to the HPLC system may change the retention time: new column, new tubing, slight changes in mobile phase, etc. Therefore it may be necessary to adjust the integration settings. Adjustments are also necessary if a new product is screened for. Again, make sure that the method used for integration is set to "Sequence Method". Open the "Preference Dialog"  and select "Sequence Method" in the "Signal/Review Options" and "Method used for Reviews of Sequence Date" section. Select "Integration" 

in the "signal window". Open the "Edit/Set Integration Events Table"  . The blue numbers in the "signals window" mark the start and stop points for the integration events". They have to be moved the appropriate position. The red lines mark the base line. Everything above the baseline will be integrated. Select the start or stop point you want to move in the "Specific Events for Signal" table. The selected start or stop marker will be shown red in the "signals window". When you move the mouse over to the "signals window" the mouse cursor will show a vertical bar. Left mouse click on the desired position to move the start or stop marker. After adjusting the start and/or stop positions press "Integrate current Chromatogram"  to check the new integration settings. To insert a new integration start and or stop position select "Set Integration"  and mark the start and stop positions. If there

was a mix-up with the start and stop positions, the start "on" and stop "off" of each maker can be changed in the "Specific Events for Signal" table. To remove a start or stop marker select the marker in the "Specific Events for Signal" table and press "Delete selected line"  . Press "Exit and save"  .

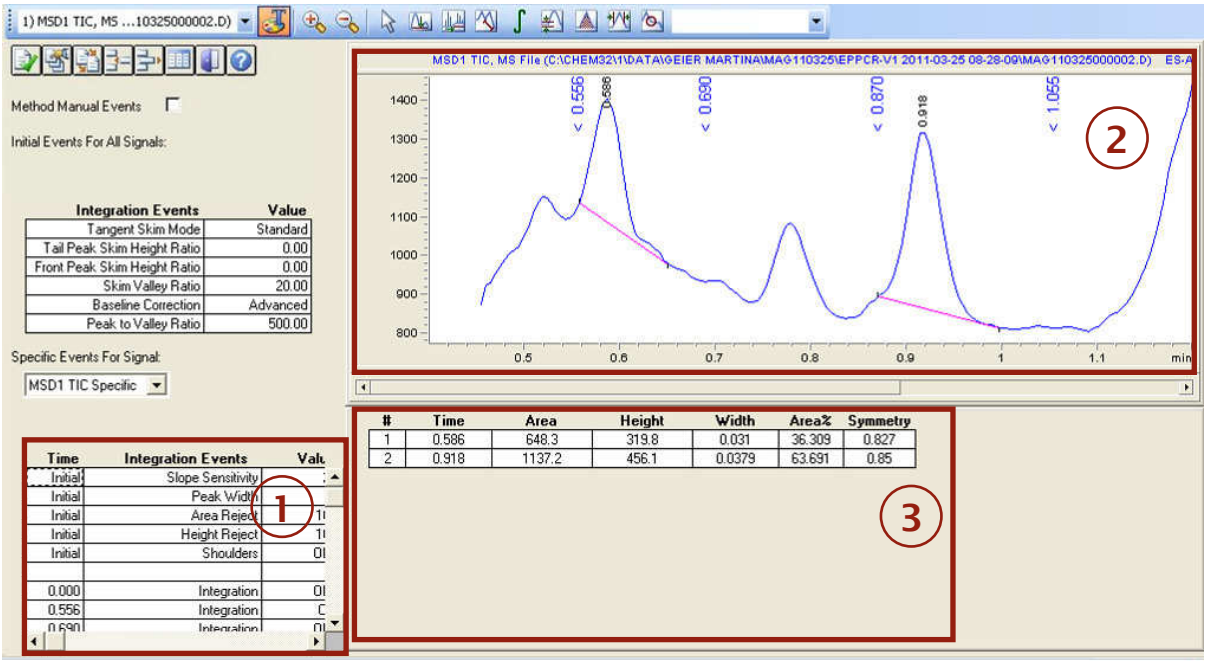


Figure 14: ChemStation: "Edit/Set Integration Events Table" screen.
"Specific Events for Signal" table (1) signals window (2), signal peak properties (3)

Now you are ready to export the signal data as described in the "Export of the data from ChemStation software" section (4.7.1).

4.7.3. Import of the data to MS Excel

Open the "HPLC-MS Data-analysis-V2.1" MS Excel file. The file contains several worksheets.

Select the first worksheet "HPLC-MS Import". Press the "Start Import" button to activate the import macro. Choose the text file which contains the signal data. Enable the "Retention time" filter. Press the "Set retention timings" button to start the retention timings setup (Figure 15).

Enter the retention times with two decimal places, of the desired signal peaks and press "OK" to accept the settings. The signal peaks retention times of a run can very at a small range. Therefore, the "Gloval Variance" can be adjusted to make sure all signal peaks are imported. The standard setting of 0.075 min. is usually fine. Press the "Import" button to start the import procedure. Close the import macro.

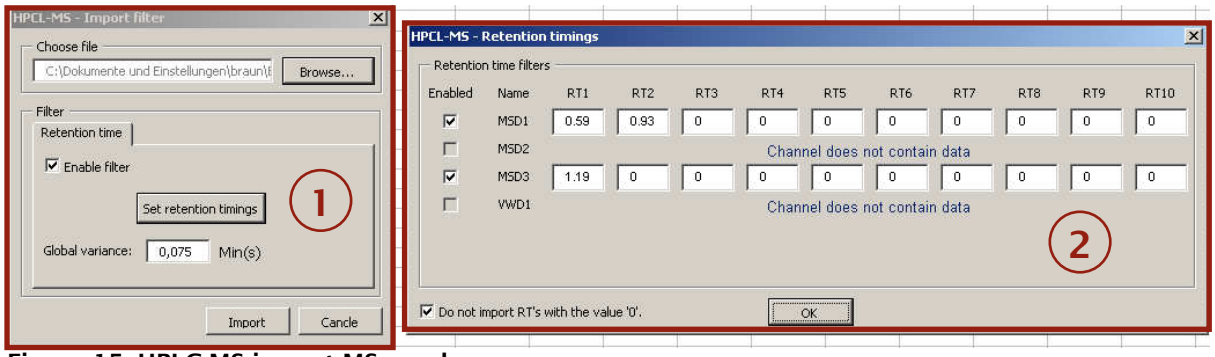


Figure 15: HPLC-MS import MS excel macro.
Macro main screen (1) Retention Timings setup screen (2)

