# HETEROLOGOUS EXPRESSION OF CYTOCHROME P450 MONOOXYGENASES IN DIFFERENT ASCOMYCETOUS YEASTS

BY

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**“I may not have gone where I intended to go, but I think I have ended up where I needed to be.”**

**— Douglas Adams**

**“The great tragedy of Science — the slaying of a beautiful hypothesis by an ugly fact.”**

**— Thomas Henry Huxley**

**“Sometimes the questions are complicated and the answers are simple.”**

**— Dr. Seuss**

**“Live as if you were to die tomorrow. Learn as if you were to live forever.”**

**— Mahatma Gandhi**

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# Chapter 1: Literature review

## 1.1. Introduction

Biocatalysis is a key area of biotechnology, as it can substantially improve production of chemicals at reduced costs while being environmentally friendly. It involves the use of biological material, whether whole cells, cellular extracts or isolated enzymes, to catalyze chemical reactions; either as steps of multistep synthetic pathways or as the entire pathway. The use of whole cell systems simplifies processes by eliminating cell fractionation or enzyme purification steps; by improving enzyme stability (and thereby also the duration of activity); and by recycling expensive cofactors within the cell. They may, however, be limited by low membrane permeability for substrate uptake, by toxicity of the product to the cell or by unwanted side reactions (Murphy, 2011; Zöllner *et al*., 2010).

Cytochrome P450 monooxygenases are biocatalysts with high potential. They can catalyze hydroxylation of non-activated hydrocarbons with exceptional specificity, by using molecular oxygen and reduced cofactors. Much of the research on P450s has been dedicated to their roles in drug metabolism and their use for drug design (Zöllner *et al*., 2010). Other applications of these enzymes include bioremediation and the biosynthesis of fine chemicals with pharmaceutical applications and organoleptic properties (Van Beilen and Funhoff, 2005; Urlacher and Eiben, 2006; Schewe *et al*., 2011; Kumar, 2010). This study will focus specifically on the potential of cytochrome P450s for their application in whole-cell biocatalysts, considering their limitations and potential solutions. As such, the study will also focus on hosts for efficient heterologous expression of cytochrome P450s.

## 1.2. The cytochrome P450 enzyme superfamily

Cytochrome P450s, (abbreviated as CYPs or P450s) comprise a highly diverse, ubiquitous family of heme-containing oxidoreductases, capable of a multitude of different reactions (Isin and Guengerich, 2007). Our interest, however, is focused only on hydroxylation reactions catalysed by these monooxygenases. P450s have been the subject of studies for over 50 years (Estabrook, 2003) and are currently the subject of research by various groups worldwide.

In a typical P450 catalysed monooxygenation reaction a reductase protein (for example cytochrome P450 reductase, CPR) transfers reducing equivalents from NAD(P)H either directly or via a mediator protein to the heme-containing P450 active site. Once reduced, the heme can then bind molecular oxygen ($O_2$) and catalyse the insertion of one oxygen atom into a carbon-hydrogen bond. The other oxygen atom is reduced to form water ($H_2O$) as a side product (figure 1.1).

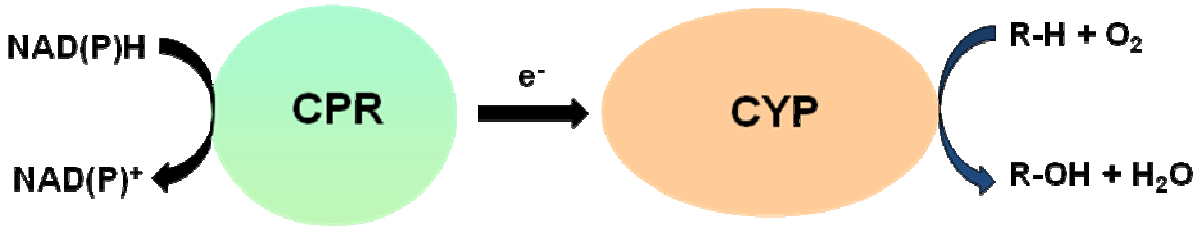



**Figure 1.1: Example of a typical monooxygenation reaction catalysed by class II cytochrome P450s.** After electron transfer from NAD(P)H via CPR (and in some cases mediator proteins), the heme-containing P450 catalyses the hydroxylation of a hydrocarbon substrate by reductively cleaving molecular oxygen ($O_2$), to yield water ($H_2O$) as a side product.

When the reduced heme binds carbon monoxide (CO), the enzyme-CO complex absorbs light differentially at 450 nm, hence their name cytochrome P (pigment)

450. A reduced P450 difference spectrum is obtained when the spectrum of the reduced enzyme is subtracted from reduced enzyme-CO complex, and can be used for P450 quantity approximation using a calculation incorporating the difference between the absorbance readings at 450 nm and 490 nm (Bernhardt, 2006).

In most cases the reducing equivalents are provided by NAD(P)H, via one or two electron transfer proteins. Different P450s use different types of electron transfer proteins. These variations have been used to classify P450s into at least 7 classes, four of which will be discussed here and are illustrated in figure 1.2. All prokaryotic P450s are soluble, cytosolic enzymes, while eukaryotic P450s are membrane-anchored by hydrophobic N-terminal regions, either in inner mitochondrial membranes (class I) or membranes of the endoplasmic reticulum (ER) (class II; microsomal P450s) (Hannemann et al., 2007).

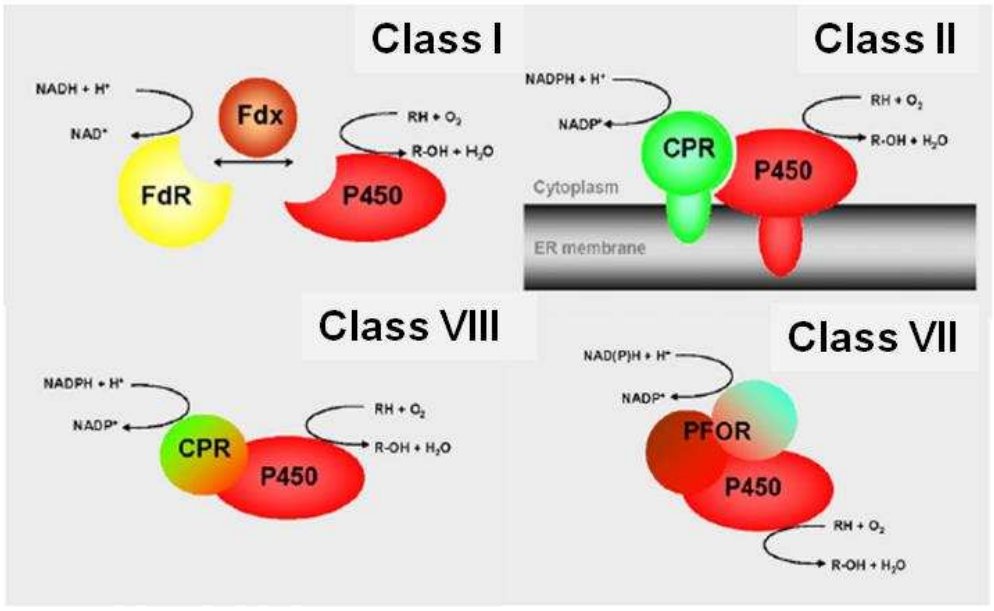



**Figure 1.2: Four different classes of cytochrome P450s based on reductase partners (Adapted from Hannemann *et al*., 2007).**

Most bacterial P450s, belong to class I, together with mitochondrial eukaryotic P450s. These are 3 component systems consisting of the P450; a [2Fe-2S]-type

iron-sulphur cluster-containing ferredoxin as a electron mediator protein; and a FAD-containing ferredoxin reductase. Microsomal eukaryotic P450s belong to class II, relying on a cytochrome P450 reductase (CPR), which contains both flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Hannemann *et al.*, 2007). Microsomal eukaryotic P450s also vary considerably in their dependence on cytochrome *b*5 as a third component in electron transfer (Yamazaki *et al*., 2002; Schenkman and Jansson, 2003).

Self-sufficient P450s are enzymes in which the P450 domain is naturally fused to a reductase domain in a single polypeptide. Class VIII self-sufficient P450s are fused to the eukaryotic class II-type CPR, for example CYP102A1 from *Bacillus megaterium* (Narhi and Fulco, 1987) and CYP505A1 from *Fusarium oxysporum* (Nakayama *et al*., 1996). Class VII P450s are fused to a reductase belonging to the phthalate dioxygenase family, which contains a FMN and a ferredoxin-like [2Fe-2S] centre. These reductases are not usually associated with P450s (Roberts *et al*., 2002).

### 1.2.1. Natural functions and further applications of P450s

In humans P450s are involved in biosynthesis of important natural compounds such as sterols, vitamins, fatty acids and eicosanoids, as well as in the metabolism of xenobiotic compounds. The human genome has 57 putative P450 genes, of which more than a quarter are confirmed to be involved in the metabolism of xenobiotics (Guengerich *et al*., 2005). Most medicinal drugs are xenobiotics; therefore P450s are key enzymes in the pharmaceutical industry for research into drug design and metabolism (O'Reilly *et al*., 2011).

P450s catalyse the initial hydroxylation of aliphatic hydrocarbons, increasing their water-solubility and functionalizing them for subsequent degradation. Their ability to catalyze initial functionalization of hydrocarbons, make them important tools

for the bioremediation of compounds such as aliphatic hydrocarbons like alkanes; bulkier and more complex compounds such as polyaromatic hydrocarbons (PAHs); halogenated compounds such as polychlorinated biphenyls (PCBs); and waste from military explosives and herbicides (O'Reilly *et al.*, 2011; Urlacher and Eiben, 2006; Kumar, 2010). Organisms with multiple P450s can be targeted for bioremediation applications.

There are three major problems when working with P450s in cell free extracts or as isolated enzymes, namely: (i) the need for constant regeneration of the required reduced co-factors; (ii) that most P450 systems require one or two electron transfer proteins; and (iii) that P450s are usually unstable under conditions applied during isolation, catalyses or storage. Nevertheless, cell-free extracts and purified P450s are often used, mostly for enzyme characterization, using small scale reactions and short reaction times. These factors, together with the additional steps involved in cell fractionation and / or enzyme purification, make the use of purified or isolated P450s impractical for industrial application (Chefson and Auclair, 2006; Behrens *et al*., 2011; O'Reilly *et al*., 2011).

### 1.2.2. Strategies for overcoming limitations of P450s

Attempts towards overcoming limitations of P450s for industrial use have largely focused on two aspects: modification of the P450 component itself to improve properties such as stability, substrate range and reaction specificity; and means to overcome the electron transfer problems.

#### Modification of P450s

Wild-type organisms can be screened for novel activities or activities of interest, based on their ability to degrade hydrophobic compounds. However an efficient screening system is required for this approach and it tends to be a great effort.

Furthermore, results may be misleading as some organisms may express P450s at low levels which fall below the detection limit of the employed assay (Furuya and Kino, 2010). Additionally, working with some wild-type hosts such as multicellular organisms (animals and plants) or pathogenic microorganisms can be very impractical (McLean *et al*., 2007).

Therefore genome mining is an alternative, where sequences of functionally characterized enzymes are used to search databases for homologues, which can then be cloned, heterologously expressed and screened for improved properties. Therefore appropriate screening techniques are also required eventually. Genome mining has been boosted by great advances in sequencing technologies which has increased the number of available sequences, including whole genomes, which are available on various online databases (Furuya and Kino, 2010; Hedeler *et al*., 2007; http://www.ncbi.nlm.nih.gov/nuccore; www.broadinstitute.org). Functions of entire cytochrome P450 complements (or CYPomes) of organisms have been predicted based on genetic information, for example the *Phanerochaete chrysosporum* CYPome consisting of 150 P450 genes, for which structural and evolutionary analyses have been performed (Doddapaneni *et al*, 2005).

Enzymes with attractive properties but certain limitations can be genetically modified to produce mutants with improved substrate specificity, product selectivity, enzyme stability or combinations of these features. The library size is dependent on the technique applied, of which there are two general routes, rational design and directed evolution. Basic overviews of these techniques are illustrated in figure 1.3.

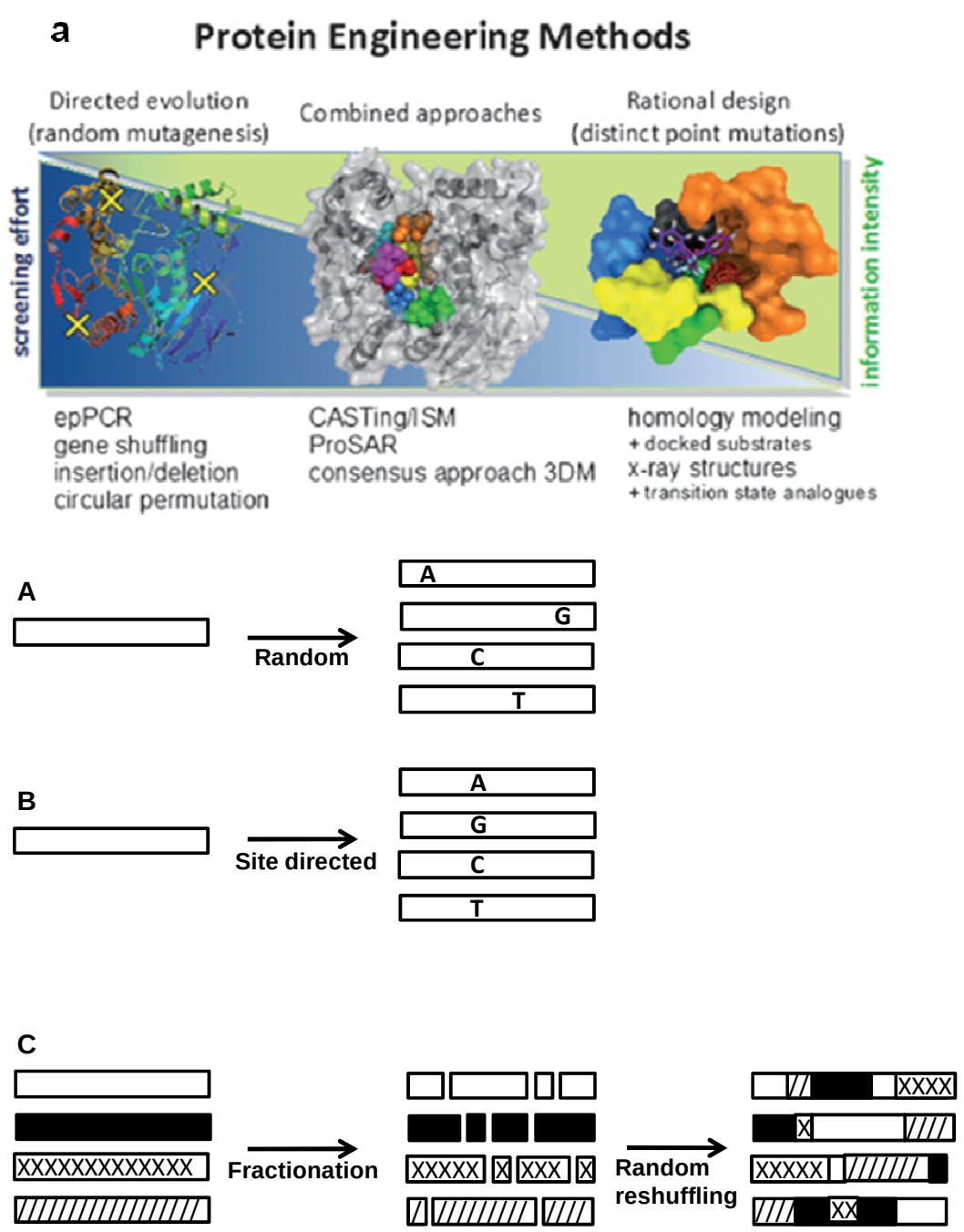



**Figure 1.3: General overview of the different routes for genetic modification of enzymes (Part 'a' adapted from Behrens *et al*, 2011).** epPCR – error-prone PCR; CAST – Combinatorial Active-site Saturation Test; ISM – Iterative Saturation Mutagenesis; ProSAR – Protein Sequence Activity Relationships; 3DM – database containing 1751 structurally related proteins.

Rational design involves site directed mutagenesis, and therefore requires extensive structural knowledge of the enzyme of interest, preferably with the 3D-structure resolved or with accurate molecular models available. Alternatively, certain conserved residues and preferentially residues which were experimentally demonstrated to be critically important, such as in the active site or regions conferring stability, can be targets for mutagenesis (Kumar, 2010; Behrens *et al*., 2011). The advantage of this technique is that specific alterations generate a limited number of mutants for screening which decreases the workload involved. A good example of this approach for CYP102A1 targeted residue F87 which was identified to be in close proximity to the heme group in the enzyme active site. Upon substrate binding, the phenyl ring moves from nearly perpendicular to the heme to within $45^{o}$ of it, forming a 'cap' over the heme and limiting substrate access (Noble *et al*., 1999; Chen *et al*., 2008). Replacing the phenylalanine with different residues led to drastic changes in substrate specificity (Noble *et al*., 1999; Graham-lorence *et al*., 1997; Oliver *et al*., 1997).

Directed evolution involves the random (or semi-random) mutagenesis of a peptide sequence. Random mutagenesis utilises techniques such as error-prone PCR to rapidly generate large and diverse libraries of mutants with no requirement of structural knowledge of the protein. The vast libraries need to be screened however; therefore a rapid and efficient screening or selection technique is required to sort through the generated mutants (Gillam, 2007; Behrens *et al*., 2011). In one example of the application of this technique CYP102A1, a fatty-acid sub-terminal hydroxylase, was converted into a short chain (C3-8) alkane hydroxylase, and further mutants even achieved conversion of ethane to ethanol (Glieder *et al.*, 2002; Meinhold *et al*., 2005). CYP102A1 has also been mutated to be able to oxidise bulkier substrates such as aromatic hydrocarbons, polyaromatic hydrocarbons (PAHs), cycloalkanes, and heteroarenes (Carmichael and Wong, 2001; Appel *et al*., 2001).

DNA recombination techniques such as DNA shuffling, involve the exchange of fragments of different genes or mutants, forming mosaic-like structures. The starting ‘parental’ DNA strands are fragmented and allowed to randomly reassemble using primerless PCR, after which the full-length sequences are amplified and cloned into expression vectors. The resultant chimeric enzymes can then be screened. DNA family shuffling is similar, but the ‘parental’ DNA strands come from homologues of the gene of interest, such as members of the same protein family or similar proteins from different organisms. The resultant mutants are therefore more likely to at least be functional and relatively stable (Harayama, 1998; Rosic *et al*., 2007). DNA shuffling (also referred to as the molecular Lego technique) was used to generate more thermostable mutants of CYP102A1, which is a useful step towards potential industrial application (Li *et al*., 2007). It has also been applied to form artificial P450–reductase fusions (Gilardi *et al*., 2002; Dodhia *et al*., 2006). A non-random gene recombination was used to combine the more thermotolerant reductase domain of CYP102A3 with the more active hydroxylase domain of CYP102A1, resulting in a chimeric enzyme with higher thermostability and longer activity retention, although with total productivity somewhat decreased (Eiben *et al*., 2007).

Usually a combination of techniques works well, as resultant mutants constructed by random mutagenesis can be templates for DNA shuffling or rational design once critical residues have been identified (Gillam, 2007; Urlacher and Girhard, 2012). Techniques combining rational design and directed evolution are called saturation mutagenesis, focused-directed evolution or semi-rational design. These techniques involve the randomization of particular amino acid residues or specific stretches of amino acids. An example is the combinatorial active-site saturation (CAST) approach, which, as the name suggests, involves mutations of residues surrounding the active site. Iterative saturation mutagenesis (ISM) can be linked to this technique, in which promising mutants from each round of

mutagenesis serve as templates for a subsequent round of mutagenesis. Statistical approaches using computer programmes, such as Protein Sequence Activity Relationships (ProSAR), analyze accumulated data concerning sequence-activity relationships, preferably obtained from different mutagenesis experiments (Figure 1.3a; (Behrens *et al*., 2011).

The mutagenic strategies mentioned above ideally require convenient, inexpensive, rapid and reliable high-throughput screening techniques. The screens should also be sufficiently sensitive and reproducible to detect even slight improvements (O'Reilly *et al*., 2011; Gillam, 2007). Some of the most frequently used assays involve fluorometric substrates (such as alkyl-derivatives of coumarin, resorufin and quinolone) (figure1.4.a) (Lussenburg *et al*., 2005; Roberts *et al.*, 2002; Kumar and Singh, 2006; Khan and Halpert, 2002; and colometric substrates (for example indole) (figure 1.4.b) (Celik *et al*., 2005; (Rosic *et al*., 2007; Li *et al*., 2000; Gillam *et al*., 1999).

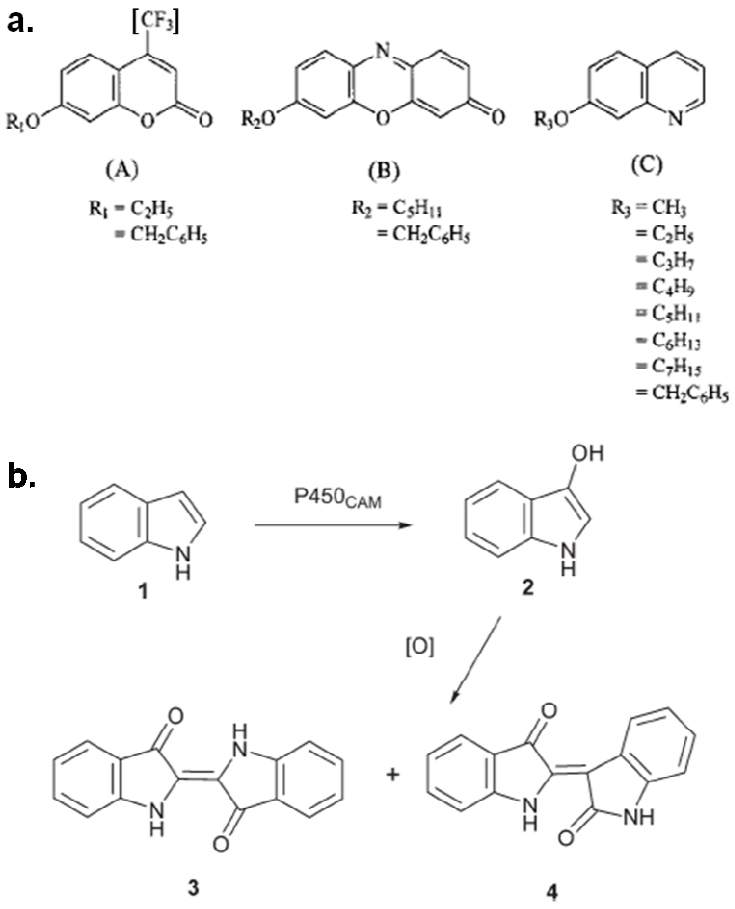



**Figure 1.4: Substrates typically used for P450 screening. a: Pro-fluorescent substrate types used in assays of P450s and their mutants, which form strongly fluorescent products (adapted from Khan and Halpert, 2002).**
**b: Conversion of indole (1) into the coloured dyes indigo (3) and indirubin (4) via the intermediate 3-hydroxy indole (2) (adapted from Celik *et al*., 2005).**

Based on the structures of these compounds, they facilitate screening for enzymes which can metabolize drug-like aromatic compounds. Schwaneberg and co-workers developed a rapid, high throughput screening technique for CYP102A1 and its mutants, which have activities toward fatty acid-type substrates. The assay is based on *p*-nitrophenoxycarboxylic acid (pNCA) substrates, which when terminally hydroxylated form ώ-oxycarboxylic acids and

the chromophore *p*-nitrophenol (pNP), which is detected spectrophotometrically at 410 nm (figure 1.7; Schwaneberg *et al*, 1999). Another interesting screen was developed using luciferin derivatives as luminogenic substrates, in which substrate metabolism liberates luciferin, which emits light when coupled to a secondary luciferase mediated reaction (Gillam, 2007). In fact, the commercially available pGlo™ system consists of membranes containing human P450s and luciferin-derived substrates for screening of compounds for modulatory effects on these enzymes (http://www.promega.com/products/cell-health-assays/adme-assays/p450_glo-cyp450-screening-systems/). As demonstrated by these examples, the intended end application of the P450 can determine the type of fluorogenic, chromogenic or luminogenic substrate analogues to use. It is however possible that potentially useful mutants which do not accept the substrate analogue employed in the screening technique, will be wasted (Gillam, 2007).

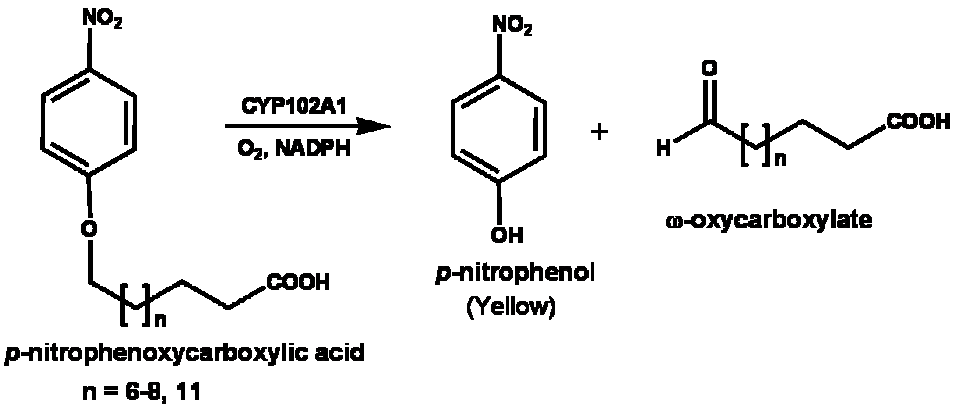



**Figure 1.7: *p*-nitrophenoxycarboxylic acid assay for fatty acid hydroxylating P450s (adapted from Schwaneberg *et al*., 1999).**

More accurate activity determination can be achieved using various analytical techniques such as gas chromatography (GC), mass spectrum (MS), high-pressure liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS) techniques, but they are generally not suitable for very high-throughput applications and are hence not preferable for initial screens.

Activities can also be detected by monitoring NAD(P)H consumption at 340nm, (Gustafsson *et al*., 2004) or $NAD(P)^+$ production in the presence of strong alkali at 360 nm (Tsotsou *et al*., 2002). An oxygen biosensor was developed (BD Biosciences, USA), which allows the monitoring of $O_2$ consumption, using an oxygen sensitive fluorophore in microtiter plates, allowing simultaneous screening of multiple samples. Oxygen quenches the ruthenium dye; therefore oxygen depletion leads to increased fluorescence of the dye (Olry *et al*., 2007). Alternatively for P450s utilizing peroxides as oxygen and reductant source, consumption of $H_2O_2$ can be monitored colorimetrically using horseradish peroxidase as a reporter enzyme (Xu *et al*., 2007), while organic peroxide consumption can be monitored by using catalase and the commercial fluorogenic dye Amplex Red (Rabe *et al*., 2008). Uncoupled cofactor or oxygen consumption upon substrate binding may lead to false positives, therefore rigorous additional control reactions are required to overcome these limitations (Furuya and Kino, 2010). For truly high-throughput screening, the screening techniques need to be applicable to whole-cells, since these also allow detection of low levels of activity due to higher stability of enzyme in the cellular environment. Additionally, time-consuming cell lysis, sub-cellular fractionation and / or enzyme purification steps are eliminated. Whole-cells are however obviously not suitable for determination of enzyme kinetics, due to interference by cellular barriers and non-specific interactions with other intracellular components (Schwaneberg *et al*., 2001; Kumar and Singh, 2006).

**Solving electron supply problems**

The requirement for expensive cofactors limits the industrial application of P450s. CYP102A1 largely solves two major problems associated with P450s, in that it is a relatively stable, single component system; but it still requires co-factor regeneration. Like many other P450s it is dependent on NADPH rather than on NADH, which is problematic because NADPH can be 4-10 times more expensive

than NADH (www.sigmaaldrich.com; Urlacher and Eiben, 2006). A CYP102A1 mutant prefers NADH over NADPH, but it is still not feasible to keep feeding NADH into a large-scale system. Coupling of a second enzymatic reaction to the P450 reaction is an efficient way of achieving co-factor regeneration in cell-free and whole cell systems. Examples include formate dehydrogenase (FDH) (Maurer *et al*., 2005), and glucose dehydrogenase (GDH). Cofactor regeneration by coexpression of GDH was found to be more efficient than adding commercially available GDH to the reaction system (Lu and Mei, 2007).

Artificial sources of reducing equivalents to bypass the NAD(P)H requirement completely have been investigated. Some P450s can use peroxides to supply electrons and oxygen through the peroxide shunt pathway, replacing the need for molecular oxygen and NAD(P)H. However these peroxygenase activities are usually inefficient and the peroxides rapidly inactivate the P450s (Cirino and Arnold, 2003). Direct chemical reduction using sodium dithionate is drastically inefficient (Schwaneberg *et al*., 2000), while direct reduction using P450s immobilized on cathodes lead to instability of the P450s, as well as poor access to the buried heme group (Kazlauskaite *et al*., 1996). This was improved by using Pt electrodes and Co(III)Sepulchrate (Co(III)sep) as an electron mediator, but Pt is very expensive, rendering the method unfeasible (Faulkner *et al*., 1995). The expensive Pt electrodes were replaced by cheap zinc dust, keeping Co(III)sep as mediator (Schwaneberg *et al*., 2000). This method was used for electron supply to an immobilized CYP102A1 variant in a bioreactor together with catalase for hydrogen peroxide removal, and the reactor could continue for 5 days with total turnover numbers of over 2000 (Zhao *et al*., 2011). Recent advances in the bioelectrochemistry of P450s, mainly focused on approaches for improving immobilization of P450s on electrodes, have been reviewed by Sadeghi and co-workers. These include combinatorial techniques of engineering both the recombinant P450s by DNA shuffling techniques and the electrodes for immobilization and electron transfer. Among the tested conditions CYP3A4

fused to the flavodoxin from *Desulfovibrio vulgaris* and immobilized on a gold electrode, was the most promising combination (figure 1.8; (Sadeghi *et al*., 2011).

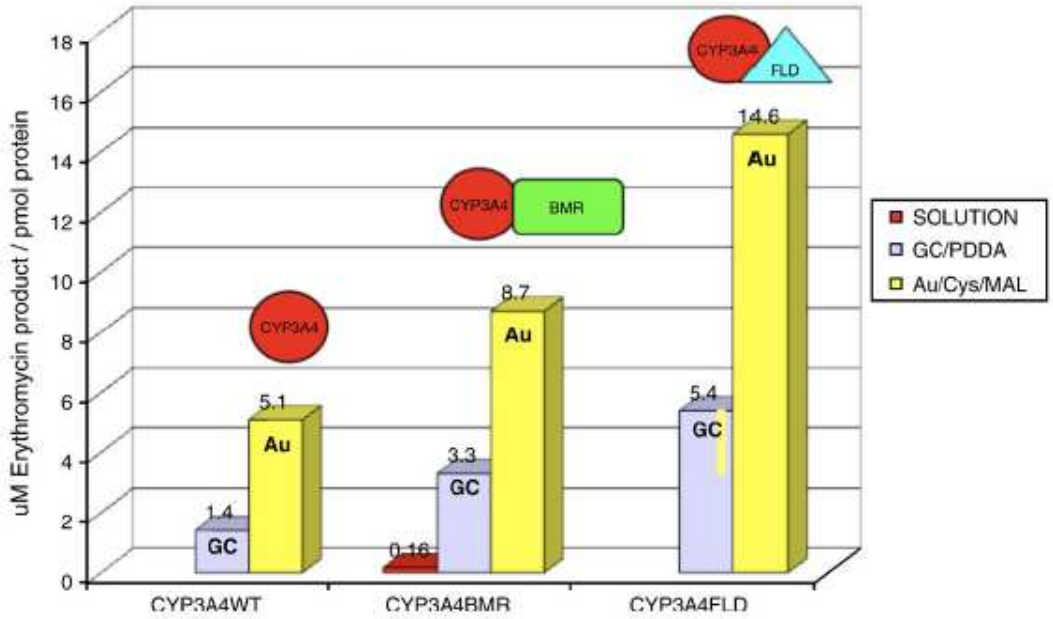



**Figure 1.8: Combinatorial approach to optimize both enzyme and immobilization electrode for activity enhancement.** CYP3A4WT – normal CYP3A4 unfused to any reductase component; CYP3A4BM3 – CYP3A4 fused to the reductase domain of CYP102A1; CYP3A4FLD – CYP3A4 fused to a flavodoxin from *Desulfovibrio vulgaris*; solution – non-immobilized enzyme; GC/PDDA – enzyme immobilized on glassy carbon electrode modified with poly-(dimethyldiallylammonium chloride) (PDDA); Au/Cys/MAL – enzyme immobilized on gold electrode modified with cystamine and maleimide (adapted from Sadeghi *et al*., 2011).

As mentioned earlier, P450 electron transfer systems consist of 1-3 components (including the P450 as final acceptor). In cases involving multi-component systems, heterologous P450 expression generally requires the coexpression of appropriate electron transfer proteins, either as homologous overexpression (in yeasts) or heterologous expression (Zöllner *et al*., 2010; Purnapatre *et al*., 2008). In *E.* coli this can be either as a polycistronic operon (Kim and Ortiz de

Montellano, 2009) or as an artificial fusion (Sibbesen *et al*., 1996). In one study, 3 components were coexpressed and fused to a hetrotrimeric dsDNA binding protein which resulted in the coexpressed proteins being in proximity of each other, allowing interaction without fusion of the components to each other (Hirakawa and Nagamune, 2010).

Self-sufficient single-component systems are either natural or artificial fusions between P450 and reductase domains (Munro *et al*., 2007; Weis *et al*., 2009).The prokaryotic CYP102A1 was the first naturally self-sufficient P450 described, and has been expressed in *E. coli* and extensively studied (Whitehouse *et al*., 2012). Its P450 domain is naturally fused to a eukaryotic type CPR. The current and most probable hypothesis is that bacteria obtained these types of genes from eukaryotic organisms via horizontal gene transfer (Kitazume *et al*., 2000). Other CYP102 family members have been identified in other *Bacillus* sp. and in *Burkholderia* sp, *Ralstonia metallidurans* and *Streptomyces avermitilis.* Some of these CYP102 family members have been expressed in *E. coli* and characterized (Gustafsson *et al*., 2004; Chowdhary *et al*., 2007; Weis *et al*., 2009; Furuya and Kino, 2010). CYP505A1 from *F. oxysporum* was the first identified eukaryotic counterpart of CYP102A1, and was subsequently expressed in *E. coli* and *S. cerevisiae* for characterization (Kitazume *et al*., 2000; Kitazume *et al.*, 2002). It is fused to the same CPR, but is the P450 component is membrane associated, unlike the soluble, cytosolic CYP102A1. More members of the CYP505 family were later identified in other fungal species, among others *Giberella monoliformis*, *G. zeae, Aspergillus oryzae*, *A. nidulans, A. fumigates, Neurospora crassa*, *Magnaporthe grisea* (Weis *et al*., 2009; Munro *et al.*, 2007) and *Phaenorocheate chrysosporum* (Doddapaneni *et al*., 2005). Some of these have been expressed in *E. coli* and characterized (Weis *et al*., 2009).

DNA shuffling was applied to fuse human P450s to the reductase domain of CYP102A1, followed by expression in *E. coli*. The resultant enzymes were

soluble and catalytically self-sufficient, exhibiting wild-type activities without addition of other proteins or detergents. Such variations of human P450s would be ideal for studying their roles in drug metabolism (Dodhia *et al*., 2006).

The uniquely organised self-sufficient P450 from *Rhodococcus* sp., P450RhF has its P450 domain fused to a reductase usually involved with phathalate dioxygenases (Roberts *et al*., 2002). This reductase domain was fused to various other P450s and the artificial fusions were successfully expressed in *E. coli* (Nodate *et al*., 2006; Kubota *et al.*, 2005). The interaction between P450s and reductase components do however vary between P450s. In one study two CYP153 enzymes were expressed in *E. coli* either with coexpression of putaredoxin (CamB) and putaredoxin reductase (CamA) from the *Pseudomonas putida* P450cam system; or fused to the reductase of P450RhF. The different combinations were tested for activities towards different substrates, and reductase preference varied greatly in each case (Fujita *et al.*, 2009).

**Whole cell biocatalysis**

As discussed in previous sections, the membrane-bound nature of many P450s, protein instability, and the requirement for electron transfer from expensive cofactors via electron transfer-proteins to P450s, all mean that large-scale applications of purified preparations of P450s are infeasible. The simplest approach to address most of these requirements is to use whole-cell biocatalysis (Behrens *et al*., 2011). Naturally many wild-type hosts are impractical to use, necessitating the application of appropriate hosts for heterologous expression of target proteins. Metabolic engineering of the host can be performed, introducing multiple proteins and / or disrupting unwanted side pathways (Waegeman and Soetaert, 2011). Improved intracellular electron recycling can for instance be obtained by coexpression of dehydrogenases, for example glucose dehydrogenase (Zhang *et al*., 2011; Lu and Mei, 2007; Zhang *et al*., 2010) or

formate dehydrogenase (Maurer *et al*., 2005). As discussed in the previous section, relevant coenzymes for electron transfer can also be coexpressed. Native enzymes may lead to overoxidation of products of hydroxylation; therefore enzymes involved can be targets for disruption or inactivation (van Beilen *et al*., 2003). Since whole cell systems also promote prolonged enzyme stability, their application overcomes most of the limitations associated with P450 applications (Urlacher and Girhard, 2012; Gillam, 2007). The rest of this review will therefore consider various hosts for heterologous expression of enzymes, particularly the cytochrome P450s.

## 1.3 Heterologous expression of cytochrome P450 monooxygenases

Most of the research on P450s for potential biocatalytic applications has been devoted to bacterial P450s, especially CYP102A1, due to their high reaction rates. For these purposes *E. coli* is the host of choice, as it readily expresses high levels of functional prokaryotic enzymes (Jung *et al*., 2011). The large number and diversity of eukaryotic P450s however represents great potential for a very broad range of reactions. Some mammalian P450s (especially CYP3A4) themselves have broad substrate ranges, as they are more multifunctional in their natural environments. Therefore they also have great potential as versatile biocatalysts (O'Reilly *et al.*, 2011).

Given the diversity of P450s present in many eukaryotes, individual eukaryotic P450s are best studied by heterologous expression. To date most of the work on expression of eukaryotic P450s has been dedicated to human P450s in studies on drug metabolism and design (Drăgan *et al*., 2011). Since merely detectable activities are often satisfactory for such applications, process optimizations for larger scale product formation have generally not been pursued. Furthermore,

due to the need for only small amounts of products, whole cell systems are scarcely used (Zöllner *et al*., 2010).

The choice of a host for P450 production is determined by the characteristics and intended downstream applications of the protein of interest. If the recombinant P450s are required as models for human drug metabolism, authenticity of the P450 is the highest priority (Gillam, 2007; Cornelissen *et al*., 2012). For larger scale chemical biosynthesis on the other hand, integrity of the protein is not important as long as maximum activities can be achieved, which is evidenced by the large amount of mutagenic studies performed on P450s for activity enhancements (Jung *et al*., 2011; Gillam, 2008).

The following sections will investigate different organisms as hosts, not only for expression of P450s, but also their potential to act as whole-cell biocatalysts. Eukaryotic P450s will receive special attention due to the limitations of *E. coli* in heterologously expressing them. The majority of eukaryotic P450s which have been heterologously expressed have been mammalian P450s, especially human, due to research on drug metabolism and their potential as biocatalysts (Gillam, 2007). As a result examples of predominantly mammalian P450s will be discussed.

### 1.3.1. Heterologous expression of P450s in *Escherichia coli*

*E. coli* was the pioneer of heterologous protein expression, and is still generally the host of choice, due to its high growth rate on cheap cultivation media, potential for high cell density cultivations (HCDC), extensive knowledge of its genetics and physiology, and the availability of various established tools for genetic manipulation (Waegeman and Soetaert, 2011; Purnapatre *et al*., 2008; Altenbuchner and Mattes, 2005). As such, various strains and vectors have been constructed and / or manipulated to further facilitate heterologous expression in

this organism, many of which are commercially available. Some will be described in the following sections.

The pET vector range (Novagen) is commonly used for heterologous expression in *E. coli*, and it allows lactose-inducible and regulatable expression when used in a strain like BL21-DE3, which contains the lysogenic lambda DE3 (Novagen). This modified prophage carries the T7 RNA polymerase under the control of the *E. coli lac* operon. In response to the presence of lactose as sole carbon source, the RNA polymerase binds very specifically to the T7 promoter, which is present in the pET vectors for regulation of expression of genes of interest. For tightly controlled induction of the promoter, isopropyl-β-D-1-thiogalactopyranoside (IPTG), an artificial analogue of lactose, can be added at a specific growth phase. Such tight regulation is required for cases in which the recombinant protein may be toxic to the cells and sufficient biomass needs to be produced prior to recombinant protein production. Alternatively when using an autoinduction medium, lactose is only utilized once other carbon sources have been depleted, at which point induction of the system commences (Altenbuchner and Mattes, 2005).

A variant of BL21 containing a pLysS vector further ensures that no leaky expression occurs (Novagen). The BL21 strain and its derivatives also have two proteases deleted to prevent degradation of recombinant proteins (Waegeman and Soetaert, 2011); Novagen). The most common vector used for expression of human P450s in *E. coli* however is the pCWori$^{+}$ plasmid, in which expression is under control of two tandem *tac* promoters. Expression levels are determined by IPTG concentrations, with derepression at low concentrations and induction at higher concentrations (Barnes *et al*., 1991; Pan *et al*., 2011).

A range of pETDuet vectors are also available, which allow co-expression of two open-reading frames (ORFs) per strain. Used in combination, these vectors can

theoretically allow the co-expression of 8 proteins in one strain (Novagen). This gives a great theoretical potential, however co-expression of too many foreign genes leads to metabolic stress and poor growth (Bentley *et al*., 2009). These vectors could however simplify coexpression of reductase proteins with the P450, as was done with CYP73A5 from *Arabidopsis thaliana* and CPRs from *Gossypium hirsutum* (Yang *et al.*, 2010). Similarly, a vector carrying a P450 from *Streptomyces peucetius* was coexpressed in *E. coli* with a pETDuet vector carrying the genes for the CamA and CamB electron-transfer system from *Pseudomonas putida* (Shrestha *et al*., 2008). Alternatively these vectors can be used for coexpression of other supplementary enzymes which can enhance P450 activities in *E. coli*, such as cofactor-regenerating dehydrogenases (Schewe *et al.*, 2008).

Plasmid maintenance itself puts metabolic strain on the cells however; with genes involved in energy metabolism and biosynthesis generally downregulated in plasmid-bearing cells (Ow *et al*., 2006). Furthermore, during long cultivations plasmid stability decreases and plasmids are lost, and overgrowth of undesirable plasmid-free bacteria may occur under non-selective conditions. This necessitates the continual maintenance of selective pressure for ensured plasmid presence, which is industrially unfavourable especially when antibiotic resistance is used. Nevertheless, episomally maintained plasmids remain the most frequently used methods for expression in *E. coli.* The other option is genomic integration of the target genes, which increases the stability of insertions over episomal plasmids, allowing prolonged cultivations without selective pressure. This strategy relies on strain auxotrophies however, and although multiple copies are achievable, they are not as high as can be achieved using episomal plasmids (Chen *et al*., 2008).

*Escherichia coli* is a very efficient host for expression of bacterial P450s, which is best exemplified by the two most studied P450s: CYP101A (P450cam) from

*Pseudomonas putida* (Kim and Ortiz de Montellano, 2009) and CYP102A1 (P450BM3) from *Bacillus megaterium.* The latter has the highest activity of any P450 described so far, and various mutants with altered substrate specificity have diversified its potential (Whitehouse *et al.*, 2011). However, even with the widened application range of bacterial mutants, eukaryotic P450s are still of interest, most notably as human P450s are consistently studied for their application in drug design (Guengerich *et al*., 2011). Numerous problems are however encountered with production of recombinant eukaryotic P450s in *E. coli*, which will be addressed in the following sections.

Eukaryotic P450s often tend to misfold (especially when expressed under strong promoters), and either get degraded by the cell through a heat-shock like stress response, or become aggregated into insoluble inclusion bodies. Difficulties in achieving high expression levels of eukaryotic P450s in *E. coli* were found to be largely associated with the N-terminal sequence. Reasons for this have been attributed to potential poor folding due to the hydrophobicity of the N-terminal membrane anchor, or to codon usage (Gillam, 2008; Purnapatre *et al*, 2008; Kaderbhai *et al.*, 2001). As a result, mutations to the N-terminal sequence, or even deletions of it, have allowed efficient expression of the P450s in *E. coli* (Gillam, 2008; Kaderbhai *et al*, 2001). While this approach is generally acceptable for biocatalytic enzymes, the compromised integrity of sequences are undesirable for human P450s intended for drug design studies.

Expression levels of P450s were also improved by fusing the P450 at the N-terminal to an *E. coli* signal sequence, eliminating the need for N-terminal sequence modifications (Pritchard *et al*., 1997; Voice *et al*., 1999). Alternatively human P450s were fused to bacterial signal peptides for targeting to the periplasmic space, and the signal peptide could later be cleaved off. This strategy resulted in expression of an unmodified, active CYP105D1 from *Streptomyces griseus* which also displayed whole cell activity, indicating that

electron donor proteins of the periplasmic space can facilitate some P450 activity (Kaderbhai *et al*, 2001).