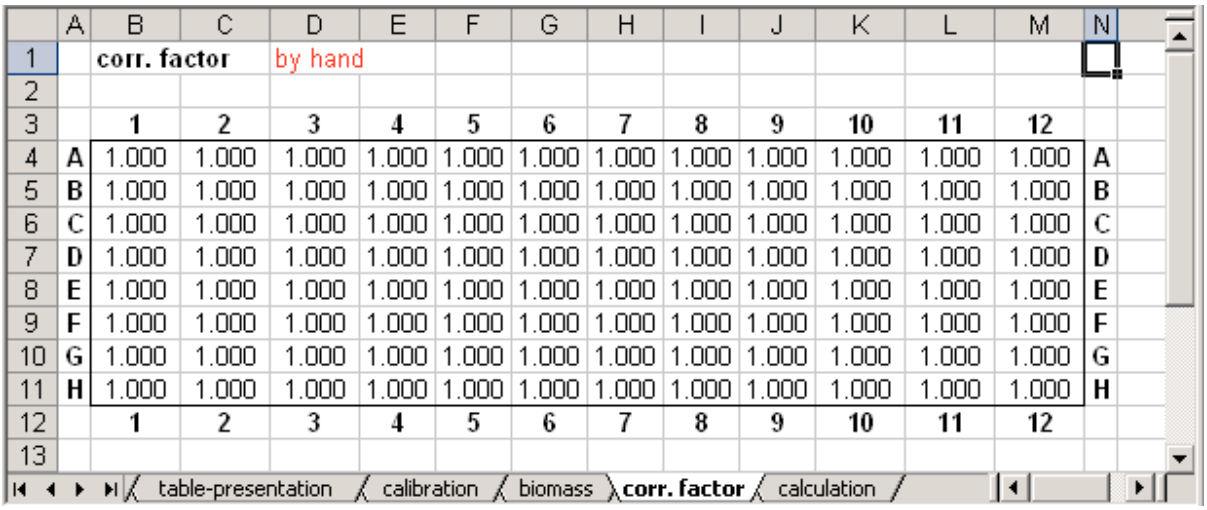
4.7.4. Data analysis in MS Excel

The data analysis excel file is set up for up to 5 signals channels to be analyzed. For optimized data handling there should be 8 rows: Position (first row), Name (second row), signal rows (third to seventh) and internal standard (eighth and last row). After successfully importing the HPLC-MS data insert the appropriate number of rows so that the internal standard is the eight and last row (Figure 16).

	A	B	C	D	E	F	G	H	I	J	K	L	M
1													
2	Import File:				Position	Name	MSD1					MSD3	
3	Start Import						RT1: 0.59 ± 0.075	RT2: 0.92 ± 0.075				RT1: 1.19 ± 0.075	
4					P2-A-01	epPCR V1	0	0				1137906	
5					P2-A-02	epPCR V1	648	1137				1371610	
6					P2-A-03	epPCR V1	264	443				1281577	
7					P2-A-04	epPCR V1	1533	1490				1503058	
8					P2-A-05	epPCR V1	980	1003				1491028	

Figure 16: HPLC-MS import: HPLC-MS Import worksheet.

Copy and paste the imported data at the correct position to the “HPLC-MS-DATA” worksheet. If OD600 data was measured, copy and paste that data at the correct position to the “OD600” worksheet, otherwise leave the values at “1”. If any correction factor needs to be considered put them at the correct position to the “corr. factor” worksheet (Figure 17), otherwise leave the values at ”1”. Enter the calibration data for each signal at the correct position to the “calibration” worksheet.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1		corr. factor		by hand										
2														
3		1	2	3	4	5	6	7	8	9	10	11	12	
4	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	A
5	B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	B
6	C	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	C
7	D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	D
8	E	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	E
9	F	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	F
10	G	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	G
11	H	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	H
12		1	2	3	4	5	6	7	8	9	10	11	12	
13														

table-presentation | calibration | biomass | **corr. factor** | calculation

Figure 17: HPLC-MS import: corr. factor worksheet

The calculations are done in the "calculation" worksheet. The peak area of the sample signals is divided by the peak area of the internal standard signal. The axis intercept is subtracted and then multiplied with the slope from the calibration. This new value is divided by the biomass and multiplied with the correction factor, if entered. The resulting unit depends if OD600 or cell dry weight in g/L was entered as biomass, µM/OD600 or µmol/g respectively.

Results are shown as convenient 96 well-plate formats in the "96Well-presentation" worksheet. Enter the desired threshold above which clones are considered to be improved. This is usually the mean value of the positive control times a desired factor. Improved clones will be shown in green (Figure 18).

		Compound:	6beta		threshold	3.0	set by hand						
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0.0	0.7	0.3	1.5	1.0	0.0	1.8	0.0	0.3	0.8	1.4	1.2	A
B	2.8	0.0	1.1	2.6	1.1	1.9	1.7	0.8	1.3	1.6	0.3	0.0	B
C	0.0	1.4	1.1	1.3	0.0	2.8	0.8	0.0	1.2	0.6	3.1	0.3	C
D	0.0	1.6	1.3	1.5	1.8	2.4	1.0	1.8	1.8	1.2	2.1	2.1	D
E	0.0	0.4	1.0	0.0	1.4	2.2	1.3	1.6	1.3	2.6	1.5	0.0	E
F	1.4	1.4	0.8	1.7	1.7	1.7	0.0	0.0	1.8	8.2	2.0	1.0	F
G	0.0	1.4	0.3	1.1	0.6	1.8	1.2	1.6	1.5	1.4	0.5	1.5	G
H	1.4	0.7	0.3	1.9	1.8	0.0	1.3	1.6	0.0	1.7	0.5	1.0	H
	1	2	3	4	5	6	7	8	9	10	11	12	

Figure 18: HPLC-MS import: 96Well-presentation worksheet

4.8. Troubleshooting

Troubleshooting

Trouble	Reason	Solution
backpressure is to high (pump module is red and shuts down)	set pressure limit of the column reached - plastic columns 200 bar - normal steal column 400 bar - special HT steal columns 600 bar column	back flush the column exchange the column exchange the in-line frit contact the person responsible for operation
fluctuation of the backpressure	probably some air is still in the tubing or in the pump it self	prolonged purging prolonged equilibration fluctuation will decrease over time and should be below plus/minus 5 bar
MS sends a shutdown signal (all modules are red)	low drying gas pressure (N2) N2 supply is connected to the liquid N2 tank, if the tank filling is below a certain point the needed pressure can not be maintained.	check if the N2 pressure is ok (gas valve between the two HPLC) check the N2 pressure in the liquid N2 tank next to the old chemistry building contact the person responsible for operation
only rack 1 and 5 can be used as input or output rack	the rack expansion is not activated	Select “More Injector” and “Configuration…” in the “Instrument” section of the menu bar Klick on “Well-Plate Handler Config.>>” and activate the “Well-Plate Rack Extansion” Press two times “OK” to confirm the settings.
software won’t start	one or several modules are not on-line MS has some electronically problems	take a look if all the HPLC modules are switched on (except the lowest one of the highest HPLC tower, this is a separate pump module which is not needed and should be switched off)

		reset the MS as taught during the instruction lesson contact the person responsible for operation
there are two rows for the same plate position e.g. two rows for P2-C-11	used integration settings gave an additional peak in the vicinity of one of the main peaks	reduce the "Global variance" during data import adjust the integration settings contact the person responsible for operation
total shutdown of all modules (all modules are red)	leak in the system	check all tubings and fittings dry the leaked liquids leaking sensor will take a while to dry (5-20 min)

5. Safety Precautions

Please follow instructions described in “acib-Mitarbeiterleitfaden Gefahrstoff- und Laborordnung”

6. Documentation

Documentation

Information	Location
HPLC-MS method development	Dissertation Braun Andreas 2012
This handbook describes various concepts of the Agilent ChemStation. It is intended to increase your understanding of how the ChemStation works.	Understanding your ChemStation – english.pdf
This presentation describes various concepts about integrating results in ChemStation. It is intended to increase your understanding of how integration in ChemStation works.	Integrating My Results in ChemStation_121008.pdf

7. References

Dong, M. W. (2006). *Modern HPLC for Practicing Scientists*. Hoboken, NJ, USA: John Wiley & Sons, Inc.

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Supplementary data 4

SOP II (*Y. lipolytica*)

Constructing a *Y. lipolytica* P450 expression strain

ACIB Protocol

Constructing a *Y. lipolytica* P450 expression strain

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Approved by:		

1. Purpose and Field of Application

A protocol for the construction of *Y. lipolytica* strains for functional expression of mammalian P450 enzymes.

This protocol can be used for expression of any mammalian P450 system in the non-conventional yeast *Y. lipolytica*. The human CYP2D6 and human CPR are described as an example.

2. Principle

For a functional mammalian P450 system not only the P450 itself but also the flavin containing cytochrome P450 reductase (CPR) is needed. The CPR transfers required electrons from NADPH to the cytochrome P450 during substrate conversion. In some cases the host own CPR can sufficiently act as electron transport chain (Schiffler et al., 2004). However, in many cases the mammalian CPRs are more effective in supplying electrons to mammalian CYPs than other CPRs (Murakami et al., 1986).

Mammalian P450 and mammalian CPR genes are cloned into a multicopy shuttle vector and integrated into the genome of the yeast expression host. Co-expression of both proteins gives a functional mammalian P450 system in the host *Y. lipolytica*. Highest expression is shown by *Y. lipolytica* clones containing multiple copies of the co-expression cassette (Nthangeni et al., 2004).

Multicopy vectors derived from the p64ICL1 vector contain the URA3 selection marker. This selection marker contains a degenerated ura3d4 promoter which gives only sufficient amounts of uracil when several copies of the vector are integrated into the genome. This allows for selection of clones with multicopy integrations or gene multiplications (Förster et al., 2007).

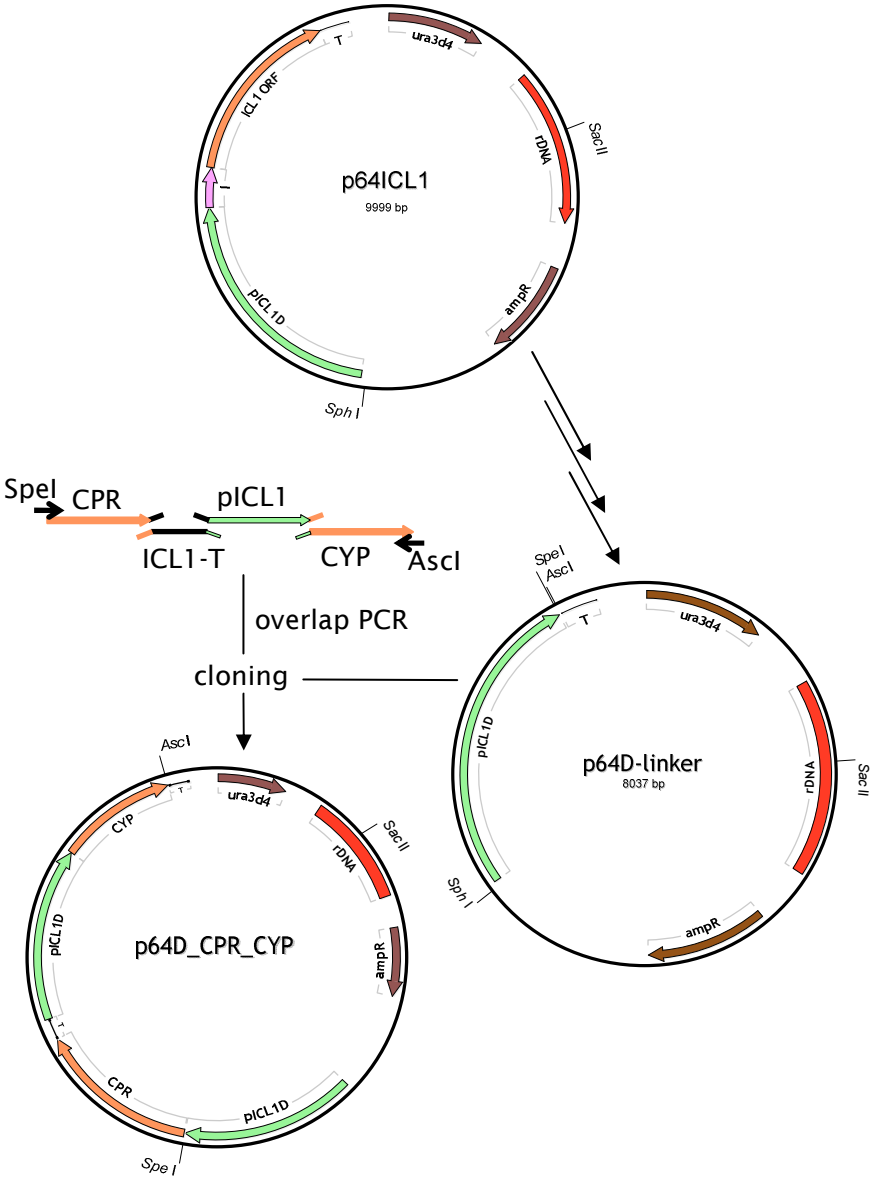

Figure 1: Schematic representation of multi copy co-expression vector construction
Construction of multi copy co-expression vectors by inserting overlap extention PCR fragments into the multi copy integrative vector p64D-linker (constructed by removing the open reading frame (ORF) as well as the ICL1 Intron) containing the selective multi copy ura3d4 marker. Green arrows, ICL1 promoter; light violette ICL1 intron; stroked goalpost, ICL1 terminator; red box, rDNA integration region; brown arrows, selection marker for E. coli or *Y. lipolytica* respectively; gene localization and orientation are indicated by orange arrows

3. Key Words, Definitions & Abbreviations

Key words: cytochrome P450, *Yarrowia lipolytica*, non-conventional yeast, co-expression

Abbreviation	Description
AmpR	ampicillin resistence gene coding for beta-lactamases
cDNA	complementary DNA, is DNA synthesized from a messenger RNA
CDW	cell dry weight
CPR	cytochrome P450 reductase
CYP	cytochrome P450
CYP2D6	human cytochrome P450 2D6
CYP3A4	human cytochrome P450 3A4
ddH2O	double distilled H2O
DNA	deoxyribonucleic acid
dNTP	desoxyribonukleosidtriphosphate
ET	electron transfer
EtOH or Eth	ethanol
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
G / C	guanidine, cytosine
Glu	glucose
GRAS	generally regarded as safe
HCPR	human cytochrome P450 reductase
HF	high fidelity
ICL1	isocistrate lyase gene 1
LB	Luria-Bertani
LiAc	lithium acetate
Lip	lipide
NADPH	nicotinamide adenine dinucleotide phosphate
NCR	NADH / NADPH cytochrome c reductase
OD600	optical density measured at 600 nm
ONC	over night culture
PCR	polymerase chain reaction
PEG	polyethylene glycol
rDNA	ribosomal DNA
RNA	ribonucleic acid
RT	room temperature
SOC	Super Optimal broth with Catabolite repression, nutrient-rich bacterial growth medium
Str	streptomycine
Suc	sucrose
Temp	temperature
Tet	tetracycline
Tgl	triglyceride
Ura	urea
WT	wildtype
Yl or *Y. lipolytica*	*Yarrowia lipolytica*
YlCPR	Yarrowia lipolytica cytochrome P450 reductase
YNB	yeast nitrogen based with out amino acids, mini-

	mal mineral salt media
YPD	yeast extract peptone dextrose, complete medium for yeast growth