Many cases of misfolding occur when strong promoters are used for heterologous expression, as the new demand for folding machinery of the cell is higher than normal, leading to stalling of the protein processing steps, which in turn activates stress responses leading to degradation or inclusion body formation (Waegeman and Soetaert, 2011). Improved protein folding can be achieved by slowing down protein production through the use of weaker promoters or lower growth temperatures. Decreased growth rates and the increased fermentation costs due to the required temperature reduction are however not preferable for industrial applications (Altenbuchner and Mattes, 2005).

An alternative option to improve folding is to increase the amount of intracellular molecular chaperone proteins, which naturally improve protein folding, through the overexpression of their genes in the expression strain. One study of four mammalian P450s showed the improvement of P450 content to be between 3 – 5 fold when the GroEL/ES chaperone was coexpressed (Wu *et al*., 2009), while it improved the P450 content of human CYP27C1 by 15-fold in another study (Wu *et al*., 2006). This approach is sometimes combined with N-terminal modifications to achieve higher expression levels, including 2210 nmol/L of human CYP2B6, which is very high for heterologous human P450 expression (Mitsuda and Iwasaki, 2006). Chaperone plasmid sets of 5 different chaperones in different combinations are commercially available from Takara Bio Inc. This system was successfully employed for the heterologous production of CYP98A3 from *Arabidopsis thaliana* without any N-terminal modifications or truncations (Rupasinghe *et al*., 2007), and for the expression of various bacterial and yeast P450s (Weis *et al*, 2009). The metabolic burden imposed on cells when

coexpressing multiple proteins (Bentley *et al*, 2009) and maintenance of multiple plasmids (Ow *et al*., 2006) should however be taken into account.

Codon optimizations of genes have been performed to allow better expression of foreign enzymes in *E. coli*, as differential codon preference can limit heterologous protein expression (Gustafsson *et al*., 2004). Protein synthesis can stall at positions where such 'rare codons' are encountered, triggering the aforementioned stress responses. Furthermore, encountering these rare codons in a gene expressed under a strong promoter, can cause decreased growth and even cell death (Zahn, 1996; Altenbuchner and Mattes, 2005). Instead of codon optimization of entire coding sequences, a more suitable approach for multiple gene expressions is the coexpression of genes encoding rare tRNAs in *E. coli*. A commercially available expression strain, Rosetta$^{TM}$2, which contains a pRARE2 plasmid carrying tRNAs for 7 codons that are rare in *E. coli* (Novagen), has been used to achieve higher levels of human P450 expression (Schumacher and Jose, 2012). Once again, the maintenance of a second vector could however increase metabolic stress to the cells, potentially slowing down growth and hence production (Ow *et al*, 2009). Codon optimization is however not always the best option, as in some cases a pause at rare codons may in fact provide time for co-translational folding to occur (Komar, 2009).

One advantage of *E. coli* as a host for P450 expression is that it lacks P450s of its own, which could interfere with interpretation of activity results obtained during heterologous P450 expressions. However, this means the organism is poorly equipped for the increased demand for heme due to heterologous expression of P450s. *E. coli* does not naturally accumulate more heme than absolutely necessary, because free heme is toxic to the cells (Harnastai *et al*., 2006). Therefore a heme precursor 5-aminolevulinic acid (5-ALA) is routinely added to P450-expressing strains, usually at approximately the point of induction, which was shown to improve P450 expression levels by 80% (Richardson *et al*., 1995)

The addition of expensive 5-ALA can be circumvented by coexpressing a glutamyl-tRNA reductase (*hemA*) with the P450s, resulting in increased yields of various P450s without the continual addition of 5-ALA (Harnastai *et al*, 2006).

*E. coli* also has no suitable reductase system to facilitate electron transfer to P450s. Human P450s can require either FAD-containing adrenodoxin (Adx) and iron-sulphur cluster-containing adrenodoxin reductase (Adr) proteins for electron supply (class I) or an FAD and FMN-containing reductase (CPR), often in cooperation with cytochrome *b*5 (classII) (Hanneman *et al*, 2007). During heterologous P450 expression the appropriate reductase component(s) are either coexpressed with the P450 (Blake *et al*., 1996), or purified preparations are added to P450 preparations to reconstitute activity (Barnes *et al*., 1991). While coexpression is a far simpler solution, it tends to lower P450 levels, as demonstrated for multiple human P450s (Iwata *et al.*, 1998; Blake *et al*, 1996). For class II human P450s cytochrome *b*5 also needs to be either added to preparations or coexpressed (Purnapatre *et al*, 2008; Schumacher and Jose, 2012).

Another problem encountered when using P450-expressing *E. coli* for whole cell biotransformations is the slow rate of uptake of some substrates by the cells, which could mask potentially important activities (Schroer *et al.*, 2010; Schumacher and Jose, 2012). Membrane permeability can be increased by using ethylenediaminetetraactetic acid (EDTA), ethylenimine, polymyxin B sulphate, sodium hexametaphosphate (Schwaneberg *et al*., 2001), polyethylene glycol (PEG) or Triton X-100 (Altenbuchner and Mattes, 2005); osmotic shock (Voice *et al*., 1999); or freeze-thaw (Iwata *et al*, 1998) prior to activity assays. In an alternative approach, cell surface display of CYP102A1 on *E. coli* cells, eliminated the need for substrate uptake by the cells. The activity obtained however required supplementation of NADPH to the cell suspension (Yim *et al*., 2010). Similarly CYP3A4 was displayed on the surface of *E. coli* cells, but CPR

and cytochrome *b*5 had to be supplied in addition to NADPH, since this is not a self-sufficient P450, rendering this approach completely unfeasible for large-scale bioconversions or screening. Coexpression and co-display of the reductase components on the same cell, coupled to the display of an electron regenerating enzyme on another set of cells, could theoretically improve this approach (Shumacher and Jose, 2012).

Combinations of the various strategies mentioned above have greatly improved eukaryotic P450 expression in *E. coli*, and many of the 57 human P450s have been expressed in this host, including some 'orphan' P450s of which the functions have not yet been established (Iwata *et al*, 1998; Wu *et al*, 2009). Of the 57, five are known to be of special importance for drug metabolism: CYP1A2, 3A4, 2D6, 2C9 and 2C19 (Purnapatre *et al*, 2008). These especially have received considerable attention (Iwata *et al*, 1998; Vail *et al*., 2005; Pritchard *et al*, 1997; Schroer *et al*, 2010). Libraries of P450s coexpressed with CPR can be prepared for screening against libraries of substrates (Schroer *et al*, 2010). Optimization studies of expression conditions have been performed, followed by upscaling, resulting in 3.5-6 fold improvements in P450 content of various human P450s (Vail *et al*, 2005).

In summary, aside from efforts to increase the amount of P450 produced, attempts to produce human P450s without sequence modifications have also been pursued (Pritchard *et al*, 1997; Kim *et al*., 2008; Schumacher and Jose, 2012). In most studies, P450s expressed in *E. coli* were assayed using solubilised membrane fractions with activity reconstituted by adding purified CPRs, often from rats; and even in some 'whole-cell' conversions the cells were disrupted prior to substrate addition, to overcome uptake limitations (Iwata *et al*, 1998; Voice *et al*, 1999).

### 1.3.2. Yeasts as alternative hosts for heterologous expression

Yeasts have the combined advantages of being unicellular organisms with the ease of manipulation and favourable growth characteristics of bacteria, which possess eukaryotic machinery for gene expression (Böer *et al*., 2007). They tend to grow faster and on simpler and far less expensive media than other eukaryotic organisms, and their applications have lower technical demands (Schroer *et al*, 2010; Sandig *et al*, 2005). Since yeasts generally have P450s of their own they tend to be well-equipped to accommodate foreign eukaryotic P450s in terms of their endoplasmic reticulum (ER) membrane environment, adequate available heme, and suitable reductase systems. Sequence modifications such as the N-terminal modifications are also not required for expression of eukaryotic P450s in yeasts (Zollner *et al*, 2010; Purnapatre *et al*, 2008). Although antibiotic resistance markers are occasionally used during yeast expression studies, auxotrophic markers are far more common (discussed in chapter 2).

Traditionally *S. cerevisiae* has been the most extensively applied yeast for heterologous gene expression, since it has also been the most extensively studied yeast. It does, however, have certain limitations in efficient recombinant protein production, such as low product yield, low plasmid stability, limited range of utilizable carbon sources and difficulties in scaling-up processes (Gellissen *et al*., 2005; Madzak *et al*., 2004). These limitations prompted investigations of various non-*Saccharomyces* yeasts as alternative hosts for recombinant protein production (Madzak *et al*, 2004; Müller *et al*., 1998); Gellisen *et al*, 2005). Most notable are the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha*; the dimorphic yeasts *Yarrowia lipolytica* and *Arxula adeninivorans*; the lactose-utilizing *Kluyveromyce lactis* and its thermophilic sister-strain *K. marxianus*; the fission yeast *Schizosaccharomyces pombe*; the amylolytic *Schwanniomyces occidentalis*; and the exceptionally halotolerant *Debaryomyces*

*hansenii*. P450 expression has, however, only been reported in four of these yeasts, which will be discussed in the following sections.

***Saccharomyces cerevisiae***

Despite the mentioned limitations, baker's yeast *S. cerevisiae* has been the most extensively applied host for heterologous P450 expression, since being the pioneer for heterologous P450 expression in yeasts (Cheng *et al*., 2006). *S. cerevisiae* has a CPR of class II P450s as well as Yah1 and arh1 proteins similar to adrenodoxin and adrenodoxin reductase respectively, of mammalian class I P450s (Schiffler *et al*., 2004). These natural reductase systems can facilitate detectable P450 activities in some cases, but activities are greatly enhanced by coexpression of reductases (either homologously overexpressed or heterologously expressed), and cytochrome *b*5 coexpression for certain human P450s (Peyronneau *et al*., 1992). In one example, for investigating multiple P450s with coexpression of CPR, human CPR was integrated into the genome of a strain, which could then be transformed with plasmids containing different P450s. The system retained 70% of episomally maintained P450 activity and 90% of chromosomally integrated CPR activity for 6 days (Cheng *et al*, 2006). Alternatively the P450 was fused to the *S. cerevisiae* CPR, which increased the activity from undetectable to 25 nmol/min/nmol$_{P450}$ (min$^{-1}$), although it decreased the P450 content from 193 pmol mg$^{-1}$ (P450 alone) to 52 pmol mg$^{-1}$ microsomal protein (fusion). Coexpression of the fusion protein with human cytochrome b5 further increased the activity to 35 min$^{-1}$ (Hayashi *et al*., 2000). Human cytochrome *b*5 was also fused to the CYP3A4 and CPR in two variations, P450-*b*5-CPR and P450-CPR-*b*5, which demonstrated activity improvements even with decreased P450 content, as well as the importance of the order of enzymes in fusion constructs (table 1.1; Inui *et al*., 2007).

**Table 1.1: Microsomal CYP3A4 content and activities in different fusions.**

| Expression product | P450 content (pmol.mg$^{-1}$) | Activity (min$^{-1}$) (Approximated) | Fold activity increase |
|---|---|---|---|
| CYP3A4 | 130 | 1.7 | 1 |
| CYP3A4-CPR fusion | 71 | 19 | 12 |
| CYP3A4-CPR-*b*5 fusion | 49 | 31 | 18 |
| CYP3A4-*b*5-CPR fusion | 45 | 62 | 37 |

An impressive application of this host demonstrating its capacity as host for numerous foreign proteins was metabolic engineering of a strain to contain an entire pathway for hydrocortisone biosynthesis from simple carbon sources. This involved the introduction of human proteins (including P450s) via chromosomal integration and episomal plasmids, as well as the disruption of unwanted side-pathways (Szczebara *et al*., 2003).

Libraries of fungal P450s from *Aspergillus oryzae* (Nazir *et al*., 2011) and *Postia placenta* (Ide *et al*., 2012) have been constructed in *S. cerevisiae* to facilitate screening for novel or desired activities. Through screening of these libraries, activities were demonstrated for 92 and 116 P450s from *A. oryzae* and *P. placenta* respectively. Another example of a fungal P450 expressed in *S. cerevisiae* is the CYP505A1 from *Fusarium oxysporum*, the first described eukaryotic counterpart of CYP102A1. It was initially characterised in *S. cerevisiae* (Kitazume *et al*, 2000), but expression in *E. coli* was required to achieve higher yields for purification and further characterisation (Kitazume *et al*, 2002). The relevance of this particular example will be clarified later on in chapter 2.

***Pichia pastoris***

This methylotrophic yeast has been developed into a highly efficient expression host, and an expression kit is commercially available from Invitrogen. One benefit

of this yeast is that it can grow to very high cell densities in large fermentors (Cereghino *et al*., 2002). Approximately 20 P450s have reportedly been expressed in this yeast, of which nearly half originate from the fungus *Phanerochaete chrysosporum*, including polyaromatic hydrocarbon (PAH) hydroxylating P450s. These were expressed and evaluated for activity towards various bulky PAHs using whole cells of this host (Syed *et al*., 2010; Syed *et al*., 2011). There are so far only two reports on heterologous expression of human P450s in this host, CYP2D6 and CYP17. For CYP2D6, human CPR was coexpressed and microsomal preparations were assayed, since initial reconstitution with human CPR could not facilitate activity (Dietrich *et al.*, 2005). Meanwhile, CYP17 was tested using whole cells containing only native reductases, but low substrate concentrations were used with maximum yield obtained after only 20 minutes (Kolar *et al*., 2007).

The majority of the reported assays of P450s expressed in *P. pastoris,* however, like *S. cerevisie* and *E. coli*, were performed using microsomal extracts or purified enzymes (Zollner *et al*, 2010).

***Schizosaccharomyces pombe***

The fission yeast *Schizosaccharomyces pombe* has been used for expression of at least 13 P450s of which 11 are mammalian, including 10 human P450s. More importantly it is so far the most extensively used host for whole-cell biotransformations using P450s (Zollner *et al*, 2010). This was demonstrated by an automated whole-cell assay system which was established for screening for inhibitors of CYP11B2, which is reliable enough to allow 1200 compounds to be tested in 2 weeks (Hakki *et al*., 2011).

This yeast also contains not only a cytochrome P450 reductase (CPR) compatible with class II P450s as in other yeasts (Zehentgruber *et al*., 2010); but also has reductase proteins similar to those of the Class I P450s, consisting of

proteins arh1 and etp1 compared to human AdR and AdX and *S. cerevisiae* arh1 and Yah1 (Ewen *et al*., 2008; Schiffler *et al*., 2004). However, coexpression of the human reductase components with human P450s still improves activities, as can be demonstrated by two examples. Firstly, through expression of relevant components in *E. coli*, CYP11A1 and CYP11B1 activities were more than 6-fold higher using AdX in combination with AdR compared to etp1 with AdR (Schiffler *et al*, 2004). Secondly, when expressed in *S. pombe,* CYP11B1 activities were more than three times higher with coexpression of human AdX and AdR compared to using only the native counterparts (Hakki *et al*., 2008).

Using whole cell activity of CYP21 and the native CPR of *S. pombe*, 250 μM of 11-deoxycortisol was produced from 1mM 17α-hydroxyprogesterone in 24h, equalling 25% conversion, while Approximately 425 μM 11-deoxycortisol was formed after 72h, equalling 43% conversion (Drăgan *et al.*, 2011). In contrast, permeabilized resting cells converted 90% of 250 μM 17α-hydroxyprogesterone to 11-deoxycortisol in 10 h (Zehentgruber *et al*, 2010). From these two examples, initial rates can be estimated as 250 $\mu M.d^{-1}$ and 1500 $\mu M.d^{-1}$, obtained using whole cells and permeabilized resting cells, respectively. Permeabilized resting cells thus gave a 6-fold improvement in activity.

CYP2C9 was carried by an episomal plasmid in a strain in which human CPR was chromosomally integrated. An overall average yield of 470 mg 4-hydroxydiclofenac was obtained in 6.25 days, which is on average 240 $\mu M.d^{-1}$. This was far higher than values obtained using other native and recombinant versions of this enzyme. The accumulative total of 6 bioreactor runs over 72h was 2.8 g (or 9 mmol) of purified 4-hydroxydiclofenac. The combination of high production rate and prolonged conversion time is impressive (Dragan *et al*, 2011).

***Yarrowia lipolytica***

*Y. lipolytica* emerged as a promising producer of foreign proteins, and the YLEX expression kit for heterologous expression in *Y. lipolytica* is commercially available from Yeastern Biotech. The high efficiency of hydrophobic substance uptake and degradation by the dimorphic yeast *Y. lipolytica* makes it particularly attractive as a host for P450 expression. This feature is partially due to the presence of 17 native P450s, 12 of which are members of the CYP52 family, which are generally classified as alkane or fatty acid hydroxylases (Thevenieau *et al*., 2007; http://drnelson.uthsc.edu/CytochromeP450.html). The high number of native P450s can complicate the use of this yeast for P450 expression, due to background activities interfering with those of cloned P450s. by comparison, *S. cerevisiae* and *P. pastoris* have only three P450s each while *S. pombe* has only two (Zollner *et al*, 2010). A breakthrough to overcome this problem has been made however, as a CYP52 null mutant strain of *Y. lipolytica* was constructed by deleting all 12 of the wild-type CYP52 genes (Takai *et al*., 2012). This might increase the use of this host for P450 expression as it has been somewhat underutilized so far for this purpose.

There are four reports of successful heterologous expression of P450s in *Y. lipolytica*, two of which are mammalian: one human and one bovine. Bovine CYP17 was expressed in *Y. lipolytica* using only the native CPR, and activities were determined for whole cell and microsomal fractions (Juretzek *et al*., 1998). Whole cell activities were however only performed on a small scale for short times. Human CYP1A1 was expressed with and without the overexpression of the *Y. lipolytica* CPR (YlCPR). A 1.5 – 2 fold increase in P450 activity was observed when the YlCPR was overexpressed, depending on the promoter used. The effects were amplified using multi-copy expression of the P450, as multi-copy P450s with overexpression of the YlCPR gave activities 6 – 12.5 fold higher than multi-copy expression without overexpression of the YlCPR. In total, activities obtained with multi-copy P450 expression and overexpression of the

YlCPR were 26 – 51 times higher than single copy P450 without YlCPR overexpression (Nthangeni *et al*., 2004). The activities were also performed on a small scale and for a short time. Therefore, both of these examples are not directly comparable to *S. pombe* results.

Larger scale whole cell biotransformations were used for CYP53B1 from the basidiomycetous fungus *Rhodotorula minuta*, which was expressed using *Y. lipolytica* with the overexpression of the YlCPR. A maximum product yield of 11.5 mM was achieved after 8.3 days, averaging at 1.39 $mM.d^{-1}$ (Shiningavamwe *et al*., 2006). In bioreactor experiments, the productivity was increased to 1.45 $mM.d^{-1}$, with 10.9 mM product accumulated in 7.5 days (Obiero, 2006). Although CYP53B1 is not of human origin, and can, therefore, not be directly compared to results obtained with human P450s in *S. pombe*, the production rates and duration of activity are nevertheless promising. Additional relevance of this example will be clarified in chapter 2.

### 1.3.3. Comparing P450 expression in different yeasts and *E. coli*

Direct comparisons between examples of P450 expression in the *same* host can be difficult, as methodologies and the interpretation and reporting of results can vary. It is thus almost impossible to make comparisons between reports of P450s expressed in different species.

With regards to reporting activities, P450 levels are given in pmol/mg membrane proteins when solubilised membrane fractions are used or nmol/L (nM) for *E. coli* whole cells. P450 levels are usually not determined when yeast whole cells are used. Activities are represented in various ways: when P450 content is known, specific activities are provided in $nmol_{product}/min/nmol_{P450}$ ($min^{-1}$); while whole cell specific activities are given as nmol product/min/g DCW. Alternatively whole cell activities are given as product concentration per day (mg/L/d or mM/d). The

omission of information such as biomass or P450 content can make data comparison really difficult.

These units can theoretically be interconverted, but this approach is often impractical. Reactions with membrane fractions are for instance generally unstable and therefore cannot continue for numerous hours; while whole cell activities may be initially delayed by rate-limiting factors such as substrate uptake, causing a non-linear relationship between product formation and reaction time. Nevertheless, comparisons between recombinant P450s produced by different hosts will be attempted in the following sections.

Since we are specifically interested in eukaryotic P450s we will focus on mammalian P450s, since they are the most commonly expressed P450s. Although non-mammalian eukaryotic P450s have also been expressed in various hosts, there is less information available on their expression in various hosts. Over 90% of drugs are metabolized by only five of the 57 human P450s. These five are: CYP1A2, 3A4, 2C9, 2C19 and 2D6 (Purnapatre *et al*, 2008). CYP3A4 was shown to be particularly active and diverse in its functions (Schroer *et al*, 2010; Purnapatre *et al*, 2008), being the most abundant P450 in the human liver and small intestine and contributing to the metabolism of about half of the drugs currently in use (Cheng *et al*, 2006). It has hence been one of the most extensively studied human P450s. Selected examples of testosterone hydroxylation by recombinant CYP3A4 are provided in table 1.2. Examples of expression of other drug metabolising P450s are provided in tables 1.3 - 1.6.

**Table 1.2: Expression of CYP3A4 in different hosts**

| Host | P450 content | | Activity | | Remarks | Reference |
|---|---|---|---|---|---|---|
| | WC (nM) | MF (pmol/mg) [1] | Specific ($min^{-1}$) | Volumetric (μM/min) | | |
| *E. coli* | 84 | - | 59.7 (WC) [2] | 0.66 | Coexpressing human CPR; no *b*5 | Iwata *et al*, 1998 |
| | 81 | 86 | 71.8 (WC) [3] | 7.18 | P450 fused to *E. coli* signal peptide; *b*5 added | Voice *et al*, 1999 |
| | | | 44.3 (MF) | 2.77 | | |
| | 263 | - | 57.8 (MF) | 2.31 | Cultivated in jar fermentor; Coexpressing human CPR; no *b*5 | Kanamori *et al*., 2003 |
| | - | 120 | 88 (MF) | 0.88 | *b*5 added | Yamazaki *et al*., 2002 |
| *S. cerevisiae* | - | 35.53 | 7.5 (MF) | 0.91 | Human CPR chromosomally integrated;P450 on episomal plasmid; no *b*5 | Cheng *et al*, 2006 |
| | - | 85 | 35 (MF) | 1.75 | P450 fused to ScCPR and human *b*5 coexpressed | Hayashi *et al*, 2000 |
| | - | 44 | 63 (MF) | - | P450 fused to human *b*5 and ScCPR (in that order) | Inui *et al*., 2007 |
| *S. pombe* | - | 34 | 10.5 (MF) | 0.67 | No coexpressed CPR or *b*5 | Palabiyik *et al*., |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | 2008 |

[1]pmol of P450 per mg of total membrane / microsomal protein

[2]Whole cells disrupted by freeze-thaw prior to assay

[3]Whole cells were osmotically shocked prior to assay

WC – permeabilized whole cells assayed; MF – solubilised membrane fraction (microsomal for yeasts) assayed.

From table 1.2 it is clear that using permeabilized whole cells of *E. coli* results in far improved volumetric activities. It is also clear how important the addition or coexpression of cytochrome *b*5 is for CYP3A4 activity. For expression in *S. cerevisiae*, the best results were obtained by creating artificial fusions between the P450 and reductase partners.

Cornelissen *et al* (2012) demonstrated how susceptible results are to interpretation. Using whole cells expressing CYP1A1 for catalysis, specific activity based on dry cell weight indicated that *E. coli* had higher activity than *S. cerevisiae*. However, when it is taken into account that *E. coli* produces far higher P450 content, it is observed that *S. cerevisiae* in fact has the better activity (table 1.3). Therefore the intended application of the system needs to be taken into consideration. For large scale whole cell biocatalysis, high productivity relative to low biomass is attractive since optimization of biomass production is not required. For screening applications on the other hand, good activity with low levels of P450 could be attractive since optimization of P450 production is unnecessary.

**Table 1.3: Relative activities of recombinant CYP1A1 produced by *E. coli* and *S. cerevisiae* (adapted from Cornelissen *et al*., 2012)**

| | | | Specific activity [b] | |
|---|---|---|---|---|
| **Strain** | **Plasmid** | **P450 content [a] (nmol/gDCW)** | **nmol/min/gDCW** | **nmol/min/nmol P450 ($min^{-1}$)** |
| ***E. coli*** | | | | |
| DH5α | pCWr1A1 | 41±2 | 429±36 | 10 |
| | pCom8-r1A1 | 7±1 | 47±3 | 7 |
| JM101 | pCWr1A1 | n.d. | 419±36 | - |
| E609 | pCWr1A1 | n.d. | 303±18 | - |
| ***S. cerevisiae*** | | | | |
| AH22 | pAMR2 | 2±1 | 163±39 | 82 |
| BY4741 | pAMR2 | 2±1 | 150±22 | 71 |

[a]Determined using cell free extracts

[b]Determined using whole cells

An important factor to consider about these results is that the rat CYP1A1 was coexpressed with human CPR in *E. coli*, while the native *S. cerevisiae* CPR was overexpressed during yeast expression of this P450. It can be plausibly speculated that there could be a better interaction between more related P450-CPR partners than less related partners.

**Table 1.4: Relative activities of recombinant CYP2D6 produced by different hosts**

| **Host** | **P450 (pmol/mg microsomal protein)** | **Activity ($min^{-1}$)** | **Ref.** |
|---|---|---|---|
| *S. cerevisiae* | 250 | 10 | Dietrich *et al*., 2005 |
| *P. pastoris* | 120 | 4.8 | |
| *E. coli* | 306 | 2.7 | |
| | 371 | 5.84 | Walsky and Obach, 2004 |

(Adapted from Dietrich *et al*, 2005).

These results demonstrate again that even though P450 content is higher in *E. coli* than *S. cerevisiae*, activity in the yeast was higher, while the *S. cerevisiae* P450 content and activity were double that of *P. pastoris*. *P. pastoris* activity was comparable to that of *E. coli* even though the P450 content was lower.

**Table 1.5: Comparison of diclofenac hydroxylase activities of recombinant CYP2C9 obtained using different hosts.**

| Host | Activity | | | Reference |
|---|---|---|---|---|
| | Specific | | Volumetric | |
| | $min^{-1}$ | $nmol/min/g_{DCW}$ | µM/min | |
| *E. coli*[1] | 40 | - | 4 | Dodhia *et al.*, 2006 |
| *S. cerevisiae*[2] | 105.9 | - | 8.47 | Niwa *et al*., 2002 |
| *S. pombe*[3] | - | 43 | 0.34 | Dragan *et al*, 2011 |

[1]Purified artificial chimera between CYP2C9 and CYP102A1-reductase domain assayed

[2]Microsomal fraction assayed

[3]Whole cell activity; P450 content not determined

In this instance *S. cerevisiae* expression outperformed the *E. coli* expression, while both had higher rates than the *S. pombe* whole cells. The reactions for the former two are however short term and yield very little total product, while the point of the *S. pombe* whole cell system is to generate much higher amounts of product over a prolonged duration (see section 1.3.4). Under the given reaction conditions, the product formed by *S. pombe* is higher than that of the other systems by a factor of $10^6$.

The most frequently expressed versions of the steroid hydroxylase CYP17 are the bovine and human enzymes. CYP17 has been expressed in a few different hosts either with coexpression of CPR, or more commonly with solubilised membrane fraction activities reconstituted with purified rat CPR; or with only native reductases. Selected examples of 17α-hydroxylation of progesterone and pregnenolone by recombinant CYP17 are provided in table 1.6.

**Table 1.6: Comparison of progesterone (P4) and pregnenolone (P5) biotransformations by recombinant CYP17 produced by different hosts**

| Host | Source | CPR supply | P450 content | | Substr. | Activity ($min^{-1}$) | | Ref. |
|---|---|---|---|---|---|---|---|---|
| | | | WC (nmol/L) | MF (pmol/mg microsomal protein) | | WC | MF | |
| *E. coli* | Hum | Reconstituted (Rat) | 300 | - | P4 | - | 8 | Imai *et al*., 1993 |
| | | | | | P5 | - | 6.5 | |
| | Bov | Reconstituted (Rat) | 400 | - | P4 | - | 28 | Gilep *et al.*, 2004 |
| | | | | | P5 | - | 17 | |
| *S. cerevisiae* | Hum | Coexpressed (Human) | - | 79 | P4 | - | 6.6 | Auchus *et al*., 1998 |
| | | | | | P5 | - | 3 | |
| | Bov | Native only | 100 | 370-400 | P4 | 19 | 7.1 | Juretzek *et al.*, 2000 |
| *Y. lipolytica* | Bov | Native only | 44 | 10-20 | P4 | 124 | 9.8 | Juretzek *et al*, 2000 |
| *P. pastoris* | Hum | Native only | 50 | 300 | P4 | 6.9 | - | Kolar *et al.*, 2007 |

P4 – progesterone; P5 – pregnenolone; Hum – human; Bov – bovine; WC – whole cells; MF – membrane fractions (microsomal for yeasts).

Considering *Y. lipolytica* activities one can see that although the microsomal P450 yields are much lower than for the other hosts, the microsomal activities are generally better, except for one report (Gilep *et al*, 2004). More outstandingly, the estimated whole cell P450 yield of *Y. lipolytica* is less than half of the *S. cerevisiae* content and about the same as *P. pastoris*, but the whole cell rate is much higher for *Y. lipolytica* than the others.

As a final example we consider non-mammalian P450s to re-emphasize how parameters can vary in different situations. In a comparative study between *E. coli* and *S. cerevisiae* for the expression of three different limonene hydroxylases from Mint, a few interesting observations were made (Table 1.6; Haudenschild *et al*., 2000). Firstly, P450 content in intact cells as well as membrane fractions were higher in *E. coli* than in *S, cerevisiae*, although the CO-difference spectrum for yeast whole cells is probably not as reliable as it is for *E. coli.* Even so, specific activities for the limonene 6-hydroxylase from spearmint (S12) and one of the limonene 3-hydroxylases from peppermint (P17) were far better in *S. cerevisiae* than in *E. coli*. These observations appear to be somewhat enzyme dependant, as another limonene 3-hydroxylases from peppermint (P2) was on the other hand more poorly expressed by *S. cerevisiae* than the other enzymes and had lower activity. When activity is higher in *S. cerevisiae*, the fold differences are greater when activities are based on P450 content, since *E. coli* produces much higher P450 contents. On the other hand, when activities are higher in *E. coli*, the biomass-based activity fold difference is drastically higher than the P450 content-based activities. This indicates that *E. coli* competes better with *S. cerevisiae* in biomass-based activities, while *S. cerevisiae* has an advantage in P450 content-based activities, points which agree with results in table 1.3 and are reiterated in section 1.3.4.

**Table 1.7: Expression of limonene hydroxyalses from plants in *E. coli* and *S. cerevisiae* (adapted from Haudenschild *et al*, 2000)**

| | | P450 content | | Specific activity | |
|---|---|---|---|---|---|
| **Host strain** | **Gene expressed** | **Intact cells (nM)** | **Supernatant / Microsomes (pmol/mg protein)** | **nmol/h/mg protein** | **$min^{-1}$** |
| *E. coli* [1] | | | | | |
| JM109 | S12-1 [2] | 280 | 400 | 247 | 10.3 |
| | S12-2 | 350 | 810 | 369 | 7.6 |
| | S12-3 | 150 | 150 | 88 | 9.8 |
| | P2-2 | 500 | 1400 | 2260 | 26.9 |
| | P17-2 | 265 | 420 | 391 | 15.5 |
| *S. cerevsisae* [3] | | | | | |
| WAT11U [4] | S12 | n.d. | 527 | 1510 | 47.9 |
| WAT21U [4] | S12 | 61 | 328 | 1675 | 85.1 |
| WAT11U | P2 | 0 | 50 | 31.8 | 10.6 |
| WAT21U | P2 | 0 | 44 | 36.2 | 13.7 |
| WAT11U | P17 | 14 | 66 | 570 | 143.8 |
| WAT21U | P17 | n.d. | 110 | 763 | 115.6 |

S12 – limonene 6-hydroxylase from spearmint; P2 and P17 – limonene 3-hydroxylases from peppermint

[1]*E. coli* membrane preparations had activity reconstituted by addition of purified recombinant spearmint cytochrome P450 reductase

[2]Numbers following the gene represent different variations of N-terminal modifications of the gene

[3] No additional CPR added to reconstitute activity in microsomal preparations, only the native CPR of the strain

[4]WAT11U and WAT21U are two strains of *S. cerevisiae* with the native CPR disrupted by either *ATR1* or *ATR2*, reductases of *Arabidopsis thaliana*

### 1.3.4. Comparing capacities of hosts for whole cell biocatalysis

The abilities of hosts to express some P450s and relative activities of the recombinant proteins were illustrated in the previous section, but most of these reactions were carried out on small scales and for short durations. If P450s are to ever be applicable for larger scale reactions however, they will need to be used in whole cell systems. The limited optimization and up-scaling of P450 reactions so far is more attributable to the problems associated with these enzymes (described in section1.2.1) than to a lack of potential applications for them. For example, mammalian P450s are mostly used for small scale reactions involving drug metabolism, despite possessing high potential for applications in pharmaceutical and fine chemical industries. Their relatively low activities, combined with the difficulties related to working with membrane-bound multi-component systems have largely dissuaded researchers from attempting to optimize mammalian P450 activities. Indeed, the self-sufficient CYP102A1, which has the highest catalytic rate of P450s described to date (Noble *et al*, 1999), has received the most attention with regards to activity optimization; since it also possesses other attractive properties in that it is a water-soluble, single-component system (Whitehouse *et al*, 2011; Maurer *et al*, 2005).

Appropriate hosts for heterologous expression of eukaryotic P450s, equipped with sufficient capacity to function as whole cell biocatalysts, need to be identified. This is because whole cell biocatalysis is so far the only realistic approach to large scale P450 reactions (Zollner *et al*, 2010). Several factors play roles in the aptitude of an organism to perform the dual role of foreign host and whole cell biocatalyst. The most notable criteria are the successful expression of target genes; lack of vulnerability towards the recombinant protein; sub-cellular facilities to accommodate eukaryotic P450s; effective substrate uptake and tolerance; efficient reductase systems; and sufficient free heme content. Additionally, the host must be able to accommodate the coexpression of foreign

reductase components (Dragan *et al*, 2011) and / or facilitators of heme production (Harnastai *et al*, 2006), if required. The strain should also be able to recycle reduced cofactors intracellularly or accommodate coexpressed enzymes for these means (van Beilen *et al*., 2003).

*E. coli* matches most of these criteria poorly, and while there are abundant options available for coexpression of assistant enzymes in this host, they tend to metabolically burden the cultures (Bentley *et al*, 2009; Ow *et al.*, 2006). Yeasts tend to be more appropriate hosts since they match most if not all of the desired criteria. In the following section we consider some of the highest whole cell P450 activities reported thus far in yeasts and *E. coli* expressing P450s.

**Table 1.8: Comparison of whole cell biocatalytic systems expressing P450s**

| **Host** | **P450** | **Activity** | | **Assay duration (h)** | **Reference** |
|---|---|---|---|---|---|
| | | **Specific ($nmol.min^{-1}.g_{DCW}^{-1}$)** | **Volumetric (μM/min)** | | |
| *E. coli* | CYP102A1 | 5000 | 10 | 0.5 | Schneider *et al*., 1998 |
| | | 160 | 32 | 1 | Gudiminchi and Smit, 2011 |
| *S. cerevisiae* | Cinnamate 4-hydroxylase | 3600 | n.c. | 0.17 | Chen and Morgan, 2006 |
| *Y. lipolytica* | CYP53B1 | 35.8 | 0.72 | 270 | Shiningavamwe *et al*, 2006 |
| *S. pombe* | CYP509C12 | 139 | 0.83 | 6 | Petrič *et al.*, 2010 |
| | CYP2C9 | 43 | 0.35 | 72 | Dragan *et al*, 2011 |

n.c. – value not calculable as biomass values were not supplied

Unsurprisingly, the best whole cell activities reported so far in *E. coli* involve the expression of CYP102A1 for pentadecanoic acid hydroxylation, in a strain which also carried a pGEc47 plasmid for facilitation of fatty acid uptake, where a rate of 5 µmol/min/$g_{DCW}$ was obtained after 30 minutes of biotransformation. This rate however decreased to 1 µmol/min/$g_{DCW}$ after 6 hours of biotransformation (Schneider *et al*, 1998). Using the same wild-type CYP102A1 in a biphasic system with hexane as the organic phase, 4-hexylbenzoic acid (HBA) was hydroxylated at a rate of only 0.16 µM/min/$g_{DCW}$ during 1 hour of biotransformation (Gudiminchi and Smit, 2011). The volumetric rate of 32 µM/min was however far higher than that of the other example, which was 10 µM/min (Schneider *et al*, 1998).

Cinnamate 4-hydroxylase (C4H) from *Arabidopsis thaliana* was coexpressed with its native CPR in *S. cerevisiae*, and the ratio between galactose (the inducer) and glucose as well as the concentration of casein hydrolysate, were optimized. The resultant activities were comparable to the *E. coli* examples, but reactions were only carried out for 10 minutes (Chen and Morgan, 2006). Meanwhile, *Y. lipolytica* expressing the benzoate-*para*-hydroxylase CYP53B1 from *Rhodotorula minuta* and overexpressing the native CPR was cultivated under optimized induction conditions, and although the total activities were not great, activity was maintained for more than a week (Shiningavamwe *et al*, 2006). In *S. pombe*, a steroid 11α-hydroxylase (CYP509C12) from *Rhizopus oryzae* coexpressed with its natural CPR partner achieved higher activities than those obtained in the *Y. lipolytica* example, but only for a period of 6 hours (Petric *et al*, 2010). When human CYP2C9 and CPR were coexpressed in *S. pombe*, activity lasted for 72 hours in a process which was superior in terms of productivity when compared to other systems for the same reaction (Dragan *et al*, 2011). Although this activity was low compared to the other examples mentioned here, it must be taken into

account that human P450s generally have low activities, and that this is a reasonably impressive result by comparison to other expressed human P450s.

## 1.4. Concluding remarks

Ultimately, the choice of host depends on the downstream application of the recombinant protein. For production of large quantities of protein, especially for purification purposes, *E. coli* is the best choice. Exceptionally high P450 production in *E. coli* achieved maximum P450 yields of more than 12 500 nmol/L after 10 hours, accounting for 11% of the dry cell mass. The activities in membrane fractions under these conditions were 375 nmol/min/nmol P450, or 37.5 μM/min (Pflug *et al*., 2007). High protein production levels in *E coli* are favoured by the high cell densities achievable with this organism. *P. pastoris* is another possibility because it can also be grown to very high cell densities and does not require sequence modifications of the P450 (Cereghino *et al.*, 2002). Hosts such as *E. coli*, *S. cerevisiae* and *P. pastoris* have generally been used for analytical scale product formation to demonstrate activity or inhibition, generally with short reaction times (5-60 min).

If the intended outcome is maximal product formation however, appropriate hosts for whole cell biocatalysis are required, since whole cell biocatalysis is the only realistic approach to large scale P450 applications currently available. As demonstrated by some of the examples in the previous section, higher levels of P450 production does not necessarily mean higher activity. Reports on whole cell systems for P450 biocatalysis are rare, but usually result in prolonged activity durations. Whole cell systems using yeasts like *S. pombe* or *Y. lipolytica* can retain activity for days, while *E. coli* reaction times rarely reach 24 hours (Kubota *et al*., 2005). An exceptional situation was the maintenance of activity for 4 days by a purified mutant of CYP102A1 in a biphasic system. A total activity of 6.67μM/min or 2.57 $min^{-1}$ was determined at the reaction time of 100h (Maurer *et*

*al*, 2005). Although this system was obviously stable and a single-component system, the problem of cofactor regeneration still makes the process unfeasible for large scale application. Therefore yeast whole cells appear to be more applicable for preparative scale product formations which require prolonged durations (Zollner *et al*, 2010).

Therefore, for large scale product formation by eukaryotic P450s in particular, a suitable host which can facilitate whole cell biocatalysis is required. The challenge is in the selection of such hosts, which is complicated by the previously mentioned inconsistencies in methodologies and reports of activities between different studies.

# Chapter 2: Introduction to the present study

## 2.1. Identification of hosts for foreign eukaryotic P450s and whole cell biocatalysis

The identification of suitable hosts based on published data is complicated, because attempting to compare reports with obvious differences in approach, methodologies and interpretations are difficult (see chapter 1). Reports generally focus on specific applications, and as a result only application-specific host selections can really be made. It is especially differences in units used to report activities that complicate comparisons (see Chapter 1, section 1.3.3). The units could theoretically be interconverted, but the results may be somewhat misleading. Furthermore, there are methodological differences between reports. Different studies using the same P450 may use different CPRs, or some researchers may coexpress reductase components while others reconstitutes activity in purified fractions (examples Peyronneau *et al*, 1992; Imaoka *et al.*, 1996). Some may add cytochrome *b*5 for class II P450s while others might not (examples Inui *et al*, 2007; Cheng *et al*, 2006). These types of variations further complicate direct comparison.

Comparisons of hosts expressing the same P450s are often subject to experimental bias, since the expression systems used are host-specific, meaning differences in factors such as the strength and regulation (constitutive vs. inducible) of promoters or gene copy numbers may differ considerably (Cornelissen *et al*, 2012; Muller *et al*, 1998). Furthermore, the better-established systems for certain yeasts may overshadow the potential of lesser-studied yeasts for which expression systems have not been optimised (Steinborn *et al*., 2006; Klabunde *et al*, 2005).

Therefore, in order to identify appropriate hosts which can facilitate whole cell biocatalysis while heterologously expressing P450s, a better means of host comparison is required. For more accurate direct comparisons to be made between hosts, consistency in methodologies will have to be maintained as far as possible. This would include culturing the candidates under similar conditions, and performing general bioconversion procedures as consistently between species as possible. Preferably, a common vector system should be used between yeasts, to ensure minimal experimental bias. While this is impossible for comparisons between yeasts and *E. coli*, it is feasible between either different yeast species or different strains of *E. coli*. Since our interest is in comparing yeast strains for the expression of eukaryotic P450s, we focused on identifying elements for a universal system for expression in different yeasts. Some of the most researched yeast species for heterologous protein production thus far will be considered, namely *S. cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Arxula adeninivorans*, *Kluyveromyce lactis*, *K. marxianus*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis*, and *Debaryomyces hansenii*.

## 2.2. Elements for heterologous expression in yeasts

Expression vectors for yeasts are generally shuttle vectors, with both bacterial and yeast elements. The bacterial portion contains a bacterial origin of replication and an antibiotic resistance marker (usually conferring resistance to ampicillin or kanamycin), required for plasmid propagation and selection in *E. coli* (Steinborn *et al*, 2006). The most important requirements of the yeast portion include elements for introduction and maintenance / replication in the host; a selection marker for identification of successful transformants; and expression cassette components, consisting of at least a promoter and terminator region for regulation of gene expression (Steinborn *et al*, 2006). The following sections will discuss yeast expression elements, with emphasis on those which are common

between species, (represented in table 2.1), to ultimately lead to a common expression system to allow direct and unbiased comparisons between different yeasts.