4. Methodology

4.1. Reagents

List of the chemicals needed

Name	Formula	MW	Purity	Supplier	Order No.	Comments
acetic acid	CH3COOH	60.05	100 %	Carl Roth GmbH, Germany	3738.2	
agar agar (Kope I)	-	-	-	Carl Roth GmbH, Germany	5210.2	10 g/L
agarose LE	-	-	-	Biozyme, Germany	840004	
ammonium acetate	CH3COONH4	77.08	≥98%	Sigma-Aldrich, Germany	A1542-250G	
ampicillin (Amp) Na-salt	C16H18N3NaO4S	371.39	≥99%	Carl Roth GmbH, Germany	K029.3	
Bacto Peptone	-	-	-	BD & Co, France	211820	
Bacto Yeast extract	-	-	-	BD & Co, France	212720	
bicin	C6H13NO4	163.2	≥98%	Carl Roth GmbH, Germany	9162.1	
carrier DNA (fisch sperm, MB grade)	-	-	-	Roche Diagnos-tics, Ger-many	11467140001	
citric acid monohydrate	C6H8O2*H2O	210.14	≥99.5	Carl Roth GmbH, Germany	3958.1	
D-glucose monohydrate	C6H12O6 * H2O	198.17	≥99.5%	Carl Roth GmbH, Germany	6780.2	
dimethylsul-	(CH3)2SO	78.13	≥99.8%	Carl Roth	4720.4	

foxid (DMSO)				GmbH, Germany	
dithiothreitol (DTT)	C4H10O2S2	154.2	≥99%	Carl Roth GmbH, Germany	6908.2
dNTP set (100 mM each)	dATP dTTP dCTP dGT	-	-	Fermentas, Germany	R0181
EDTA disodium dihydrate	C10H14N2Na2O8*2H2O	372.24	≥99%	Carl Roth GmbH, Germany	8043.2
ethanol	C2H5OH	46.07	abs.	J.T. Baker, Netherlands	8006
ethylene glycol	HOCH2CH2OH	62.07	≥99.8	Sigma-Aldrich, Germany	32.455-8
formic acid	HCOOH	46.02	≥98%	Carl Roth GmbH, Germany	4724.2
glycerol	C3H8O3	92.1	≥98	Carl Roth GmbH, Germany	7530.4
hydrochloric acid	HCl	36.46	37%	Carl Roth GmbH, Germany	9277.2
lithium acetate dihydrate	C2H3LiO2*2H2O	102.02	≥99.0%	Sigma-Aldrich, Germany	62393-100G
magnesium chloride hexahydrate	MgCl2*6H2O	203.3	≥98%	Carl Roth GmbH, Germany	A537.1
magnesium sulphate heptahydrate	MgSO4*7H2O	246.48	≥99%	Carl Roth GmbH, Germany	T888.2
PEG 4000	-	-	-	Sigma-Aldrich, Germany	81242-1KG
Phenylmethanesulfonyl fluoride (PMSF)	C7H7FO2S	174.19	≥99.0%	Sigma-Aldrich, Germany	78830-5G
phosphoric acid	H3PO4	98.00	≥85%	Carl Roth GmbH, Germany	6366.1

Phusion High-Fidelity DNA Polymerase	-	-	-	NEB, England	M0530L
potassium chloride	KCl	74.56	≥99%	Carl Roth GmbH, Germany	P017.1
potassium dihydrogenephosphate	KH2PO4	136.09	≥99%	Carl Roth GmbH, Germany	3904.1
potassium hydrogenephosphate	K2HPO4	174.18	≥98%	Carl Roth GmbH, Germany	6875.1
potassium hydroxide	KOH	56.11	≥85%	Carl Roth GmbH, Germany	6751.1
restriction enzymes	SpeI (BcuI) AscI (SgsI) SacII (Cfr42I)	-	-	Fermentas, Germany	ER1251 ER1891 ER0201
sodium acetate	CH3COONa	82.03	≥99%	Carl Roth GmbH, Germany	6773.2
sodium chloride	NaCl	58.44	≥99.8%	Carl Roth GmbH, Germany	9265.1
sodium hydroxide	NaOH	40.00	≥98%	Carl Roth GmbH, Germany	P031.2
sorbitol (sorbit)	C6H14O6	182.18	≥98%	Carl Roth GmbH, Germany	6213.2
Tris	C4H11NO3	121.14	≥99.3%	Carl Roth GmbH, Germany	AE15.3
tri-sodium citrate dihydrate	C6H5Na3O7*2H2O	294.1	≥99%	Carl Roth GmbH, Germany	3580.1
Trypton / Peptone	-	-	-	Carl Roth GmbH, Germany	8952.2
Yeast extract	-	-	-	Carl Roth GmbH, Germany	2363.2
YNB	-	-	-	BD & Co, France	291920

4.2. Solutions

List of the solutions needed to perform the method

Name	Ingredients	Comments
ammonium acetate (pH 5.0, 10 mM)	0.7708 g ammonium acetate, acetic acid till pH 5.0, to 1 L with ddH2O	
ampicillin Stock (Amp)	100 mg/mL dissolved in H2O → 100 µg/mL final concentration	
BEDS solution	1.63 g bicin (→ 10 mM), NaOH till pH 7.3, 192 g sorbitol (→ 1 M), 30 mL ethylene glycol, 50 mL DMSO, to 1 L with ddH2O	
D-glucose solution	20%: 220 g D-glucose monohydrate, to 1 L with ddH2O	
dithiothreitol (DTT)	1 M: 1.542 g dithiothreitol, to 10 mL with ddH2O 2 M: 3.08 g dithiothreitol, to 10 mL with ddH2O	
dNTP mix (2 mM each)	25 µL dATP, 25 µL dTTP, 25 µL dCTP, 25 µL dGT, to 1250 µL ddH2O	
EDTA disodium dihydrate (pH 8.0)	1 M: 3.72 g EDTA disodium dihydrate, NaOH till pH 8.0, to 10 mL with ddH2O 0.2 M: 3.72 g EDTA disodium dihydrate, NaOH till pH 8.0, to 50 mL with ddH2O	
ethanol (75%)	30 mL ethanol (100%), to 40 mL with ddH2O	
LB medium (Luria-Bertani)	10 g tryptone, 5 g yeast extract, 5 g NaCl, to 1 L with ddH2O solid form by adding 15 g/L agar	
lithium acetate (pH 6.0, 100 mM)	10.2 g lithium acetate dehydrate, acetic acid till pH 6.0, to 1 L with ddH2O	
PEG4000 (40%)	4 g PEG4000, to 10 mL with lithium acetate (100 mM)	
potassium phosphate buffer (KPi-buffer, different pH)	pH 6.5, 1 M: 57.39 g K2HPO4, 90.57 g KH2PO4, to 1 L with ddH2OpH 7.4, 1M: 139.69 g K2HPO4, 26.95 g KH2PO4, to 1 L with ddH2O + KOH for phosphoric acid till desired pH	
SOC	20 g bacto peptone, 5 g bacto yeast extract, 0.58 g NaCl, 0.18 g KCl, 2 g MgCl2 x 6H2O, 2.46 g MgSO4 x 7H2O, 100 mL D-glucose (3.81g D-glucose monohydrate in 100 mL), to 1L with ddH2O	autoclave D-glucose separately
sodium acetate	4.92 g sodium acetate, to 20 mL with ddH2O	

(3 M)		
sodium citrate buffer (pH 4.0, 500 mM)	65.56 g citric acid monohydrate, 27.62 g tri-sodium citrate monohydrate, NaOH till pH 4.0, to 1L with ddH2O or 105.08 g citric acid monohydrate, NaOH till pH 4.0, to 1L with ddH2O	
sorbitol (1 M)	18.2 g sorbitol, to 100 mL with ddH2O	
TE-Buffer (pH 8.0)	1.21 g tris (→ 10 mM), 0.37 g EDTA disodium dehydrate (→ 1 mM), HCl or NaOH till pH 8.0, to 1 L with ddH2O	
Tris-HCl (pH 7.0, 100 mM)	12.1 g Tris diluted, to 1 L with ddH2O pH adjusted with 1 M HCl	
YNB Difco (yeast nitrogen base , composition)	5 g ammonium sulfate, 2 µg biotin, 0.4 mg calcium pantothenate, 2 µg folic acid, 2 mg inositol, 0,4 mg niacin, 0.2 mg p-aminobenzoic acid, 0.4 mg pyridoxine hydrochloride, 0.2 mg riboflavin, 0.4 thiamine hydrochloride, 0.5 mg boric acid, 40 µg copper sulfate, 0.1 mg potassium iodide, 0.2 mg iron chloride, 0.4 mg manganese sulfate, 0.2 mg sodium molybdate, 0.4 mg zinc sulfate, 1 g potassium dihydrogene-phosphate, 0.5 g magnesium sulfate, 0.1 g sodium chloride, 0.1 g calcium chloride, to 1 L with H2O	Supplier: BD Biosciences
YNB solution (10x)	67 g YNB Difco, to 1 L with ddH2O	sterilization by filtration
YNBG (different glucose concentrations)	1%: 100 mL YNB (10x), 50 mL D-glucose (20%) and 200 mLKPi-Buffer (1M, pH 6.5), to 1 L with ddH2O 0.6%: 100 mL YNB (10x), 30 mL D-glucose (20%) and 200 mL KPi-Buffer (1M, pH 6.5), to 1 L with ddH2O solid form by adding 15 g/L agar	autoclave D-glucose separately
YPD (pH 4.0)	20 g tryptone (peptone), 10 g yeast extract, 100 mL D-glucose (20%), optional: 100 mL sodium citrate buffer (pH 4.0, 500 mM) to 1 L with ddH2O solid form by adding 15 g/L agar	autoclave D-glucose separately

4.3. Materials

Description of materials needed to perform the method

Name	Supplier	Order No.	Comments
Cellstar Tubes (15 mL)	Greiner bio-one, Germany	188271	Greinis
Cellstar Tubes (50 mL)	Greiner bio-one, Germany	210261	Greinis
CloneJET™ PCR Cleaning Kit	Fermentas, Germany	# K1232	
Cuvettes (10x4x45mm)	Sarstedt, Germany	67.742	
Electroporation Cuvettes (2mm gap)	cellprojects Lt., UK	EP-102	
GeneJET™ Plasmid Miniprep Kit	Fermentas, Germany	# K0702	
MF-Milipore Membrane Filters (0.025µm)	Millipore, Germany	VSWP01300	
PCR Reaction Tubes (0.2 mL)	Biozyme Scientific GmbH, germany	711068	
Petri Dish	Greiner bio-one, Germany	633180	
Reaction tubes (1.5 mL)	Greiner bio-one, Germany	616 201	Eppis
Reaction tubes (2 mL)	Eppendorf AG, Germany	0030125.150	Eppis
Serological Pipette (10 mL)	Sarstedt, Germany	86.1254.001	
Serological Pipette (25 mL)	Greiner bio-one, Germany	760 180	
Serological Pipette (5 mL)	Sarstedt, Germany	86.1253.001	
Tips, 10 µL	Biozyme Scientific GmbH, Germany	720031	
Tips, 1000 µL	Greiner, Germany	740290	

Tips, 200 µL	Biozyme Scientific GmbH, Germany	760201	
Toothpicks	PAPSTRAR, Germany	12713	bought at a local vendor
Wizard ® SV Gel and PCR Clean-Up System	Promega, USA	# A9282	

Primers for construction of co-expression Vector containing CYP2D6-WT and HCPR-WT

name	Sequence	info
T_ol_pD_rv	CAAGTATGTCCATCTCGAG-GATCCGTAAAGTCACGATAGCTTAAC	ICL1-Terminator overlap with ICL1-Promoter
pD_ol_T_fw	CTATCGTGACTTTACGGATCCTCGA-GATGGACATACTTGTATCGTC	
p_ol_2D6WT_rv	GTGCTTCTAGCCCCATTTTTTGTATGCTTGG TCAGTCTACTGG	ICL1-Promoter overlap with 2D6WT
2D6WT_ol_p_fw	CTGACCAAGCATACAAAAAATGGGGCTA-GAAGCACTGGTG	
HCPR-WT_ol_T_rv	GCTAAACAAACTGCCTAGCTCCA-CACGTCCAGGG	WT Human CPR overlap with ICL1-Terminator
T_ol_HCPR-WT_vw	GGACGTGTGGAGCTAGGCAGTTTGTTTAG-CAAAATATATTTAACG	
HCPR-WT_SpeI_fw	AAAAACTAGTGCCACCATGGGA-GACTCCCACGTGG	Amplification of the whole overlap extension PCR fragment
2D6_WT_rv	TTTTGGCGCGCCCTAGCGGGGCACAGCAC	

***Y. lipolytica* strain for co-expression of CYP2D6-WT and HCPR-WT**

Y. lipolytica strains	Genotype	Phenotype	Reference
H222-S4	MATA, ura3-302a	Ura-, Alk+, Tgl+, Lip+, Eth+, Glu+ ,Suc+	(Mauersberger et al., 2001) strain collection Institute of molecular biotechnology Nr. 3595

Plasmids used for construction of co-expression Vector containing CYP2D6-WT and HCPR-WT

Plasmid	Description	Marker gene (selection in yeast)	Reference
ID30915411	cDNA wild-type human cytochrome P450 2D6 (2D6-WT)		BioCat GmbH, Germany
ID7262313	cDNA wild-type human cytochrome P450 reductase (HCPR-WT)		
pJet1.2	Cloning vector		Fermentas, Germany
p64D-linker	Cloning and expression vector	ura3d4 (mc)	(Braun et al., unpublished) EC22 strain collection Institute of molecular bio-technology Nr. 6220

4.4. Apparatus

Description of the instruments needed to perform the test and specifications

Name	Supplier	Comments
BioPhotometer	Eppendorf AG, Germany	
Centrifuge 5810	Eppendorf AG, Germany	
Gene PulserTM	BIO-RAD, USA	
GeneAmp®PCR System 2700	Applied Biosystems, USA	
HT MiltronII shaker	Infors AG, Swiss	
Pipetboy	Eppendorf AG, Germany	
PowerPacTM Basic + Sub-Cell GT	Biorad, USA	
Thermomixer comfort	Eppendorf AG, Germany	
Vortex-Genie 2	Scientific Industries Inc, USA	
Waterbath	LAUDA, Germany	
Nanodrop 2000	Thermo Scientific, USA	
Centrifuge 5415 R + A-4-62 rotor	Eppendorf AG, Germany	

pipettes: 0.1 – 2,5 µl, 0.5 – 10 µl,	Eppendorf AG, Germany
pipettes: 2 – 20 µl, 20 – 200 µl, 100 – 1000 µL	Gilson Inc., USA

4.5. Procedure

4.5.1. Multicopy, co-expression shuttle vector construction

To have each gene under the control of their own promoter the inserts are constructed by overlap extension PCR. The constructed insert is ligated between ICL1 promoter one terminator into the P64D-linker mutli-copy plasmid (Figure 1).