**Table 2.1: Common elements used for integrative transformation of yeasts**

| **Integration target** | | | |
|---|---|---|---|
| **rDNA element** | **Source** | **Species** | **Reference** |
| 25S; 18S | *A. adeninivorans* | *A. adeninivorans*<br>*D. hansenii*<br>*D. polymorphus*<br>*H. polymorpha*<br>*P. pastoris*<br>*S. cerevisiae* | Rösel and Kunze, 1998<br>Terentiev *et al*., 2004a |
| | *H. polymorpha* | *H. polymorpha*<br>*A. adeninivorans*<br>*P. stiptilis*<br>*S. cerevisiae* | Klabunde *et al*., 2002<br>Klabunde *et al*., 2003 |
| | *K. lactis* | *K. lactis* | Maullu *et al*., 1999 |
| | *K. marxianus* | *K. marxianus* | Hong *et al*., 2007 |
| | *P. pastoris* | *P. pastoris* | Marx *et al*., 2009 |
| | *S. cerevisiae* | *S. cerevisiae* | Klabunde *et al*, 2005 |
| | *Y. lipolytica* | *Y. lipolytica* | Le Dall *et al*., 1994 |
| **Selective markers** | | | |
| **Auxotrophic markers** | | | |
| **Marker gene** | **Auxotrophy** | **Species** | **Reference** |

| | | | |
|---|---|---|---|
| *URA3* | Uracil | *D. hansenii* | Maggi and Govind, 2004 |
| | | *H. polymorpha* | Kiel *et al.*, 1999 |
| | | *K. lactis* | van Ooyen *et al*., 2006 |
| | | *K. marxianus* | Hong *et al*, 2007 |
| | | *P. pastoris* | Lin Cereghino *et al*., 2001 |
| | | *S. pombe* | Bähler *et al.*, 1998 |
| | | *S. cerevisiae* | Mumberg *et al*., 1995 |
| | | *Y. lipolytica* | Juretzek *et al*., 2001 |
| *LEU2* | Leucine | *A. adeninivorans* | Wartmann *et al.*, 2003b |
| | | *H. polymorpha* | Agaphonov *et al*., 1999 |
| | | *K. lactis* | Van Ooyen *et al*, 2006 |
| | | *K. marxianus* | Hong *et al*, 2007 |
| | | *S. cerevisiae* | Mumberg *et al*, 1995 |
| | | *S. pombe* | Iacovoni *et al*., 1999 |
| | | *Y. lipolytica* | Muller *et al*, 1998 |
| *TRP* | Tryptophan | *A. adeninivorans* | Steinborn *et al*., 2007 |
| | | *H. polymorpha* | Cheon *et al*., 2009 |
| | | *K. lactis* | Van Ooyen *et al*, 2006 |
| | | *K. marxianus* | Hong *et al*, 2007 |
| | | *S. cerevisiae* | Mumberg *et al*, 1995 |
| | | *Schw. occidentalis* | Piontek *et al*., 1998 |
| *ADE* | Adenine | *H. polymorpha* | Cheon *et al*, 2009 |
| | | *P. pastoris* | Lin Cereghino *et al*., 2001 |
| | | *S. pombe* | Fujita *et al*., 2005 |
| | | *Schw. occidentalis* | Klein and Favreau, 1988 |
| *LYS2* | Lysine | *A. adeninivorans* | Wartmann and Kunze, 2000 |
| | | *K. marxianus* | Abdel-Banat *et al.*, 2010 |
| | | *Y. lipolytica* | Madzak *et al*, 2004 |
| *HIS* | Histidine | *P. pastoris* | Invitrogen |
| | | *S. cerevisiae* | Mumberg *et al*, 1995 |

| | | | |
|---|---|---|---|
| | | *S. pombe* | Fujita *et al*, 2005 |
| **Antibiotic markers** | | | |
| **Marker gene** | **Antibiotic** | **Species** | **Reference** |
| *HPH* | Hygromycin B | *A. adeninivorans*<br>*D. hansenii*<br>*H. polymorpha*<br>*K. lactis*<br>*P. pastoris*<br>*S. cerevisiae*<br>*S. pombe*<br>*Y. lipolytica* | Rosel and Kunze, 1998<br>Steinborn *et al.*, 2006<br>Kang *et al*., 2001<br>Van Ooyen *et al*, 2006<br>Nett *et al*., 2005<br>Goldstein and McCusker, 1999<br>Hentges *et al*., 2005<br>Cordero Otero and Gaillardin, 1996 |
| *KanMX* | G418 / Geneticin | *H. polymorpha*<br>*K. lactis*<br>*K. marxianus*<br>*P. pastoris*<br>*S. cerevisiae*<br>*S. pombe* | Sohn *et al.*, 1999<br>Van Ooyen *et al*, 2006<br>Ribeiro *et al*., 2007<br>Lin-cereghino *et al.*, 2008<br>Goldstein and McCusker, 1999<br>Bähler *et al.*, 1998 |
| **Constitutive promoters** | | | |
| **Gene** | **Role** | **Species** | **Reference** |
| *TEF1* | Translational elongation factor | *A. adeninivorans*<br>*P. pastoris*<br>*S. cerevisiae*<br>*Y. lipolytica* | Terentiev *et al.*, 2004a<br>Ahn *et al.*, 2007<br>Mumberg *et al*, 1995<br>Muller *et al*, 1998 |
| *AaTEF* | TEF from *A. adeninivorans* | *A. adeninivorans*<br>*D. hansenii*<br>*D. polymorphus* | Terentiev *et al*., 2004b |

| | | | |
|---|---|---|---|
| | | *H. polymorpha*<br>*P. pastoris*<br>*S. cerevisiae* | |
| *GAP* | Glyceraldehydes-3-phosphate dehydrogenase | *H. polymorpha*<br>*K. marxianus*<br>*P. pastoris*<br>*S. cerevisiae* (as 'GPD') | Heo *et al.*, 2003<br>Hong *et al*, 2007<br>Waterham *et al.*, 1997<br>Mumberg *et al*, 1995 |

### 2.2.1. Types of plasmids for expression in yeast hosts

Vectors used for introduction and maintenance of foreign genes in yeast hosts can be either episomal or integrative. Episomally maintained plasmids for yeasts are rare, the most common type being the 2µ plasmid of *S. cerevisiae* (Veit and Fangman, 1988), Invitrogen). Expression vectors based on the 2µ plasmid are the most commonly used for expression in *S. cerevisiae*, and are predominantly limited to this species. There is a similar set of vectors for *Kluyveromyces* species, based on the pKD1 vector from *K. drosophilinarum*, with comparable copy-numbers to the 2µ plasmid in *K. lactis* (Bianchi *et al*., 1987). Vectors based on pKD1 have been applied for expression in *K. lactis* and *K. marxianus* without the need for maintained selective pressure (Bianchi *et al*, 1987; Bergkamp *et al*., 1993). These vectors are however limited to *Kluyveromyces* species, and have low copy numbers in *K. marxianus* (Fonseca *et al*., 2008).

Vectors have been constructed based on autonomously replicating sequences (ARS) present in various yeasts (table 2.2). These vectors are however mitotically unstable, which can be remedied by inclusion of centromeric sequences (CEN), at the expense of copy number reduction to below that of pKD or 2µ plasmids (Romanos *et al.*, 1992; Heus *et al*., 1990; Juretzek *et al*., 2001). The ARS-based vectors are also not applicable to all yeasts. For example, no

ARS have been identified for *A. adeninivorans*, and attempts to use the ARS from *S. cerevisiae* and *H. polymorpha* in *A. adeninivorans* have been unsuccessful (Böer *et al*., 2009). The *Hansenula polymorpha* ARS (*HARS*) has the ability to initially replicate episomally, and later integrate into the genome to allow higher copy numbers with improved mitotic stability (Gellissen and Hollenberg, 1997).

**Table 2.2: Use of vectors based on autonomously replicating sequences (ARS) used for yeast transformation**

| **ARS** | **Source** | **Species** | **Reference** |
|---|---|---|---|
| *ARS* | *D. hansenii* | *D. hansenii* | Maggi and Govind, 2004 |
| *HARS1* [1] | *H. polymorpha* | *H. polymorpha* | Kim *et al*., 2003 |
| *KARS2* | *K. lactis* | *K. lactis*<br>*K. marxianus* | Heus *et al*, 1990<br>Fonseca *et al*, 2008 |
| *PARS* | *P. pastoris* | *P. pastoris* | Cregg *et al*., 1985 |
| *ARS* | *S. cerevisiae* | *S. cerevisiae* | Da Silva and Srikrishnan, 2012 |
| *ARS1* | *S. pombe* | *S. pombe* | Iacovini *et al*, 1999 |
| *SwARS* | *Schw. occidentalis* | *Schw. occidentalis* | Piontek *et al*, 1998 |
| *ARS* | *Y. lipolytica* | *Y. lipolytica* | Juretzek *et al*, 2001 |

Integrative vectors are preferable for stability of the recombinant strain produced, especially under non-selective conditions. Integration is preferably homologous, and the specific genes used are often functional copies of genes to complement pre-instated auxotrophic markers (discussed further in next section). Examples include the *URA* gene in *K. lactis* and the *LEU* gene in *H. polymorpha* (Iwata *et al*., 1998; Agaphonov *et al*, 1999). These genes are often present in single genomic copies, meaning that only a single copy of the integrant used will be introduced.

A different type of single-locus integration target is the *AOX1* gene of *Pichia pastoris*. Integration in this gene disrupts the *AOX1* gene encoding the more active of two alcohol oxidases involved in methanol metabolism in this yeast (Invitrogen, Ilgen *et al*, 2005). The resulting mutant depends on the less effective alcohol oxidase (*AOX2*) for methanol utilization, resulting in far slower growth on methanol as sole carbon source than wild-type cells. This feature allows simple identification of transformants based on the slow growing phenotype on methanol (methanol utilization slow, $Mut^S$). More importantly, the strong *AOX1* promoter is still active for expression of foreign genes fused to it, but requires far lower methanol concentrations due to its inefficient metabolism, hence reducing fire risks during larger scale applications (Invitrogen, Ilgen *et al*, 2005).

Some *Yarrowia lipolytica* strains have a unique single locus for homologous recombination, which is a homologous sequence from the pBR family of plasmids integrated into the genome, serving as a docking site for pBR-based plasmids (Le Dall *et al*, 1994). Another unique recombination target for certain strains of *Y. lipolytica* is based on the Ylt1 retrotransposon, which contains long terminal repeats (LTRs) termed zeta elements. Zeta elements can be incorporated into expression vectors, allowing homologous recombination into the corresponding genomic sequences. High copy numbers could potentially be reached, as multiple copies of the zeta elements occur within the genome (Juretzek *et al*, 2001). An interesting feature of this system is that it facilitates stable, random genomic integration in strains which do not contain zeta elements, a feature which has been used for random mutagenesis in such strains (Thevenieau *et al*, 2006; (Mauersberger *et al*., 2001).

A popular option is the use of ribosomal DNA (rDNA) elements as integration targets. Even though the exact numbers of repeating units of rDNA vary between species, all species have large amounts of repeats as potential targets for multiple copy integration of genes (Klabunde *et al*, 2002; van Ooyen *et al*, 2006)

or the co-integration of multiple genes into the same genome. In one study using *H. polymorpha*, co-integration of four vectors containing the same rDNA elements and auxotrophic markers but different reporter genes, resulted in the successful co-expression of three of the genes (Klabunde *et al*, 2002). In another study, a polyhydroxyalkanoate biosynthetic pathway consisting of three genes was introduced into *A. adeninivorans*, using two vectors containing the same rDNA elements but different auxotrophic markers (Terentiev *et al*., 2004a). The use of rDNA sequences as integration targets has been applied to various yeasts (table 2.1).

Another advantage of using rDNA elements as integration targets is the universality of rDNA among yeasts, due to its ubiquitous role in protein synthesis. The majority of the rDNA sequence is highly conserved among yeast species, meaning elements from one species may be used in various species, as was proven for rDNA sequences from *H. polymorpha* and *A. adeninivorans* (table 2.1; Klabunde *et al*, 2003; Terentiev *et al*., 2004b).

### 2.2.2. Markers for selection of transformants

Two types of markers are generally used for transformant selection, namely auxotrophic markers and antibiotic resistance markers. The use of auxotrophic markers requires strains which have pre-introduced disruptions of key genes involved in the synthesis of key compounds, such as uracil or leucine. These auxotrophies can be restored by complementation with integrants containing functional versions of the disrupted genes (Wartmann *et al*., 2003b; Madzak *et al*, 2004; van Ooyen *et al*, 2006). The most commonly used auxotrophic markers for heterologous expression in yeasts are the *URA* and *LEU* markers, complementing deficiencies in synthesis of uracil and leucine respectively (Table 2.1). Various others exist, but tend to be more specific to certain species.

A promoter-truncated version of the *Y. lipolytica URA* marker (*ura3d4*) was produced to facilitate multi-copy integration, since more copies of the deficient marker are required to restore auxotrophy. It was found that this deficient marker was the determinant factor for multi-copy integration in *Y. lipolytica*, irrespective of the integration target used (Le Dall *et* al, 1994; Madzak *et al*, 2004). Multi-copy integration was similarly achieved in *A. adeninivorans* using the *ATRP1* marker under transcriptional control of a truncated *LEU* promoter (Steinborn *et al*., 2007). Defective promoters have also been used for dominant antibiotic resistance markers, in which increasing antibiotic concentration combined with truncation of the promoter increased copy numbers (Kang *et al*, 2001). Markers from one species are often used for complementation of auxotrophies in another species, for example the *S. cerevisiae URA3* marker used in *K. marxianus* (Almeida *et al.*, 2003). If the marker from one species is poorly expressed in a different species however, then a similar effect is observed as with promoter deficient markers, where multi-copy integrations occur for compensation (Agaphonov *et al*, 1999).

It is important to note here (as mentioned for *Y. lipolytica* in the previous section), that higher copy numbers are dependent on the marker used rather than the integration target. Theoretically, the high numbers of repeats of major rDNA units in yeasts and the zeta elements of *Y. lipolytica* could result in very high copy numbers. Logically though, the physiological function of rDNA subunits will not be compromised by maintaining unnecessary integrants. It can be deduced that the strain will only maintain as many copies of an integrant as is necessary to confer prototrophy or antibiotic resistance, depending on the marker employed. To illustrate this point, it was observed in *Y. lipolytica* that 60 copies of the integrant were present prior to induction of expression of a heterologous gene. After induction however, the copy number deamplified until it stabilized around 10-15 copies (Le Dall *et al*, 1994). Other reports of copy numbers up to 25 using a promoter-defective antibiotic marker (Kang *et al*, 2001) and 40 using a promoter-

defective auxotrophic marker (Klabunde *et al*, 2002) obtained in *H. polymorpha*, were not coupled to heterologous expression. Using the corresponding promoter-defective auxotrophic marker described for *H. polymorpha* in *A. adeninivorans* coupled to heterologous expression resulted in integrants with only 8 copies (Steinborn *et al*, 2007). Although the species are different, the results resemble those obtained with *Y. lipolytica*.

The prerequisite of an auxotrophic strain, which is not always available, is however inconvenient. In addition, auxotrophic strains tend to grow slowly or produce lower amounts of recombinant proteins, which may limit their industrial application (van Ooyen *et al*, 2006). Antibiotic resistance markers can, therefore, be convenient since they have no genotypic or phenotypic prerequisites for receptor strains (other than vulnerability to the antibiotic in question). Antibiotic tolerance / susceptibility vary between strains, but sensitivity to geneticin / G418 and / or hygromycin B seem to be quite common amongst species (table 2.1). Antibiotic resistance markers are undesirable for industrial applications, especially when expensive antibiotics have to be regularly supplied to maintain selective pressure (van Ooyen *et al*, 2006), but can be quite useful for screening studies.

A solution to selection marker problems is marker rescue by means of a system like the Cre-*lox*P which has been applied in, amongst others, *K. lactis* and *K. marxianus* (Ribeiro *et al*, 2007). It involves the Cre site-specific recombinase from phage P1 which recombines specific *lox*P recognition sequences (illustrated in figure 1.9). The homologous regions of the gene to be deleted (for example *LAC* encoding β-galactosidase, in figure 1.9) flank the *lox*P sequences, which in turn flank a marker, (for example *HPH* for hygromycin resistance, figure 2.1). The Cre recombinase combines the *loxP* sites, removing the marker gene and leaving a *lox*P scar which disrupts the target gene. The simultaneous benefits of such a system are the ability to delete various genes using the same system, and

ultimately be left with an antibiotic resistance-free strain when the marker is an antibiotic resistance gene. This strategy has been applied to various other yeasts (Ribeiro *et al*, 2007).

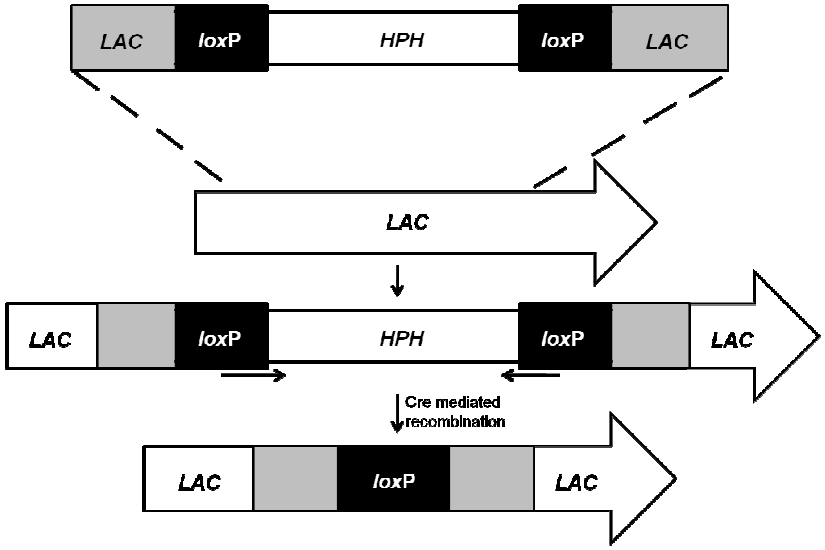



**Figure 2.1: Schematic representation of the utilization of the Cre-*lox*P system for gene disruption and marker recovery.**

Auxotrophic and antibiotic resistance markers can also be used in combination to allow insertion of multiple genes into the same genome, for example when three genes of the polyhydroxyalkanoate biosynthetic pathway from *Ralstonia eutropha* were simultaneously expressed in *A. adeninivorans*, using both the *LEU* and *HPH* markers (Terentiev *et al*., 2004a). Different auxotrophic markers can also be used together to allow insertion of multiple genes into the same genome, for example during coexpression of CYP53B1 and YlCPR in *Y. lipolytica* performed in our group, both *URA* and *LEU* markers were used (Theron, 2007). In *S. pombe* a reductase gene and *URA* marker were integrated into the *leu* locus of the genome of an *ura*$^-$ strain, simultaneously complementing the *ura* mutation and disrupting the *leu* gene. P450 genes were then introduced on episomal plasmids containing the *LEU* marker for complementation (Drăgan *et al*., 2011).

In *K. marxianus* the *URA3* gene was recoverable for re-use in multiple integrations, using the URA blaster system which employs two *Salmonella hisG* repeats surrounding the *URA3* marker. Mitotic recombination of the bacterial repeats results in removal of the marker, allowing selection of sub-sequential transformations using the same marker. Strains which have lost the *URA* gene are selected on plates containing 5-fluoro-ortic acid, which is toxic to *URA* containing cells. The only potential problem with this system is that the integration targets are random (Pecota *et al*., 2007). A similar strategy has been used for other yeasts, for example *S. pombe* (Siam *et al.*, 2004). The principle of this system is similar to the abovementioned Cre-*lox*P system for marker recovery.

Other interesting species-specific markers are generated by expression of foreign genes in hosts, similar to the case of antibiotic resistance genes. For example the *SUC2* gene encoding an invertase involved in sucrose metabolism in *S. cerevisiae*, was used as a selection marker for *Y. lipolytica* which cannot normally utilize sucrose (Madzak *et al*, 2004). Another example is the expression of the *amdS* gene from *Aspergillus nidulans* in *K. lactis*, conferring the ability to convert acetamide into ammonia as nitrogen source (Read *et al*., 2007).

### 2.2.3. Promoters

Yeast promoters can basically be divided into two classes, constitutive (table 2.1) and inducible (table 2.3) promoters. Constitutive promoters naturally regulate expression of ‘house-keeping’ genes involved in maintenance of regular cellular activities, and are hence constantly expressed (Madzak *et al*, 2004; Ilgen *et al*, 2005). Naturally, these tend to be conserved amongst different strains, such as the promoter of the translational elongation factor (TEFp) (table 2.1). Such promoters therefore have high potential for regulation of gene expression in foreign hosts.

Inducible promoters tend to be quite species specific, and based on unique physiological or metabolic properties of a species, such as the ability to utilize unusual carbon sources. Examples include the inducible promoters *AOX* and *MOX*, involved in methanol metabolism in *P. pastoris* and *H. polymorpha* respectively (Gellissen, 2000); and the *POX2* and *ICL1* promoters involved in the hydrocarbon utilization in *Y. lipolytica* (Juretzek *et al*., 2000). Commonly used species specific promoters are listed in table 2.3.

**Table 2.3: Important species-specific promoters commonly used for heterolgous gene expression in yeasts [1, 2]**

| Yeast species | Promoter | Inducer | Physiological role | Reference |
|---|---|---|---|---|
| *A. adeninivorans* | *GAA* | Maltose | Glucoamylase, metabolism of polysaccharides like starch | Wartmann and Kunze, 2000 |
| | *AINV1* | Expression increased using sucrose | Invertase, metabolism of sucrose, inulin and raffinose | Böer *et al.*, 2004b |
| | *AHOG1* | High osmolarity | Osmotolerance of cells | Böer *et al*., 2004a |
| | *AHSB4* | Constitutive, active under high salt concentrations | Histone B4 expression | Wartmann *et al*., 2003a |
| | | | | |
| *H. polymorpha* | ***MOX*** | Methanol induction<br>Glycerol derepression | Alcohol oxidase, methanol metabolism | Kang *et al*, 2001 |
| | *FMD* | Methanol induction<br>Glycerol derepression | Formate dehydrogenase, methanol metabolism | van Dijk et al., 2000 |
| | *DHAS* | Methanol induction<br>Glycerol derepression | Dihydroxyacetone synthase, methanol metabolism | |
| | *TPS1* | Temperature, high expression at 44 $^{o}$C | Trehalose biosynthesis | Amuel *et al.*, 2000 |
| | *PMA* | Constitutive | Plasma membrane $H^{+}$-ATPase | Cox *et al.*, 2000 |

| | | | | |
|---|---|---|---|---|
| *K. lactis* | ***LAC4*** | Lactose, galactose | β-galactosidase for lactose utilization | Muller *et al*, 1998 |
| | *ADH4* | Ethanol | Alcohol dehydrogenase | Saliola *et al*., 1999 |
| | *GAL7* | Galactose, lactose | Galactose metabolism | Maullu *et al*, 1999 |
| | *KmINU* | Sucrose | Inulinase gene of *K. marxianus* | Rocha *et al.*, 2011 |
| | Various *Sc* promoters | Various | Promoters from *S. cerevisiae* genes | Van Ooyen *et al*, 2006 |
| | | | | |
| *K. marxianus* | ***INU*** | Sucrose | Inulinase, metabolism of inulin, sucrose | Rocha *et al*, 2011 |
| | *PCPL3* | Constitutive | Purine-cytosine permease | Ball et al., 1999 |
| | *ScGAL1* | Galactose | Galactose metabolism in *S. cerevisiae* | Almeida *et al*., 2003 |
| | | | | |
| *P. pastoris* | ***AOX1*** | Methanol<br>Strictly no glucose | Major alcohol oxidase, methanol metabolism | Invitrogen |
| | *DHAS1* | Methanol | Dihydroxyacetone synthase, methanol metabolism | Tschopp *et al.*, 1987 |
| | *FLD1* | Methanol as C-source + $NH_4SO_4$ as N-source *or* Methylamine as N-source + glucose as C-source | Formaldehyde dehydrogenase, methylated amine and methanol metabolism | Shen *et al.*, 1998 |

| | | | | |
|---|---|---|---|---|
| *S. cerevisiae* | ***GAL1*** | Galactose | Galactose metabolism | Invitrogen |
| | *ADH1* | Constitutive | Alcohol dehydrogenase, ethanol production | Da Silva and Srikrishnan, 2012 |
| | *ADH2* | Glucose repressible | Alcohol dehydrogenase, ethanol production | |
| | *PGK* | Glucose | Phosphoglycerate kinase in glycolytic pathway | Cheng *et al*, 2006 |
| | | | | |
| *S. pombe* | ***nmt1*** | Thiamine repressible | | Petrescu-Dănilă *et al*., 2009 |
| | *nmt185* | Temperature shift | Truncated version of *nmt1* | Kumar and Singh, 2006 |
| | *ADH4* | Constitutive | Alcohol dehydrogenase, ethanol metabolism | Muller *et al*, 1998 |
| | *INV* | Sucrose | Invertase, sucrose metabolism | Iacovoni *et al*, 1999 |
| | *HSP16* | Heat, ethanol $Cd^{2+}$ and oxidative stress factors | Heat shock protein | Fujita *et al*, 2006 |
| | | | | |
| *Schw. occidentalis* | *AMY1* | Starch | α-amylase, starch metabolism | Suthar and Chattoo, 2006 |

| | | | | |
|---|---|---|---|---|
| | *GAM1* | Maltose | Glucoamylase, starch metabolism | Piontek *et al*, 1998 |
| | *ScADH1* | Constitutive | Ethanol production in *S. cerevisiae* | |
| | | | | |
| *Y. lipolytica* | ***XPR2*** | Peptones (pH above 6) | Alkaline extracellular protease | Juretzek *et al*, 2001 |
| | ***POX2*** | Fatty acids and their derivatives; alkanes | Peroxisomal acyl-CoA oxidase 2 | Juretzek *et al*, 2000 |
| | ***ICL1*** | Fatty acids and their derivatives; alkanes; ethanol and acetate | Isocitrate lyase | |
| | *G3P* | Glycerol | Glycerol-3-phosphate dehyhdrogenase | |
| | hp4d | Growth phase dependant, max at stationary phase | Hybrid promoter, derived from p*XPR2* | Madzak *et al*, 2004 |
| | *RPS7* | Constitutive | Ribosomal protein S7 | Muller *et al*, 1998 |

**[1] Constitutive promoters listed in table 2.1 are not included.**

**[2] Most commonly used promoters given in bold, unless they are listed in table 2.1.**

## 2.3. CoMed wide-range expression system

After successful transformation of various strains using rDNA elements of *H. polymorpha* and *A. adeninivorans* (table 2.1), Klabunde *et al* (2003) designed an expression system based on *A. adeninivorans* components for application in various yeasts. The vector contained 25S rDNA elements for integration and the TEF promoter for gene regulation (both from *A. adeninivorans*), as well as an *E. coli*-derived hygromycin resistance gene *hph*. Using this system they achieved successful heterologous expression in *A. adeninivorans*, *S. cerevisiae*, *D. hansenii*, *D. polymorphus*, *H. polmorpha*, and *P. pastoris* (Terentiev *et al*., 2004b). The *A. adeninivorans*-based system formed the basis of the CoMed system, which was developed for the purpose of screening of different yeasts under the same set of conditions.

The basic vector for the CoMed system consists of the bacterial moiety for sub-cloning in *E. coli*, and multiple cloning sites for insertion of combinations of ARS (for species capable of maintaining episomal plasmids), rDNA targeting regions for chromosomal integration, selectable markers and promoters for regulation of expression. A variety of these elements are available, flanked by appropriate restriction sites for convenient insertion into the vector (figure 2.2) (Steinborn *et al*, 2006; Pharmedartis). In this way customized vectors can be built according to specific requirements. One could initially build a generalized vector for parallel use in various hosts, as described earlier (Terentiev *et al*., 2004b). Once an appropriate candidate has been identified, the specific elements of the vector could be exchanged for more relevant ones to that specific species, tailoring the vector toward specific needs (Steinborn *et al*, 2006; Pharmedartis). Some examples which prove its application have been discussed (Terentiev *et al*, 2004b; Klabunde *et al*, 2003; Böer *et al*., 2007; Gellissen *et al*., 2005; Steinborn *et al*, 2006).

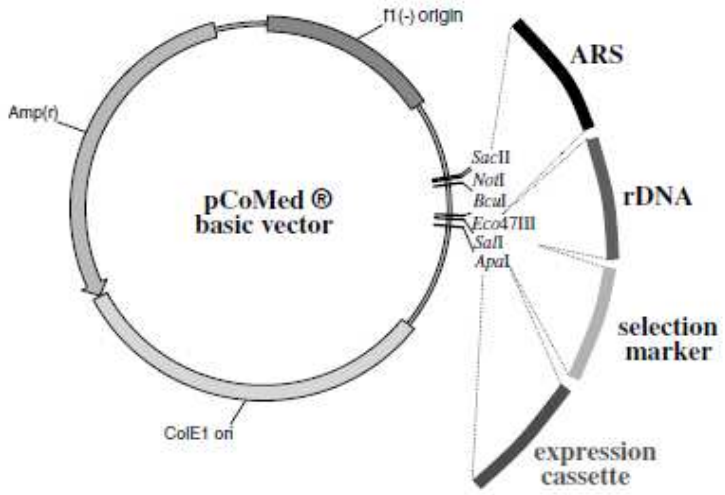



**Figure 2.2: Design of the CoMed$^{TM}$ vector system (from Steinborn *et al*, 2006).**

## 2.4. A wide-range expression system developed at the UFS

To compare different yeast hosts under the same conditions, a broad-range vector system was developed in our research group. This wide range vector is compiled of more diverse components than the CoMed$^{TM}$ system, as components from a variety of yeasts were used in the vector assembly. Therefore the vector should have less bias toward any particular host, facilitating a less biased selection of promising hosts (Albertyn *et al*, 2011). The components of the vector will be briefly described (see also table 2.4 and figure 2.3). During the descriptions, the corresponding names of the elements as designated in figure 2.3 are given in brackets.

The bacterial portion of the vector consists of genes required for plasmid replication and kanamycin resistance (kan). The hph gene from *E.coli* which confers resistance to hygromycin B, under the control of ScTEFp, was used to select for successful yeast transformants. All of the yeast species tested in this study showed significant sensitivity to hygromycin B. This dominant selection marker was chosen to exclude the requirement of corresponding auxotrophic strains for each of the yeast species. Since many yeast species do not maintain episomal plasmids, genomic integration was

targeted. rDNA was chosen as the targeting region for chromosomal integration as it is predominantly well conserved and has been proven to work well in different yeasts (Terentiev *et al*, 2004b; Steinborn *et al*., 2005). The rDNA regions were obtained from *Kluyveromyces marxianus*, and include the external transcribed spacer (ETS), 18S, internal and external transcribed spacer (ITS) and 5.8S elements (Steinborn *et al*, 2005).

The constitutive *Y. lipolytica* TEF promoter (YlTEFp) was chosen to control expression in the hosts so that no special induction conditions were required. Preliminary studies showed that the YlTEFp worked reasonably well in all of the hosts tested. The choice of terminator is less important and the *K. marxianus* inulinase (INU) terminator (KmINUt) was selected. Between YlTEFp and KmINUt there is an inulinase secretion signal sequence from *K. marxianus* (KmINUsigP), which can be optionally used or removed, depending on which restriction sites are used. Genes of interest can be introduced using either the *Xho*I or *Avr*II restriction enzymes or both in combination. The eukaryotic portion can be separated from the bacterial portion prior to yeast transformation by digestion with *Not*I (figure 2.3).

We have access to a number of yeast strains in the MIRCEN culture collection at the University of the Free State, South Africa. Representative strains for *S. cerevisiae, H. polymorpha, K. marxianus, Y. lipolytica*, *A. adeninivorans* various strains of *Candida* sp. and *Pichia* sp., *Debaromyces hansenii* and *Schwaniomyces occidentalis* were initially selected to test different antibiotic resistance markers and promoters. These early investigations decreased the list of yeasts that gave promising results with this vector to *S. cerevisiae, H. polymorpha, K. marxianus, Y. lipolytica*, *A. adeninivorans*, *Candida deformans* and *Kluyveromyces lactis* (Albertyn *et al*, 2011).

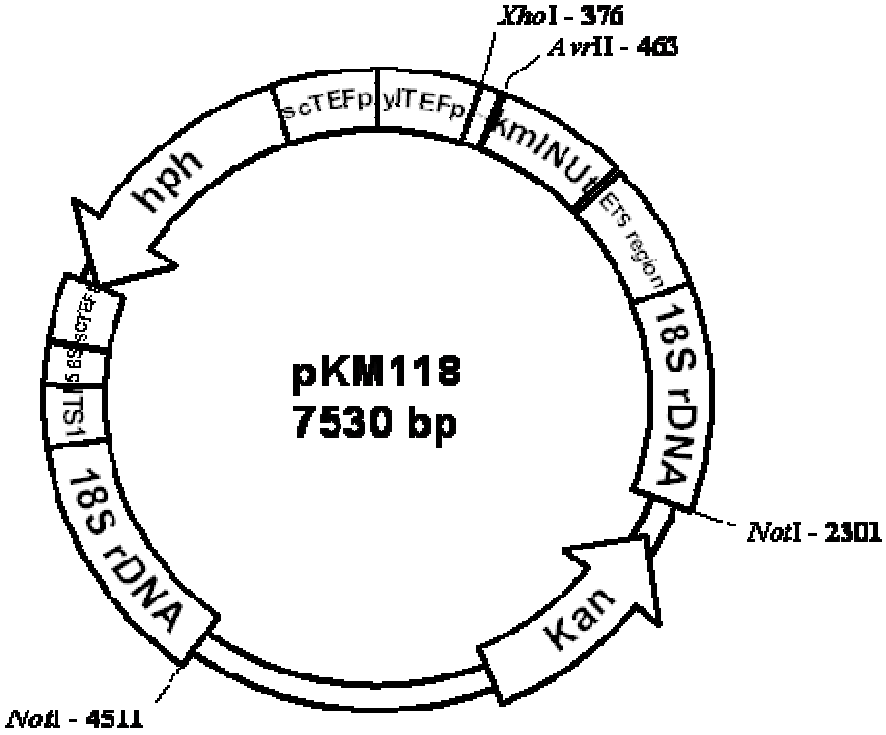



**Figure 2.3: Illustration of the broad range expression vector pKM118.**

**Table 2.4: Basic components of the broad range vector used in this study**

| **Component** | **Gene (according to fig.1.11)** | **Product / function** | **Source** |
|---|---|---|---|
| **Marker (*E. coli*)** | Kan | Neomycin phosphotransferase / kanamycin resistance | - |
| **Marker (Yeast)** | hph | Hygromycin phosphotransferase / hygromycin resistance | *E. coli* |
| **Integration target** | 18S rDNA | Ribosomal DNA | *K. marxianus* |
| **Expression cassette** | | | |
| **Promoter** | ylTEFp | Promoter for translational elongation factor | *Y. lipolytica* |
| **Terminator** | kmINUt | Terminator of inulinase gene | *K. marxianus* |

## 2.5. Aims of this study

As discussed earlier, whole cell applications of P450s are more feasible for large-scale applications, therefore one would ideally want to screen various yeasts not only for their abilities to actively and efficiently produce P450s, but also their potential as P450-containing whole cell biocatalysts. A wide range vector system as described above would facilitate convenient, parallel screening of various yeasts for these criteria under the same set of conditions. The method of gene transfer and destination, transfromant selection and induction conditions can all be consistent. This can be a starting point for identifying relative activities of various hosts. Such a starting point will also be useful for initial expression attempts of different P450s in the same host, as variations in the expression of different P450s within the same host are probable.

To evaluate the wide-range expression system developed at the UFS, various P450s were selected as reporter enzymes. Two self-sufficient P450s fused to CPRs and two conventional class II P450s, requiring CPR partners, were selected. While native yeast CPRs can facilitate activity, co-expression of CPRs enhances P450 activities (Schiffler *et al*, 2004; Nthangeni *et al*, 2004). Therefore various P450 reductases (CPR) were also selected for comparison by coexpression with the class II P450s.

The self-sufficient P450s chosen were the widely studied bacterial CYP102A1 (P450BM3) from *Bacillus* megaterium, and its first identified eukaryotic counterpart, CYP505A1 (P450foxy) from *Fusarium oxysporum*, both of which are sub-terminal fatty acid hydroxylases. Since the natural substrates of fatty acids are unsuitable for use in yeast whole cell systems because of native β-oxidation, alternative substrates were used. 4-Hexylbenzoic acid (HBA) was identified as a non-natural substrate for CYP102A1 by our group (Gudiminchi and Smit, 2011). The structure of HBA resembles natural fatty acids to some extent, but the compound is not completely utilized by the host cell. Furthermore, the presence of the benzoic acid group allows convenient monitoring of activity using thin-layer chromatography (TLC) on plates with a UV

fluorescence indicator (in our case F254) embedded in the stationary phase (in our case silica). Upon excitation of the indicator compound by UV light, UV-absorbing compounds quench the fluorescence of the indicator, and appear as dark spots on illuminated backgrounds. It was anticipated that this substrate could also be used to test for CYP505A1 activity. The structures of the substrate and products obtained using CYP102A1 are illustrated in figure 2.4.

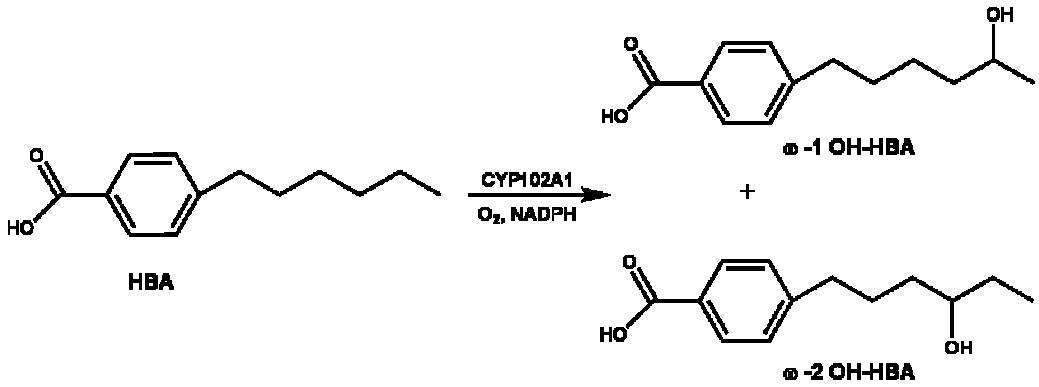



**Figure 2.4: Structure of 4-hexylbenzoic acid (HBA) and the products formed by CYP102A1 activity (Gudiminchi and Smit, 2011).**

The class II P450s selected were CYP53B1 from *Rhodotorula minuta* and CYP557A1 from *Rhodotorula retinophila.* The cDNAs for these P450s were available from previous studies (Shiningavamwe, 2004). The CYP557A1 gene encodes a putative fatty acid and alkane hydroxylase, although activity has not yet been proven for this enzyme, nor has its preferred substrate range been experimentally established.

Benzoate-*para*-hydroxylase activity of CYP53B1 (figure 2.5) can be screened for by using its natural substrate benzoic acid (BA), since none of the yeasts used for this study exhibit any significant wild-type activity toward this substrate. Furthermore, as described for HBA, convenient and reasonably rapid TLC analysis can be used to monitor activity due to high UV absorption of the benzoic acid and especially its hydroxylated product *para*-hydroxy benzoic acid (pHBA). The CYP53B1 has previously been successfully expressed in *Y. lipolytica* in our group (Shiningavamwe, 2004; Obiero, 2006; Theron, 2007).

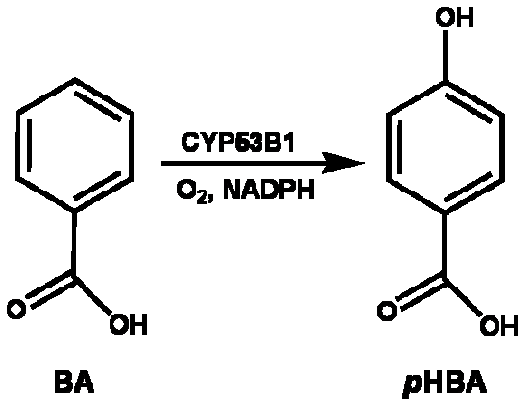



**Figure 2.5: Hydroxylation of benzoic acid (BA) to *p*-hydroxybenzoic acid (pHBA) by CYP53B1**

Coexpression of reductase enzymes have demonstrated increased activities of P450s (discussed in section 1.3.3). Our group has previously coexpressed the CPR from the ascomycetous *Y. lipolytica* (YlCPR) to improve expressed P450 activity in *Y. lipolytica* (Shiningavamwe, 2004; Theron, 2007). We would therefore also coexpress the YlCPR with CYP53B1 and CYP557A1 in this study. Additionally, we wanted to investigate the effect of coexpression of reductases from two basidiomycetes: *R. minuta*, (from which CYP53B1 also originates), and *U. maydis*. The *U. maydis* CPR was chosen because *U. maydis*, a fungus related to *Rhodotorula* sp., contains a P450 proven to be a fatty acid terminal hydroxylase which shares high similarity to CYP557A1 (Hewald *et al.*, 2005; Teichmann *et al*., 2007).

Therefore, by using our wide-range vector construct and the discussed reporter genes, we intended to compare P450 expression in terms of whole cell activity of different yeasts expressing P450s. Whichever yeast shows the most promise as such a host, can then be further studied for optimisation of activities. After preliminary screening rounds only *S. cerevisiae, H. polymorpha, K. marxianus, Y. lipolytica*, *A. adeninivorans* were selected for the study represented in this thesis. Since P450 expression in at least three of these hosts has not been reported, there exists the possibility of identifying alternative hosts to ultimately improve the rather limited sector of eukaryotic P450 research, particularly for biocatalytic purposes.

# Chapter 3: Materials and methods

## Part A: Molecular techniques for preparation of recombinant yeasts

### 3.1. Materials

#### 3.1.1. Enzymes, kits, general chemicals and reagents

Restriction endonucleases, T4 DNA ligase, T4 polymerase, polynucleotide kinase (PNK) and DNA molecular weight markers were all supplied by Fermentas.

Antarctic phosphatase and dNTPs were supplied by New England Biolabs.

KAPA HiFi DNA polymerase and associated reagents were supplied by KAPA Biosystems, Expand reverse transcriptase was provided by Roche. EconoTaq and the pSMART HC Kan vector was supplied by Lucigen. Sequencing reagents were supplied by AB biosystems.

BioFlux Biospin gel extraction kits and Biospin plasmid DNA extraction kits for DNA / RNA extraction and purification were supplied by Separations Scientific.

Oligonucleotide primers (Table 3.1) were designed and analysed using the 'Oligo Analyzer' tool on the Integrated DNA Technologies (IDT) web page (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) using default settings. Primers were synthesized and supplied by IDT-DNA or Bioneer.

Hygromycin B was supplied by HyClone, while G418 / geneticin and kanamycin sulphate were supplied by Sigma-Aldrich.

TRIzol solution was supplied by Invitrogen.

Other chemicals were, unless otherwise specified, obtained from Fluka, Merck, or Sigma-Aldrich.

**Table 3.1: List of primers used during this study**

| Primer name | Sequence (5' – 3') | Binding site |
|---|---|---|
| Fox505F1 | ATGGCTGAATCTGTCCCTATTCCTGAG | CYP505A1 start |
| Fox505R1 | CGCTAATCGAAAACATCAGTAGCAAAACGC | CYP505A1 end |
| HisFoxF1 | CTCGAGATGGGCAGCAGCCATCATCATC | 5' End His-tag |
| FOFOXINTFWD1 | CTCGGATGCTCCTAGCCACGGCTTC | CYP505A1 internal |
| FOFOXINTREV1 | GGTCTTCGGGAAGGTTCTGCTTGGC | CYP505A1 internal |
| FOXINTFWD2 | GCAAGGTCCAGAAAGAAGTCGACG | CYP505A1 internal |
| FOXINTREV2 | GTGACGATTTCACCCTTCTTGACG | CYP505A1 internal |
| FOXINTFWD3 | CCC GTTGTCATCGTCACTGC | CYP505A1 internal |
| FOXINTREV3 | CGAGTACCGCCACGCTTCTC | CYP505A1 internal |
| FOXINTFWD4 | CCTATCTGGCCATGCTCCCTC | CYP505A1 internal |
| FOXINTREV4 | CAGAGAGCGAAGGAGCGTCC | CYP505A1 internal |
| ylTEF-1F | AATGGTACCAGAGACCGGGTTGGCGG | TEF promoter |
| 557A1-1R | CCTAGGTCATCGTCGGGATATTGTTACCCAG | CYP557A1 |
| 53B1-1R | CCTAGGCTAGGCATCAATGGATCTGCG | CYP53B1 |
| ylCPR-1R | CCTAGGCTACCACACATCTTCCTGGTAG | YlCPR |
| R_minuta CPR-1R | GCTAGCCTAACTCCAGACATCGAGGAGGAGACG | RmCPR |

| | | |
|---|---|---|
| U_maydis CPR-1R | CCTAGGTTAGGACCATACATCGAGAAGCAGCCTCG | UmCPR |
| SL1 | CAGTCCAGTTACGCTGGAGTC | pSMART vector |
| SR2 | TGACTGGTAAATTTAGTATGGACTGG | pSMART vector |

### 3.1.2. Strains of bacteria, fungi and yeasts used in this study

All yeast strains were obtained from storage under liquid nitrogen at -70$^0$C in the MIRCEN yeast culture collection of the University of the Free State (UFS), South Africa. The cultures were frozen in LN broth containing glycerol (7% v/v final concentration). The yeasts were revived by streaking on YM agar plates supplemented with a vitamin solution. Media are described in the following section. The yeast strains are summarized in table 3.2.

**Table 3.2: Yeast strains used as hosts for heterologous expression during this study**

| Species | Strain | Genotype |
|---|---|---|
| *Saccharomyces cerevivsiae* | W3031A(a) | *MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2* |
| *Yarrowia lipolytica* | CTY003 (E150 derivative) | *MatB his1 ura3-302 leu2-270 URA3 LEU2 xpr2-322* |
| | CTY029 (FT120 derivative) | *MatA ura3-302 leu2-270 URA3 LEU2 xpr2-322 pox1-6::lox pJMp21::CPR* |
| *Kluyveromyces marxianus* | UOFS Y1185 | Wild-type |
| | Isolate 2.1 | |
| *Hansenula polymorpha* | UOFS Y1507 | |
| *Arxula adeninivorans* | UOFS Y1220 | |
| | UOFS Y1226 | |
| *Fusarium oxysporum* | MRC3239 | |

Plasmid manipulations were performed using *Escherichia coli* strain XL-10-Gold [*Tet*$^{r}$ *D(mcrA) 183 D(mcrB-hsdSMR-mrr)173end A1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacI*$^{q}$*ZDM15 Tn10 (Tet*$^{r}$*) Amy Cam*$^{r}$ *;* Stratagene].

Gene expressions were performed using *E. coli*,strain BL21 (DE3) [F–, *omp*T, *hsd*SB (rB–, mB–), *dcm*, *gal*, λ(DE3); Stratagene].

### 3.1.3. Reporter genes

The *Bacillus megaterium* CYP102A1 gene was generously provided by Professor Vlada Urlacher, University of Dussledorf, Germany, in a pET28(b) plasmid.