Empty multicopy shuttle vector p64D-linker is isolated from the *E.coli* clone EC22 (Strain collection Institute of molecular Biotechnology Nr.: 6220) according to the GeneJET™ Plasmid Miniprep Kit manual. The vector backbone is prepared by cutting the shuttle vector with restriction enzymes *Spe*I and *Asc*I over night and refurbished by Wizard ® SV Gel and PCR Clean-Up System.

Vector backbone preparation

cutting reaction mixture
50 µL Vector (whole miniprep)
10 µL SgsI (AscI)
10 µL BcuI (SpeI)
8 µL Tango Buffer (10X)
2 µL deion. H2O
ON at 37

Primers described in the "materials" section are for the construction of a human CYP2D6-WT and human CPR-WT expression strain.

The "preoverlap" fragments for the overlap extension PCR are prepared by PCR reaction. For cleaning, the whole PCR reaction is applied to a 1% agarose gel and electrophoretically separated by applying 80 V for 2-3 hours. Subsequently, the PCR fragments are isolated by Wizard ® SV Gel and PCR Clean-Up System.

Preoverlap PCR reaction

PCR mixture	PCR program
1 µL template	98°C 30''
5 µL forward primer (5µM)	(98°C 5'' – 58°C 20'' – 72°C 35'') x 30

5 µL reverse p (5µM)	72˚C 10’
5 µL dNTPmix (2mM each)	4˚C ∞
10 µL Phusion Buffer HF 5x	
23,5 µL deion. H2O	
0,5 µL Phusion Polymerase (NEB)	

The overlap extension PCR is done in two steps. The first step is the actual overlap PCR. All fragments are added to the PCR reaction mix and the PCR is run for 20 cycles using Phusion Polymerase. After the first step flanking primers are added to the reaction and the PCR is run for another 35 cycles. This PCR construct is again subject to cleaning by applying it to a 1% agarose gel and electrophoretically separate it by applying 80 V for 2-3 hours. Subsequently, the overlap construct is isolated by Wizard ® SV Gel and PCR Clean-Up System.

Overlap PCR reaction

PCR mixture	PCR program
1 µL ol-temp HCPR-WT_ol_T	98˚C 30’’
1 µL ol-temp ol_HCPR-WT_T_ol_pD	(98˚C 5’’ – 69˚C 20’’– 72˚C 3’) x 20
1 µL ol-temp ol_T _pD_ ol_2D6WT	72˚C 5’
1 µL ol-temp ol_p_2D6WT (long)	4˚C ∞
5 µL dNTPmix (2mM each)	
10 µL Phusion Buffer HF 5x	
30,5 µL deion. H2O	
0,5 µL Phusion Polymerase (NEB)	
+ (after 20 cycles)	
5 µL Primer HCPR-WT_SpeI_fw (5µM)	
5 µL Primer 2D6_WT_rv (5µM)	98˚C 30’’
4 µL Phusion Buffer HF 5x	(98˚C 5’’ – 69˚C 20’’ – 72˚C 3’) x 35
2 µL dNTPmix (2mM each)	72˚C 5’
3,5 µL deion. H2O	4˚C ∞
0,5 µL Phusion Polymerase (NEB)	

The overlap extension PCR fragment is then cloned into pJet1.2 according to manufacturer’s instructions using the CloneJET™ PCR Cloning Kit. Ligation is done at RT for 30 min. Afterwards the ligase is inactivated by incubation at 65˚C for 10 min. The ligation reaction mix is desalted by applying the mix 30 min to milipore dialysis membranes floating on ddH2O. The mix is then directly used for transformation into electro competent *E.Coli* cells. 80 µL competent cells are towed on ice. 2.5 µL desalted ligation mix is added and incubated on ice for 2-5 min. The cell mixture is transferred to an electroporation cuvette. The cuvette is pulsed by with Gene Pulser™ by applying a current of 1.25 kv/mm while resistance and ca-

pacitance are kept constant at 200 Ω and 25 µF, respectively. Subsequently, 920 µL SOC-media are added and the cell suspension transferred to a 1.5 mL eppi. The cell suspension is incubated 1 h at 37˚C and afterwards aliquots are plated on LB-Amp agar plates. The plates are incubated at 37˚C over night. Positive clones are picked, plasmid isolated according to the GeneJET™ Plasmid Miniprep Kit manual and send for sequencing to confirm the sequence.

pJet cloning

ligation reaction mix	
5,0 µL Buffer (2) 0,5 µL pJet1.2 vector (3000bp, ca 25 ng) 1,0 µL overlap extension PCR fragment (6000bp) 3,0 µL H2O deion. 0,5 µL T4 DNA ligase	30 min at RT → heat inactivation at 65˚C for 10 min → desalting via Milipore Membrane (30 min) → 2,5 to 5 µL used for transformation in to e-competent E.Coli Top10F' cells

After confirming the sequence, the co-expression insert is prepared by cutting the pJet vector with restriction enzymes *Spe*I and *Asc*I over night and refurbished by applying it to a 1% agarose gel and electrophoretically separate the insert by applying 80 V for 2-3 hours. Subsequently, the insert is isolated by Wizard ® SV Gel and PCR Clean-Up System by Wizard ® SV Gel and PCR Clean-Up System.

The concentration of the co-expression insert and the multicopy vector backbone is determined using the Nanodrop 2000. The concentrations for the ligation are adjusted in the way that the molar ration between vector backbone and insert is 1:3. The ligation, transformation and isolation are done as described above for the pJet cloning.

Ligation reaction of p64D-HCPR-WT-2D6WTlg

ligation reaction mixture	
2µL Ligase Buffer Fermentas (10x → 1x) 1µL T4 DNA Ligase Fermentas 1,0 µL Insert (Ol-PCR fragment, ca. 140 ng) 2,5 µL Vectorbackbone (ca. 50 ng) 13,0 µL H2O	→ ON at RT → 10 min at 65˚C heat inactivation → desalting with Millipor membrane filter →5µL, transformation direct into electro competent cells (E.Coli)

The co-expression cassette is linearized with the restriction enzyme *Sac*II. For optimal functionality of the restriction enzyme a second cutting site is recommended. This can be achieved by adding a small oligonucleotide sequence containing a *Sac*II cutting site. Linearization is done over night at 37˚C. The restriction enzyme is inactivated by incubation at 65˚C for 10 min and refurbished by Wizard ® SV Gel and PCR Clean-Up System. To prepare

sufficient linearized co-expression cassette several cleaned cutting reactions are pooled together and concentrated by DNA precipitation.