The cDNAs of CYP53B1 and CYP557A1 from *Rhodotorula minuta* and *Rhodotorula* sp. respectively, as well as for the reductase (CPR) genes from *Yarrowia lipolytica, Rhodotorula minuta* and *Ustilago maydis* were already available in our group (Shiningavamwe, 2004; Labuschagne, unpublished results).

An artificially synthesised gene for CYP505A1 was obtained from GeneArt. The cDNA for CYP505A1 was also obtained from mRNA extracted from *Fusarium oxysporum*.

Plasmid maps were constructed and modified using the pDRAW32 program version 1.1.112 of ACACLONE software, based on sequencing information and information obtained from NCBI databases (http://www.ncbi.nlm.nih.gov/).

The vector system used in this study was constructed in our research group by Dr. Michel Labuschagne, using DNA from cultures obtained from the UFS culture collection (sections 1.5 and 2.3).

## 3.2. General methods

### 3.2.1. General techniques

Standard genetic techniques were used, as described in Sambrook and Russel (2001).

All DNA purifications from solutions or agarose gels were performed using the Bioflux DNA Gel Band extraction kit using the manufacturer's recommendations.

Polymerase chain reaction (PCR) amplification was performed using Kapa Hi-Fi HotStart DNA polymerase (Kapa biosystems), except for basic PCR screening in which case EconoTaq (Lucigen) was used. General PCR conditions used in this study are described in table 3.3.

Sub-cloning of all PCR products were performed using pSMART HC Kan vector (Lucigen).

Genomic DNA isolation was performed using the method described by Labuschagne and Albertyn (2007).

**Table 3.3: Standard PCR profiles used in this study**

| **Step** | **KAPA HiFi** | | **EconoTaq** | | **Number of cycles** |
|---|---|---|---|---|---|
| | **Temp.** (°C) | **Duration** (s) | **Temp.** (°C) | **Duration** (s) | |
| Initial denaturation | 95 | 120 | 94 | 120 | 1 |
| Denaturation | 98 | 20 | 94 | 30 | |
| Annealing | 55[a] | 15 | 55[a] | 30 | 10 |

| | | | | | |
|---|---|---|---|---|---|
| Elongation | 72 | 90[b] | 72 | 90[b] | |
| Final elongation | 72 | 300 | 72 | 300 | 1 |

[a] 55°C given as an example, annealing temperature was determined by the melting temperatures of the specific primers used (based on information from the IDT-DNA Oligoanalyzer tool).

[b] Elongation time was dependant on the expected amplicon size, approximately 30 seconds / 1kb.

### 3.2.2. Cultivation media and conditions

*E. coli* strains carrying plasmids were grown in Luria – Bertani (LB) broth, which contained, per litre distilled water, 5 g yeast extract, 10 g sodium chloride, and 10 g tryptone; or on LB plates with additional 20 g agar per litre water. The LB plates and broth were supplemented with 30 µg / ml kanamycin to elicit selective pressure. Cells cultured in test tubes containing 5 ml LB broth supplemented with 30 µg / ml kanamycin were incubated at 37°C on a rotary shaker at 130 rpm for 16 hours. Plates were incubated at 37°C for 16h.

Yeast strains were grown in YPD broth containing, per litre of distilled water, 10 g yeast extract, 20 g peptone and 20 g glucose. For selective YPD agar plates the media was supplemented with 20 g agar and 400 mg hygromycin B per litre distilled water. For selection of G418 resistant *K. marxianus* transformants, YPD agar plates containing 75 mg per litre distilled water were used. *K. marxianus* transformants carrying genes under the control of the inulinase promoter were grown in YPS broth, containing, per litre of distilled water, 10 g yeast extract, 20 g peptone and 20 g sucrose. Cultures were generally grown at 28°C in test tubes or Erlenmeyer flasks on a rotary shaker with a speed of 130 rpm; or on selective plates. The cultivation broth occupied a volume equal

to one tenth of the maximum volumetric capacity of the flask to facilitate oxygen transfer.

Fungal strains were grown in YM medium containing per litre of distilled water, 10 g glucose, 5 g peptone, 3 g malt extract, and 3 g yeast extract. YM plates contained an additional 20 g agar per litre distilled water. For RNA extraction, *F. oxysporum* strain TVN498 was cultivated in a medium based on the medium described by Shoun and co-workers (Shoun *et al*., 1989). The medium contained 2 $g.L^{-1}$ $NaNO_3$, 2 $g.L^{-1}$ peptone, 3% v/v glycerol, 0.2 $g.L^{-1}$ $MgSO_4.7H_2O$, 2 $mg.L^{-1}$ $CoCl_2.6H_2O$, 2 $mg.L^{-1}$ $FeSO_2.7H_2O$, and 10 mM potassium phosphate buffer, pH8.

Fungal strains were grown for 48h at 28$^{o}$C on a rotary shaker at 130 rpm.

### 3.2.3. General cloning, transformation of *E. coli*, and plasmid isolation

Blunt-ended PCR amplicons generated by KAPA HiFi polymerase were phosphorylated using polynucleotide kinase (PNK, Fermentas) according to the manufacturer's instructions, before being ligated for 1 hour at room temperature to the pSMART HC Kan blunt cloning vector (Lucigen) for sub-cloning.

DNA was digested using restriction endonucleases (Fermentas) according to specifications supplied by the supplier. DNA fragments obtained from digestion of PCR amplicons were electrophoresed in 1% w/v agarose gels containing GoldView Nucleic Acid Stain (SBS Genetech) in TAE electrophoresis buffer (40 mM Tris, 2 mM EDTA, 20 mM glacial acetic acid; pH 8.5). DNA samples were visualized using a ChemiDoc XRS (Bio-Rad Laboratories) for documentation purposes, or a DarkReader$^{TM}$ transilluminator (Bio-Rad Laboratories) for excision of fragments from agarose gels for purification. DNA purification from agarose gels was achieved using a BioFlux Biospin gel extraction kit. Purified DNA was quantified using a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, USA).

When products required blunting T4 DNA polymerase (Fermentas) was used according to the supplier's instructions, at 11$^{o}$C for 20 minutes before heat inactivation at 75$^{o}$C for 10 minutes. Blunted vector backbones were dephosphorylated using Antarctic phosphatase (New England Biolabs) according to the supplier's instructions, at 37$^{o}$C for 1 hour before heat inactivation at 65$^{o}$C for 10 minutes. Appropriate fragments were ligated together for 1 hour at room temperature before the ligation mixture was used to transform *E. coli*.

Competent *E. coli* cells were prepared and transformed according to the methods described by Inoue *et al,* (1990). Transformed cells were plated on LB agar supplemented with 30 µg.mL-1 kanamycin and incubated at 37$^{o}$C for 16 hours. Positive transformants which were selected using colony PCR, were inoculated into 5 mL LB media supplemented with 30 µg.mL-1 kanamycin for plasmid propagation.

When possible, colony PCR using EconoTaq was used as a preliminary screen for successful transformants or correct orientation of the insert in the vector.

### 3.2.4. Plasmid Extraction

Plasmid mini-preparations were performed according to the lysis by boiling method described by Sambrook and Russell (2001). The DNA was resuspended in 50 µl TE buffer (10 mM Tris-Hcl, 1 mM EDTA; pH8) containing 50 µg / ml RNase and incubated at 37$^{o}$C for 1 hour. Thereafter mini-preparations were subjected to restriction analysis to confirm that cloning was successful or whether inserts in vectors were in the correct orientation.

Alternatively plasmid DNA was extracted with a BioSpin Plasmid DNA Extraction Kit (Separation Scientific) according to instructions of the supplier.

### 3.2.5. DNA Sequence analyses

DNA sequencing was performed at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State. The BigDyeTM Terminator Cycle Sequencing Kit v3.1 (Applied 42 Biosystems) was used for sequencing of both strands of plasmid DNA, according to the manufacturer's instructions. Sequencing primers were at final concentrations of 0.32 pmol / µl, while plasmid DNA concentrations of 15 – 30 ng / µl were used as sequencing templates in 10 µl reactions. A standard PCR program was used for all the reactions (Table 2.4).

**Table 3.4: Standard PCR program for sequencing reactions**

| Step | Temperature | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 96℃ | 1 min | 1 |
| Denaturation | 96℃ | 10 sec | 25 |
| Annealing | 60℃ | 5 sec | |
| Elongation | 60℃ | 4 min | |

Post-reaction clean-up of the samples (10 µl) was done using an ethanol-EDTA precipitation method. Millipore quality water (1 volume) was added to samples, followed by EDTA to a final concentration of 25 mM. Ethanol was added to a final concentration of 70% v/v, followed by vortexing, and incubation at room temperature for 20 minutes. Samples were pelleted at 14 000 x *g* at 4$^{o}$C for 20 minutes. Ethanol (70% v/v, 60µl) was added to the pellets prior to further centrifugation at 14 000 x *g* at 4$^{o}$C for 10 minutes. Pellets were dried completely using an Eppendorf Concentrator Plus, and refrigerated at 4$^{o}$C until sequencing.

Sequencing was performed with a 3130*xl* Genetic Analyzer (Applied Biosystems). Genes inserted into the pSMART-HC plasmid were sequenced using plasmid specific primers SL1 and SR2 and gene specific internal primers (Table 2.1).

Nucleotide sequences were analyzed using Vector NTI Advance 10. Nucleotide sequences were compared with sequences in the database at NCBI using BLAST (http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi). Nucleotide and amino acid sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalw2) and virtual translations of DNA sequences were performed using EMBOSS Transeq (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html).

### 3.3. Cloning CYP505A1 from *Fusarium oxysporum* TVN489

*F. oxysporum* strain TVN498 was cultivated for 48h at 28$^{o}$C in a medium based on the medium described by Shoun and co-workers (1989; section 2.2.2). After 48h growth the cultures were harvested. The pelleted mycelia were frozen with liquid nitrogen and ground into a fine powder using a mortar and pestle. Total RNA extraction was performed using TRIzol (Intvitrogen) according to the supplier's instructions and the method described by Labuschagne and Albertyn (2007). Total extracted RNA was resuspended in 50 µl of formamide or DEPC-treated $H_2O$ (0.1%w/v). Samples were treated with 5% v/v formaldehyde, 50% v/v formamide ethidium bromide and 1x NBC buffer (final concentrations of 50 mM boric acid, 1 mM sodium citrate and 5 mM NaOH, pH7.5); and evaluated using a 1% denaturing agarose gel containing 4% formaldehyde.

To obtain cDNA, total RNA was used as a template for RT-PCR using Expand reverse transcriptase (Roche) according to the supplier's instructions. cDNA was amplified further using PCR program described in table 3.3.

Genomic DNA was extracted according to the method described by Labuschagne and Albertyn (2007).

PCR amplicons of genomic DNA and cDNA copies of the gene for CYP505A1 were phosphorylated using polynucleotide kinase (PNK, Fermentas) and ligated into the

pSMART-HC blunt-end cloning plasmid. The resulting ligation mixtures were used to transform competent *E. coli* cells for propagation. Propagated plasmids were extracted from *E. coli* and prepared for sequencing.

### 3.4. Construction and modification of expression vectors

#### 3.4.1. Available vectors

**Broad range vector system**

The broad range vector used in this study (pKM118; figure 2.1) was constructed in our group using components from *K. marxianus, Y. lipolytica* and *S. cerevisiae* and a backbone vector carrying the genes necessary for replication and selection during sub-cloning in *E. coli.* The vector components were described in detail in section 1.5.

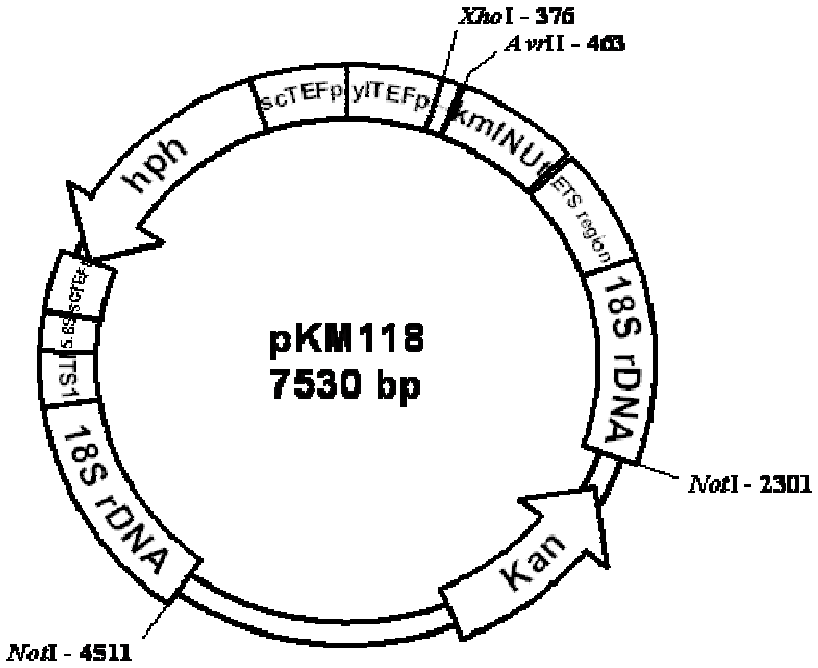



**Figure 3.1: Illustration of the broad range expression vector pKM118.** The components were described in section 1.5. The locations of the *Xho*I and *Avr*II restriction sites for insertion of genes of interest, as well as the *Not*I restriction sites for liberation of the yeast integration cassette, are provided.

Variations of this vector were created by introducing one (pKM173, 'A' in figure 3.2) or two (pKM177, 'B' in figure 3.2) I-*Sce*I sites into the vector (figure 3.2). pKM177 which has two I-*Sce*I sites, has a *Afe*I site instead of an *Avr*II site for cloning of inserts.

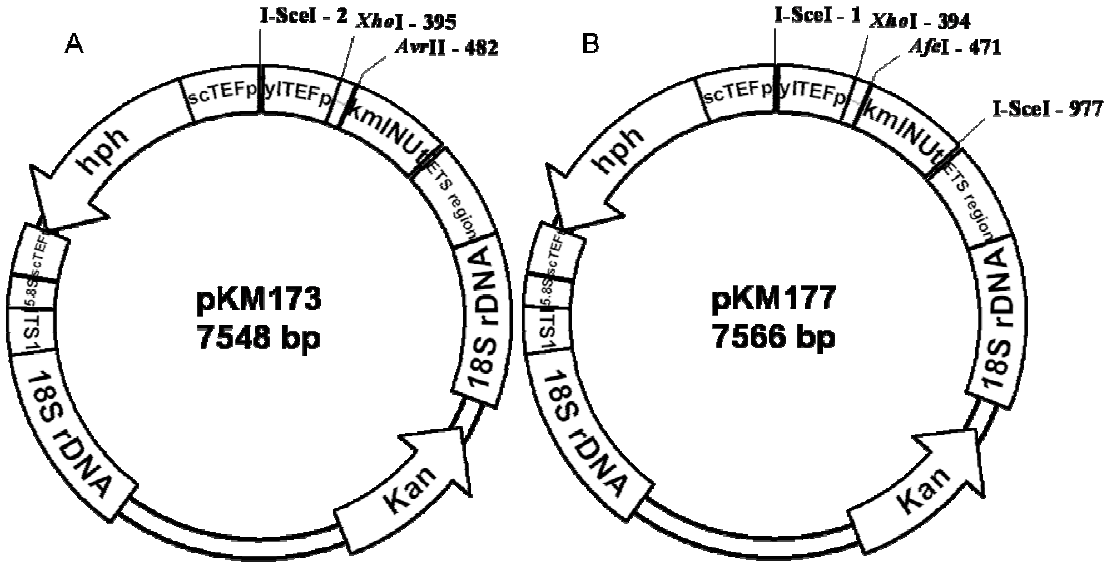



**Figure 3.2: Illustration of pKM173 and pKM177, modifications of pKM118 containing I-*Sce*I site(s).**

Expression vector pKM118 was used for initial expression of CYP102A1 and CYP505A1. pKM173 and pKM177 were used for simultaneous coexpression of two genes, or expression of two copies of the same gene. pKM118, pKM173, pKM177 and pKM63 (next sub-section) are referred to as 'empty vectors' when they were used to transform yeasts to obtain controls which do not express foreign P450 or CPRs (Chapter 4).

### Specific vector for expression in *K. marxianus*

The *K. marxinus*-specific vector was identical to the broad range vector except for two differences: the *Y. lipolytica* TEF promoter was replaced by the inulinase (INU) promoter

from *K. marxianus* isolate 2.1 (Km 2.1 PINU); and the hygromycin resistance marker *hph* was replaced by *kanMX* conferring resistance to G418 / geneticin (figure 3.3).

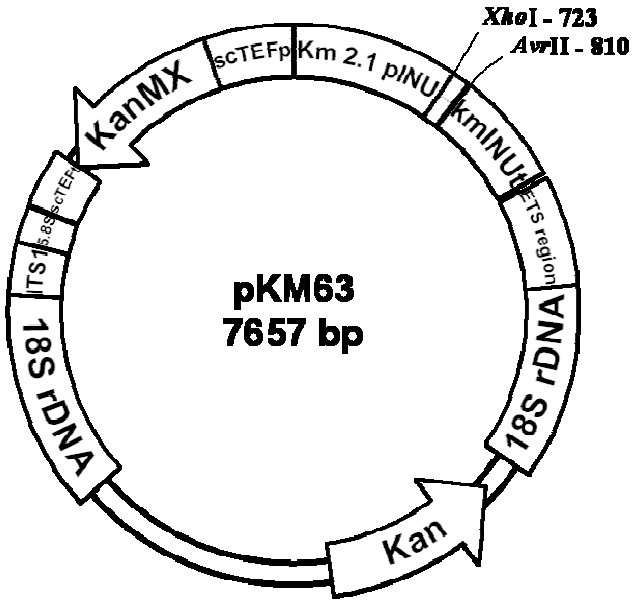



**Figure 3.3: Illustration of pKM63, a variant of pKM118 specific for expression in *K. marxianus*.**

**Vectors containing genes encoding reporter enzymes**

The artificially synthesized CYP505A1 was provided by the supplier in a pMK-RQ vector, while CYP102A1 was present in a pET28a vector. The artificially synthesized CYP505A1 was also cloned into pET28b by Dr. van Marwijk for expression in *E. coli*, which was later used to obtain N-terminally His-tagged CYP505A1 for yeast expression (figure 3.4).

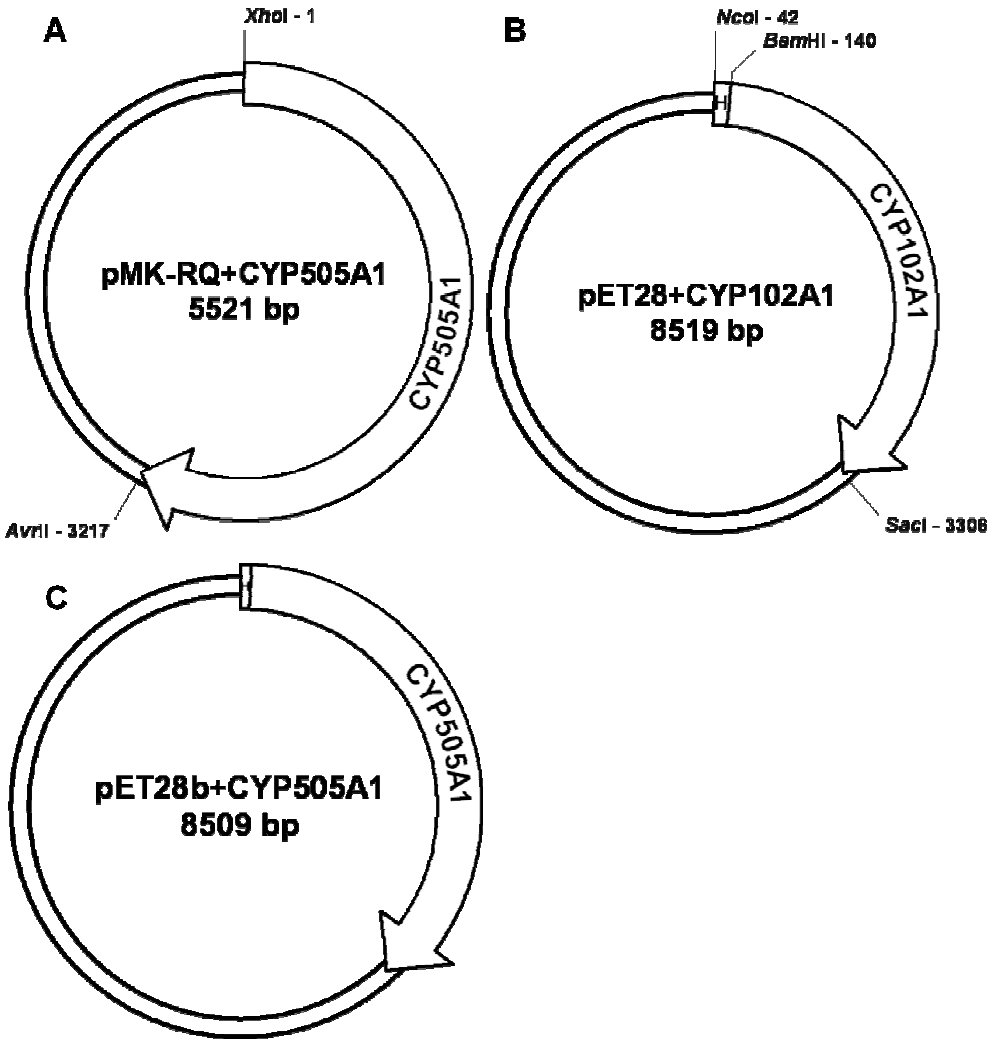
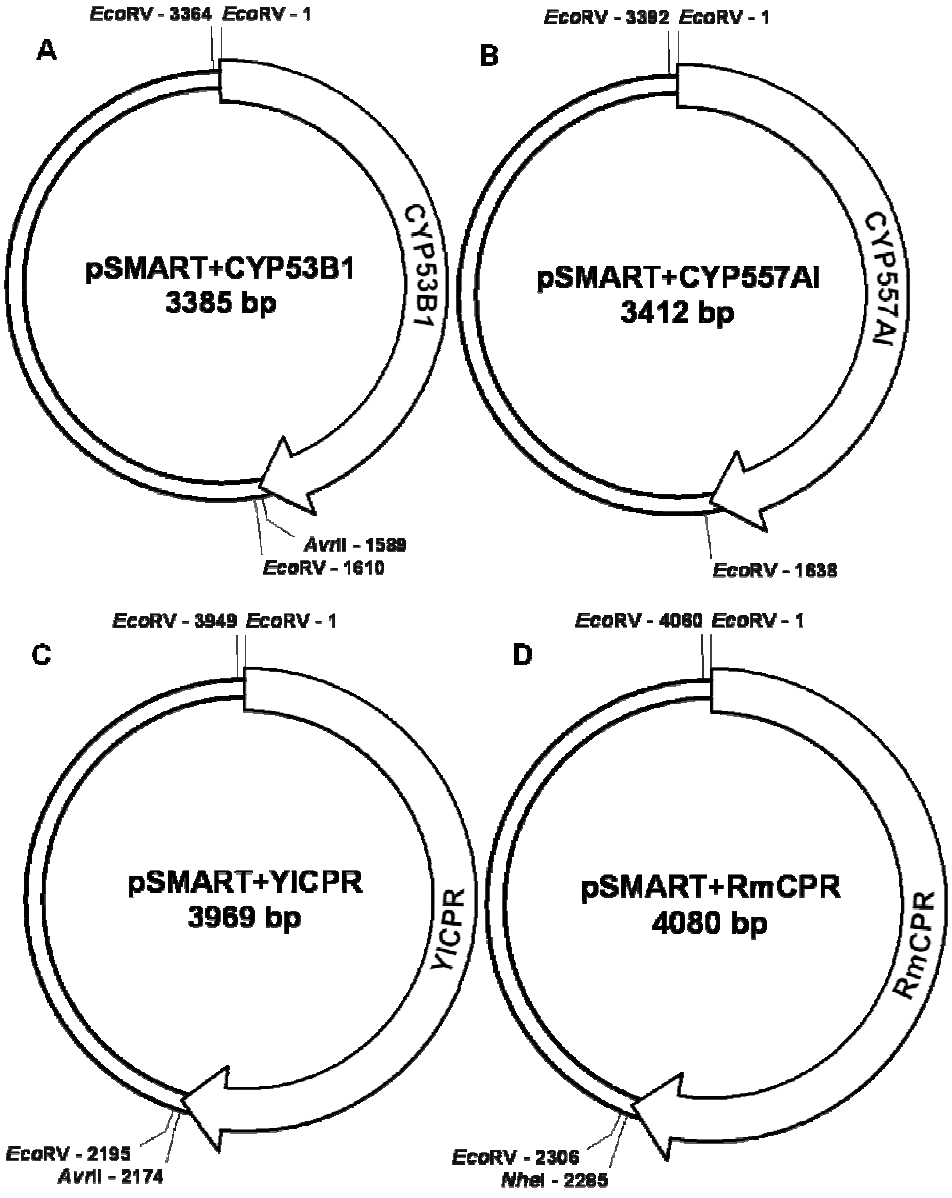



**Figure 3.4: Cloning vectors pMK-RQ+CYP505A1 (A) and pET28+CYP102A1 (B); and the *E. coli* expression vector pET28b+CYP505A1 (C).** CYP102A1 in pET28 is fused to a histidine tag (labeled as 'H') at the N-terminal side, which can be included if *Nco*I is used or excluded if *Bam*HI is used.

CYP53B1 and CYP557A1, as well as the three CPRs tested in this study, were all present in pSMART vectors (figure 3.5).


A
EcoRV - 3364 EcoRV - 1
pSMART+CYP53B1
3385 bp
CYP53B1
AvrII - 1589
EcoRV - 1610
B
EcoRV - 3392 EcoRV - 1
pSMART+CYP557A1
3412 bp
CYP557A1
EcoRV - 1638
C
EcoRV - 3949 EcoRV - 1
pSMART+YlCPR
3969 bp
YlCPR
EcoRV - 2195
AvrII - 2174
D
EcoRV - 4080 EcoRV - 1
pSMART+RmCPR
4080 bp
RmCPR
EcoRV - 2306
NheI - 2285

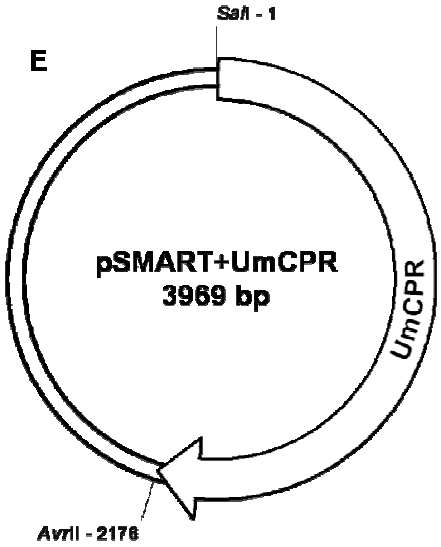
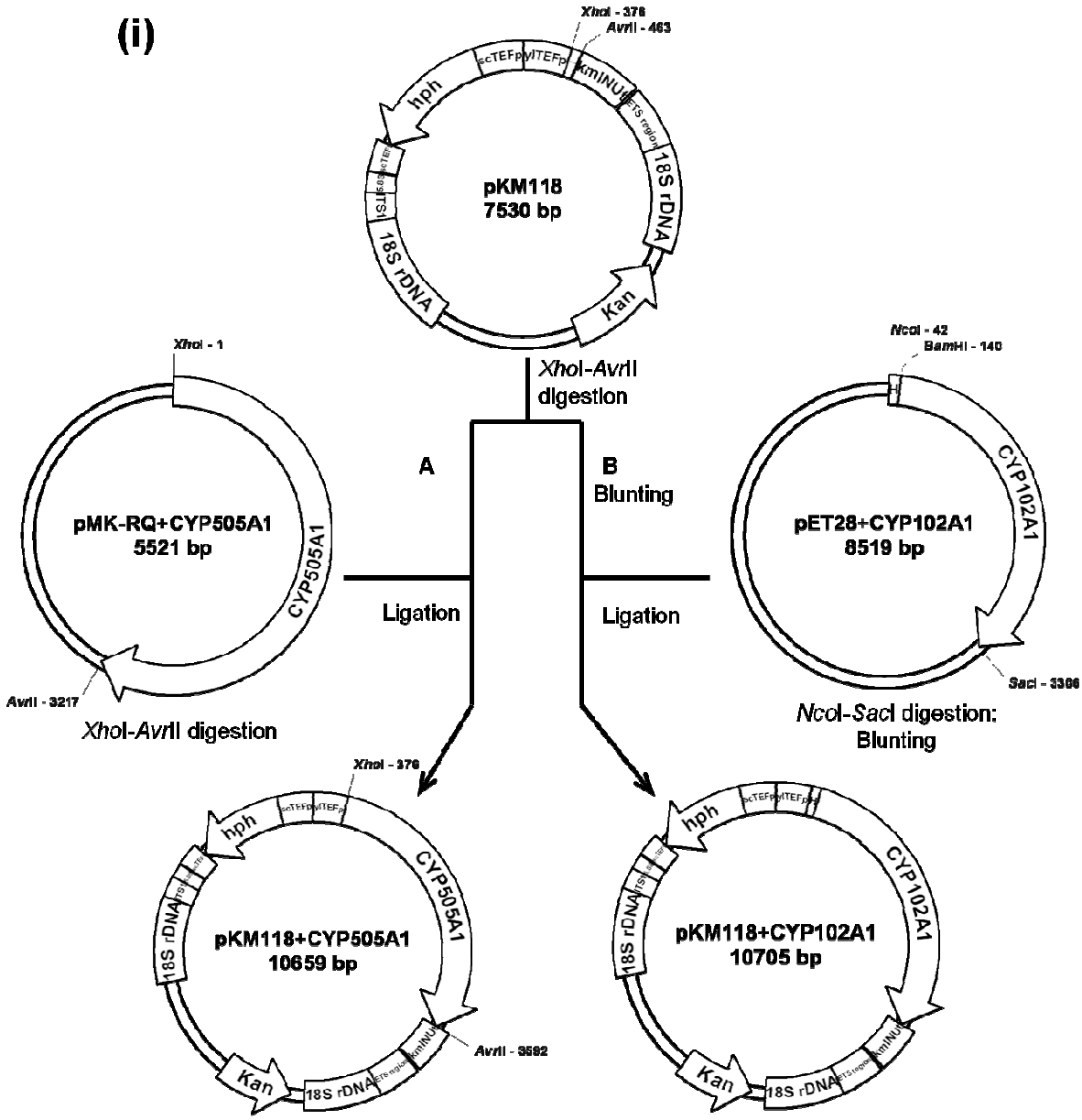



**Figure 3.5: pSMART cloning vectors containing CYP53B1 (A), CYP557A1 (B), YlCPR (C), RmCPR (D), and UmCPR (E).**

### 3.4.2. Expression vector construction

**Expression vectors for fused P450s**

pMK-RQ+CYP505A1 was digested with *Xho*I and *Avr*II, and the liberated CYP505A1 gene was ligated into pKM118 (broad-range vector) or pKM63 (*K. marxianus* specific vector) which was opened by digestion with the same enzymes (figure 3.6 A). pET28+CYP102A1 was digested with either *Nco*I and *Sac*I to keep the His-tag, or *Bam*HI and *Sac*I to exclude the His-tag. The liberated fragment was treated with T4 polymerase to generate blunt ends on both sides. The resultant blunt-ended fragment was ligated into pKM118 / pKM63 which was digested with *Xho*I and *Avr*II, treated with T4 polymerase for blunting, and then treated with Antarctic phosphatase to dephosphorylate the vector backbone to prevent self-ligation (figure 3.6 B). The restriction enzyme recognition sequences are destroyed during blunting.


(i)
pKM118
7530 bp
XhoI - 376
AvrII - 463
hph
ScTEFp
VITEFp
kmNUT
18S rDNA
Kan
pMK-RQ+CYP505A1
5521 bp
CYP505A1
XhoI - 1
AvrII - 3217
XhoI-AvrII digestion
XhoI-AvrII digestion
A
B
Blunting
Ligation
Ligation
pET28+CYP102A1
8519 bp
CYP102A1
NcoI - 42
BamHI - 140
SacI - 3366
NcoI-SacI digestion:
Blunting
pKM118+CYP505A1
10659 bp
AvrII - 3582
pKM118+CYP102A1
10705 bp

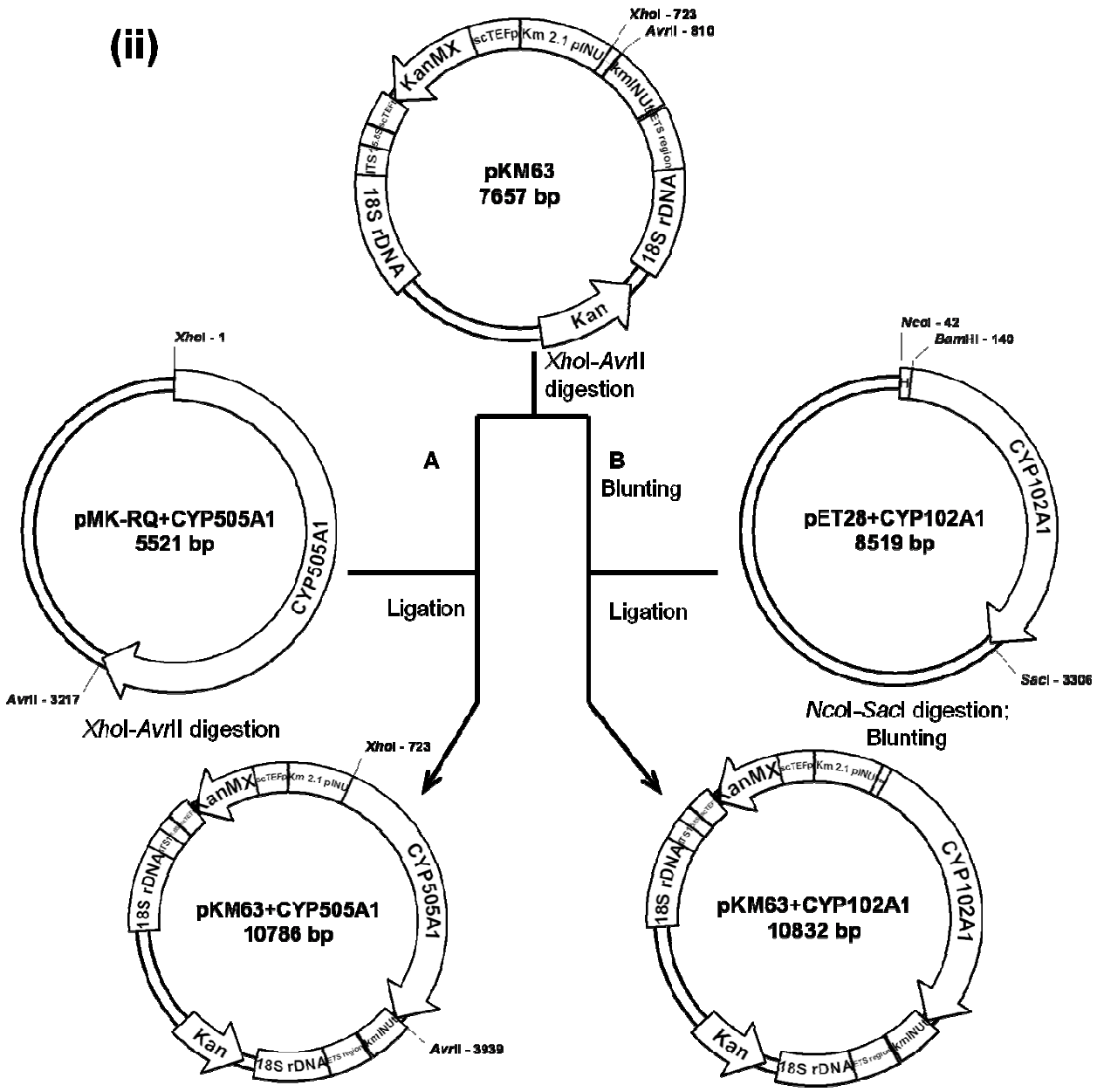
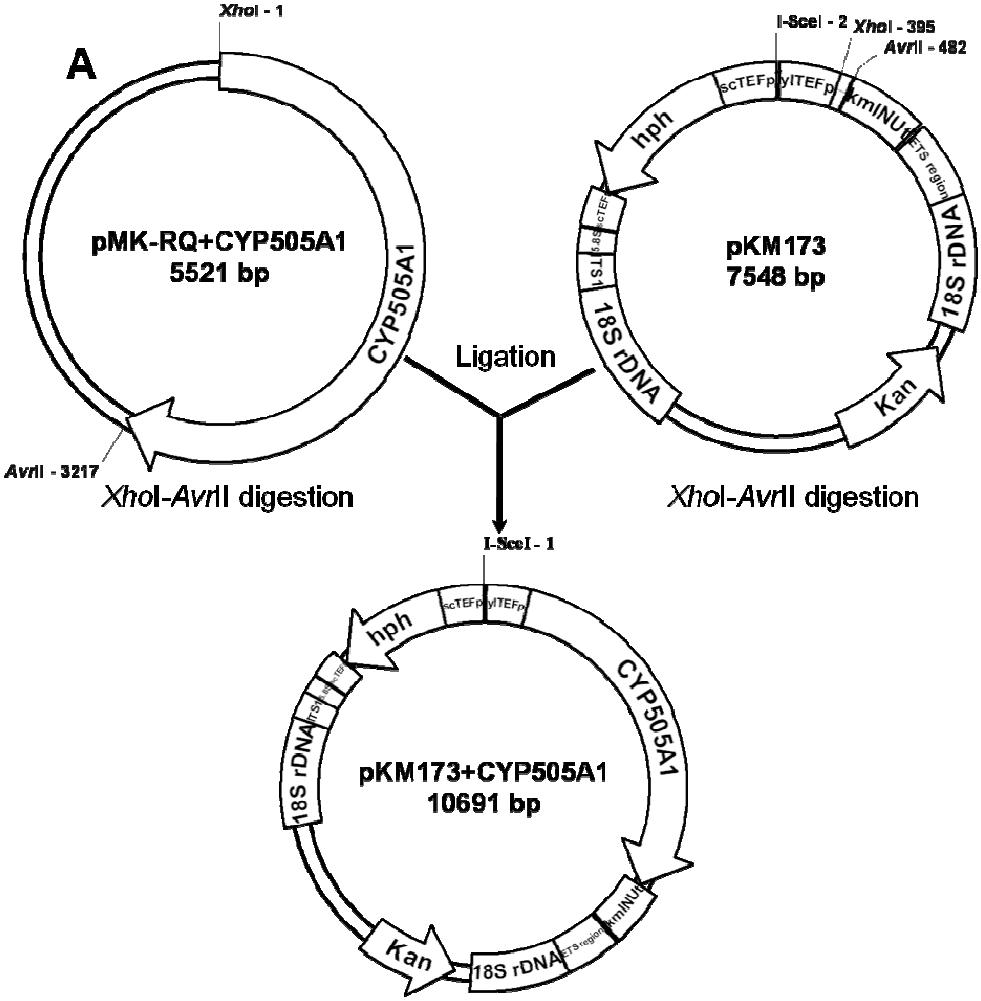
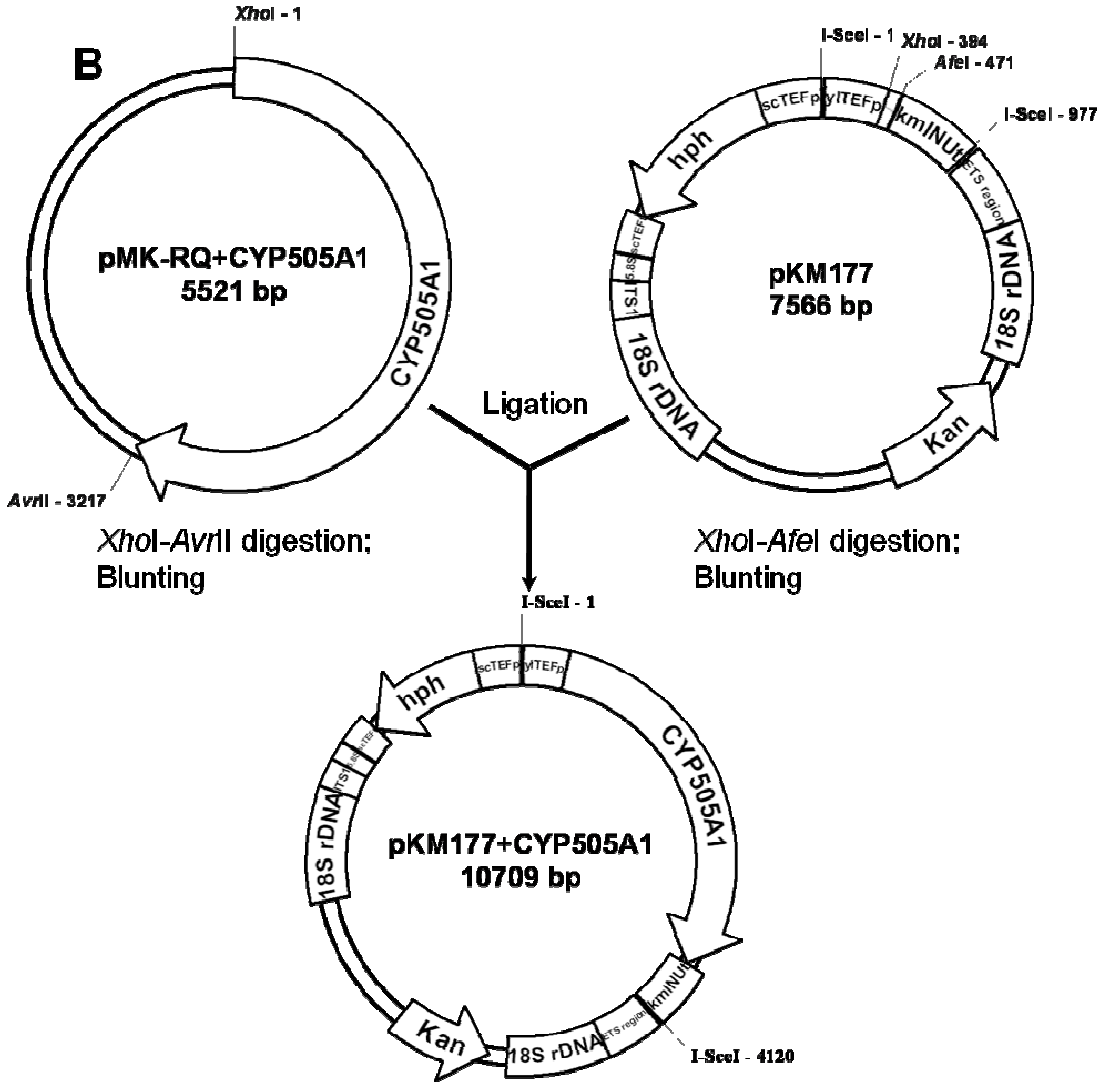



**Figure 3.6: Construction of expression vectors for CYP505A1 (path A) and CYP102A1 (path B) into pKM118 (i) and pKM63 (ii).** CYP102A1 can be cloned from pET28+CYP102A1 with or without the N-terminal His-tag depending on the restriction enzyme used, but only the His-tagged forms are included is depicted in this figure.

**Expression vector carrying double CYP505A1 cassettes**

pMK-RQ+CYP505A1 was digested with *Xho*I and *Avr*II, and the liberated CYP505A1 gene was ligated into pKM173 which was opened by digestion with the same enzymes; resulting in pKM173+CYP505A1 (figure 3.7 A). pMK-RQ+CYP505A1 was also digested with *Avr*II, followed by treatment with T4 polymerase. The blunted product was digested with *Xho*I, and the resultant product was ligated to pKM177 which had been digested with *Xho*I and *Afe*I (the latter produces a blunt-end); resulting in pKM177+CYP505A1 (figure 2.7B). The expression cassette containing CYP505A1 was removed from pKM177 by I-SceI digestion, and ligated into pKM173 which had been opened by digestion with I-SceI; resulting in pKM173+2xCYP505A1 (figure 3.7C).




B
XhoI - 1
pMK-RQ+CYP505A1
5521 bp
CYP505A1
AvrII - 3217
XhoI-AvrII digestion;
Blunting
I-SceI - 1
XhoI - 384
AfeI - 471
I-SceI - 977
hph
scTEFp
yTEFp
kmlNUT
18S rDNA
Kan
pKM177
7566 bp
Ligation
pKM177+CYP505A1
10709 bp
I-SceI - 4120

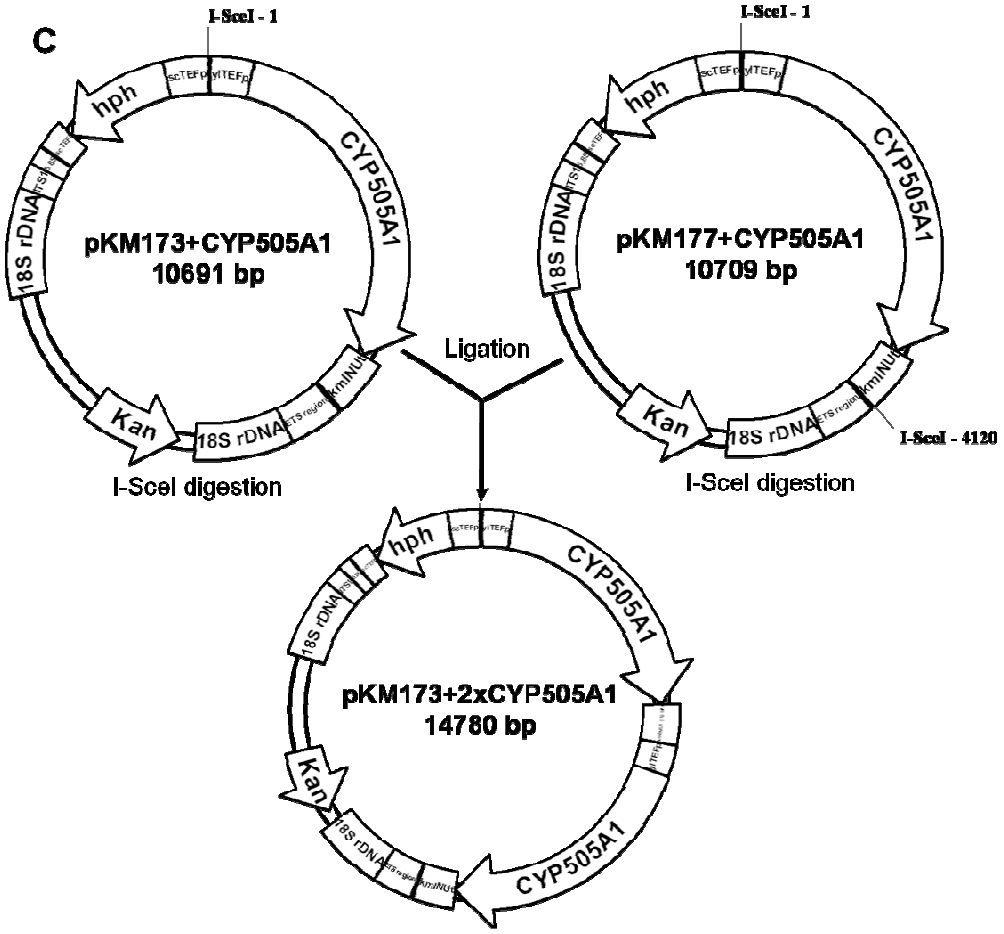



**Figure 3.7: Construction of expression vectors pKM173+CYP505A1 (A) and pKM177+CYP505A1 (B), as initial vectors for the ultimate construction of the double CYP505A1 expression cassette-containing pKM173+2xCYP505A1 (C).**

A N-terminally His-tagged version of CYP505A1 was PCR amplified from pET28b+CYP505A1 using HisFoxF1 and Fox505R1, and was similarly cloned into pKM173 and pKM177 to obtain a single and double CYP505A1 expression cassette.

**Expression vectors for non-fused class II P450s and CPRs**

CYP53B1 was liberated from pSMART+CYP53B1 by digestion with *Eco*RV and *Avr*II, followed by treatment with T4 polymerase to blunt the *Avr*II cohesive end (*Eco*RV digestion already results in a blunt end). CYP557A1 was released from

pSMART+CYP557A1 by digestion with *Eco*RV. The resulting blunt fragments were ligated (individually) to pKM177, which had been digested with *Xho*I and *Afe*I, prior to treatment with T4 polymerase (figure 3.8).

pKM173 was digested with *Xho*I, followed by treatment with T4 polymerase. The resultant blunted open vector was then digested with *Avr*II, resulting in one blunt-end and one *Avr*II-compatible cohesive end. YlCPR was released from pSMART+YlCPR using *Eco*RV and *Avr*II, while RmCPR was released from pSMART+RmCPR using *Eco*RV and *Nhe*I, which gives cohesive ends compatible with those generated by *Avr*II digestion. Both the YlCPR and RmCPR were ligated into the prepared pKM173 vector (figure 3.9).

UmCPR was removed from pSMART+UmCPR by digestion with *Sal*I and *Avr*II, and directly ligated to pKM173 which was opened by digestion with *Xho*I and *Avr*II, since *Sal*I digestion gives cohesive ends compatible with cohesive ends resulting from *Xho*I digestion (figure 3.9).

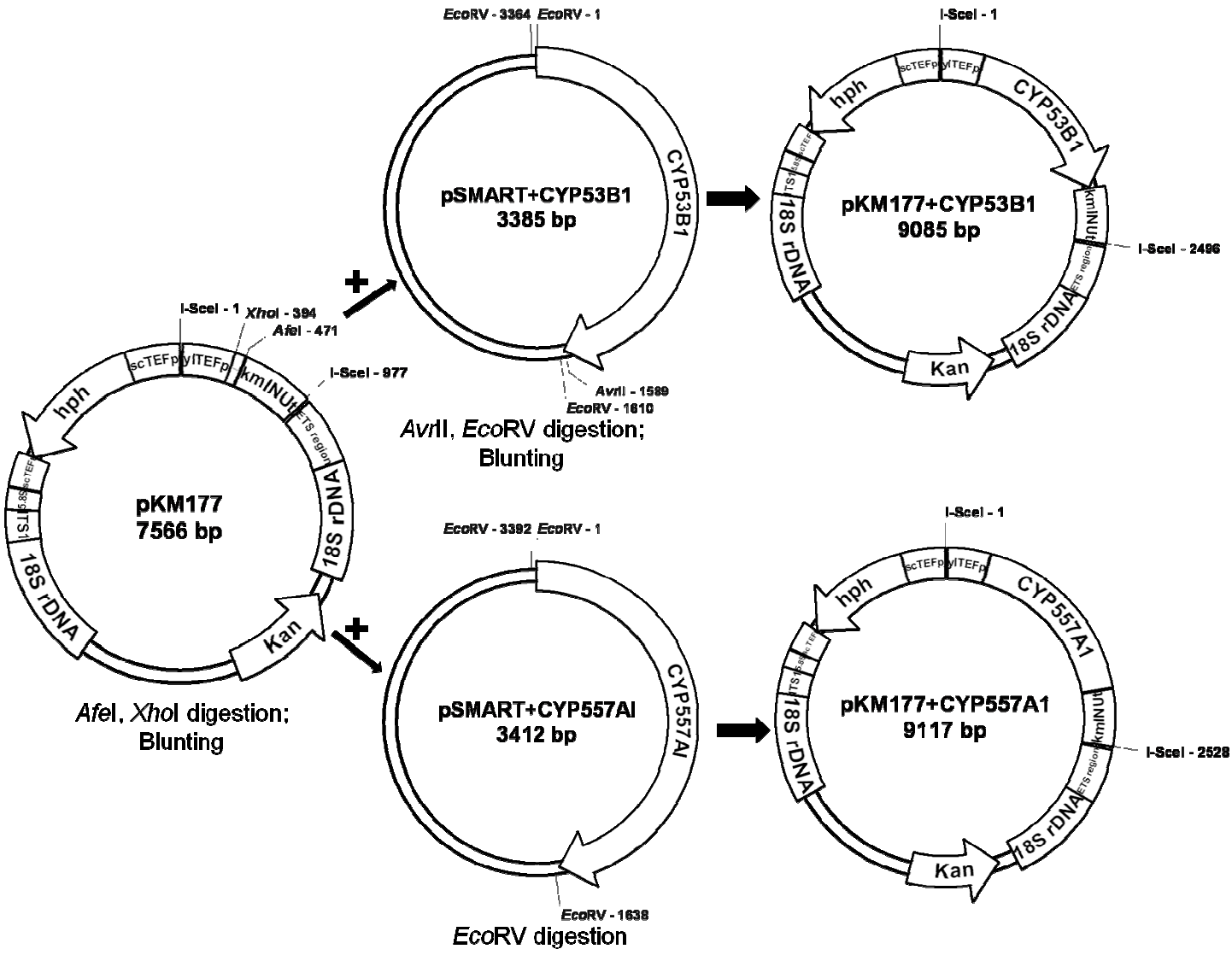



**Figure 3.8: Construction of expression vectors for non-fused P450s CYP53B1 and CYP557A1.**

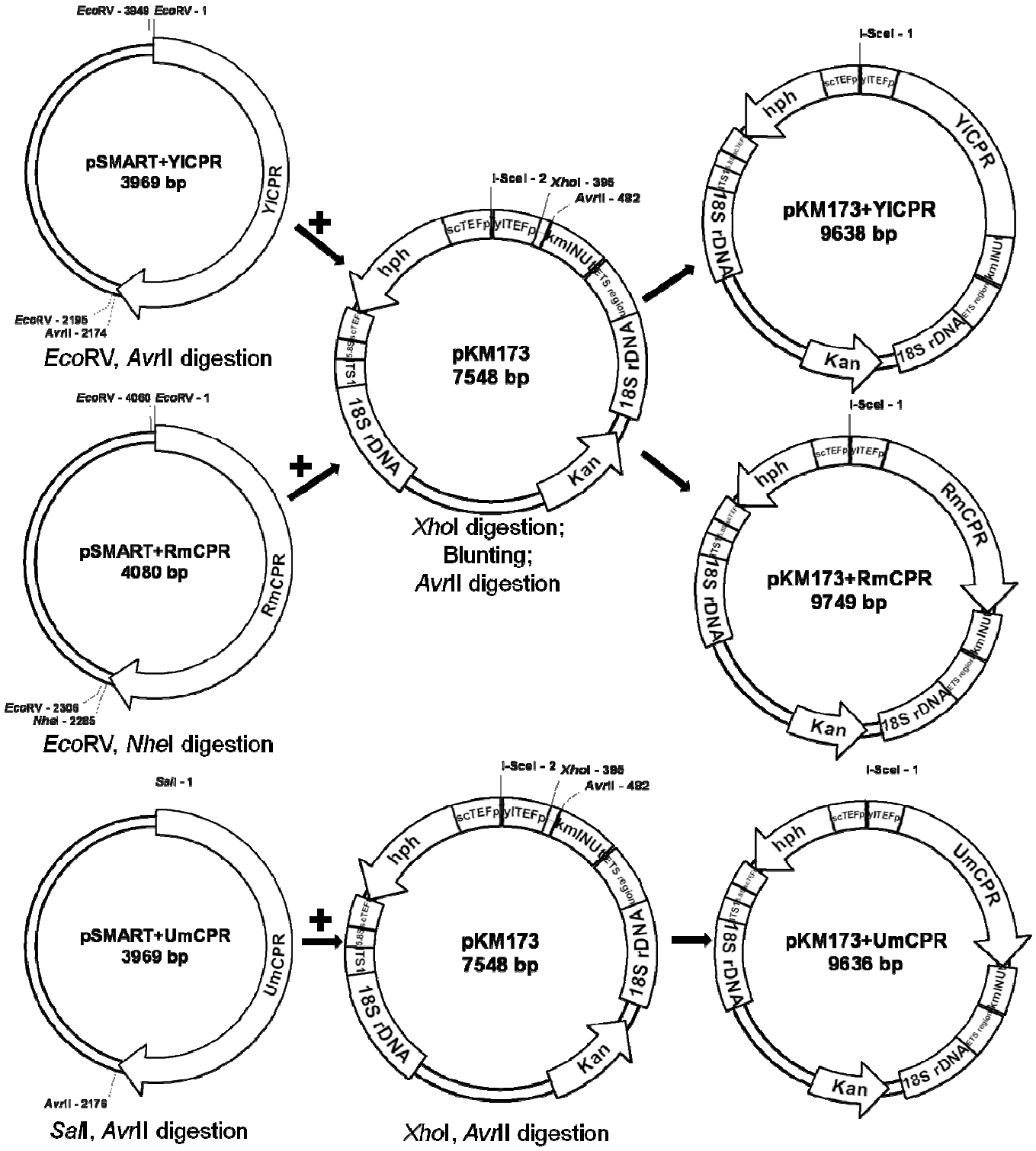
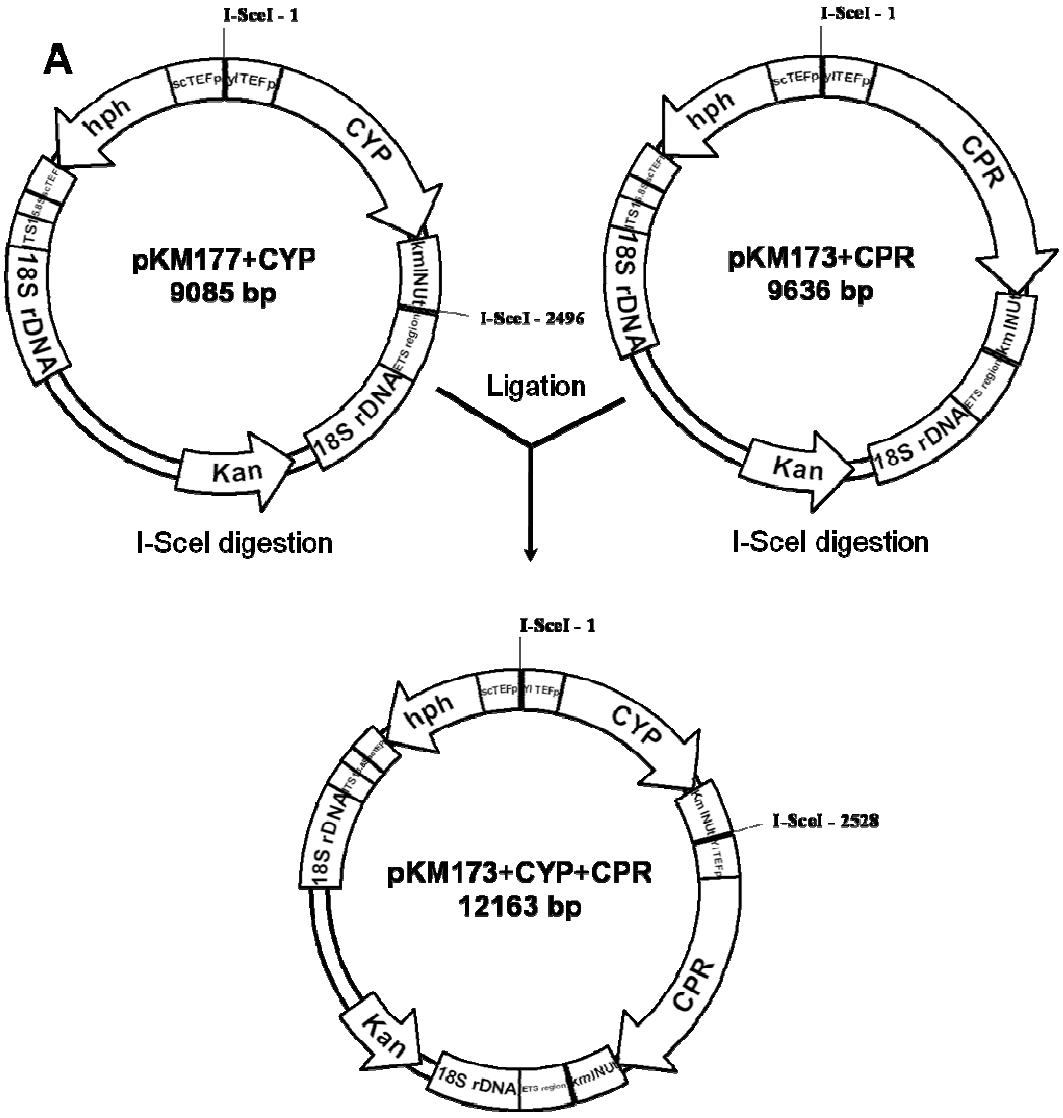



**Figure 3.9: Construction of expression vectors for YlCPR, RmCPR and UmCPR.**

**Vectors for coexpression of non-fused P450s and CPRs**

The expression cassettes for the CPRs (figure 3.9) contain one I-SceI site, and serve as backbone vectors for insertion of different P450s. The expression cassettes containing CYP53B1 and CYP557A1 were removed from pKM177+CYP53B1 and CYP557A1 respectively, by digestion with I-SceI. The resulting fragments were individually ligated into pKM173+YlCPR, pKM173+RmCPR and pKM173+UmCPR vectors, which had been opened by digestion with I-SceI (figure 3.10A).



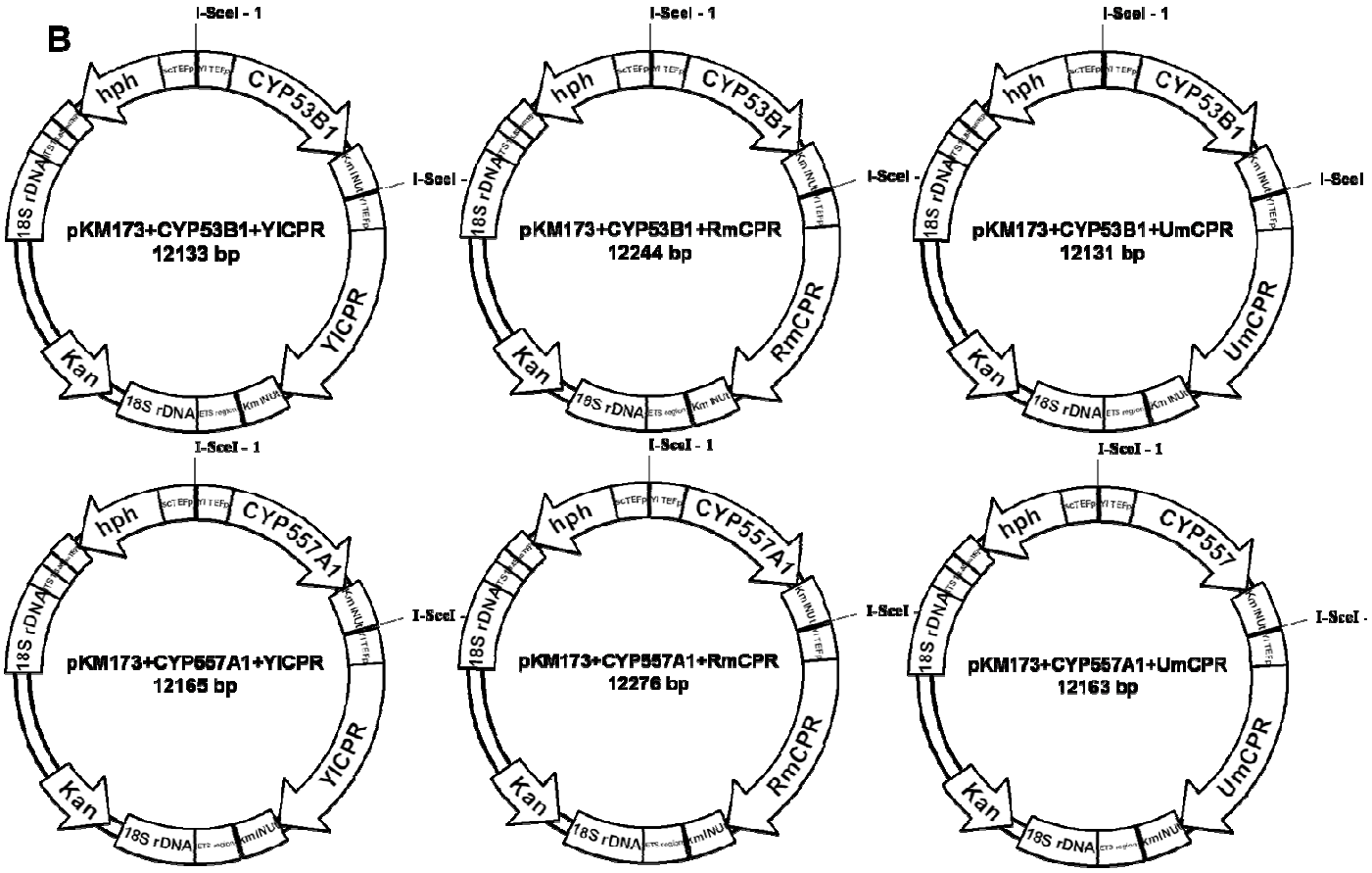



**Figure 3.10: Example of the strategy for construction of vectors for co-expression of P450s and CPRs (A), and the final co-expression constructs obtained (B).**

### 3.5. Transformation of yeast strains

Prior to yeast transformation vectors were digested with *Not*I to separate the yeast integration portion from the bacterial fragment.

#### *Kluyveromyces marxianus*

*Kluyveromyces marxianus* was transformed according to a modified version of the method described by Chen *et al* (1997). Cells (800 µl) from a *K. marxianus* culture in exponential phase were harvested at 13 200 x *g* for 1 minute. *Not*I digested vector (~1 µg) was added to the pellet, before resuspension in 100 µl one step buffer (OSB) (250 ng / µl salmon sperm carrier DNA; 100 mM lithium acetate ; 45% v/v polyethylene glycol

(PEG) 4000; 100 mM dithiothreitol (DTT)). The resultant resuspension was incubated at 42$^{o}$C for 1 hour, before addition of 900 µl of YPD, followed by recovery for 2 hours at 37$^{o}$C. After harvesting at 5200 x *g* for 5 minutes, approximately 900 µl of supernatant was removed and the pellet was resuspended in the remaining supernatant and spread onto selective YPD plates containing either 75 mg/L G418 / geneticin or 400 mg/L hygromycin B. The plates were incubated at 37$^{o}$C until colonies formed.

**Other yeast strains**

Yeast strains were transformed using a modified version of the method described by Lin-Cereghino *et al* (2005). An overnight grown yeast culture was used to inoculate 50 ml YPD to an $A_{600nm}$ of 0.15 – 0.2. The resultant culture was incubated until an $A_{600nm}$ of 0.8 – 1 was reached, at which point the cells were harvested at 500 g for 5 minutes. The pellet was resuspended in 10 ml BEDS solution (10 mM bicine-NaOH, pH8.3; 3% v/v ethylene glycol; 5% v/v DMSO (dimethyl sulfoxide); 1M sorbitol) supplemented with 100 mM DTT. The cell suspension was incubated for 5 minutes at 30$^{o}$C, before harvesting at 500 g for 5 minutes. The pellet was resuspended in 1 ml BEDS and aliquoted in 100 µl amounts for transformation.

*Not*I digested DNA (~1 µg) was added to the competent cells along with 50 µg of carrier DNA, before 1.4 ml of solution 2 (40% v/v PEG 1000; 200 mM bicine-NaOH, pH8.4) was added. The mixture was vortexed for 1 minute prior to incubation at 30$^{o}$C for 1 hour. Cells were then heat shocked at 37$^{o}$C for 10 minutes, prior to harvesting at 5200 rpm for 5 minutes. The pellet was resuspended in 500 µl solution 3 (150 mM NaCl; 10 mM bicine-NaOH, pH8.4) and 500 µl YPD; followed by recovery at 30$^{o}$C for 2 hours. After harvesting at 5200 g for 5 minutes, approximately 800 µl supernatant was removed and the pellet was resuspended in the remaining supernatant. The resuspension was streaked on selective YPD plates containing 400 mg/L hygromycin B until colonies formed.

### 3.6. Confirmation of genomic integration of heterologous cytochrome P450 genes

Selected transformants were subjected to three rounds of re-streaking on selective plates, prior to culturing for genomic DNA extraction. Genomic DNA was isolated from each transformant according to the method described by Labuschagne and Albertyn (2007). Genomic DNA was used as a template for PCR using EconoTaq (settings described table 3.3) using primers for the genes themselves, or combinations of the promoter forward primer and specific gene reverse primer (primers listed in table 3.1).

Transformants testing positive for genomic integration were cultivated until mid-exponential phase before being stored with glycerol (15% v/v final concentration) at -80$^{o}$C until required for biotransformation studies.

## Part B: Biotransformations using recombinant yeasts

### 3.7. General chemicals

General chemicals were, unless otherwise specified, obtained from Fluka, Merck, or Sigma-Aldrich. Hygromycin B was supplied by (HyClone).

### 3.8. Strains

The preparation of all transformants used for activity assays was described above in section A of this chapter.

### 3.9. Cultivation media and conditions for yeasts

YPD broth contained, per litre of distilled water, 10 g yeast extract, 20 g peptone and 20 g glucose. For selective YPD agar plates the media was supplemented with 20 g agar and 400 mg hygromycin B per litre distilled water. For selection of G418 resistant *K.*

*marxianus* transformants YPD agar plates containing 75 mg G418 per litre distilled water were used. *K. marxianus* transformants carrying genes under the control of the inulinase promoter were grown in YPS broth, containing, per litre of distilled water, 10 g yeast extract, 20 g peptone and 20 g sucrose.

Cultures were generally grown at 28$^{o}$C on plates or in Erlenmeyer flasks on a rotary shaker with a speed of 130 rpm. The cultivation broth occupied 10% of the maximum volumetric capacity of the flask to facilitate oxygen transfer. Initial screening was performed using 5 ml YPD in 25 ml test tubes.

### 3.10. General biotransformation procedures using yeast transformants

#### 3.10.1. General biotransformation procedure using growing cells

Selected yeast transformants (section A) were obtained from storage at -80$^{o}$C and revived by streaking on selective YPD agar plates supplemented with hygromycin B (400 mg.L$^{-1}$). Cells were transferred from overnight growth on selective plates to YPD broth (5 ml in 25 ml test tubes or 25 ml in 250 ml flasks, unless specified otherwise), and incubated at 28$^{o}$C on a rotary shaker (130 rpm). Cells were grown for 48h to stationary phase before addition of substrates, which were dissolved in DMSO as a co-solvent (1% v/v final concentration DMSO in cultivation media). Final substrate concentrations were generally 5 mM unless otherwise stated. After substrate addition cells were re-incubated at 28$^{o}$C and samples were taken regularly.

Various approaches were followed to increase substrate concentration. In one approach, higher substrate concentrations were added to 48h old cultures. In another approach, 5 mM substrate was added to 48h old cultures, and re-added at 24h intervals in 5 mM or 2.5 mM concentrations, until a total combined addition of 15 mM was reached. In another approach, 5 mM substrate was added to 48h old cultures, and after re-incubation for 24h, cells were harvested at 4000 g for 10 min, washed and

resuspended in 200 mM phosphate buffer or 0.9% NaCl solution, and 5 mM substrate was re-added to the resuspension.

Hexylbenzene and nonylbenzene were added to final concentrations of 20 mM, while R-(+)-Limonene and *p*-Cymene were added to final concentrations of 30 mM. These substrates were added together with DMSO as a co-solvent.

### 3.10.2. General biotransformation procedure using growing cells in buffered YPD broth

Cells were cultured as described in the previous section, but 100 mM or 200 mM potassium phosphate buffer was added to the YPD either at inoculation or at substrate addition. The pH values of the cultures were monitored using universal pH indicator strips (Merck).

### 3.10.3. General biotransformation procedure using resting cells from YPD broth

Cells were transferred from overnight growth on selective plates to YPD broth (50 ml in 500 ml flasks), and incubated at 28$^{o}$C on a rotary shaker (130 rpm). Cells were grown for 48h to stationary phase before being harvested at 4000 g for 10 minutes. Cells were washed with 50 mM potassium phosphate buffer (pH8), before being resuspended in a resuspension buffer (200 mM potassium phosphate buffer, pH8; 16% v/v glycerol, 100 µg.ml$^{-1}$$FeSO_4.7H_2O$) at a concentration of approximately 333 $g_{WCW}.L^{-1}$. 2ml reaction mixtures were set up in 40ml amber bottles, containing 200 mM potassium phosphate buffer (pH8), 36 mM glucose, 5mM substrate, 1% v/v DMSO, 8% v/v glycerol, 50 µg.ml$^{-1}$$FeSO_4.7H_2O$, and cell suspension of 41.8 $g_{DCW}.L^{-1}$ (*A. adeninivorans*) or 33.5 $g_{DCW}.L^{-1}$ (other species). Reaction mixtures were incubated for 24h on a rotary shaker at 28$^{o}$C and 130 rpm.

### 3.10.4. General biotransformation procedure using resting cells from a chemically defined medium

Cells were transferred from overnight growth on selective plates to YPD broth (25 ml in 250 ml flasks), and incubated at 28$^{o}$C on a rotary shaker (130 rpm). Cultures were grown for 24h before they were used as inoculums (10% v/v) for a chemically defined medium, consisting of 20 g.L$^{-1}$ glucose, 10 g.L$^{-1}$ $(NH_4)_2$.SO4, 0.8 g.L$^{-1}$ $MgSO_4.7H_2O$, 0.1 g.L$^{-1}$ $NaSO_4$, 0.4 g.L$^{-1}$ $CaCl_2.2H_2O$, 2.7 mg.L$^{-1}$ KI, 0.27 g.L$^{-1}$ $(NH_4)_2FeSO_4.6H_2O$, 0.11 g.L$^{-1}$ $MnSO_4.H_2O$, 0.53 mg.L$^{-1}$ $NiCl.6H_2O$, 11 mg.L$^{-1}$ $CuSO_4.5H_2O$, 0.08 g.L$^{-1}$ $ZnSO_4.7H_2O$, 2.7mg.L$^{-1}$ $NiSO_4.6H_2O$, 2.7mg.L$^{-1}$ $CoCl_4.6H_2O$, 2.7mg.L$^{-1}$ $Na2Mo_2.2H_2O$, 2.7 mg.L$^{-1}$ boric acid, 0.8 mg.L$^{-1}$ D(+)-Biotin, 0.53 g.L$^{-1}$ Thiamine-HCl, and 200 mM potassium phosphate buffer (pH8); (modified from Knoll et al., 2007).

Cells were cultured for a further 24h in the chemically defined medium, before being harvested at 4000 g for 10 minutes. Cells were washed with 50mM potassium phosphate buffer (pH8), before being resuspended in a resuspension buffer (200 mM potassium phosphate buffer, pH8; 16% v/v glycerol, 100 µg.ml$^{-1}$ $FeSO_4.7H_2O$) at a concentration of approximately 333 $g_{WCW}.L^{-1}$. Reaction mixtures were set up in 40ml amber bottles, containing 200 mM potassium phosphate buffer (pH8), 36 mM glucose, 5mM substrate, 1% v/v DMSO, 8% v/v glycerol, 50 µg.ml$^{-1}$$FeSO_4.7H_2O$, and cell suspension of 41.8 $g_{DCW}.L^{-1}$ (*A. adeninivorans*) or 33.5 $g_{DCW}.L^{-1}$ (other species). 2 ml reaction mixtures were incubated in 40 ml amber bottles for 24h on a rotary shaker at 28$^{o}$C and 130 rpm.

### 3.11. Expression of CYP505A1 in *E. coli* BL21 (DE3)

*E. coli* BL21 cells carrying pET28(b) were used to inoculate 5 ml LB medium (see section 3.2.2) supplemented with 30 µg / ml kanamycin and grown overnight at 30°C. 1 ml of this pre-culture was used to inoculate main cultures of 50 ml LB supplemented with 30 µg / ml kanamycin in a 250 ml shake flask and incubated at 37°C to an optical

density (OD) reading of 0.8. At that point, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1 mM 5-aminolevulinic acid (5-ALA) was added, followed by incubation at 20°C until 24 h after the inoculation of main culture (approximately 22 h post-induction). Cells were harvested for 10 min at 4000 *g* and resuspended to a final biomass of approximately 333 $g_{WCW}$ / L and reactions were set up as in section 3.10.3 with a final biomass concentration of approximately 167 $g_{WCW}$ / L and final substrate (HBA) concentration of 0.5 mM.

## 3.12. Investigation of sub-cellular localization of CYP505A1 in *Arxula adeninivorans*

Transformants of *A. adeninivorans* expressing CYP505A1 were cultivated as described for biotransformation using harvested cells from chemically defined medium (section 3.1.8). After harvesting the cells the pellet was washed with 10 mM Tris buffer (pH7.5) containing 0.65 M sorbitol, 0.1 mM DTT and 0.1 mM EDTA. The cells were then resuspended in a lysis buffer consisting of 10 mM Tris buffer (pH7.5), 2 M sorbitol, 0.1 mM DTT, 1 mM EDTA and 0.25 mM PMSF.

The resuspended cells were disrupted using three passages through the Constant Systems Cell Disrupter using 15 kPsi. The lysate was centrifuged for 10 minutes at 4000 g. The resultant pellet represented cellular debris and incompletely lysed cells. The supernatant was then ultracentrifuged at 12000 g for 30 minutes. The resultant pellet fraction represented the mitochondrial fraction, and the supernatant was further ultracentrifuged at 87000 g for 2 hours. The resultant supernatant represented the soluble fraction, with the membrane representing the microsomal fraction. Pellets were resuspended in a resuspension buffer consisting of 100 mM Tris buffer (pH7.5), 20% v/v glycerol and 0.1 mM DTT.

All fractions were used for activity assays in amber bottles, with the reaction mixtures containing 50 mM Tris buffer (pH7.5), 1 mM NADPH, 250 µM hexylbenzoic acid (HBA),

6U glucose 6-phosphate dehydrogenase, 8 mM glucose 6-phosphate, and 50% v/v relevant fraction. 2ml reaction mixtures were incubated in 40 ml amber bottles for 24h on a rotary shaker at 28$^{o}$C and 130 rpm.

### 3.13. Testing the effect of 5-aminolevulinic acid addition on P450 activity

Cells were cultured in YPD or chemically defined medium (sections 3.10 – 3.13), but with the addition of 0.5 mM 5-aminolevulinic acid (5-ALA) from the point of inoculation. Further cultivation, substrate addition and sampling times were the same as in sections 3.10 – 3.13.

### 3.14. Testing the effect of 1, 10-phenanthroline addition on P450 activity

Cells were cultured in YPD medium as described in the sections above, but with the addition of 1 mM 1, 10-phenanthroline 1 hr prior to substrate addition. Further cultivation after substrate addition and sampling times were the same as in section 3.10.

### 3.15. Sample extraction and analysis

After substrate addition, samples (500 µl) were taken at regular intervals and acidified using hydrochloric acid (5M) to a pH below 3. Ethyl acetate (300 µl) containing myristic acid (0.5 mM), as an internal standard, was added to the samples. After thorough vortexing, samples were centrifuged for 10 minutes at 14000 rpm. The upper organic layer was collected, before the process was repeated. The collected fractions were pooled for concentration using an Eppendorf Concentrator Plus, before further analysis.

For the assays carried out in amber vials using resting cells, the entire reaction mixture volume (2ml) was extracted using proportionate amounts of ethyl acetate containing myristic acid (0.5 mM) as an internal standard.

Analysis was done using thin layer chromatography (TLC) and / or gas chromatography (GC). For TLC aliquots (5-10 µl) of organic extracts of samples and standards were spotted on Alugram® silica gel $F_{254}$ TLC plates (Merck) and the plates were developed using a mobile phase containing di-n-butyl ether: formic acid: distilled water (90:7:3 v/v/v). The plates were dried and viewed under UV light. Aromatic compounds appeared as UV absorbing spots. TLC plates were analyzed using a BioRad Gel Doc EQ system with Quantity One® version 4.5 software.

Organic acids were methylated prior to GC analysis using equal volumes of a trimethylsulfonium hydroxide (TMSH) preparation (Obiero, 2006). GC analyses were done on samples (1µl) using a Hewlett-Packard 5890 series II gas chromatograph equipped with a 30 M x 0.53mm Chrompack® CP wax 52 CB column, with carrier gas, $H_2$, at 5ml/min., split ratio of 1:40, inlet temperature at 200$^{o}$C, initial temperature at 120$^{o}$C for 5 min, increased at 10$^{o}$C/min to 250$^{o}$C for 30 minutes. Flame ionisation detector (FID) temperature was at 300$^{o}$C. For GC-MS analysis of products, methylation was coupled to silylation of samples. Samples were dried in a vacuum rotor, before being resuspended in TMSH (200µl), and incubated for one hour at room temperature. The samples were again dried, before resuspension in (BTSFA) (100µl) and pyridine (100µl), and incubated at room temperature for 40 minutes. Samples were again dried, before being resuspended in ethyl acetate and injected on the GC-MS (Gudiminchi and Smit, 2011).

### 3.16. Biomass and pH determination

During cultivation, 2 ml samples were collected in pre-weighed tubes for biomass determination. Cells were harvested at 13 000 x *g* for 5 minutes and washed with 0.9% w/v NaCl solution, before wet cell weight (wcw) determination. Tubes were then baked at 100$^{o}$C for 24h before dry cell weight (dcw) determination.

pH values of the cultures were monitored using universal pH indicator strips (Merck).

### 3.17. CO-difference spectrum analysis of in *K. marxianus* transformants expressing CYP102A1

Cells were cultivated as described for biotransformations using resting cells from YPD broth (section 3.12). The cell pellets were resuspended to a concentration of approximately 333 $g_{WCW}.L^{-1}$ (corresponding to approximately 67 $g_{DCW}.L^{-1}$), in 50 mM potassium phosphate buffer (pH8). F8 Maxisorp Nunc-Immuno™ Modules (Nunc A/s, Denmark) were used for CO-spectrum determination. Duplicate samples of 200 µL were added into wells of two different microtiter strips, and one strip was saturated with carbon monoxide by exposure to carbon monoxide flow for 5 min. The samples of both microtiter strips were reduced by adding a few grains of sodium dithionite, followed by mixing for 10 s. The spectrum between 400 nm and 500 nm was recorded using a Spectramax M2 Microtiter Plate Reader (Molecular Devices Corporation, USA) with the non-carbon monoxide saturated well serving as the reference, until a stable A450–490 difference was obtained.

# Chapter 4: Results and discussion

## Part A: Molecular techniques for preparation of recombinant yeasts

### 4.1. Cloning of CYP505A1 from *Fusarium oxysporum* MRC3239

The initial intention was to clone the gene encoding wild-type CYP505A1 from a strain of *Fusarium oxysporum* which was available to us. Following expression of the gene, activities could be compared to published data on this enzyme (Kitazume *et al*, 2000). Due to the presence of introns, the cDNA of the gene would be synthesised by reverse transcription of mRNA.

Total RNA was extracted from *Fusarium oxysporum* strain MRC3239 according to the TRIzol method described by Labuschagne and Albertyn (2007) and the supplier's recommendations. The isolated RNA was visualised on a 1% denaturing agarose gel containing 4% formaldehyde (figure 4.1).

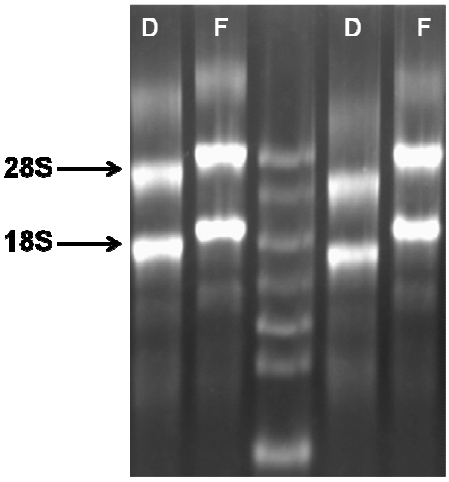



**Figure 4.1: Extracted RNA resuspended in DEPC-treated water (D) and formamide (F), and visualized on a 1% denaturing agarose gel containing formaldehyde and ethidium bromide.** The 28S and 18S ribosomal RNA subunits are indicated.

Reverse transcription was then carried out using Expand reverse transcriptase (Roche) to obtain the corresponding cDNA, which was then further amplified using Kapa-HiFi polymerase according to the PCR program settings listed in table 3.3 (figure 4.2). Genomic DNA (gDNA) was also extracted from *F. oxysporum* according to the method described by Labuschagne and Albertyn (2007); and the genomic DNA copy of the CYP505A1 gene was amplified using Kapa-HiFi polymerase and the same PCR settings as for the cDNA (figure 4.2).

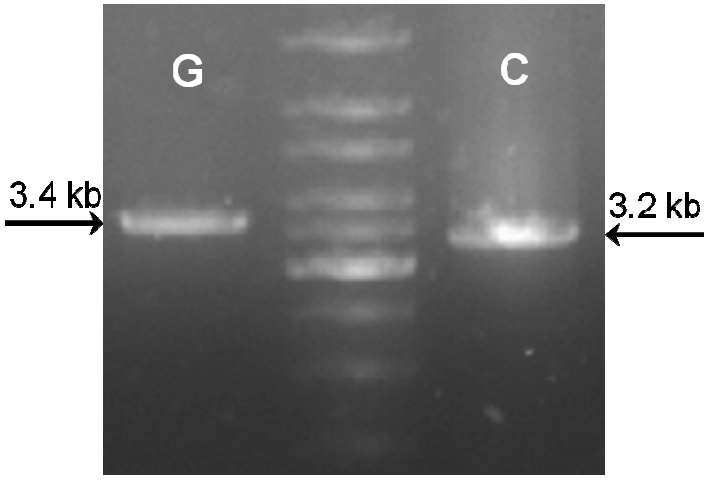



**Figure 4.2: Genomic (G) and complementary (C) DNA versions of the CYP505A1 gene.** The two forms are not clearly distinguishable from each other using regular agarose gel electrophoresis due to the small size difference of approximately 200 base pairs (approximate band sizes indicated on either side of the gel).

Since Kapa HiFi polymerase produces blunt-ended amplicons, both forms were ligated into the pSMART (Lucigen) cloning vector after being phosphorylated by polynucleotide kinase (PNK) (Fermentas). After plasmid propagation in *E. coli* cells and subsequent purification, the inserts within the pSMART vectors were subjected to sequencing.

The resultant sequences were compared to reference sequences from the NCBI database (Accession No. AB030037.1 at http://www.ncbi.nlm.nih.gov/) and the Broad Institute database (Accession No. FOXG_0.4152.2 at http://www.broadinstitute.org/). Sequence analysis of two cDNA and gDNA clones revealed differences compared to

both references, although the sequences were closer to the references from the Broad Institute. The MRC3239 sequence shared 99% identity with the sequence from the Broad Institute database, and 97% identity with the sequence from the NCBI database, which was therefore also 97% identical to the sequence from the Broad Institute database. Several of the differences occurred in intronic regions and would hence not necessarily affect the amino acid sequence.

The most important differences in the coding sequence were deletions of an adenine residue at position number 7 and cytosine residues at positions 2504 and 2506 in the MRC3239 DNA sequence. Although the net effect of the deletions resulted in a polynucleotide which was still a multiple of 3, the sequence alterations lead to a premature stop codon at nucleotide residues 88-90, leading to the formation of a peptide of only 29 amino acids.

For further investigation of the other differences, these deletions were corrected *in silico* and the rest of the coding sequence was virtually translated. The resultant translated sequence revealed a full-length protein comparable to the reference protein sequences, with 99% identity to the reference sequences, which shared 98% identity with each other. The differences between the 3 sequences are listed in table 4.1.

**Table 4.1: Differences between amino acid sequences of MRC3239 and references**

| **MRC3239** | **Broad Institute** | **NCBI** |
|---|---|---|
| A142 | A142 | T142 |
| N164 | N164 | Y164 |
| D287 | D287 | E287 |
| V367 | A367 | V367 |
| S490 | S490 | L490 |
| T492 | T492 | A492 |
| A494 | T494 | A494 |
| G496 | G496 | G missing |

| | | |
|---|---|---|
| N575 | N575 | D574 |
| E579 | E579 | D578 |
| T640 | T640 | I639 |
| A790 | A790 | D789 |
| F925 | F925 | L924 |

The translated MRC3239 sequence is again more similar to the reference sequence from the Broad Institute, except for 2 instances namely a valine residue instead of an alanine at position 367 and an alanine instead of a threonine at position 494. These two differences are also shared with the NCBI sequence.

Although the cDNA sequence could be corrected by site-specific mutagenic PCR, the two rounds of PCR required increased the risk of further mutagenesis to the sequence, especially considering the length of the sequences. To circumvent these problems, the gene was artificially synthesised by GeneArt according to the NCBI reference sequence.

### 4.2. Transformation of yeast strains

#### 4.2.1. Vectors and yeast strains

Three broad-range vectors, pKM118, pKM173 and pKM177, were constructed in our group to facilitate heterologous expression in multiple yeasts (Albertyn *et al*, 2011). The most important features of these vectors are rDNA regions for integration into host chromosomal DNA by homologous recombination; a gene conferring hygromycin resistance as a selection marker; and a translation elongation factor (TEF) promoter to drive constitutive expression.

Three variations of the broad range vector for expression in various yeasts are available, namely pKM118, pKM173 and pKM177. pKM173 and pKM177 are derivatives

of pKM118 containing one and two I-SceI sites respectively. The self-sufficient P450s CYP102A1 and CYP505A1 were cloned into pKM118. To obtain two tandem expression cassettes containing CYP505A1, pKM173 and pKM177 were used. Class II P450s were cloned into pKM177 while P450 reductases (CPRs) were cloned into pKM173. To obtain vectors for the coexpression of P450s and CPRs expression cassettes from pKM177 were ligated into linearized pKM173 vectors which already contained CPRs. pKM173 and pKM177 were also used to obtain two tandem expression cassettes containing CYP505A1.

Additionally, there is a vector (pKM63) which was more suitably designed for *K. marxianus*, which carries a sucrose-inducible *K. marxianus* inulinase (INU) promoter. CYP102A1, CYP505A1, and a combination of CYP53B1 and YlCPR were also cloned into pKM63.

The expression vectors constructed (described in more detail in section 3.4) for comparison of reporter P450 activities in different yeasts are listed in table 4.2.