Linearization reaction of p64D-HCPR-WT-2D6WTlg (14020bp)

reaction mixture	
50 µL Miniprep (100ng-300ng) 10 µL SacII (10U/µL) 10 µL Buffer B (10X) 0.5 µL Oligo-SacII (→5µM) 29,5 µL deion. H2O	ON at 37° → 65°C heat inactivation for 10 min → PCR-cleanup kit (promega) dilution with 50 µL H2O (optional: → pool together) → precipitation (several hours) → control gel

For DNA precipitation, 1/10 Volume of 3 M sodium acetate (end concentration 0,3 M) is added the DNA solution and gently mixed. After adding two volumes prechilled ethanol (100%) precipitating DNA is visible. The mixture is gently mixed and incubate for several hours at -20°C (over night is also possible). The DNA is separated by centrifugation at 4°C and 16000g for 10 min. The supernatant is discarded and the DNA pellet washed with one volume prechilled 75% ethanol. Again the DNA is separated by centrifugation at 4°C and 16000g for 10 min. The supernatant is discarded and the pellet dried fro 15 min at ~ 40 °C. The DNA is redissolved with 50 µL sterile ddH2O.

4.5.2. DNA transformation

Transformation can be achieved by the lithium acetate method (Barth et al., 1996) or by a adjusted condensed electroporation protocol (Lin-Cereghino et al., 2005).

4.5.2.1. Lithium acetate method

Preparation of competent cells

In the morning 5 mL YPD pH 4.0 are inoculate with some *Y. lipolytica* cells (Strain: H222-S4, ura-) in a 100-ml flask and shake at 28 °C and 220 rpm. In the evening OD600 is measured and 4 times 10 mL YPD pH 4.0 in 250 mL flasks are inoculated to OD600 = 0.001, 0.004, 0.008 and 0.016 (1x10^4, 5×10^4 , 1×10^5, and 2×10^5 cells/ml) respectively. The cells are grow overnight at 28 °C and 220 rpm. The next morning, the culture that has an OD600 = 5-6 (9×10^7 and 1×10^8 cells/ml) is further used. ***Important:*** This density is critical

Cells are washed twice with 10 ml TE-buffer (prewarmed at 28°C). Cells are resuspended to an OD600 = 3 (5×10^7 cells/ml) with 0.1 M LiAc pH 6.0 (prewormed at 28°C) and incubate for 1 h while gentle shaking (60-100 rpm) at 28 °C. Cells are concentrated by centrifugation at 2000 g and 28°C for 5 min and resuspending in one-tenth of the volume with LiAc buffer

to obtain around OD = 30 (5×10^8 cells/ml) These competent cells can by directly used for transformation.

Note: Competent cells can be stored at -80°C. However, to obtain highest transformation efficiencies, competent cells should be prepared fresh. Especially when going for multicopy integration

Transformation

All operations are performed on ice. 0.5-1 µg of transforming DNA (linearized Vector) in 1-5 µL are placed at the bottom of a 2 ml reaction tube. The DNA is heat shocked at 95°C for 5min and placed immediately on ice. 5 µL denatured carrier DNA (heat shocked for 5min at 95°C and place on ice) is added to the transforming DNA. 100 µL competent cells are added and gently mixed and incubated at 28°C for 15 min without shaking. ***Attention:*** the cells are very sensitive, cut of the tip of the tips.

Cells are again gently mixed and 0.7 ml 40 % PEG 4000 is added, the mixture gently mixed and incubate for 1 h at 28 °C, while shaking (rotary shaker at 100 rpm). The cell suspension is heatshocked at 39 °C for 10 min in a water bath. Afterwards 1.2 ml 0.1 M LiAc buffer pH 6.0 is added in two portions and gently mixed. Aliquots of 200 µL are plated on selective YNBG agar plates and incubated at 28°C. *Y. lipolytica* transformants are selected for ura+ phenotypes.

Single copy transformants appear after 1–2 days while multicopy transformants appear after 2-3 weeks. Positive transformants are transferred to fresh YNBG agar plates.

4.5.2.2. Condensed electroporation protocol

Preparation of competent cells

5-10 mL of YPD pH 4.0 in a 100-ml flask are inoculate with some *Y. lipolytica* cells (strain: H222-S4, ura-) and incubated at 28 °C and 220 rpm over night. The next day 50 mL YPD pH 4 in 200 mL buffled flask are inoculated to an OD600 of 0.15 – 0.20. Cells are grown to an OD600 of 0.8 – 1.0 at 28°C and 120 rpm. ***Note:*** based on generation time of 120 to 150 min, *Y. lipolytica* should take 5-6 h

Cells are harvested by centrifuge at RT and 500g for 5 min. The supernatant is discarded and the cell pellet resuspended in 9 mL of ice-cold BEDS solution supplemented with 1 mL DTT. The cell suspension is incubated at 28°C and 100 rpm for 5 min. ***Note:*** Gentle agitate with the hand till the solution feels warm works also quite well.

The cell suspension is again centrifuged at RT and 500g for 5 min at RT and resuspended in 1 mL (0.02 volumes) of BEDS solution without DTT.

Note: if the OD600 is below 0.8 one can reduce the volume of BEDS accordingly (e.g. 0.6 – 0.8 → ~ 0.8 mL, and so on).

These competent cells can be directly used for transformation.

Note: Alternatively, cells can be stored at -80˚C. Freeze cells slowly in small aliquots. Cells can be stored for at least 6 months at -80˚C. However, to obtain highest transformation efficiencies, competent cells should be prepared fresh

Transformation

5-15 µL linearized plasmid DNA (0.5 – 1 µg for single copy, 4-8 µg for multi copy) are mixed with 80 - 100 µL of competent cells in an electroporation cuvette and incubated for 2 min on ice. Electroporation is performed using Gene Pulser with following settings: 1.5 (single copy) – 2.0 (multicopy) kV charging voltage, 200 Ω resistance and 25 µF capacitance. Immediately after electroporation cell suspensions are resuspended in 1 mL cold 1.0 M sorbitol. Aliquots are plated on selective YNBG agar plates and incubated at 28˚C. *Y. lipolytica* transformants are selected for ura+ phenotypes.

Optional: After electroporation and adding 1 mL cold 1.0 M sorbitol, incubate the cell suspension for 15 min at 28 ˚C

Single copy transformants appear after 1–2 days while multicopy transformants appear after 2-3 weeks. Positive transformants are transferred to fresh YNBG agar plates.

4.5.3. Cultivation and Induction

Pre culture:

50mL YNBG1 in a 300 mL flask are inoculated with some cells of a positive *Y. lipolytica* transformant from an agar plate. The cells are grown for 18-24 h at 28°C while shaking at 220 rpm.

Main culture:

250 mL YNBG0.6 in a 1 L flask are inoculated to a starting OD600 = 0.2.

Optional: Centrifuge preculture at 1000 g for 5 min and resuspend cells in 5 mL YNBG0.6.

The cell suspension is incubated at 28°C and 200 rpm till complete glucose depletion which takes roughly 17-20 h. After glucose depletion cells are further incubated for another 2-4h (starvation phase).

Induction:

The heterologue protein production of genes under the control of the ICL1 promoter is started by adding EtOH to a concentration of 1%. Subsequently, after 8 h and 18-20 h additional EtOH is added to a concentration of 1%. Cells are harvested after 20-24 hours of induction.

This cells can be used for whole cell conversion experiments as well as for microsome isolation.

4.6. Calculations

4.6.1. Cell count

The cleaned thoma chamber is breathed on and the glass plate put onto it by slightly pressing with the thumbs the sides of the glass plate so that the small carved lines of the grid slightly above the edge of the glass plate (Figure 2). A small amount of cell culture is taken and diluted by the factor of 100. 10 µL of this diluted cell culture is placed on top of the glass plate edge where the solution can flow onto the carved grid. The carved gird can only hold a defined amount of cell solution. Exceeding cell solution will pure out below. Wait for a few minutes till the cells have settled. Count the cells in 5 of the smaller squares (LQ) (Figure 3).