**Table 4.2: Expression vectors constructed during this study.**

| Starting vector | Genes inserted | Final construct name | Applicable yeast |
|---|---|---|---|
| pKM118 | None | pKM118 | All |
| | CYP102A1 | pKM118+CYP102A1 | |
| | CYP505A1 | pKM118+CYP505A1 | |
| pKM177 | CYP53B1 | pKM177+CYP53B1 | |
| | CYP557A1 | pKM177+CYP557A1 | |
| | CYP505A1 | pKM177+CYP505A1 | |
| pKM173 | None | pKM173 | |
| | UmCPR | pKM173+UmCPR | |
| | RmCPR | pKM173+RmCPR | |
| | YlCPR | pKM173+YlCPR | |
| | CYP505A1 | pKM173+CYP505A1 | |

| | | | |
|---|---|---|---|
| pKM173+UmCPR | CYP53B1 | pKM173+CYP53B1+UmCPR | |
| | CYP557A1 | pKM173+CYP557A1+UmCPR | |
| pKM173+RmCPR | CYP53B1 | pKM173+CYP53B1+RmCPR | |
| | CYP557A1 | pKM173+CYP557A1+RmCPR | |
| pKM173+YlCPR | CYP53B1 | pKM173+CYP53B1+YlCPR | |
| | CYP557A1 | pKM173+CYP557A1+YlCPR | |
| pKM173+CYP505A1 | CYP505A1 | pKM173+2xCYP505A1 | |
| pKM63 | None | pKM63 | *K. marxianus* |
| | CYP102A1 | pKM63+CYP102A1 | |
| | CYP505A1 | pKM63+CYP505A1 | |
| | CYP53B1+YlCPR | pKM89 | |

YlCPR – CPR from *Y. lipolytica*; RmCPR – CPR from *R. minuta*; UmCPR – CPR from *U. maydis*

The yeast strains used are listed in table 3.2. The strains are all wild-type isolates except for the *S. cerevisiae* W3031A(a), a commonly used laboratory strain; and the two *Y. lipolytica* strains. The CTY003 strain is derived from *Y. lipolytica* E150 by restoring the *ura* and *leu* auxotrophic markers. The CTY029 strain was derived from FT-120 by restoring the *ura* and *leu* auxotrophic markers. FT-120, itself a W29 derivative, which had its β-oxidation disrupted by deletion of genes for six peroxisomal acyl-CoA oxidases.

### 4.2.2. Yeast transformation

Constructed expression vectors (table 4.2) were digested with *Not*I to release the yeast integration cassette from the bacterial portion, prior to transformation of yeast strains. The transformations were performed using not only expression vectors containing genes of interest, but also relevant controls. In every case, an ‘empty’ vector, i.e. one not carrying a gene for a P450 or CPR was used to generate negative control strains. In

the cases where CPRs were coexpressed with the P450s, vectors containing each P450 and each CPR alone were used to generate further controls.

All yeast strains were transformed using a modified version of the method described by Lin-Cereghino *et al* (2005) except for *K. marxianus,* which was transformed using a modified version of the method described by Chen *et al* (1997). This was due to the fact that the method of Lin-Cereghino *et al* (2005) uses DMSO during the preparation of competent cells to increase cell permeability, but DMSO was shown to reduce the transformation efficiency of *K. marxianus*, possibly due to toxicity (Antunes *et al*., 2000). Transformation efficiencies using either method were generally in the range of 10 – 100 colony forming units (CFU) per µg DNA.

### 4.3. Confirmation of genomic integration of foreign DNA

Transformants were plated out on selective media, usually containing 400 µg/ml hygromycin B, or in cases of *K. marxianus* with pKM63 or its derivatives cloned 75 µg/ml G418 / geneticin. Once colony formation was observed, a selection of at least five transformants in each were selected in each case and re-streaked onto fresh selective media. A total of three rounds of re-streaking on fresh selective media were performed for stability (Pignède *et al*., 2000), before further analysis of the strains. Since the wild-type strains of all the yeasts tested cannot grow in the presence of the relevant antibiotics, colony formation on the selective media was an initial indication of successful integration, although further confirmation was pursued for the sake of thoroughness.

Colony PCR was initially attempted for the yeast transformants, but the results using whole cells were unreliable due to very low reproducibility (data not shown). Therefore, to be completely confident of the results obtained, genomic DNA was extracted for each selected transformant and used as a template for PCRs. Combinations of the TEFp forward primer and gene specific reverse primers were generally used (figure 4.3). This

combination was particularly useful when a CPR was coexpressed with a P450, as reverse primers for both genes were included, allowing the presence of both genes to be demonstrated (figure 4.3 B).

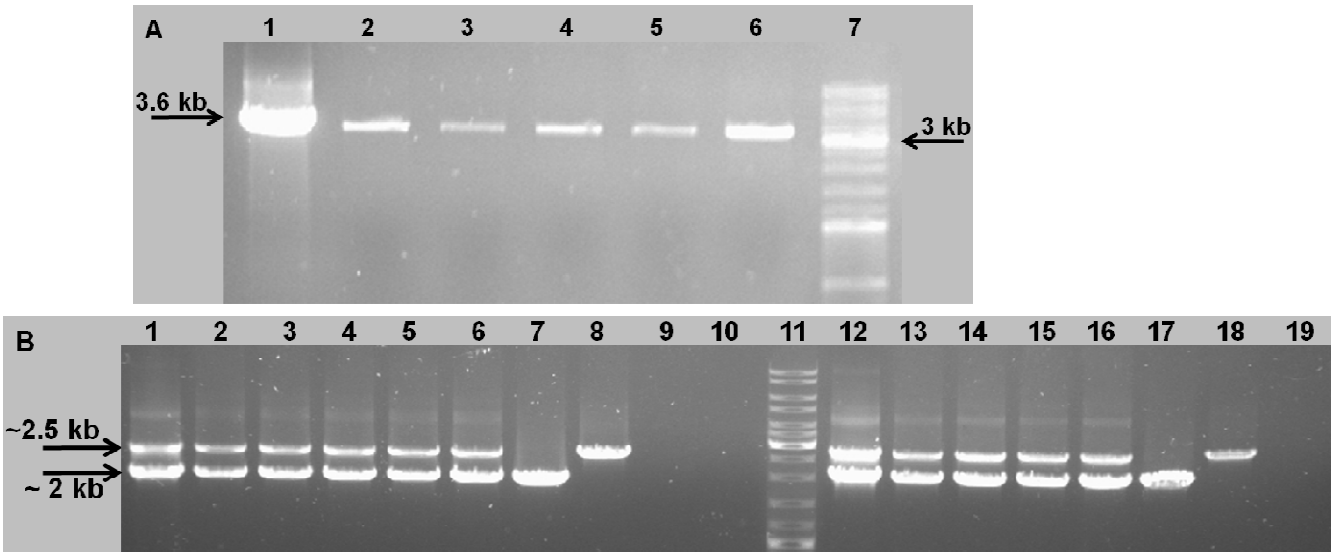



**Figure 4.3: Agarose gels of examples of PCR screening for confirmation of integration of foreign DNA into the genomic DNA. In this case, genomic DNA from transformants of *A. adeninivorans* was used as the templates.**

A – Expression of P450-CPR fusions, in this case CYP505A1 which is ~3.2 kb plus the TEF promoter which is ~0.4 kb, resulting in an amplicon of ~3.6 kb.
Lane 1 is a control in which vector DNA was used as a template; lanes 2-6 are genomic DNA test samples; and lane 7 is the DNA ladder.

B – Coexpression of P450s and CPRs, in this case RmCPR in combination with CYP53B1 (lanes 1-10) and CYP557A1 (lanes 12-19). RmCPR is ~2.1 kb, therefore in combination with the TEF promoter the amplicon is ~2.5 kb. The P450s are ~1.5 kb, therefore in combination with the TEF promoter the amplicons are ~2 kb.

Lanes 1-6 are test samples of transformants with RmCPR and CYP53B1 cloned. Lanes 12-16 are test samples of transformants with RmCPR and CYP557A1 cloned. Lanes 7 and 17 are samples from transformants with only CYP53B1 and CYP557A1 cloned, respectively. Lanes 8 and 18 are samples from a transformant with only RmCPR cloned. Lanes 9 and 19 are samples from transformants in which an empty vector was integrated. Lane 10 is a PCR negative control in which no template was added to the PCR mixture. Lane 11 is the DNA ladder.

The success rate of integration was not always as high as depicted in figure 4.3, but transformants were screened using PCRs until at least 4 representatives of each containing the relevant integrant were identified. Once genomic DNA integration was satisfactorily confirmed, aliquots of cultures of 4-5 transformants from each gene / gene combination of interest were stored with 15% final concentration glycerol at -80$^{o}$C until required for biotransformation studies.

## Part B: Biotransformations using growing cells of recombinant yeast strains expressing class II P450s

### (i) CYP53B1

The benzoate-*para*-hydroxylase CYP53B1 from *Rhodotorula minuta*, had previously been heterologously expressed in *Y. lipolytica* and evaluated for activity during a doctoral study in our group (Shiningavamwe, 2004), and we later used it as a reporter enzyme for heterologous protein expression studies (Obiero, 2006; Theron, 2007). CYP53B1 hydroxylates benzoic acid (BA) at the *para*-position on the aromatic ring, yielding *para*-hydroxybenzoic acid (pHBA; figure 2.5). It is a convenient reporter enzyme because its natural substrate allows convenient TLC detection of activity, due to UV absorption properties of the substrate and product on TLC plates which contain a UV fluorescence indicator (in our case F254) embedded in the stationary phase (in our case silica). Upon excitation of the indicator compound by UV light, UV-absorbing

compounds quench the fluorescence of the indicator, and appear as dark spots on illuminated backgrounds.

None of the strains tested in this study possess detectable natural activities toward the substrate (BA) or product (pHBA), although minor conversions of low concentrations of pHBA were reported for the LS3 strain of *A. adeninivorans* (Sietmann *et al.,* 2010). Due to our previous experience with this enzyme, it was used to establish biotransformation systems, which could later be applied to other enzymes.

Since CYP53B1 is not a self-sufficient P450, their activities are dependent on the natural P450 reductase enzymes (CPRs) within the yeast hosts. Studies have however shown that coexpression of CPRs greatly enhances P450 activities, even in yeasts which already posses reductase components of their own (Schiffler *et al*, 2004; Nthangeni *et al*, 2004). Therefore, coexpression of CPRs from *Yarrowia lipolytica* (YlCPR), *Rhodotorula minuta* (RmCPR) and *Ustilago maydis* (UmCPR) were investigated.

### 4.4. Initial screening of transformants of different yeast species with CYP53B1 and different CPRs cloned

The general parameters for comparative biotransformations using the different species had to be defined at the onset, to keep conditions throughout the screening as equal and impartial as possible. It was decided that 48 h growth at 28°C would be used for comparison of the different species, since in our group Dr. Labuschagné had determined that extracellular levels of the *Thermomyces lanigunosis* xylanase cloned into the different yeasts using the wide range vector was approximately constant in all the yeasts in samples taken between 48 h and 96 h growth, while it was lower in samples taken after 24 h growth (Albertyn *et al*, 2011). Furthermore, it was anticipated that all species would have reached the stationary growth phase after 48 h, allowing for comparisons at a uniform growth phase.

*K. marxianus* (UOFS Y1185), *S. cerevisiae* (W3031A(a)), *H. polymorpha* (UOFS Y1507), *Y. lipolytica* (CTY003) and *A. adeninivorans* (UOFS Y1220) were transformed with the vectors pKM173+CYP53+YlCPR, pKM173+CYP53+RmCPR, pKM173+CYP53+UmCPR, pKM177+CYP53, pKM173+YlCPR, pKM173+RmCPR, pKM173+UmCPR and pKM173. Four or five transformants from each transformation, which were confirmed by gDNA PCR to contain the relevant foreign DNA, were used for initial screening for transformants with the highest activity. Appropriate control transformants were prepared by using an empty vector without cloned genes, vectors containing only the individual P450 reductases (CPRs), and a vector containing only CYP53B1. Test tubes containing 5ml YPD were inoculated from YPD agar plates containing hygromycin B, and the substrate concentration used was 5mM. Screening results obtained with the different yeasts are described in the next sections, in which the most promising transformants which were selected for further studies are numbered. All reported activities are from samples taken after 24 h biotransformation unless specifically indicated otherwise.

### 4.4.1. *Kluyveromyces marxianus*

Results from the initial screen for benzoate *para*-hydroxylase activity in *K. marxianus* transformants are shown in figure 4.4.

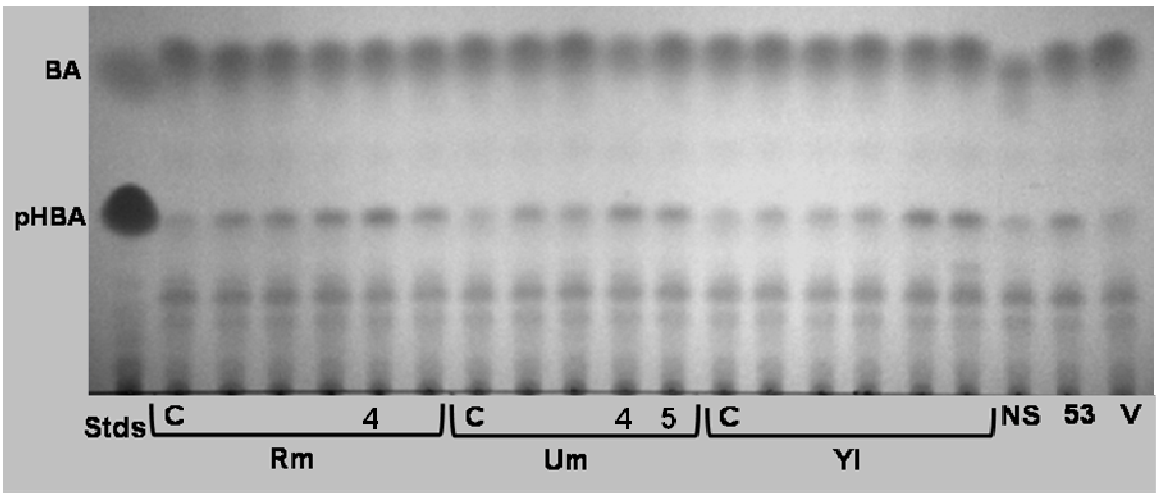



**Figure 4.4: TLC analysis of the initial screen of *K. marxianus* transformants expressing CYP53B1 with or without coexpression of CPRs, and a negative control transformed with an empty vector.** Stds – benzoic acid and *para*-hydroxybenzoic acid standards, RmCPR – coexpressing CPR from *Rhodotorula minuta*, UmCPR – coexpressing CPR from *Ustilago maydis*, YlCPR – coexpressing CPR from *Yarrowia lipolytica*, C – expressing the reductase only, NS – no substrate added to culture of a test transformant, 53 – expressing only the CYP53B1, V – transformed with a vector without a gene for either P450 or CPR. Transformants used for further studies are numbered.

The results showed that CYP53B1 was successfully expressed by *K. marxianus* when under control of the *Y. lipolytica* TEF promoter and that whole-cell biocatalysis can be achieved using this host. This is, to our knowledge, the first report on expression of a P450 in this host. Due to low activities, co-extracted metabolites from the cells lead to background 'noise' which interfered with result interpretation. This is illustrated in the 'NS' lane, in which sample from a culture to which substrate was not added, was analyzed. Even in the absence of substrate (and therefore product) spots were observed with similar retention factor (RF) values to the substrates and products. In such cases analyses must be performed with much care.

In the initial screen some samples from transformants coexpressing CPRs did appear to be better than the transformant expressing only CYP53B1. There was however considerable variations between transformants, which may be attributed to growth differences or perhaps copy number variations, since neither factor was taken into account for the initial screening. Since the transformants coexpressing CYP53B1 and CPR had such variation, there could also be such variations in transformants expressing only CYP53B1. Therefore using only one such transformant as a control may be misleading, rendering observations made using only one such transformant insufficient for deriving conclusions. Hence four more transformants expressing only CYP53B1 were compared to the one used in the initial screen (figure 4.5).

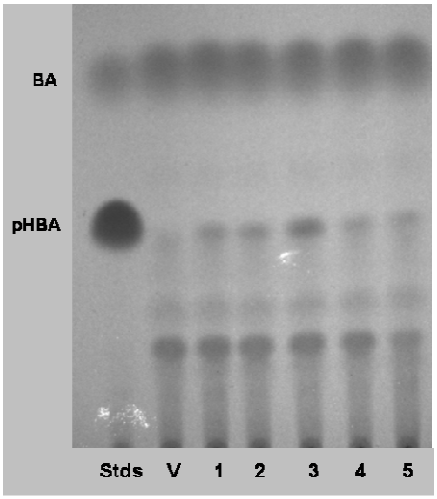



**Figure 4.5: TLC analysis of the comparison of *K. marxianus* transformants with CYP53B1 cloned and a control with no P450 cloned.** Stds – benzoic acid and *para*-hydroxybenzoic acid standards, V – transformed with a vector without a P450 or CPR, 1-5 – transformants expressing only CYP53B1.

Figure 4.5 shows that transformant number 1 which was used in the initial screen (figure 4.4) was in fact a lot less active than transformant number 3, although it was not the least active. This reinforces the proposal that using only one such transformant for experimental control can lead to inaccurate deductions being made from results obtained. It was therefore decided to use more transformants expressing only CYP53B1 for the initial screening of other strains, in order to correctly distinguish whether or not

better activities are obtained when CPRs were coexpressed. Transformants 2 and 3 from this transformation were chosen for further investigations (section 4.5).

### 4.4.2. *Saccharomyces cerevisiae*

Results for the initial screening for benzoate *para*-hydroxylase activity in *S. cerevisiae* transformants are shown in figure 4.6.

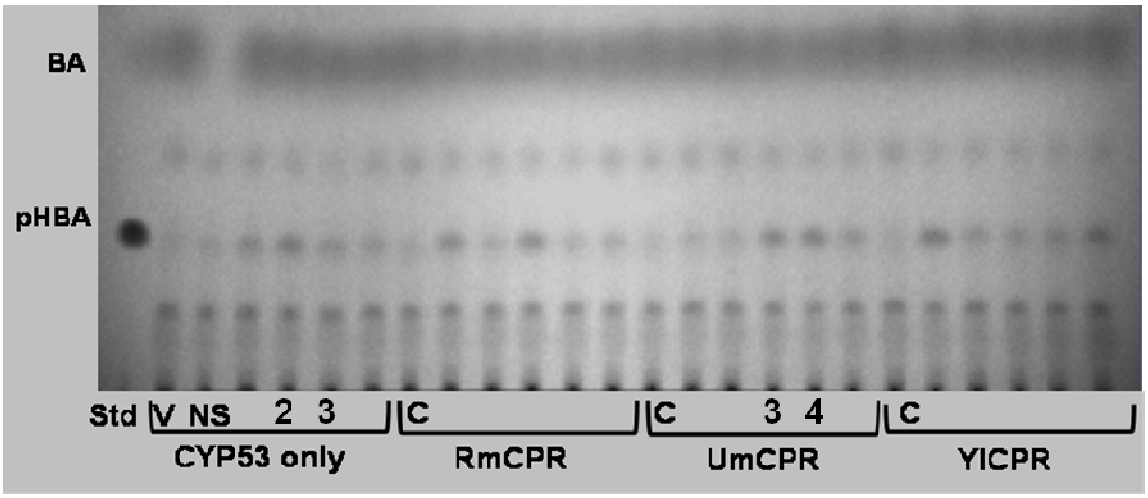



**Figure 4.6: TLC analysis of the initial screen of *S. cerevisiae* transformants expressing CYP53B1 with or without coexpression of CPRs, and a negative control.** Stds – *para*-hydroxybenzoic acid (pHBA) standard, V – transformed with a vector without a P450 or CPR, NS – no substrate added to culture of a test transformant, CYP53 only – expressing only the P450 and no foreign CPR, RmCPR – coexpressing CPR from *Rhodotorula minuta*, UmCPR – coexpressing CPR from *Ustilago maydis*, YlCPR – coexpressing CPR from *Yarrowia lipolytica*, C – expressing the foreign reductase only. Transformants which were selected for further studies are numbered.

As observed for *K. marxianus*, other co-extracted compounds with Rf values similar to pHBA, as seen in the extract from the culture with no substrate added (NS), interfere with the interpretation of results. There are however samples in which the formation of

pHBA can clearly be observed, indicating that the P450 was successfully expressed in this host using the broad-range vector and that whole-cell biocatalysis could be achieved using this host . Samples from transformants coexpressing CPRs appeared to be slightly better than those expressing only CYP53B1, although these results are difficult to interpret.

### 4.4.3. *Hansenula polymorpha*

Results for the initial screening for benzoate *para*-hydroxylase activity in *H. polymorpha* transformants are shown in figure 4.7.

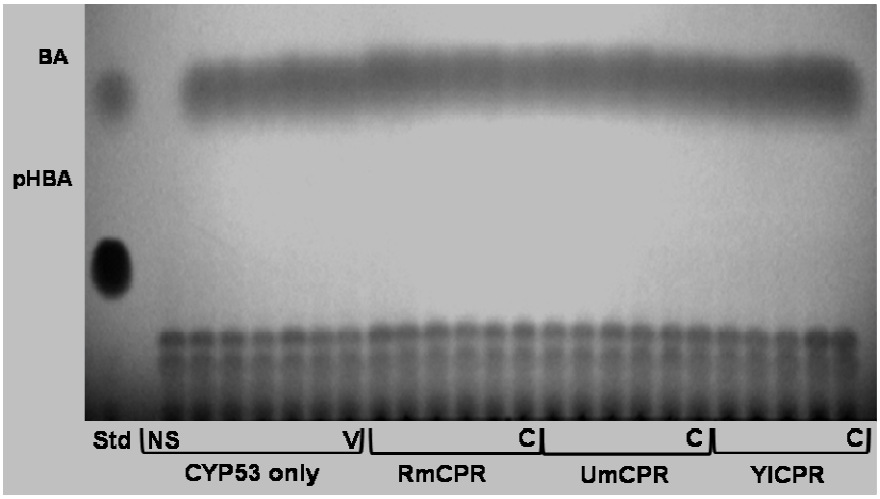



**Figure 4.7: TLC analysis of the initial screen of *H. polymorpha* transformants expressing CYP53B1 with or without coexpression of CPRsl.** Std – benzoic acic and *para*-hydroxybenzoic acid standards, V – transformed with a vector without a P450 or CPR, NS – no substrate added to culture of a test transformant, CYP53 only – expressing only the P450 and no foreign CPR, RmCPR – coexpressing CPR from *Rhodotorula minuta*, UmCPR – coexpressing CPR from *Ustilago maydis*, YlCPR – coexpressing CPR from *Yarrowia lipolytica*, C – expressing the foreign reductase only.

It is evident from figure 4.7 that absolutely no activity could be observed in any of the transformants. These results were surprising because of the promising results observed with this yeast in heterologous expression of other enzymes, although those results

were generally obtained with strong inducible promoters, unlike the constitutive promoter used in this study (Kang and Gellissen, 2005). Dr. M. Labuschagne also obtained poor results with this strain when expressing xylanase from *Thermomyces lanigunosis* and vannilyl alcohol oxidase (VAO) from *Penicillium simplicissimum*, although those activities were at least comparable to those obtained with *S. cerevisiae*.

### 4.4.4. *Yarrowia lipolytica* (strain CTY003)

Results for the initial screening for benzoate *para*-hydroxylase activity in *Y. lipolytica* CTY003 transformants are shown in figure 4.8.

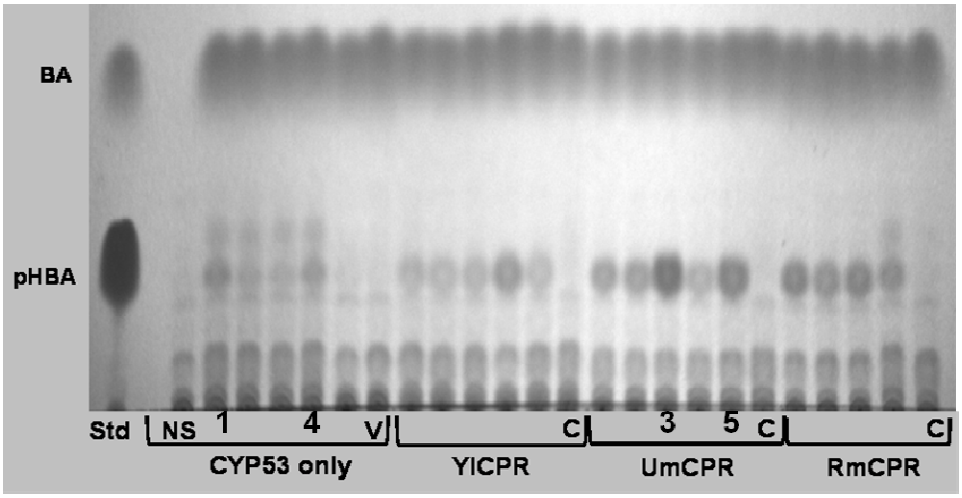



**Figure 4.8: TLC analysis of the initial screen of *Y. lipolytica* transformants expressing CYP53B1 with or without coexpression of CPRs, and a negative control.** Stds – benzoic acic and *para*-hydroxybenzoic acid standard, V – transformed with a vector without a P450 or CPR, NS – no substrate added to culture of a test transformant, CYP53 only – expressing only the P450 and no foreign CPR, YlCPR – coexpressing CPR from *Yarrowia lipolytica*, UmCPR – coexpressing CPR from *Ustilago maydis*, RmCPR – coexpressing CPR from *Rhodotorula minuta*, C – expressing the foreign reductase only. Transformants which were selected for further studies are numbered.

Firstly, we clearly observed whole-cell CYP53B1 activity in *Y. lipolytca* transformants, as expected from experience (Shiningavamwe *et al*., 2006). In previous cases however, strong inducible promoters were used, whereas we observed activity using the constitutive TEF promoter in our broad-range vector in this case. The differences between transformants with and without coexpressed CPRs, and between the different coexpressed CPRs, were highly pronounced in this yeast. In this case, coexpression of the *Y. lipolytica* CPR was in actual fact overexpression of the natural CPR, and is the only such case in this entire study. It was therefore even more intriguing to see that the other two CPRs actually increased the P450 activity even more than the YlCPR did. The significance of the choice of CPR used to assist P450 activity was starting to become clear.

### 4.4.5. *Arxula adeninivorans*

Results obtained by the initial screening for benzoate *para*-hydroxylase activity in *A. adeninivorans* transformants are shown in figure 4.9.

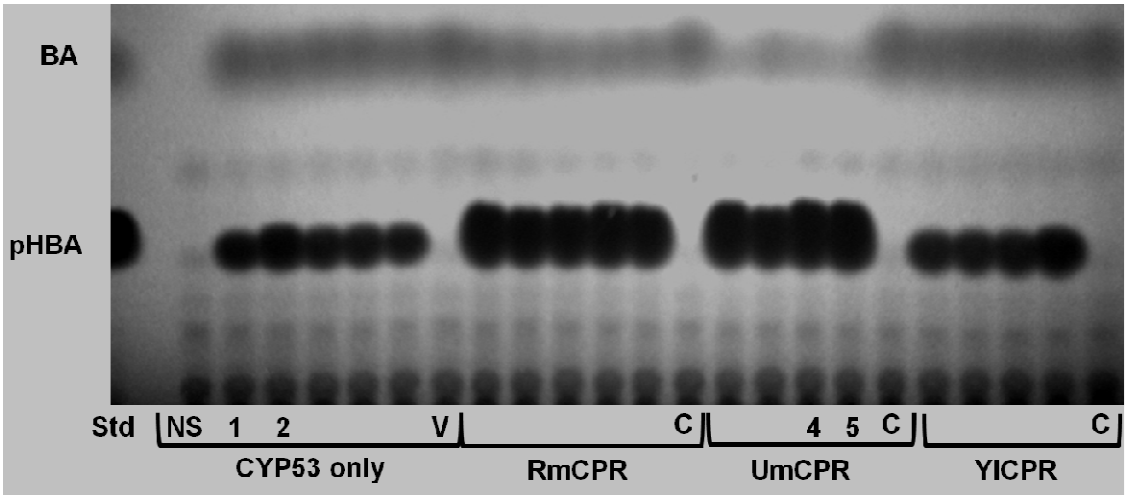



**Figure 4.9: TLC analysis of the initial screen of *A. adeninivorans* transformants expressing CYP53B1 with or without coexpression of CPRs.** Std – benzoic acic and *para*-hydroxybenzoic acid standard, V – transformed with a vector without a P450 or CPR, NS – no substrate added to culture of a test transformant, CYP53 only – expressing only the P450 and no foreign CPR, RmCPR – coexpressing CPR from

*Rhodotorula minuta*, UmCPR – coexpressing CPR from *Ustilago maydis*, YlCPR – coexpressing CPR from *Yarrowia lipolytica*, C – expressing the foreign reductase only. Transformants which were selected for further studies are numbered.

High yields of *p*-hydroxybenzoic acid were obtained as proof that CYP53B1 was successfully expressed and that whole-cell biocatalysis can be achieved using this host. This is, to our knowledge, the first report on P450 expression in this yeast. The Benzoate *para*-hydroxylase activities in the *A. adeninivorans* transformants were much higher than in any of the other yeasts. The differences between activities when the different CPRs were coexpressed were also more pronounced. The variation between transformants was less, which increased the reproducibility of the results. There did not appear to be much improvement observed in transformants coexpressing YlCPR compared to those expressing only CYP53B1. The differences made by the basidiomycetous CPRs on the other hand were huge, as can be seen not only by higher product formation but also by the drastic reduction of substrate in these samples, particularly those from transformants coexpressing UmCPR and CYP53B1 (figure 4.9). These were clearly the most promising results obtained thus far, as even the transformants expressing only CYP53B1 appeared better than any of the transformants of other yeasts.

### 4.5. Direct quantitative comparisons between selected transformants

Transformants with the highest activities (numbered in Figs. 4.4, 4.6, 4.8 and 4.9) were selected from the initial screens for direct comparisons between the different yeasts, specifically using quantitative data for more accurate comparisons. Growth curves were constructed to confirm that all of the species which would be tested for further biotransformations do in fact reach stationary phase by 48 h of cultivation (figure 4.10).

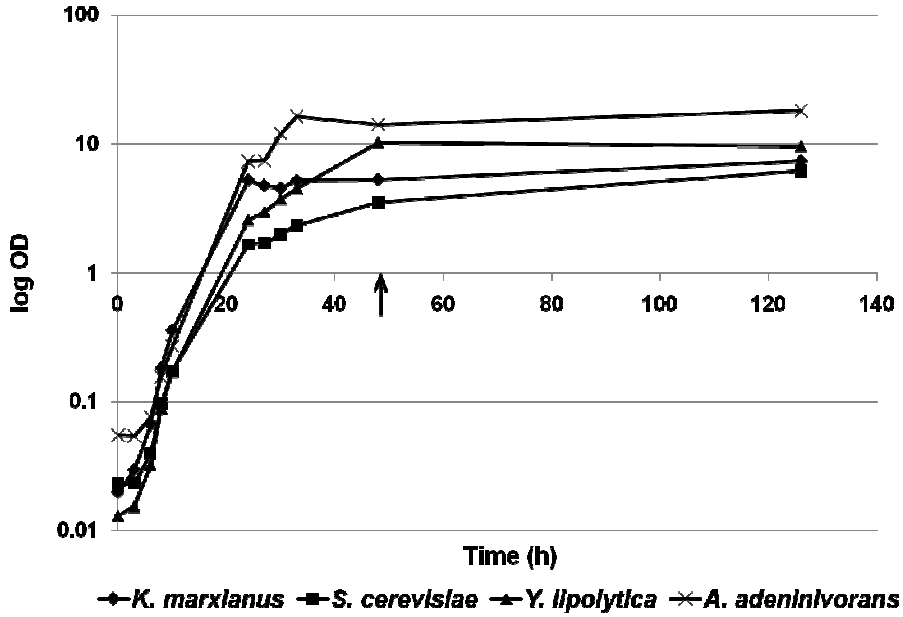



**Figure 4.10: Growth curves for the yeast species for which comparative biotransformations would be performed.** The point chosen for substrate addition (48 h of growth) is indicated by an arrow.

### 4.5.1. General pH and biomass values of cultures during cultivation under specified conditions

The general pH and biomass ranges of the different species, obtained under the specified biotransformation conditions, are presented in table 4.3.

**Table 4.3: Typical biomass concentrations of transformants of the different yeast strains at the point of substrate addition and pH ranges at the first and last activity samples.**

| | **pH value** | | **Biomass (g/L DCW)** |
|---|---|---|---|
| | **0h** $^{+}$ | **96h** $^{++}$ | **0h** $^{+}$ |
| *K. marxianus* | 5.5-6.5 | 5.5-6 | 9-11 |
| *S. cerevisiae* | 5.5-6 | 5 | 4-7 |
| *Y. lipolytica* (CTY003) | 6.5 | 8-8.5 | 17-20 |

| | | | |
|---|---|---|---|
| *A. adeninivorans* | 6-8 | 9 | 16.5-18.5 |

[+] - 0h sample was taken immediately before substrate addition, i.e. after 48 h growth.

[++]- 96h sample was taken 96 h after substrate addition.

The pH of the 0 h sample of *A. adeninivorans* varied more than any other host, with values ranging between 6 and 8. This pH variation at the time of substrate addition did however not appear to significantly affect the activities between biotransformations.

### 4.5.2. Direct comparison between transformants of different yeast strains expressing only CYP53B1

Two transformants of each host transformed with pKM177+CYP53 which showed the highest pHBA formation in the TLC assays were compared for benzoate *para*-hydroxylase activity, relying only on their natural CPRs. The two most promising transformants in each case were selected because exact copy numbers were not determined, since suitable markers for quantitative PCR were not available for all the yeasts (Terentiev *et al*., 2004b). The initial comparative biotransformations were carried out as described in section 4.4, except 50 ml YPD was used in 500 ml flasks. BA and pHBA were quantified using gas chromatography (GC), and growth was taken into account so that specific rates could be determined. All of the results are averages of at least duplicate cultures.

It was observed that the pH in cultures of the different yeast species differed after 48 h growth (the point of substrate addition), with *S. cerevisiae* and *K. marxianus* often having pH values below 6 (table 4.3). The effect of pH on whole cell activity was thus also investigated, since organic acids are more toxic at lower pH and this might explain the relatively poor activity observed with *K. marxianus* and *S. cerevisiae*. At substrate addition, potassium phosphate buffer (pH 8) was also added to final concentrations of 100 mM and 200 mM to two sets of cultures. Although biotransformations were continued for 96 h, only product formation after 24 h of biotransformation and specific

activity calculated using the yield at 24 h are shown in Figure 4.11, while changes in pH during the biotransformation phase is shown in Figure 4.12.

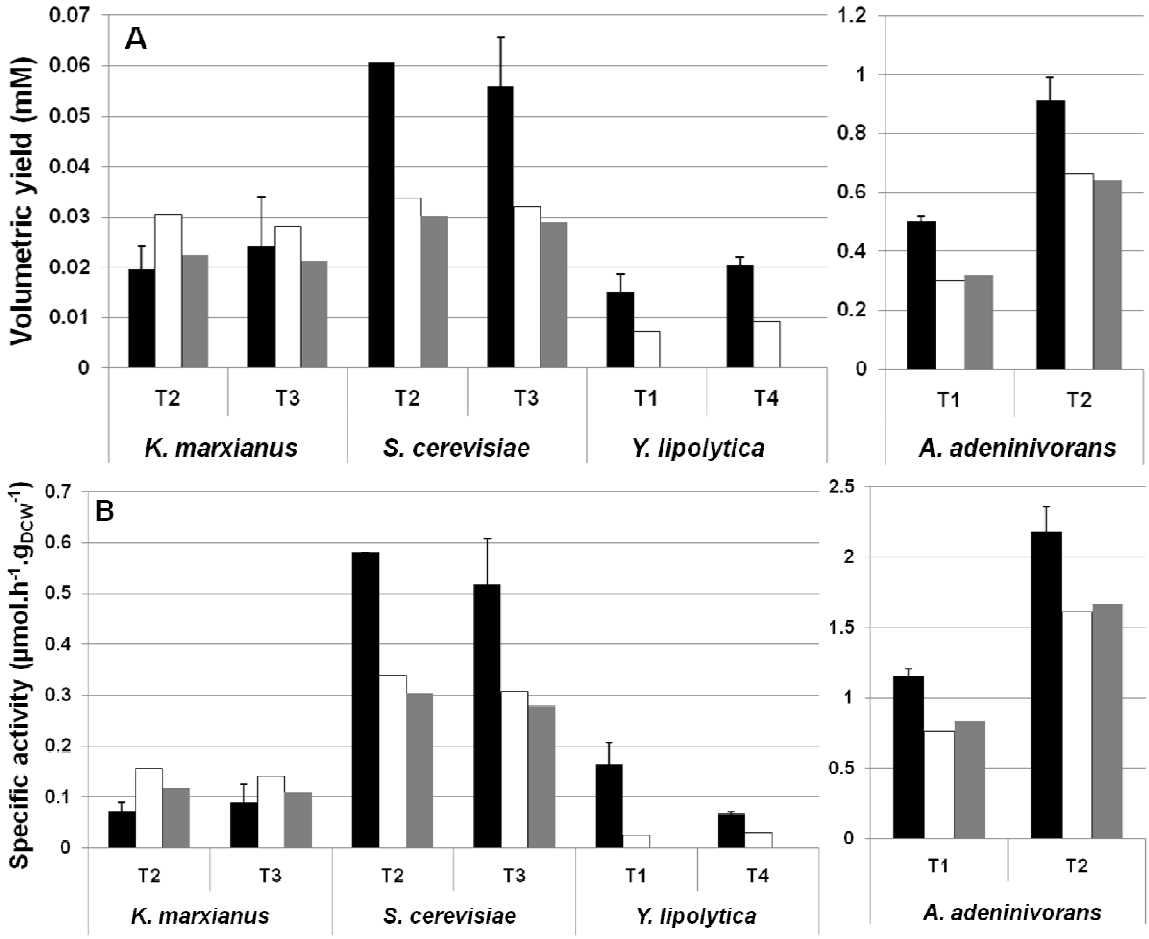



**Figure 4.11: pHBA production (A) and specific activity (B) of transformants of different yeasts transformed with pKM177+CYP53 after growth in YPD.** Black columns –YPD without buffer; white columns – YPD containing 100 mM potassium phosphate buffer, pH8; gray columns – YPD containing 200 mM potassium phosphate buffer, pH8. Buffer was added just before substrate addition after 48 h growth. Values for cultures without buffer added are averages of duplicate cultures. Buffered cultures were not done in duplicate.

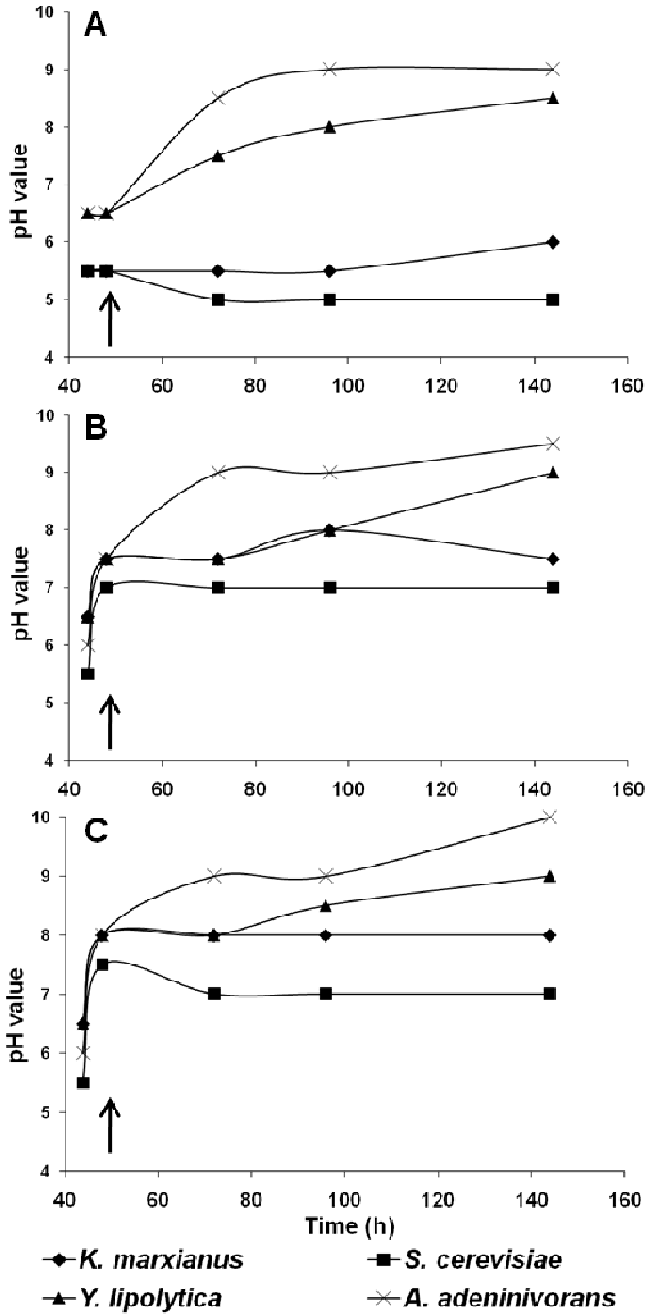



**Figure 4.12: Graph representing pH values during cultivation of transformants in cultures grown in (A) un-buffered YPD, (B) YPD containing 100 mM phosphate buffer (pH8), and (C) YPD containing 200 mM phosphate buffer (pH8).** The culture age is given on the x-axis, and the buffer was added, along with substrate, after 48 h of growth (indicated by the arrows).

The results for the cultures without buffer clearly demonstrate how much better the activity achieved with *A. adeninivorans* transformants is than the other hosts tested, especially if one notes the different ranges of the Y-axes. The next best activity was observed with *S. cerevisiae*, particularly when considering specific activity, given the low biomass of this organism compared to the other tested yeasts. *K. marxianus* performed slightly better than *Y. lipolytica*.

One can also clearly observe a nearly two-fold difference in activity between the two transformants of *A. adeninivorans,* the biggest difference between transformants of any given host. This difference hints that one transformant has double the copy number of the other one. The higher the copy number of the transformant with lowest activity, the more unlikely it is that other transformants will have exactly double that copy number, i.e. it is more likely to find transformants with 1 and 2 copies than to find ones with for example 3 and 6 copies. Therefore these transformants most probably contain 1 and 2 copies, respectively, which would agree with the findings of Rosel and Kunze, who reported that 1-3 stably integrated copies could be obtained in transformants when using hygromycin resistance while targeting rDNA for integration (Rosel and Kunze, 1998). This means that the superior performance of *A. adeninivorans* transformants is unlikely to be due to higher copy numbers being obtained in this host, since other yeasts cannot have less than 1 integrated copy. It is quite likely that transformants of *S. cerevisiae* and *Y. lipolytica* contained 2 or 3 copies of the vector, since two transformants with higher activities than the minimum could in each case be selected from TLC.

The addition of buffer had, contrary to expectation, a negative effect on activity of all the yeasts, with the exception of *K. marxianus*. The more concentrated 200 mM buffer was in most cases possibly more detrimental than 100 mM. Only *K. marxianus* activity increased marginally in the presence of buffer. The pH values monitored throughout the cultivations are represented in figure 4.12. The pH values of the buffered cultures did not drop below 6.5-7, which was higher than in the un-buffered cultures of *K. marxianus*

and *S. cerevisiae*. The pH values of these cultures remained stable, while the cultures of *Y. lipolytica* and *A. adeninivorans* still raised the pH as in the un-buffered cases. pH values were generally maintained higher for the more concentrated buffer, generally leading to lower activities.

Since *S. cerevisiae* had the lowest pH in un-buffered YPD, we expected that perhaps the activity would increase in the presence of the buffer, if the low pH was affecting the toxicity of the substrate and / or product (Obiero, 2006; revisited in section 4.6). The elevated pH did not however increase the activity. The optimal pH for *S. cerevisiae* is below neutral, and alkalinization of the medium induces stress responses from the cells. One such response is towards oxidative stress due to reactive oxygen species generated by high pH, while another involves ensuring iron is free for physiological functions (Ariño, 2010). It is possible that increasing the pH may cause similar effects in the other yeasts. We hoped that such stress response effects would not affect our results much, since the cells were no longer actively growing and P450 production had already occurred, but obviously the high pH stress did affect the results.

### 4.5.3. Quantification of the effect of coexpression of different CPRs in *A. adeninivorans*

Since the effect of co-expressed CPR was the clearest and most consistent in *A. adeninivorans*, because the activities in this host were far better than in the other ones tested and hence observed easier, we chose to quantify the effect of coexpression of the different CPRs in this host. Two transformants that displayed the highest activity in the TLC assays were again selected and biotransformations were carried out as described in the previous section. Product formation after 24 h of biotransformation and the specific activities based on that data are shown in Figure 4.13.

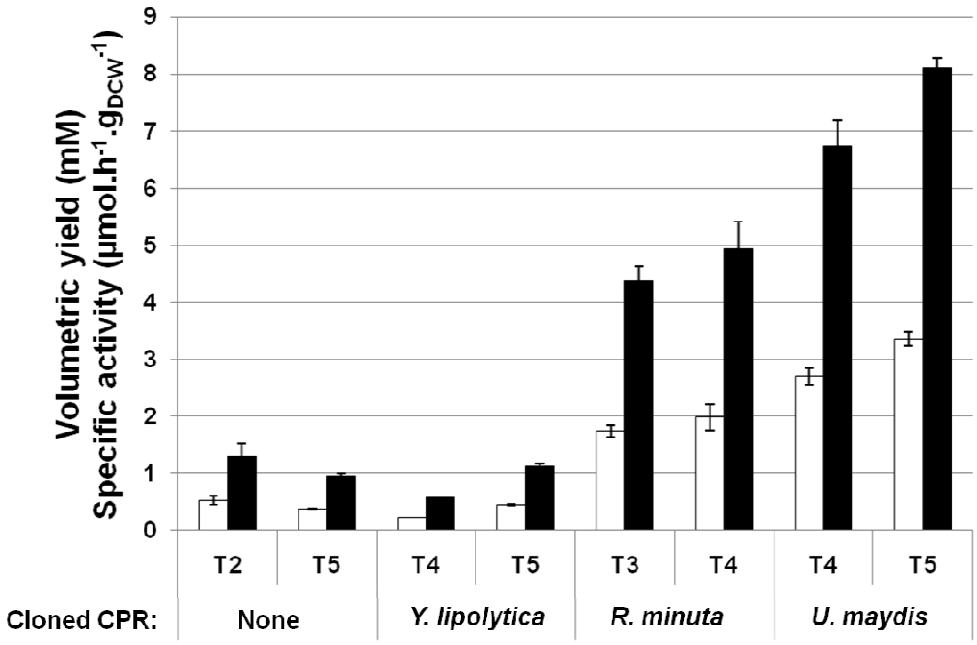



**Figure 4.13: pHBA production (white columns) and specific activity (black columns) of transformants of *A. adeninivorans* transformed with vectors pKM177+CYP53, pKM173+CYP53+YlCPR, pKM173+CYP53+RmCPR and pKM173+CYP53+UmCPR after growth in YPD.**

The results obtained from this comparison of different CPRs are indeed very interesting, as the basidiomycetous CPRs drastically enhance the P450 activity, while the ascomycetous CPR from *Y. lipolytica* (YlCPR) in fact reduces the activity. The CPRs from the basidiomycetes might be more compatible with CYP53B1, since this P450 is also of basidiomycetous origin. However, a puzzling aspect is that the CPR from *R. minuta* (RmCPR) should then theoretically be the best suited, since the CYP53B1 also originates from this organism. This is clearly not the case however, as the CPR from *U. maydis* (UmCPR) enhances the activity the most.

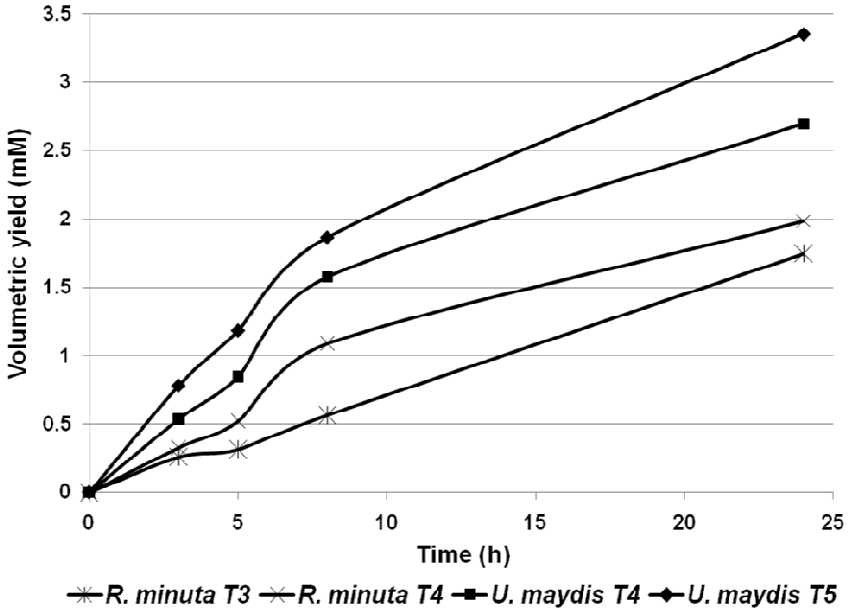



**Figure 4.14: Progress curves of pHBA production over 24 h by *A. adeninivorans* transformants transformed with pKM173+CYP53B1+RmCPR and pKM173+CYP53B1+UmCPR.**

Progress curves were constructed from the biotransformation data obtained from transformants coexpressing RmCPR and UmCPR with CYP53B1, since these transformants displayed the best activities. These progress curves demonstrate that in cases where the best activities occurred, pHBA production was still linear relative to elapsed time after 24 h of biotransformation. In all other cases in which activities were lower, activities were only really detectable after about 24 h of biotransformation. Since the rates were so low in those cases, it is difficult to obtain linear rates to use in specific activity determination, but one can safely assume that those activities are also still linear at 24 h. Therefore, to once again keep consistency between different experiments, specific activities were calculated using pHBA yield at 24 h as a relatively safe estimation. One can however see that during the progression of pHBA formation by transformants with high activities (figure 4.14) the initial rate could probably be more accurately determined during the first 8 h of biotransformation. Therefore, in these cases we could actually be underestimating the best specific activities, although they

remain far superior to other activities in any case. This point is reiterated again in section 4.5.5.

### 4.5.4. Effect of coexpression of CPR from *U. maydis* (UmCPR) on recombinant CYP53B1 activity in different hosts

In the initial TLC screens it was obvious that UmCPR was the best reductase for *A. adeninivorans* and *Y. lipolytica*, and quite likely also for *S. cerevisiae* and *K. marxianus*, although the differences were not as apparent in the latter two cases. As explained in the previous chapter, the P450 and CPR are carried on the same vector, each under a copy of the same constitutive promoter; therefore equal copies of P450 and CPR were integrated in each case. Two transformants of each host transformed with pKM173+CYP53+UmCPR which showed the highest pHBA formation in the TLC assays were selected and their BA conversions compared in a biotransformation experiment carried out as described in the previous two sections. The product formations and specific activities are compared with the results described in section 4.5.2 (non-buffered cultures) in figure 4.15.

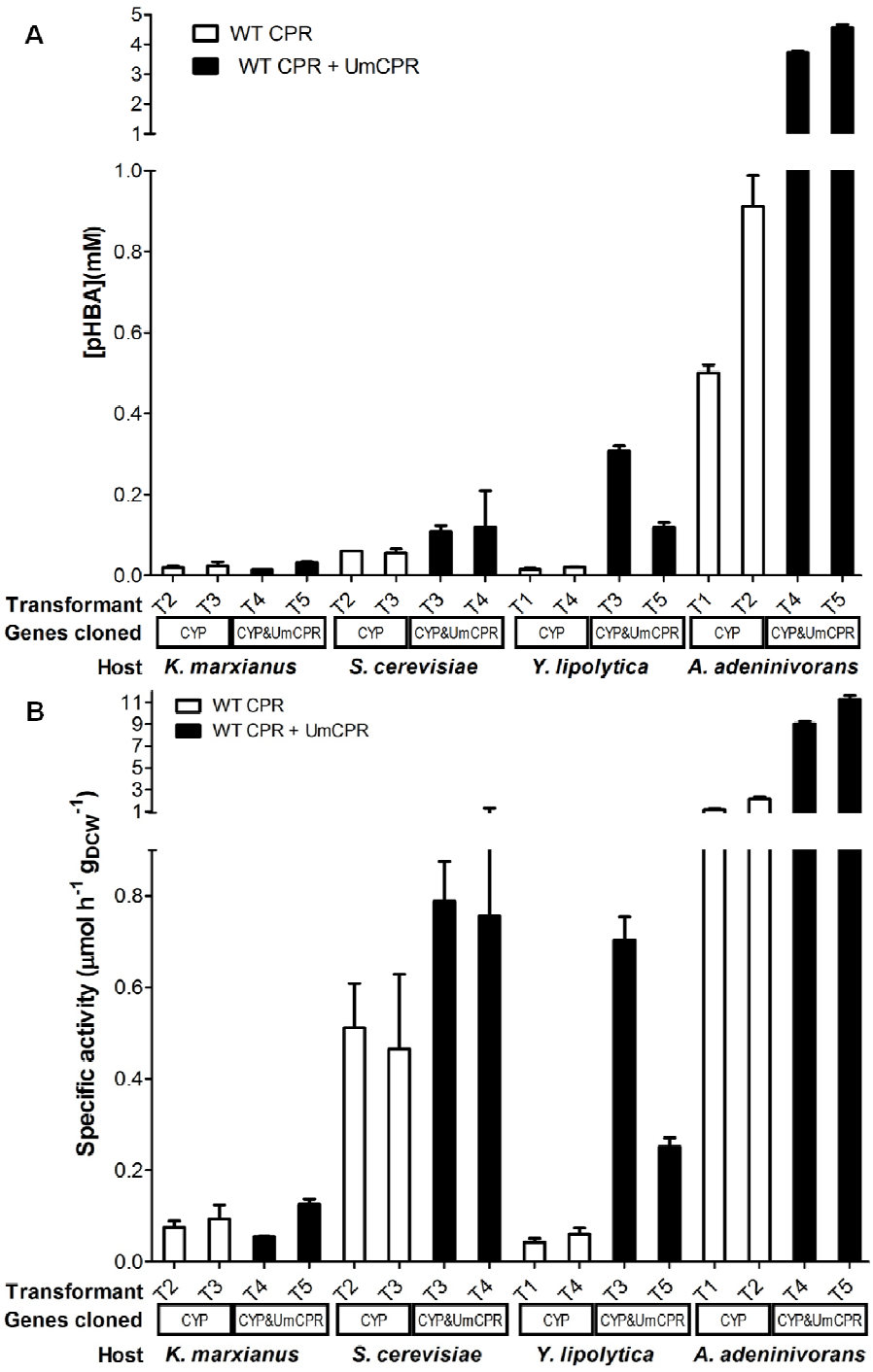



**Figure 4.15: pHBA production (A) and specific activity (B) of transformants of different yeasts transformed with pKM177+CYP53 (white columns) compared to yeasts transformed with pKM177+CYP53+UmCPR (black columns).** The represented values are averages of at least duplicate cultures.

From the results it is clear that the UmCPR does not improve the CYP53B1equally in all of the yeasts. Comparisons are complicated by the observation that one pKM173+CYP53+UmCPR transformant of both *K. marxianus* (T5) and *Y. lipolytica* (T3) probably contained two or in the case of *Y. lipolytica* perhaps three copies of the integrated vector. The most important observation is that the expressed activity in *Y. lipolytica* is raised so much that in one case (T3) it surpasses *S. cerevisiae* in terms of volumetric yield. When considering specific activity, *S. cerevisiae* is still slightly better, but drastically less so than without coexpression of the UmCPR.

*A, adeninivorans* again displayed undisputedly the highest activities. The highest volumetric yield obtained by a single culture of a single transformant of *Y. lipolytica* coexpressing CYP53B1 and UmCPR was 0.317 mM, while the lowest activity obtained by a single culture of a single transformant of *A. adeninivorans* expressing only CYP53B1 was 0.486 mM. Similarly, the highest specific activity obtained by a single culture of a single transformant of *S. cerevisiae* coexpressing CYP53B1 and UmCPR was 0.849 $\mu mol.h^{-1}.g_{DCW}^{-1}$, while the lowest activity obtained by a single culture of a single transformant of *A. adeninivorans* expressing only CYP53B1 was 1.124 $\mu mol.h^{-1}.g_{DCW}^{-1}$.

### 4.5.5. Effect of 5-aminolevulinic acid addition on recombinant CYP53B1 activity in different hosts

When expressing hemeproteins such as P450s in *E. coli*, 5-aminolevulinic acid (5-ALA), a precursor for heme, is routinely added to the cells, usually at the point of induction. This is because free heme is toxic to *E. coli* cells and is hence not accumulated. Supplying heme itself is inefficient due to the impermeability of the membranes to heme (Harnastai *et al*, 2006). One would expect that these problems would not be as prominent for yeasts, but in case heme availability is limited within the cells, particularly when the heme supply is likely to be exhausted due to native hemeproteins, we tested the effect of addition of 5-ALA on CYP53B1 activity. There have been reports of 5-ALA

supplementation to yeast cultures, for example to *S. cerevisiae* (Cheng *et al*, 2006) and *P. pastoris* (Syed *et al*, 2011), although in these cases cultures with 5-ALA supplementation were not compared to ones without supplementation.

Transformants coexpressing CYP53B1 and UmCPR were used, since they had higher activities in most cases. Since there is no induction point when using a constitutive promoter such as the TEF promoter, 5-ALA (0.5 mM) was added at the point of inoculation to one of two sets of cultures. Biomass variation was not radically different between cultures grown in the presence or absence of additional 5-ALA (data not shown).

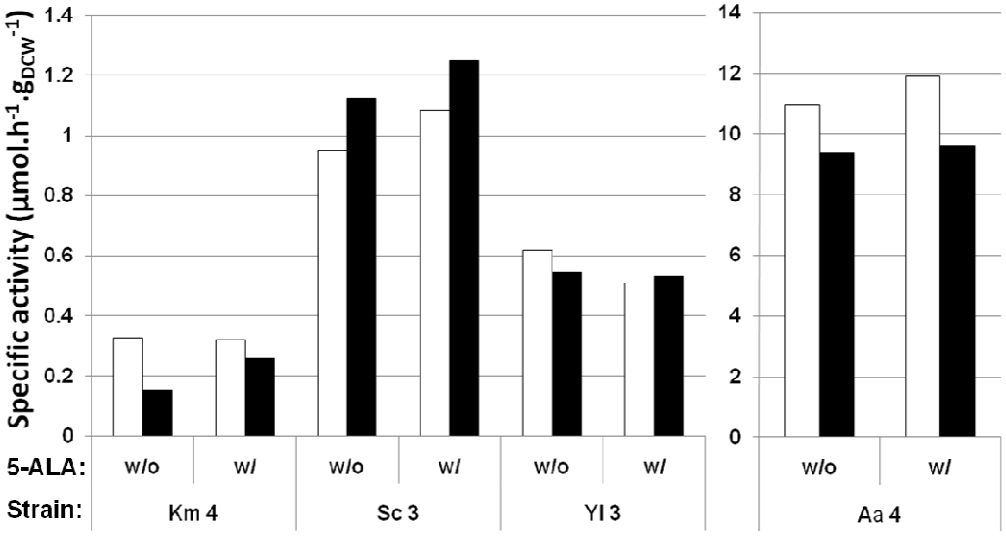



**Figure 4.16: Graph representing CYP53B1 activity in the absence (w/o) and presence (w/) of added 5-ALA.** Activities were monitored by calculating specific activities for samples taken after 9 h (white columns) and 24 h (black columns) biotransformation.

This was an exploratory experiment in which we only searched for major differences in activity, and as such the experiments were not done in duplicate. As demonstrated by figure 4.16 however, the addition of 5-ALA to the cultures made no significant difference to CYP53B1 activity in the transformants of all species except *K. marxianus.* The *K.*

*marxianus* specific activity calculated at 24 h was 67% higher when 5-ALA was supplemented, but even so it was still far lower activity than observed with the other species.

It is possible that in the other yeasts either heme content is not a limiting factor; or the heme is used for other cellular functions; or there could be degradation or limited uptake of 5-ALA; or combinations of these possibilities. These results however ruled out the possibility that insufficient heme production might explain the large differences in benzoate *p*-hydroxylase activity observed in the different yeasts.

The results presented in figure 4.16 reiterate that when using the 24 h time point for specific activity determination, we actually underestimate the activities of *A. adeninivorans* and *K. marxianus*, since the rates calculated at 9 h were better in these cases. The underestimation is however not responsible for *K. marxianus* having the lowest activity, since it is still far lower at 9 h as well. Instead, it really only re-emphasizes the superiority of the *A. adeninivorans* transformants.

### 4.6. Increasing substrate (benzoic acid (BA)) concentration in cultures of *A. adeninivorans* coexpressing CYP53B1 and UmCPR

The major question regarding this part of the work was answered, as we identified the host which most efficiently expressed CYP53B1 to be *A. adeninivorans*. Secondly, it was demonstrated that the activity could be further enhanced by coexpressing foreign CPRs, particularly the one from *U. maydis* (UmCPR).

Next we then wanted to see if we could increase the substrate concentration to achieve higher amounts of product. The highest activity previously obtained in our group using CYP53B1, under optimized conditions in shake flasks and in a bioreactor, was 5 $\mu mol.h^{-1}\ g_{DCW}^{-1}$ (Shiningavamwe *et al*, 2006; Obiero, 2006). This was achieved using a *Y. lipolytica* strain containing multiple copies of CYP53B1 under the strong inducible POX2

promoter, with an additional copy of the *Y. lipolytica* CPR under control of the ICL promoter. In the current report, using *A. adeninivorans* coexpressing CYP5B1 and UmCPR (transformant 5) under control of a constitutive promoter and under non-optimised shake flask conditions, we already obtained double the reported activity. In the *Y. lipolytica* studies however, the BA concentration was 41 mM, and Obiero (2006) reported that BA and pHBA concentrations of up to 160 mM were not toxic to *Y. lipolytica* if added to 36 h cultures with pH maintained at 8.

In contrast, the maximum concentration of benzoic acid used in the experiments discussed so far was only 5 mM. The next aim was to gradually increase the substrate concentration until conversion was hindered. Initially, higher substrate concentrations were added after 48 h of cultivation (figure 4.17).

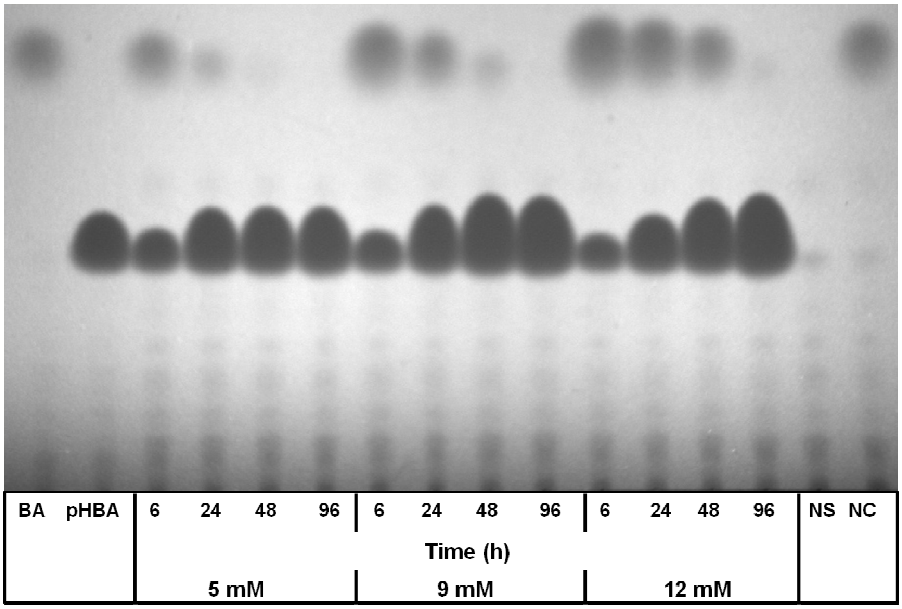



**Figure 4.17: TLC analysis of the effect of increased substrate concentrations on CYP53B1 activity in *A. adeninivorans* pKM173+CYP53B1+UmCPR transformant 5.** BA and pHBA represent substrate and product standards respectively; NS represents a control where no substrate was added to the test transformant; and NC is the negative control transformant not expressing any foreign P450 or CPR.

It is demonstrated by Figure 4.17 that as the substrate concentration increases, the time taken to convert nearly all of the substrate increases, but within 96h 12 mM BA is nearly completely converted to pHBA. From 15 mM conversion started to become less efficient. When much higher concentrations were used, i.e. 40 mM, the pH dropped too drastically, and no conversion occurred (data not shown). YPD buffered with potassium phosphate buffer (pH8) was then used to counteract acidification by the higher BA concentrations, and 41 mM BA was added (figure 4.18).

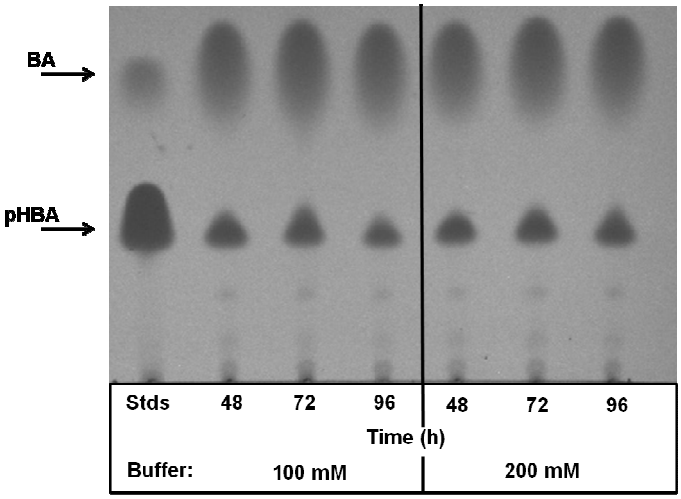



**Figure 4.18: TLC analysis of the conversion of 41 mM BA by *A. adeninivorans* coexpressing CYP53B1 and UmCPR (transformant 5).**

Only a limited percentage of conversion occurred, but even so the amount of pHBA produced was probably within the range of highest amounts in figure 4.17. As observed earlier though (figure 4.11), the buffer actually reduces activity. Therefore it was unclear how much the low conversions were attributable to high BA concentration and how much to buffer presence.

In our experiments, benzoic acid (BA) was added in the co-solvent DMSO and buffer was added to counteract acidification. The undissociated (associated) benzoic acid (pKa 4.19) was therefore introduced to the cultures, which then dissociated upon entering the medium with higher pH, releasing $H^+$-ions into the media and thereby

acidifying it to levels potentially detrimental to cell physiology. In the report on heterologous CYP53B1 activity in *Y. lipolytica*, an aqueous solution of benzoic acid (BA) adjusted to pH 8 with NaOH was added to the cultures (Obiero, 2006). At this high pH the benzoic acid dissociates, forming its corresponding salt with the sodium ions from the NaOH base. Addition of this benzoate salt to cultures avoids the acidification effects encountered in our case, provided that the culture pH remains sufficiently high to keep the dissociation-association equilibrium in favor of the salt-form. When pH was controlled in the reported cases, it was also done through the addition of NaOH to cultures. Therefore alkalinization of aqueous substrate solutions through addition of a strong base, like NaOH could potentially improve the tolerance to higher BA concentrations, but it was not investigated in our study.

Instead we decided to rather feed the substrate at fixed intervals. To test whether the feeding strategy would work, three culture conditions were set up. In all three cases, 5 mM would initially be added to 48 h old cultures. In the first case, ((i) in plate ‘A’ in figure 4.19), further 5 mM amounts were added at 24 h intervals. In the second case, ((ii) in plate ‘A’ in figure 4.19), further 2.5 mM amounts were added at 24 h intervals. In the third case ((iii) in plate ‘B’ in figure 4.19), cells were harvested after 24 h of biotransformation. The pellet was resuspended in potassium phosphate buffer, and another 5 mM substrate was added followed by re-incubation. In this way most of the substrate and product were removed from the cells, to eliminate the possibility of feedback inhibition.

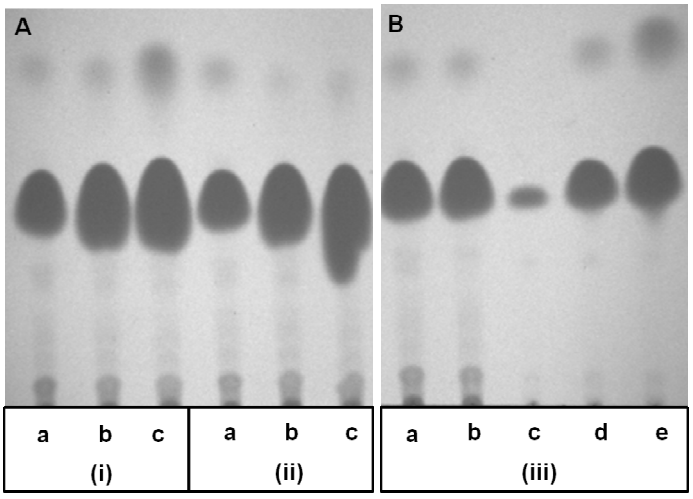



**Figure 4.19: TLC plates representing substrate feeding strategies for biotransformation of BA using a transformant of *A. adeninivorans* coexpressing CYP53B1 and UmCPR.**

Plate A: Culture (i) a – 24 h after initial addition of 5 mM BA, another 5 mM BA was added at this point; b – 24 h after 'a' another 5 mM BA was added, i.e. 48 h after the initial addition. c – Sample at 48 h after 'a', i.e. 72 h after the initial addition.

Culture (ii): a – 24 h after initial addition of 5 mM BA, another 2.5 mM BA was added at this point; b – 24 h after 'a' another 2.5 mM BA was added, i.e. 48 h after the initial addition. c – Sample at 48 h after 'a', i.e. 72 h after the initial addition.

Plate B: Culture (iii): a – 24h after initial addition of 5 mM BA, cells were harvested.

b – Sample from supernatant after harvesting. c – Sample from the resuspended cells after harvesting; to which another 5 mM BA was added. d – 24 h after 'c' another 5 mM BA was added. e – Sample at 24h after 'e'.

Evidently it is unnecessary to harvest the cells, as feedback inhibition does not seem to be a problem; in fact resuspended cells resulted in lower activity. Re-addition of either 5 mM or 2.5 mM does not seem to make much difference, although less total substrate was added in culture (ii) than in culture (i) in this case (7.5 mM as opposed to 10 mM), which may explain why addition of 2.5 mM appears to be better. With this knowledge, the feeding strategy was refined.

An accumulative substrate concentration of 15 mM was selected for the sake of comparison of three cultures. The first culture (A) received a once off addition of 15 mM after 48 h hours of growth. The second culture (B) received three additions of 5 mM each at 24 h hour intervals, starting after 48 h of culturing. The third culture (C) received six additions of 2.5 mM each at 12 h hour intervals, starting after 48 h of culturing. Samples were taken at regular intervals for 120 h of the biotransformation phase.

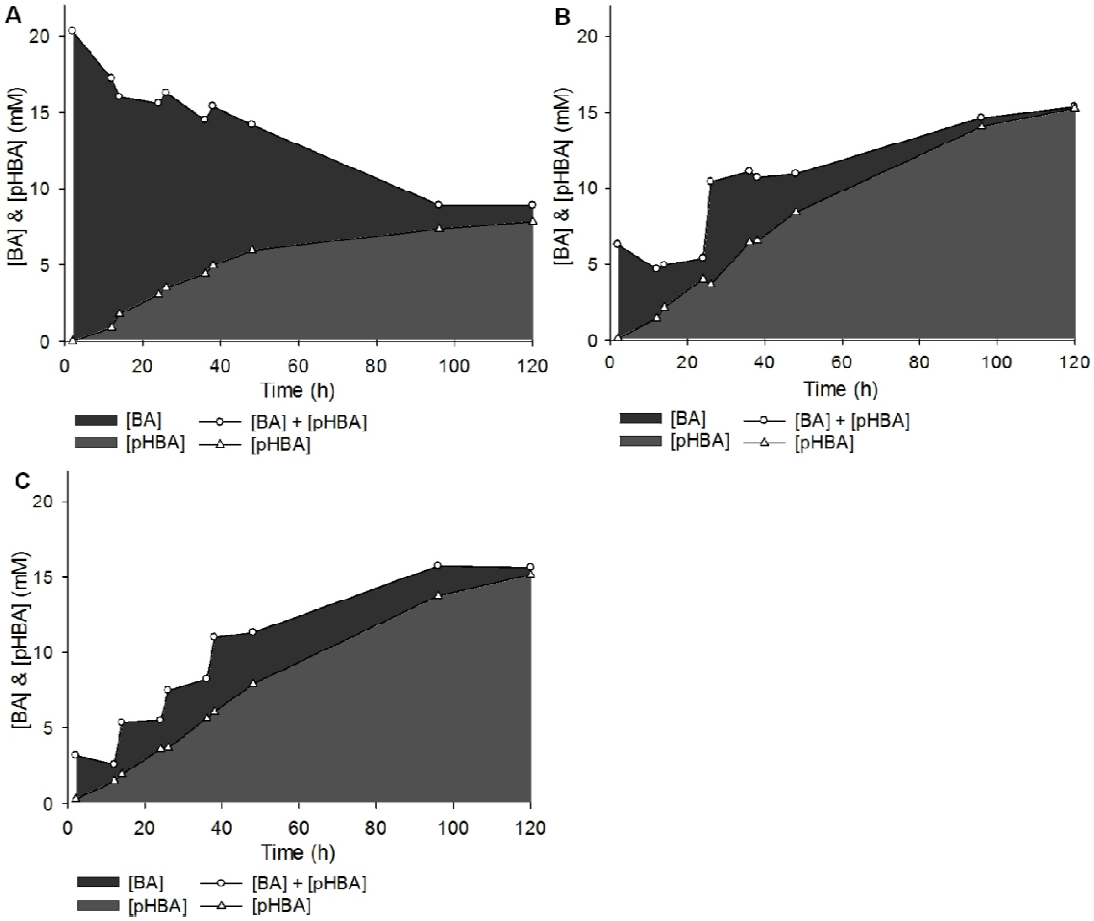



**Figure 4.20: Charts representing relative BA (black) and pHBA (gray) concentrations obtained by using the three different strategies.** Time '0' represents 48 h cultivation time, at which point the first BA additions took place. A - 15 mM BA added at 48 h of cultivation. B – Three additions of 5 mM BA at 24 h intervals, starting at 48 h of cultivation. C – Six additions of 5 mM BA at 12 h intervals, starting at 48 h of cultivation. The $2^{nd}$ addition in B and the $2^{nd}$ – $4^{th}$ additions in C are observable as spikes in BA concentration.

As depicted in figure 4.20, both of the true feeding strategies used (cultures B and C), worked really well, as the full 15 mM was recovered at 120 h after the initial substrate addition. The strategy in culture B appeared slightly better than the one used for culture C, although the differences were marginal. It does however mean that fewer additions are required, which reduces the experimental steps and contamination risk involved.

While all of the added substrate is accountable for in cultures B and C as product (pHBA) at 120 h, only 60% is accounted for in culture A. The total recovery (BA+pHBA) in culture A was fine initially but gradually decreased over time. This is a problem associated with substrate and / or product recovery, and does not necessarily mean that pHBA production is very much worse than in the other two cases, as the pHBA contributed 87% of the total recovery (BA+pHBA). This is still however lower than the 100% conversions in cultures B and C, indicating that the feeding strategies are probably better options.

The reasons for the decreased recovery in culture A are unclear, but experimental error is ruled out because the trend was conserved in samples from multiple time points. In addition, the cultures were run in parallel, with the sample collection, processing, and analysis of all samples being performed in parallel to each other. Yet the recovery problem was limited to culture A. One possibility is that the cultures are put under less pressure when the substrate is introduced in regular, small installments. Direct addition of the bulk amount possibly causes stress to the cell, perhaps causing the cells to stores some of the substrate and product in separate sub-cellular locations to reduce the impact of the high concentration. If the cells are not completely lysed and fractionated during our simple extraction technique, perhaps not all of the substrate and product are recovered. In other work done in our group, a similar situation was encountered, whereby further extraction procedures were required to be able to account for all of the substrate and product from an experiment (Gudiminchi, personal communication).

A common toxicity limiting action of microorganisms is pumping excess substances out of the cell using efflux pumps (Del Sorbo *et al*., 2000), but if the compounds were simply rejected from the cells they would have been detected in the supernatant. The same argument stands for if all of the substrate was not taken up by the cell, or if cell lysis occurred. The volatility of these compounds is negligible, ruling out evaporation from the medium after being excluded from cells.

Another possibility is that under such high benzoic acid concentrations, pathways for degradation of pHBA are activated. Such pathways were described for *A. adeninivorans* by Sietmann and co-workers (2009), where the LS3 strain could grow on low concentrations of pHBA but not on BA. Once the CYP53B1 was heterologously expressed in our case, it could have converted the BA to pHBA, which may then have been subsequently degraded over time.

We can however conclude that when using the simple and reasonably rapid extraction procedure employed during this study, more product can be recovered from cultures to which substrate was added in intervals. Therefore the feeding strategies also represent the simplest methods for convenient recovery of maximum product yields. Final substrate concentrations higher than 15 mM could possibly be achieved by applying such feeding strategies, but higher substrate concentrations were not investigated in this study. Certainly optimization by carefully controlling the environmental parameters in a bioreactor would lead to even better advances in conversions. The potential of this system is therefore highlighted by considering that these experiments were carried out rather basically and with minimal experimental control in shake flasks.

### 4.6.1. Investigating *p*-hydroxybenzoic acid (pHBA) degradation by *A. adeninivorans* transformant transformed with an empty vector

*A. adeninivorans* LS3 was reported to be able to degrade pHBA among other hydroxylated aromatic hydrocarbons (Sietmann *et al*, 2009). We decided to investigate whether this phenomenon was responsible for the results in figure 4.20, although we did not observe any noticeable decrease in the pHBA produced by our transformants in other cases. The reported concentrations which were tested were 50 mg/L and 100 mg/L; while if 5 mM was produced in this research, it converts to 690 mg/L. We decided to first test approximately 10% of those concentrations (10 mg/L and 70 mg/L) to test for any significant degradation. A control transformant transformed with the expression vector devoid of genes for P450s or CPRs was used. The pHBA was treated as a substrate in a standard biotransformation using buffered (100 mM potassium phosphate buffer, pH8) and non-buffered YPD medium.

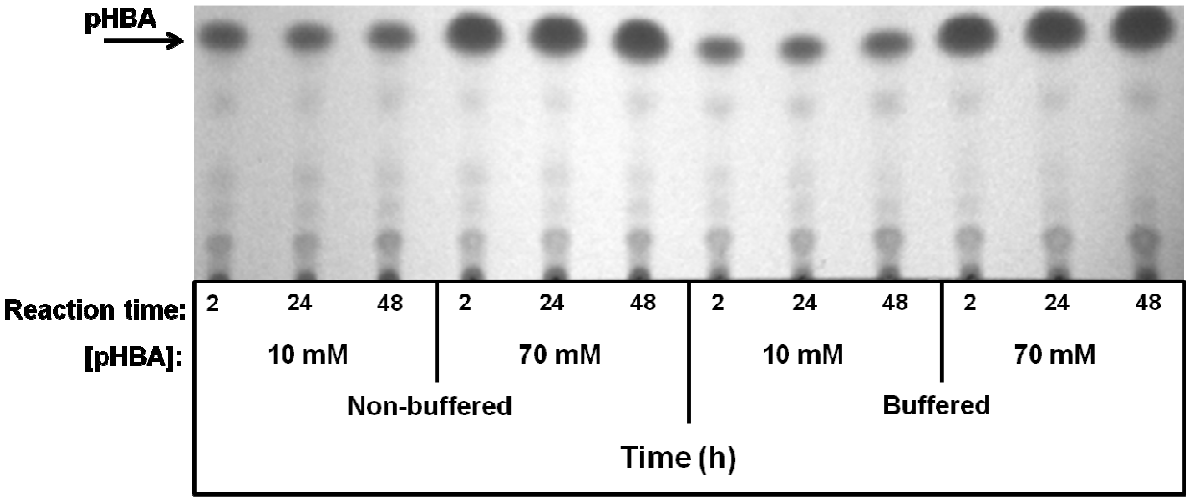



**Figure 4.21: TLC analysis of the degradation of pHBA by the transformant of *A. adeninivorans* used in this study.** Sampling times, substrate (pHBA) concentration, and buffer presence are all indicated.

For this experiment, buffer was added from inoculation when it was included. The buffered cultures had lower growth than the un-buffered cultures, but no further effect

on biomass was observed under different pHBA concentrations. The pH values of all cultures during this experiment remained around a value of 8 or higher, irrespective of buffer presence or pHBA concentration. Although the TLC analysis in figure 4.21 is not quantitative, significant differences would be detectable, but none were observed. Whether or not pHBA degradation could possibly be triggered by high BA concentrations was not investigated here, but we confidently report our general results on pHBA production without fear of significant degradation by wild-type enzymes.

## 4.7. Further investigation using *Kluyveromyces marxianus*

### 4.7.1. Variation of induction conditions using an inducible *K. marxianus* inulinase (INU) promoter

Although the aim if this research was to test the wide-range expression system for the expression of the P450s in different yeasts, experiments were also conducted on *K. marxianus* using an alternative vector which was available for this yeast. This vector, pKM63, contains a sucrose-inducible inulinase promoter from *K. marxianus*. Dr. M. Labuschagne constructed a strain of *K. marxianus* isolate 2.1 by transformation with the pKM63 vector carrying CYP53B1 and YlCPR both under the control of copies of the *K. marxianus* inulinase promoter. A control transformant, which had been transformed with an empty vector, was also included in the experiment.

The general parameters for comparative biotransformations using the different species were initially defined as 48 h growth at 28℃ prior to substrate addition. However, comparable and often better growth has been reported for *K. marxianus* at 37°C (Rocha *et al*, 2011; Rech *et al*., 1999), in fact the thermotolerance of this organism is one of its most appealing features for industrial application (Fonseca *et al*, 2008). Due to the industrial relevance of higher reaction temperatures, we compared activities at both 28℃ and 37℃. Additionally, we wanted to test the effect of reducing the cultivation time before substrate addition from 48h to 24h for both temperatures, to see if the overall

process time could be reduced, especially since this organism has higher growth rates (figure 4.10) and has in fact been described as ‘the fastest-growing eukaryote’ (Groeneveld *et al*., 2009), another one of its most appealing features.

Cells were cultured in 25 ml YPS medium in 250 ml flasks at either 28℃ or 37℃ for either 24h or 48h before the substrate was added, and samples were taken after 24 h incubation. The inulinase promoter responds to sucrose as a carbon source, but since sucrose was used as the sole carbon source from the beginning of the cultivations, constitutive expression was mimicked during these experiments. For these tests the final substrate concentration was only 1mM. These results are only indicative of volumetric yield, as biomass was not taken into account (figure 4.22).

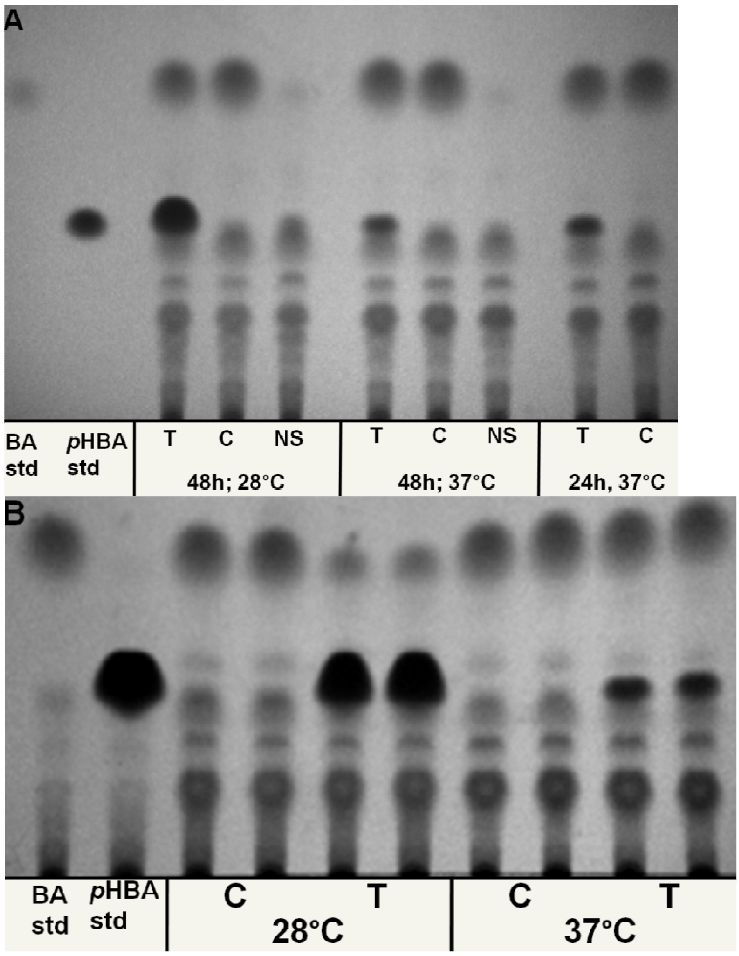



**Figure 4.22: TLC analysis of the effects of different parameters on CYP53B1 activities of *K. marxianus* transformants.** BA std – benzoic acid standard, pHBA std – *para*-hydroxybenzoic acid standard, T – test transformant transformed with pKM63+CYP53B1+YlCPR, C – control transformant transformed with pKM63, NS – no substrate added to culture of test transformant.
Plate A: Cells were cultured for 48h at 28℃ and 37℃, and 24h at 37℃ prior to substrate addition. Plate B: Cells were cultured for 24h at 28℃ and 37℃. Samples were taken after 24 h of biotransformation in all cases.

Better pHBA yields were observed in cultures grown at 28℃, confirming this as the preferable incubation temperature. For *K. marxianus* however, adding substrate after 24 h cultivation seems to be a better option than 48 h growth. The growth curve of this yeast on sucrose resembled that observed on glucose illustrated in figure 4.10, in that

stationary phase was reached at 24 h growth rather than the 48 h required for most other yeasts tested (data not shown).

Although the pHBA produced in these cases were not quantified, they clearly appear to be better than those obtained using the TEF promoter (figures 4.4 and 4.15). From figure 4.23(A) one can see that certainly more than 10% (i.e. 0.1 mM) and possibly closer to 50% (i.e. 0.5 mM) of the 1 mM BA is converted to pHBA. Using the TEF promoter on the other hand, *K. marxianus* transformants could not produce 0.05 mM pHBA from 5 mM BA. An important difference however, is that the transformants containing the INU promoter-driven CYP53B1 were assayed sooner after being transformed than those containing the TEF promoter-driven CYP53B1. Dr. Labuschagne demonstrated that *K. marxianus* had high instability of homologously integrated DNA (Albertyn *et al*, 2011), while similar findings have also been reported in literature (Kooistra *et al*., 2004; Abdel-Banat *et al*, 2009).

The effect of supplementing additional sucrose to the cultures was also evaluated, to determine whether additional sucrose would increase activity. The culture initially contained 2% v/v sucrose, and an additional 2% v/v was added after either 12 h or 24 h growth at 28℃. Substrate was added after 24 h grow th. Cultures were incubated for a further 24 h before samples were taken.

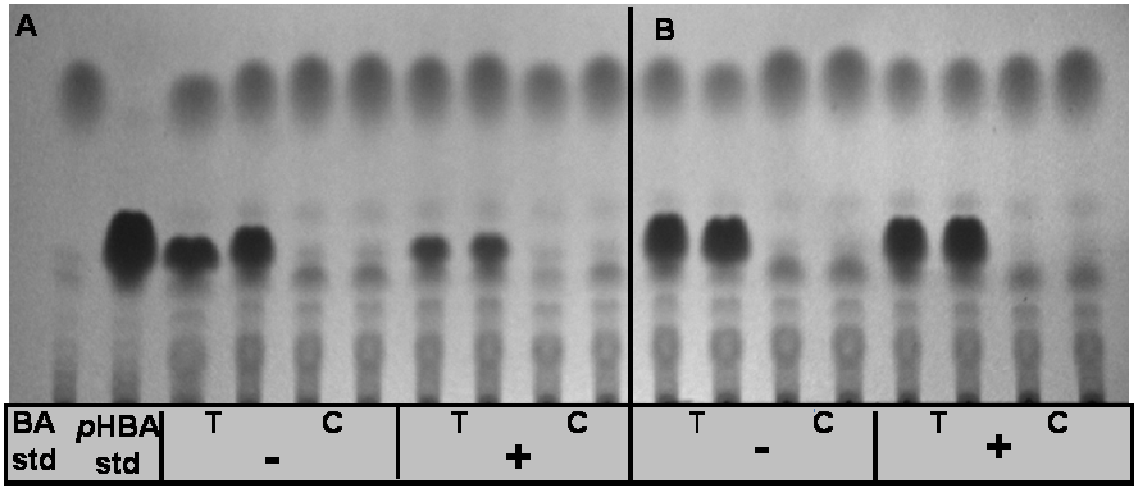


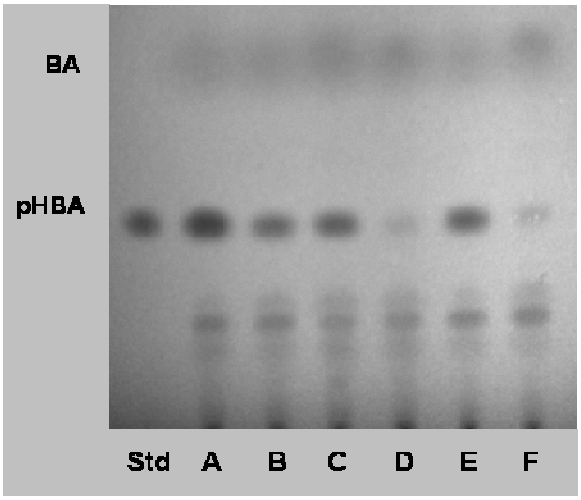

**Figure 4.23: TLC analysis of the effects of supplementing additional sucrose to the culture of *K. marxianus* expressing CYP53B1.** BA std – benzoic acid standard, pHBA std – *para*-hydroxybenzoic acid standard, T – test transformant, C – control transformant, '-' indicates that no additional sucrose was added, while '+' indicates that sucrose was supplemented. Extra sucrose (2%) was added after 12 h (A) or 24 h (B) growth in YPS medium.