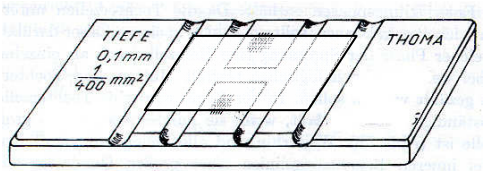

Figure 2: THOMA chamber

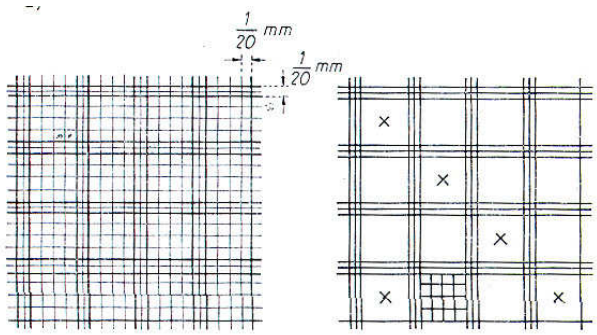

Figure 3: Close-up of the grid

5 small squares equates to 0.02 µL. To get the cell count per mL, the counted cells in this 5 small squares are multiplied with the factor 50000 and multiplied with the dilution factor, in our case 100.

Example: total cells in 5 small squares is 17 → 8.5*10^8 cc/mL

4.6.2. Transformation rate

Colonies are counted of a plate where an aliquot of 200 µL has been plated. The colony numbers are multiplied by 5 and divided by the µg DNA used for the transformation. This gives a transformation frequency (rate) of colonies per µg DNA.

5. Safety Precautions

Please follow instructions described in “acib-Mitarbeiterleitfaden Gefahrstoff- und Laborordnung”

6. Documentation

Lab books AGB-1-3 (Braun Andreas)
Dissertation Braun Andreas 2012

7. References

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Curriculum Vitae

Europass Curriculum Vitae

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Work experience

Dates	2008 - 20012
Occupation or position held	scientific researcher in the framework of the EU-Project OXYGREEN (Project Nr. 212281)
Main activities and responsibilities	Independent scientific research, planning and executing of complex research tasks in the area of molecular biotechnology and analytical chemistry in the framework of the PhD. responsible person for HPLC-MS including training of new co-worker
Name and address of employer	Graz University of Technology Institute of molecular biotechnology Petersgasse 14, 8010 Graz, Austria
Type of business or sector	University
Dates	07.2006 – 09.2006 07.2005 – 09.2005 02.2005 08.2004-09.2004
Occupation or position held	analytic and quality management, Internship
Main activities and responsibilities	routine analytics, establishing and validating of new analytical methods, relations with suppliers, training of co-workers
Name and address of employer	Kraft Food Austria “Suchard”, Bludenz, AUT
Type of business or sector	Manufacturer of confectionery
Dates	Summer 2004
Occupation or position held	Lab assistant, Internship
Main activities and responsibilities	synthesis of silicon compounds
Name and address of employer	Dr. Maschner, Institute of inorganic chemistry, University of applied technology, Graz, AUT
Type of business or sector	Research

Education and training

Dates	2008 – 2012
Title of qualification awarded	PhD in engineering sciences (Biotechnology, Biochemistry and food chemistry)
Principal subjects/occupational skills covered	engineering sciences with major in Biotechnology, biochemistry and food chemistry Dissertation Title: Expression of human liver cytochrome P450 enzymes in the non-conventional yeast Yarrowia lipolytica for the evaluation of two-liquid biphasic whole-cell biotransformation of steroids
Name and type of organisation providing education and training	Graz University of Technology, Graz, AUT
Level in national or international classification	ISCED 6
Dates	2005-2008
Title of qualification awarded	Master of science in chemical engineering
Principal subjects/occupational skills covered	chemical engineering with major in biochemistry, biotechnology and food chemistry Thesis Title: 'Random mutagenesis of industrially important esterase APLE (alternative pig liver esterase)', Protein engineering that contributes to the understanding of the structure-function relationship.
Name and type of organisation providing education and training	Graz University of Technology, Graz, AUT
Level in national or international classification	ISCED 5A
Dates	2002-2005
Title of qualification awarded	
Principal subjects/occupational skills covered	chemical engineering
Name and type of organisation providing education and training	Graz University of Technology, Graz, AUT
Level in national or international classification	ISCED 5A
Dates	1997-2002
Principal subjects/occupational skills covered	chemical engineering with major in Environmental engineering
Name and type of organisation providing education and training	higher technical education institute HTL, Dornbirn, AUT
Level in national or international classification	ISCED 3A
Dates	1993 – 1997
Title of qualification awarded	trained worker
Principal subjects/occupational skills covered	chemical laboratory worker
Name and type of organisation providing education and training	vocational industry school, Dornbirn, AUT JM Fussenegger Textil, Dornbirn, AUT
Level in national or international classification	ISCED 3C

Personal skills and competences

Mother tongue(s) **German**

Other language(s)

Self-assessment	Understanding				Speaking				Writing	
European level (*)	Listening		Reading		Spoken interaction		Spoken production			
English	C2	Proficient user	C2	Proficient user	C1	proficient user	C1	proficient user	B2	Independent user

(*) Common European Framework of Reference for Languages

Social skills and competences

- supervision: During my diploma thesis I took care of two trainees from abroad.
- team work: I was involved in various types of teams during my internships and studies.
- communication qualities: as researcher in the framework of an international EU-project I was able to practice my presentation and communication skills at the regularly project meetings

Organisational skills and competences

- sense of organization (organized a big school event for 1500 people)
- leadership qualities: during my Master thesis and PhD I was overseeing exchange students from abroad (USA, Croatia). Additionally I was involved in teaching and tutoring seminars and lab courses. In the summer semester of 2011 I lectured applied Bioinformatics at the university of applied sciences Graz
- independence: as research associate I planned and executed independently complex scientific tasks

Technical skills and competences

- high throughput screening: For my diploma thesis I used a modern automated picking system to generate ordered screening arrays
- Bioreactors: during my PhD I worked with Sartorius and DasGIP fermentation systems
- HPLC-MS: during my PhD I worked and was responsible for maintenance of the HPLC-MS system additionally I was responsible for training of new co-workers
- GC-MS: at my current employment I am responsible to setting up a new GC-MS system (Shimadzu) for routine analytics

Computer skills and competences

- good command of Microsoft Office tools (Word, Excel and PowerPoint)
- good knowledge of Bioinformatics tools (VNTI, PyMOL, SeqMen, …)
- good knowledge of Internet tools (FTP, …)

Deutsche Fassung:
Beschluss der Curricula-Kommission für Bachelor-, Master- und Diplomstudien vom 10.11.2008
Genehmigung des Senates am 1.12.2008

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die vorliegende Arbeit selbstständig verfasst, andere als die angegebenen Quellen/Hilfsmittel nicht benutzt, und die den benutzten Quellen wörtlich und inhaltlich entnommene Stellen als solche kenntlich gemacht habe.

Graz, am 4/7/2012 ..... Braun Andreas .....
(Unterschrift)

Englische Fassung:

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

4/7/2012 ..... Braun Andreas .....
date (signature)