The additional sucrose did not improve activity, and in fact had an inhibitory effect on activity when added after only 12 h of growth, probably due an accumulative effect due to incomplete consumption of the first addition. Excessive glucose or sucrose concentrations cause preferential utilization of these carbon sources at the expense of other cellular responses, including uptake of lower priority carbon sources, which would include benzoic acid in our case (Verstrepen *et al*., 2004).

### 4.7.2. Testing different induction and cultivation conditions for *K. marxianus* using the TEF promoter in the broad range vector

We decided to confirm whether or not the effects of the different conditions observed using the INU promoter are conserved when using the TEF promoter. For these tests, a promising transformant (number 4) of *K. marxianus* transformed with pKM+CYP53B1+RmCPR was selected from the initial screen (figure 4.4). In addition to

comparing substrate addition times and cultivation temperatures, various culture volumes were also investigated for different aeration / oxygen availability (figure 4.25).




| | Growth temperature ($^{o}$C) | Time of substrate addition | Culture volume / flask volume |
|---|---|---|---|
| **A** | 28 | 24h | 20ml / 100ml flask |
| **B** | 28 | 48h | 20ml / 100ml flask |
| **C** | 28 | 48h | 10ml / 100ml flask |
| **D** | 28 | 48h | 5ml / test tube |
| **E** | 37 | 24h | 10ml / 100ml flask |
| **F** | 37 | 48h | 10ml / 100ml flask |

**Figure 4.25: TLC analysis of the comparison of CYP53B1 activity in *K. marxianus* under different conditions.** Std – *para*-hydroxybenzoic acid standard, A-F cultures cultivation parameters tested, as described in the table below the figure.

The general screening conditions are represented by 'D', and the general biotransformation conditions for further studies are represented by 'C' in terms of the medium: flask-capacity ratio. It is obvious that using test tubes resulted in far poorer results than using flasks, but they are still acceptable for initial screening of transformants though, since activities of all transformants are proportionately lower, meaning that relative differences are still distinguishable. Cultivating at 28°C was again preferred over 37°C, and adding substrate after 24h growth again seemed preferable

over addition at 48h at 37°C for this organism. Doubling the culture volume from 10% volumetric capacity to 20%, thereby decreasing the aeration in the flask appeared to be beneficial when the substrate was added 24h earlier than usual. This was however more likely attributable to the addition time than the medium volume, since culture B had lower activity than culture C.

These studies can benefit further optimization of *K. marxianus* biotransformations. However, for direct comparisons between yeasts, the conditions of growing the cultures at 28°C for 48h prior to substrate addition, in a volume of 10% of the volumetric capacity of the flask, were maintained.

## (ii) CYP557A1

### 4.8. Screening for activity in *A. adeninivorans* transformants coexpressing CYP557A1 and CPR(s)

cDNA for the CYP557A1 gene was synthesized from mRNA of *Rhodotorula retinophila* using reverse transcriptase PCR during a doctoral study in our research group, and expressed in *Y. lipolytica* (Shiningavamwe, 2004). The deduced protein sequence has 17-22% identity with the CYP52 family of fatty acid and alkane hydroxylases in yeasts, and 28-33% identity with putative P450s from *Ustilago maydis*. One of the latter have through deletion studies been implicated in fatty acid hydroxylation (Hewald *et al*., 2005). So far the preferred substrate range and indeed activity for this protein have not been identified. Since the mRNA used for cDNA synthesis was extracted from cells grown in environments saturated with limonene and decane vapors, it is possible that these could be substrates for this enzyme (Shiningavamwe, 2004).

We decided to try our broad-range expression system to investigate activity of CYP557A1. We were particularly optimistic about using *A. adeninivorans* which gave such promising activities for CYP53B1, from a fellow *Rhodotorula* species member,

*Rhodotorula minuta*. Since the best results for CYP53B1 activity were obtained by coexpression with the UmCPR in *A. adeninivorans*, we decided to initially focus our efforts to identify activity of this P450 on transformants of *A. adeninivorans* transformed with pKM173+CYP557+UmCPR. Substrates to be tesed for the CYP557A1 activity screening, included hexylbenzene, nonyloxybenzoic acid, nonylbenzene, limonene and *p*-cymene.

### 4.8.1. Evaluating CYP557A1 activity in transformants coexpressing UmCPR using various substrates

As in the case of CYP53B1 integration of the cloned CYP557A1 and UmCPR into the rDNA of transformants of *A. adeninivorans* was confirmed with PCR. A control transformant transformed with an empty vector, and another control transformant with only UmCPR cloned were compared to test transformants with CYP557A1 and UmCPR cloned. Since *A. adeninivorans* is an alkane utilizing yeast, it possesses wild type enzymes (most likely P450s) with the ability to hydroxylate not only alkanes but also limonene (Van Rensburg *et al*., 1997), probably also fatty acids, hexylbenzoic acid, nonyloxybenzoic acid, alkylbenzenes and *p*-cymene. Other alkane-utilizing yeasts such as *Yarrowia lipolytica* (Fickers *et al*., 2005) and *Candidia tropicalis* (Lu *et al*., 2010) can also degrade substrates such as fatty acids, hexylbenzoic acid, nonyloxynenzoic acid and alkylbenzenes as well (Shuping, 2007; Ramarobi, 2008). If the transformant expressing only UmCPR showed equal or better activity than transformants with CYP557A1 cloned, the improvement would be attributable to the expressed UmCPR only, which would be benefitting the wild-type activities toward these substrates.

Biotransformations with transformants were carried out as with CYP53B1 transformants with substrates added after 48 h growth at 28°C to YPD grown cultures.

The three alkylbenzenes (hexylbenzene, nonylbenzene and *p*-cymene) and the aliphatic monoterpene (R-(+)-limonene) were subsequently tested. Relative productivities were

determined by considering the ratios of area of product to area of internal standard. Standard curves were not constructed and actual concentrations of products were therefore not determined, since in these preliminary experiments the goal was only to find a substrate that will reveal large differences between tests and controls. Calculated concentrations would merely change the values obtained, not affect the relative trends between samples.

Out of concern that over extended reaction times native enzymes would be induced and compete for the substrates; the sampling in these initial experiments was done at 3h after substrate addition. Due to similarities in substrate structures hexylbenzene and nonylbenzene will be discussed together, while *p*-cymene will be discussed with R-(+)-limonene.

**Hexylbenzene and nonylbenzene**

The expected products from hexylbenzene and nonylbenzene were respectively phenylacetic acid (PAA) and benzoic acid (BA), because if terminal hydroxylation occurs on the alkyl chains of these substrates, the resultant alcohols could be further oxidized within the cells to the corresponding acids, and the acids would then be broken down by the β-oxidation pathway of the cell (Ramarobi, 2008). We therefore used standards of these two compounds as references for GC analysis. PAA and BA were eventually also the only products detected in GC analysis of samples from the biotransformations of hexylbenzene and nonylbenzene. The relative amounts of PAA and BA produced by the different transformants and controls are compared in Figure 4.26.

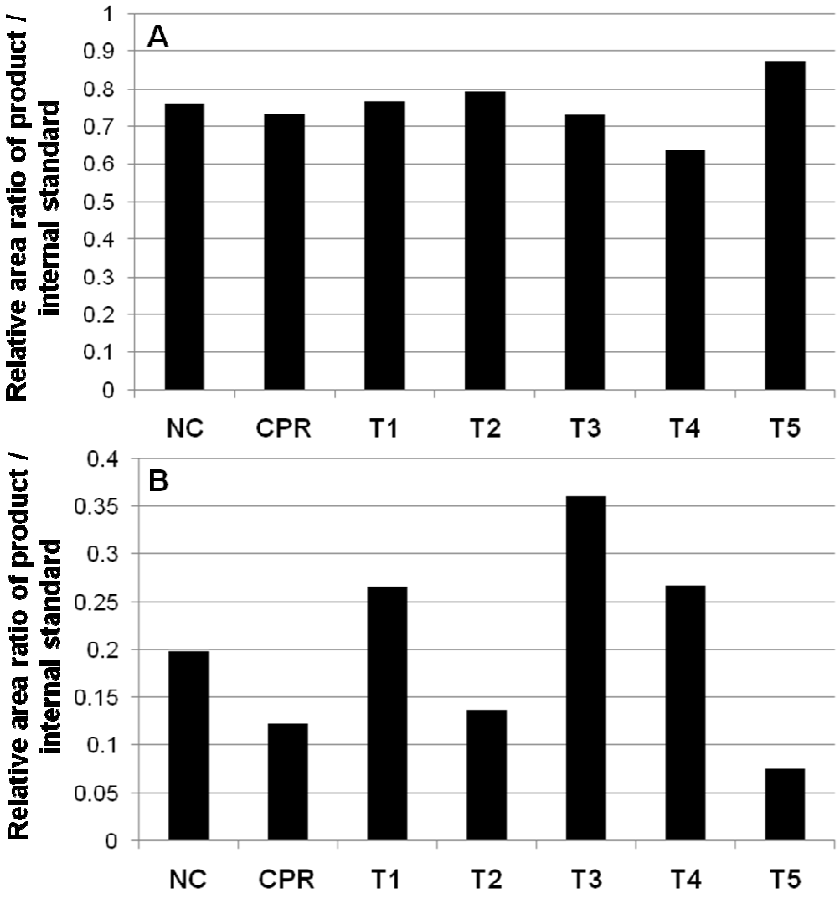



**Figure 4.26: PAA and BA production from hexylbenzene (A), and nonylbenzene (B) by *A. adeninivorans* transformants with CYP557A1 and UmCPR cloned.** The ratios of the relative areas of products, (phenylacetic acid (PAA) in A and benzoic acid (BA) in B), internal standard (myristic acid) are plotted on the Y-axis. NC – transformant with no foreign P450 or CPR cloned; transformant with only UmCPR cloned; T1-5 – transformants with CYP557A1 and UmCPR cloned.

The results require cautious treatment, as experiments were not done in duplicate, nor were biomass values considered. For hexylbenzene conversion to PAA (A), transformant 5 seemed slightly more promising than the other transformants. For nonylbenzene conversion to BA (B) on the other hand, transformant 3 appeared the most promising. It is difficult to explain why PAA production from hexylbenzene by the

different strains was so similar, while there was such a large variation in BA production from nonylbenzene.

### *p*-Cymene and R-(+)-limonene

Hydroxylation of limonene can occur at various positions, but we used perillic acid (PA), which is the further oxidized form of the C7 hydroxylated product (figure 4.27A), as a reference standard. *p*-Cymene has a similar structure to limonene, and the product of hydroxylation at the same position as limonene gives *p*-isopropylbenzoic acid (pIBA) as a product (4.27B). Careful inspection of GC chromatograms revealed PA and pIBA as the only products formed from limonene and *p*-cymene. No obvious peaks were also observed in chromatograms from test transformants which were absent in controls. The relative amounts of PA and pIBA produced by the different transformants and controls are compared in Figure 4.28.

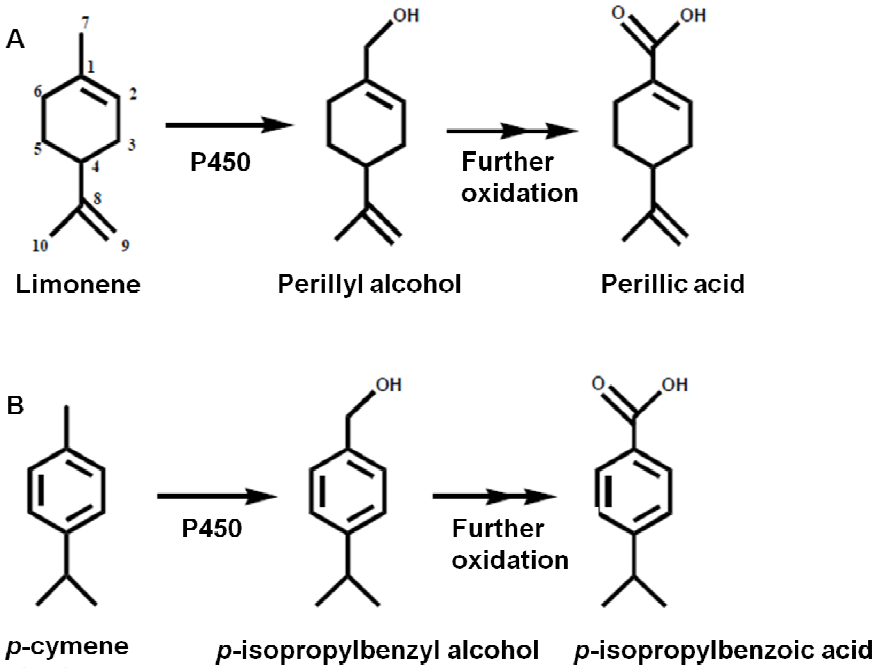



**Figure 4.27: Chemical structures of limonene (A) and *p*-cymene (B) and the expected product formation after hydroxylation.**

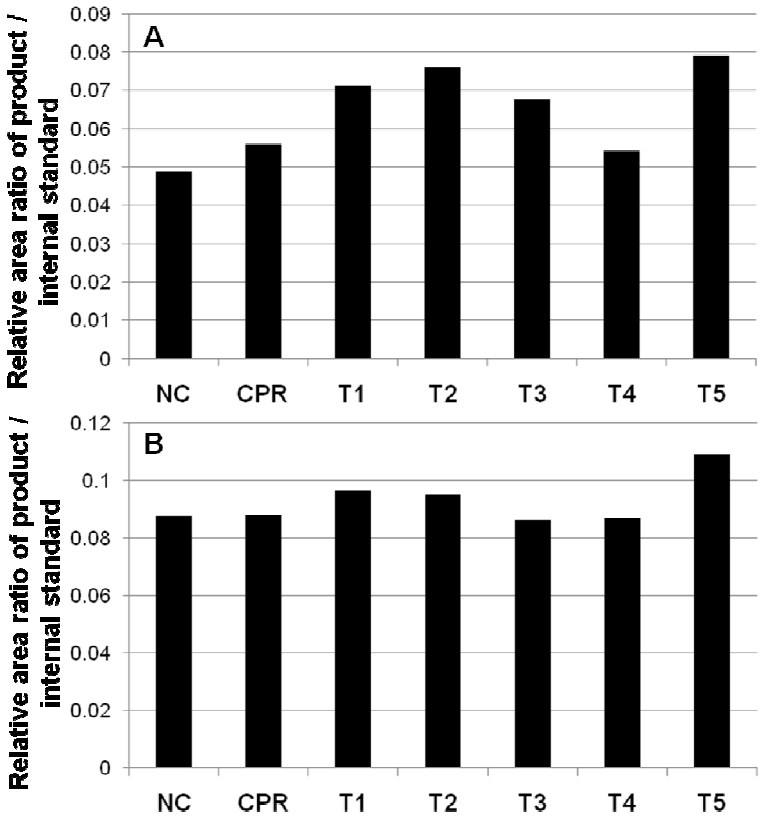



**Figure 4.28: PA (A) and pIBA (B) production from limonene and p-cymene by *A. adeninivorans* transformants with CYP557A1 and UmCPR cloned.**The ratios of the relative areas of products, (PA in A and pIBA in B), over internal standards are plotted on the Y-axis. NC – transformant with no foreign P450 or CPR cloned; CPR transformant expressing only UmCPR; T1-5 – transformants with CYP557A1 and UmCPR cloned.

*A. adeninivorans* has previously been reported to convert limonene to perillic acid (van Rensburg *et al*, 1997). It was anticipated that CYP557A1 activity would result in significantly higher PA formation in test strains when compared to control strains. Differences between transformants and controls were unfortunately disappointingly small. It was however promising that the largest differences were observed with limonene and it was also very promising that the same transformant, transformant 5 produced the most product from limonene, p-cymene and hexylbenzene.

Although the screening was not performed in duplicate and that biomass was not taken into account, the fact that one transformant gave the most promising results for 3 of the 4 tested substrates, was assumed to be non-coincidental. The other explanation would require this transformant to have had significantly higher biomass production than the other transformants during every assay. The second most promising strain for biotransformation of hexylbenzene and limonene was transformant 2; therefore transformants 2 and 5 were used for further analysis.

These transformants were compared to the controls used before, as well as an additional control transformant with CYP53B1 and UmCPR cloned. The idea was that the latter control will show whether a cloned CYP that does not have limonene hydroxylase activity, will have a negative effect on the wild-type activity due to reduced energy and resources, including CPR.

Limonene bioconversion was tested with three cultures of each transformant and samples were taken after 4 h biotransformation. A standard curve for perillic acid was constructed to quantify PA, and biomasses were determined so that specific activities could be determined.

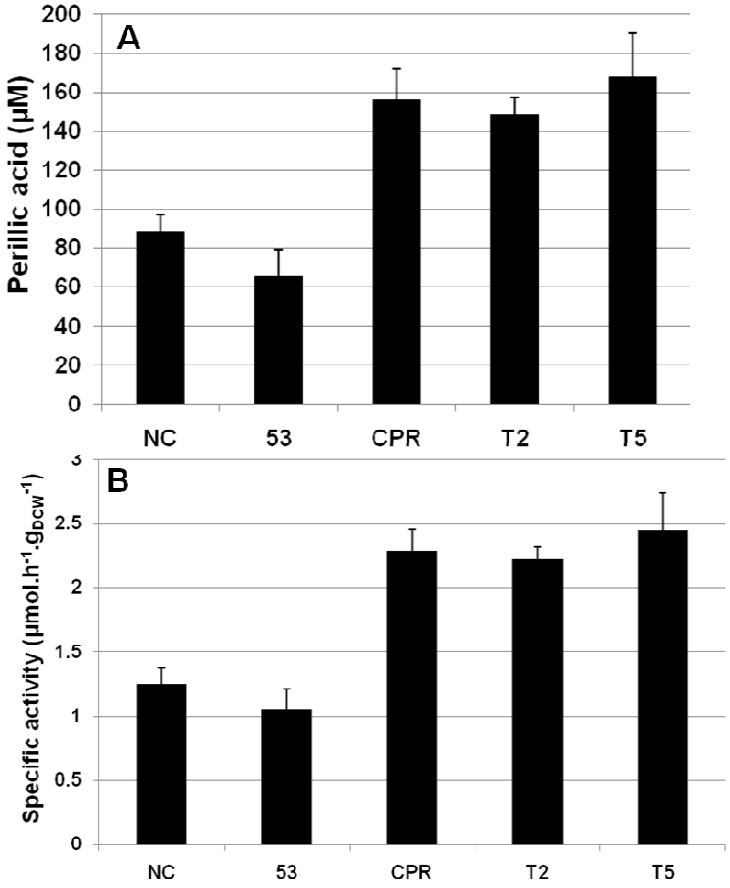



**Figure 4.29: Yields (A) and specific activities (B) of PA production from limonene by *A. adeninivorans* transformants.** NC – transformant with no P450 or CPR cloned; 53 – transformant with CYP53B1 and UmCPR cloned; CPR – transformant with only UmCPR cloned; T2 and T5 – transformants with CYP557A1 and UmCPR cloned.

Firstly, the test transformants did exhibit better activity than the NC control transformant, but so did the transformant expressing only the UmCPR. This indicates that the improved activity is probably exclusively due to the effect of the foreign CPR improving electron transport to native enzymes. Although there were differences between the CPR control and the test transformants, they were disappointingly lower than would be expected for efficient utilization of the substrate by CYP557A1.

The expression of CYP53B1 which does not have activity towards limonene did in fact decrease the conversion of limonene, even though it was coexpressed with UmCPR,

with activity much lower than that of the strain with only UmCPR cloned. This result can be interpreted in one of two ways. It might be considered as an indication that CYP557A1 is expressed and indeed has activity towards limonene, because if like CYP53B1 it did not have activity towards limonene the wild type activity towards limonene would have been similarly reduced in the CYP557A1 transformants. Alternatively it might be an indication that CYP557A1 is not sufficiently expressed to have an impact on the wild type activity.

### 4.8.2. Evaluating CYP557A1 activity towards limonene in transformants coexpressing RmCPR

We decided to also test transformants coexpressing RmCPR as well as those expressing only CYP557A1. Any activity improvements observed in transformants with only CYP557A1 cloned would indicate expression of active CYP557A1, discounting the improvement of wild-type activity caused by the cloned CPR. Results from the initial screen are shown in figure 4.30.

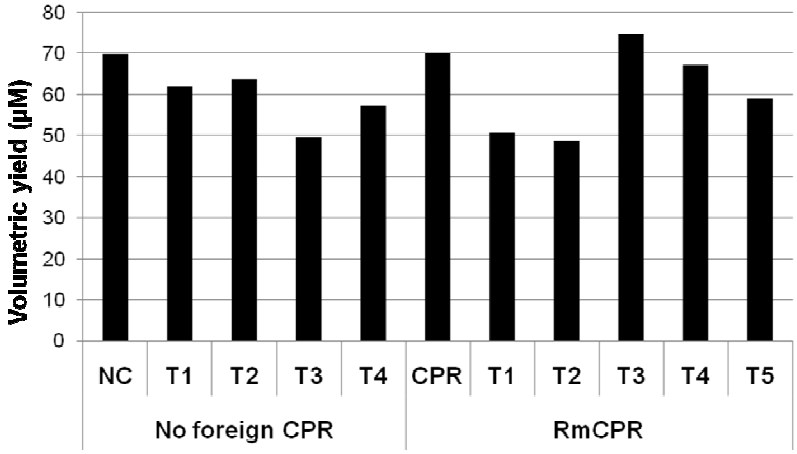



**Figure 4.30: PA production from limonene by *A. adeninivorans* transformants.** NC - transformant with no foreign P450 or CPR cloned; CPR – transformant with only RmCPR cloned; T1-5 – transformants with either only CYP557A1 (‘no foreign CPR’) or CYP557A1 and RmCPR (‘RmCPR’) cloned.

Specific transformants were selected from the initial screen (figure 4.30) for more thorough investigation in triplicate cultures (figure 4.31).

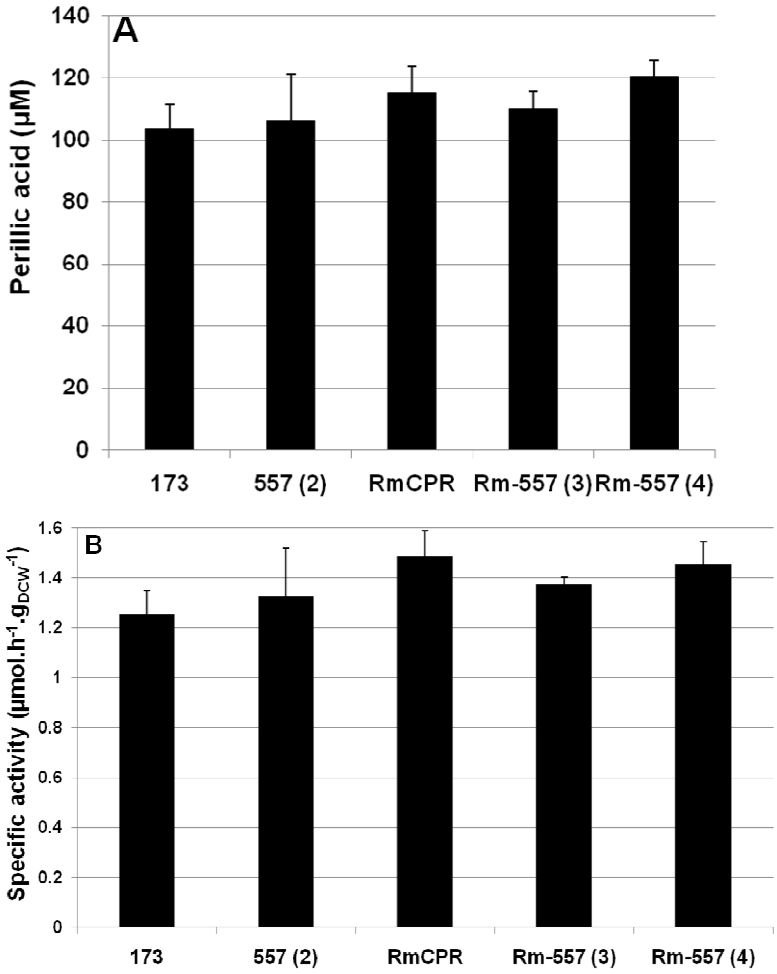



**Figure 4.31: Yields (A) and specific activities (B) of PA production from limonene by *A. adeninivorans* transformants.** 173 – transformant with no P450 or CPR cloned; 557 (2) – transformant with only CYP557A1 cloned; RmCPR – transformant with only RmCPR cloned; Rm-557 (3) and (4) – transformants with CYP557A1 and RmCPR cloned.

The results of figure 4.31 resemble those obtained previously (figure 4.29), in that all transformants where a P450, a CPR or both were cloned had higher activities than the transformant without any cloned CPR or P450. The major factor contributing the higher activity however still seems to be the cloned CPR rather than the cloned P450. Some

transformants had lower activities than the control (figure 4.30), as in the case when a transformant with CYP53B1 cloned was tested for limonene conversion (figure 4.29), which may indicate that the CYP557A1 is functionally expressed but limonene is not a substrate for it.

An interesting observation is that while the specific activity of the control transformant was equal in experiments involving either cloned CPR, the specific activities of transformants containing the UmCPR (with or without CYP557) were consistently higher than transformants containing the RmCPR (with or without CYP557). This most likely demonstrates that the UmCPR is assisting the native *A. adeninivorans* P450s better than RmCPR, again suggesting that this CPR is a superior CPR, as discovered with CYP53B1 (section 4.5.3).

The overall conversion of limonene seemed rather low overall, with the highest amount of perillic acid obtained in this case within 4 h by a transformant without any cloned P450 or CPR was 103 µM; while 167 µM was produced by a transformant with both CYP557 and UmCPR cloned. However, the previously report on limonene biotransformation by wild-type *A. adeninivorans* CSIR Y-1149 was 360 µM after at least 24 h, which actually makes our conversions appear quite promising (van Rensburg *et al*, 1997).

More investigations into CYP557A1 activity in *A. adeninivorans* were performed, including screening with 4-hexylbenzoic acid (HBA) and nonyloxybenzoic acid (NOBA); and biotransformations using resting cells. Although the best chance for obtaining activity for CYP557A1, based on results for CYP53B1, would probably be in *A. adeninivorans,* a considerable amount of work was done on expression of CYP557A1 in *K. marxianus* as well. All of the substrates tested with *A. adeninivorans* transformants, i.e. HBA, NOBA, hexylbenzene and nonylbenzene, were also tested for *K. marxianus*; but none of that work could conclusively prove active expression of this enzyme either (data not shown).

It is uncertain whether or not the CYP557A1 is being correctly expressed, or whether the activity was not detected because the wrong substrates were tested. More work is required to answer these questions, possibly including expression in *E. coli*. Provided that active protein will be produced, the activity could be monitored without concern of interference by wild-type enzymes. Alternatively, purified enzymes could be considered, but as discussed in chapter 1 this is a difficult route to follow when working with P450s, particularly class II P450s. Since neither of these approaches fall within the aims of this study however, further work with this enzyme was abandoned.

### 4.9. Effect of 1, 10-phenanthroline on whole cell activity of an *A. adeninivorans* transformants

1, 10-phenanthroline was reported to inhibit the promoter of CYP1A1 in mouse hepatoma cells, while stabilizing already synthesized mRNA and protein products of CYP1A1 (Chou *et al*., 2010). Supposing that native P450s could be induced by substrate addition to *A. adeninivorans* cultures, we proposed that adding 1, 10-phenanthroline just before the substrate could theoretically suppress induction of wild-type P450s, while stabilizing already produced heterologous enzymes. That is of course assuming that the 1, 10 phenanthroline will have similar effects in our system as in the case of CYP1A1. Preliminary work done in our group indicated that this may be the case (Coetzee, personal communication).

If successful, this approach would result in minimized background activities which interfere with the interpretations of CYP557A1 results. To test this phenomenon however we would need to use a transformant with confirmed activity, therefore we chose the pKM173+CYP53B1+UmCPR transformant 5. The 1, 10-phenanthroline was added 1h prior to substrate addition, to allow time for it to take effect. Both 1, 10-phenanthroline and benzoic acid were added in DMSO, so that the final DMSO

concentration was maintained as 2% v/v whether or not 1, 10 phenanthroline was added.

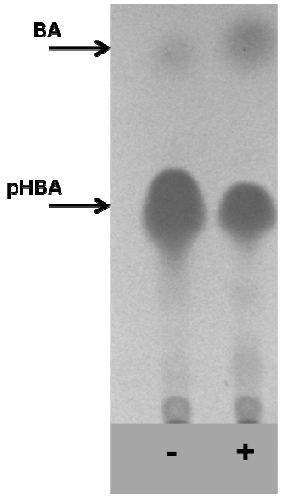



**Figure 4.31: TLC analysis of the effect of 1, 10-phenanthroline on CYP53B1 activity in whole cells of *A. adeninivorans* transformants.** '-' indicates the culture to which 1, 10-phenanthroline was not added, as opposed to '+' which indicates the culture to which 1, 10-phenanthroline was added.

At the time of addition, the biomass required for biotransformation was already produced, therefore any negative effects on the cell viability caused by 1, 10-phenanthroline should not make a difference to the activity of the enzymes. The 1, 10-phenanthroline addition however displayed in fact the opposite effect from what we expected, lowering the conversion of benzoic acid to *p*-hydroxybenzoic acid. This was probably due to inhibitory effects to other cellular components which may indirectly benefit CYP53B1 activity, such as dehydrogenases involved in NADPH regeneration. Therefore addition of this inhibitor was unlikely to assist in CYP557A1 activities either, but it was nevertheless attempted in *A. adeninivorans* transformants with CYP557A1 and UmCPR cloned, with no convincing results (data not shown).

## Part C: Recombinant strains expressing self-sufficient P450s

Both CYP102A1 (P450BM3) and CYP505A1 (P450Foxy) are self-sufficient P450s comprising of natural fusions between cytochrome P450 and P450 reductase (CPR) domains. They share 36% amino acid identity and both are sub-terminal fatty acid hydroxylases. The biggest differences are in their origins, as P450BM3 is prokaryotic, since it was isolated from the bacterium *Bacillus megaterium*; while P450Foxy is eukaryotic, as it was isolated from the fungus *Fusarium oxysporum.* The current and most likely explanation is that bacteria obtained these types of genes from eukaryotes *via* horizontal gene transfer (Kitazume *et al*, 2000).

Naturally P450BM3 is a soluble cytosolic enzyme in *B. megaterium*, while P450Foxy is membrane bound in the endoplasmic reticulum (ER) of *F. oxysporum*. Furthermore, they show different preferences in chain-length of fatty acid substrates, and the catalytic rate of BM3 is higher than that of P450Foxy. Fatty acids are impractical substrates when using whole cell yeast systems for biotransformations, because of they are rapidly degraded by β-oxidation. As discussed in chapter 1, an assay using *para*-nitrophenoxycarboxlic acids (pNCA) as a non-natural substrate was developed (Schwaneberg *et al*, 1999). These substrates are recognised as analogs for fatty acids, and the chromophore *p*-nitrophenol (*p*NP) is released. The success of these substrates encouraged our group to test the commercially available compound hexylbenzoic acid (HBA) as a potential non-natural substrate analog for P450BM3. Gudiminchi and Smit (2011) showed that HBA is indeed converted by P450BM3 *via* sub-terminal hydroxylations of the alkyl chain. The benzoic acid component of the substrate absorbs UV light and can be used for detection using TLC plates containing a fluorescence indicator as described in part B (Gudiminchi and Smit, 2011). Van Rooyen (2005) and Shuping (2007) demonstrated that alkane degrading yeasts such as *Y. lipolytica* and *Candida tropicalis* terminally hydroxylate HBA to produce, after oxidation, the intact (4-(5-carboxypentyl)benzoic acid, which is then further degraded *via* β-oxidation to accumulate a short-chain dioic acid (4-(5-carboxymethyl)benzoic acid; which is not

further degraded. These two products are completely different from the sub-terminal hydroxylation products formed by CYP102A1 and that we expected from P450Foxy.

All activities reported in the following sections are from samples taken after 24 h biotransformation, unless specifically indicated otherwise.

### 4.10. Expression of CYP505A1 in *E. coli* and biotransformation of 4-hexylbenzoic acid (HBA)

Since 4-hexylbenzoic acid was shown to be a non-natural substrate for CYP102A1, we anticipated that it could also be a substrate for CYP505A1 (Gudiminchi and Smit, 2011). Since that work was conducted using *E. coli*, and CYP505A1 has also been actively expressed in *E. coli* (Kitazume *et al*, 2002), we decided to first test for HBA conversion by CYP505A1 in *E. coli*. CYP505A1 had previously been cloned into the pET28(b) vector and pET28b+CYP505A1 was used to transform *E. coli* BL21 by Dr. J. van Marwijk. After IPTG induction in the presence of 5-ALA, cells were harvested and reactions were set up in amber bottles using resting cells at a concentration of approximately 167 $g_{WCW}$ / L and final HBA concentration of 0.5 mM. Reactions were run for 0, 2, 4, 6 and 24 h intervals, before being stopped by addition of hydrochloric acid (HCl). The results are shown in figure 4.32.

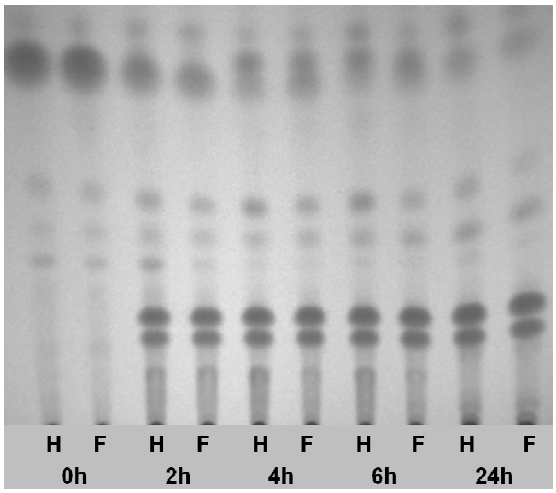



**Figure 4.32: TLC analysis of HBA conversion by *E. coli* transformed with pET28(b)+CYP505A1.** H – N-terminally His-tagged CYP505A1, F – normal CYP505A1. Reaction durations are given in hours.

The two products which were formed were confirmed to be the same as those formed by CYP102A1, namely the sub-terminally hydroxylated products ω-2 hydroxyhexylbenzoic acid (ω-2 OH-HBA) and ω-1 hydroxyhexylbenzoic acid (ω-1 OH-HBA). These products are in a 1:1 ratio however, contrary to the 7:1 ω-2 OH-HBA: ω-1 OH-HBA ratio observed with CYP102A1 (Gudiminchi and Smit, 2011). Since HBA was recognized as a substrate by CYP505A1 however, we could proceed with this substrate for activity assays of CYP102A1 and CYP505A1 cloned into yeasts.

### 4.11. Initial biotransformations using growing cells

*Kluyveromyces marxianus* (UOFS Y1185), *Saccharomyces cerevisiae* (W3031A(a)), *Hansenula polymorpha* (UOFS Y1507), *Yarrowia lipolytica* (CTY003) and (CTY029) and *Arxula adeninivorans* (UOFS Y1220) were transformed with the vectors pKM118+CYP102A1 and pKM118+CYP505A1, while *K. marxianus* was additionally transformed with pKM63+CYP102A1 and pKM63+CYP505A1. Two to five transformants from each transformation, which were confirmed by gDNA PCR to

contain the relevant foreign DNA, were tested for activity towards HBA. A control transformant was prepared by using pKM118 without genes for P450.

Due to promising results obtained with growing cells displaying CYP53B1 activity, the same method was initially used for biotransformation of HBA using strains with CYP102A1 or CYP505A1 cloned. Cultures were again grown for 48 h in YPD (or YPS for *K. marxianus* INU promoter) before HBA was added and biotransformations were allowed to continue for 24 h.

### 4.11.1. Evaluating expression of CYP102A1 and CYP505A1 in *Kluyveromyces marxianus* strains by comparing the YlTEF or KmINU promoters

Given the results obtained with CYP53 expression in *K. marxianus*, initial work was done using *K. marxianus* transformants with CYP102A1 cloned under either the YlTEF promoter in the broad range vector (pKM118), or the *K. marxianus*-specific inulinase (INU) promoter in the *K. marxianus*-specific vector (pKM63). When the KmINU promoter was used, sucrose was the main carbon source, while glucose was the main carbon source when the YlTEF promoter was used. A vector coding for a His-tagged version of CYP102A1 also prepared for comparison with wild-type CYP102A1.

Transformants from two strains of *K. marxianus* containing either His-tagged or normal versions of CYP102A1 under control of the INU promoter were tested for their abilities to hydroxylate 2 mM HBA (figure 4.33).

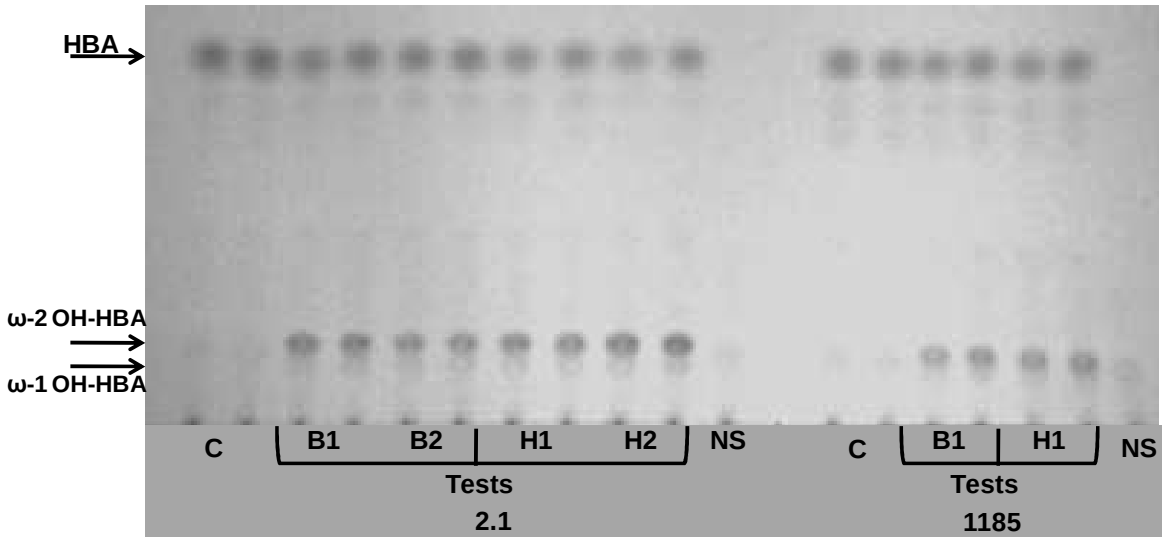



**Figure 4.33: TLC analysis of HBA conversion by transformants of *K. marxianus* isolate 2.1 and UOFS Y1185 transformed with pKM63+His-CYP102A1 or pKM63+CYP102A1. Samples were not concentrated prior to TLC analysis.** C – Control transformed with pKM63; B – transformant with CYP102A1 cloned; H – transformant with His-tagged CYP102A1 cloned; NS – No substrate added to a test transformant.

From the work in *E. coli* we recognized the highest spot as HBA, and the other two spots as presumably ω-2 OH-HBA (higher, more intense spot) and ω-1 OH-HBA (weaker spot). Differences between tests and controls were more obvious when using non-concentrated samples, but activities were not always as easily detectable. Therefore generally samples were concentrated so that low activities would not be mistaken for complete absence of activity.

The two strains showed comparable CYP102A1 activities and *K. marxianus* UOFS Y1185 was used for further work. For comparison of the KmINU and YlTEF promoters during expression of CYP102A1, 5 mM HBA was added to 48 h YPD (for TEF promoter) or YPS (for INU promoter) cultures and incubated for 24 h before extraction (figure 4.34).

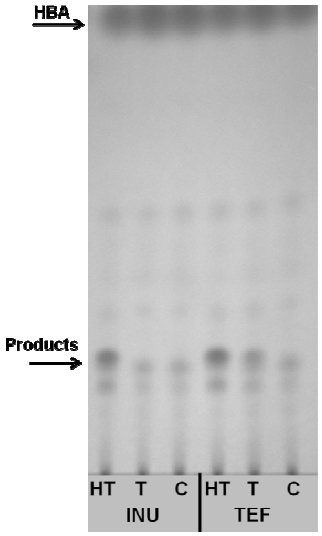



**Figure 4.34: TLC analysis of HBA biotransformation using *K. marxianus* UOFS Y1185 transformants with CYP102A1 cloned under the KmINU and YlTEF promoters.** C – Control transformant not expressing any foreign P450; T – test transformant with CYP102A1 cloned; HT – test transformant with His-tagged CYP102A1 cloned.

There are spots of obvious higher intensity in the tests than in the controls, although it becomes more difficult to differentiate between products and other compounds extracted from the cultures when the cloned hydroxylase activity is low. The TEF promoter compared favorably with the INU promoter; in fact it actually seemed better. The His-tag did not appear to negatively affect activity; in fact activity seemed better when the enzyme was His-tagged, although growth differences were not considered in this case. We decided to continue further with only the His-tagged version, as we can eventually use the His-tag for Western blot analysis using anti-His antibodies to determine protein levels between transformants or species.

To compare CYP505A1 expression under the control of both promoters, and to CYP102A1 expression, 5 mM HBA was added to 48 h YPD (for YlTEF promoter) or YPS (for KmINU promoter) cultures and incubated for 24 h before extraction (figure 4.35).

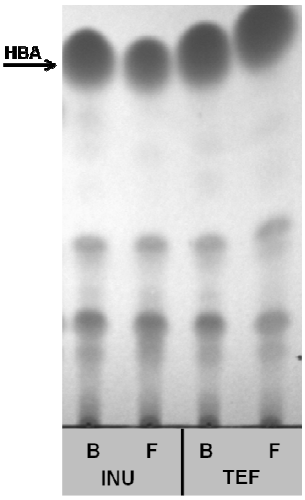



**Figure 4.35: TLC analysis of HBA biotransformation using *K. marxianus* transformants with CYP102A1 and CYP505A1 cloned under the KmINU and YlTEF promoters.** INU – inulinase promoter from *K. marxianus*; TEF – translational elongation factor promoter from *Y. lipolytica*; B – CYP102A1 (P450BM3); F – CYP505A1 (P450Foxy).

Similar activities were observed between the two P450s in terms of the products formed, although when using the YlTEF promoter, CYP102A1 activity appeared to be slightly better than CYP505A1 activity, while CYP505A1 had higher activity using the INU promoter. Overall though, expression of both P450s using the YlTEF promoter is comparable with the KmINU promoter.

### 4.11.2. CO-spectrum for CYP102A1 expressed by *K. marxianus*

Whole-cell CO-spectrum was obtained using a transformant of *K. marxianus* UOFS Y1185 with pKM63+CYP102A1 cloned. Cells were cultivated for 48h as in for biotransformations, before being harvested and resuspended to an approximate concentration of 67 $g_{DCW}.L^{-1}$), in 50 mM potassium phosphate buffer (pH8). Aliquots were reduced with sodium dithionate, and test samples were saturated with carbon monoxide. Absorbance values of samples were determined from 400-500 nm and the difference between absorbance at 450 nm and 490 nm calculated.

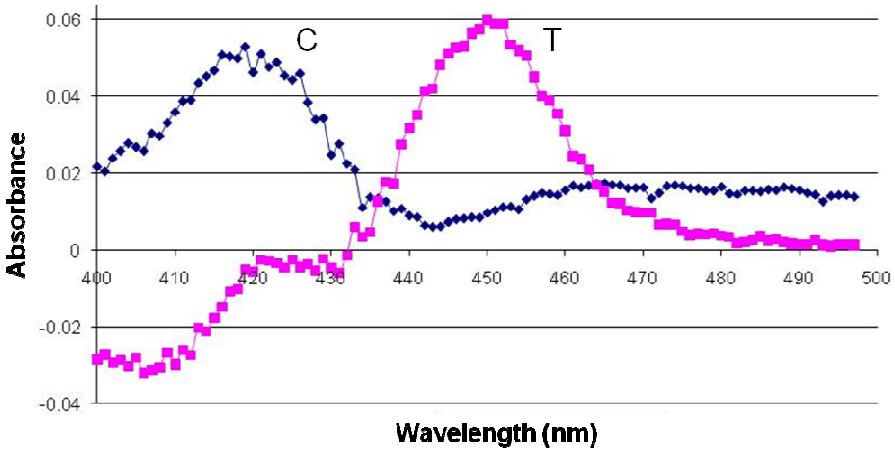



**Figure 4.36: Graph representing the reduced carbon monoxide spectrum (CO-spectrum) obtained using whole cells of transformants of *K. marxianus* UOFS Y1185 transformed with pKM63+CYP102A1.** C – Sample reduced with sodium dithionite only; T – Sample reduced with sodium dithionite and saturated with carbon monoxide.

The reproducibility of these results was however poor, possibly due to the instability of *K. marxianus* transformants (Albertyn *et al*, 2011). This was demonstrated when the same spectrum was repeated for the same enzyme after reviving the strain about four months after the above spectrum was attained, we obtained a smaller and less defined peak at 450 nm figure 4.37. Numerous attempts to demonstrate CO-difference spectrum for the various strains in later experiments were unsuccessful, which was not surprising since whole cell P450 spectra has very seldom been described for yeasts (Gillam *et al*, 2007).

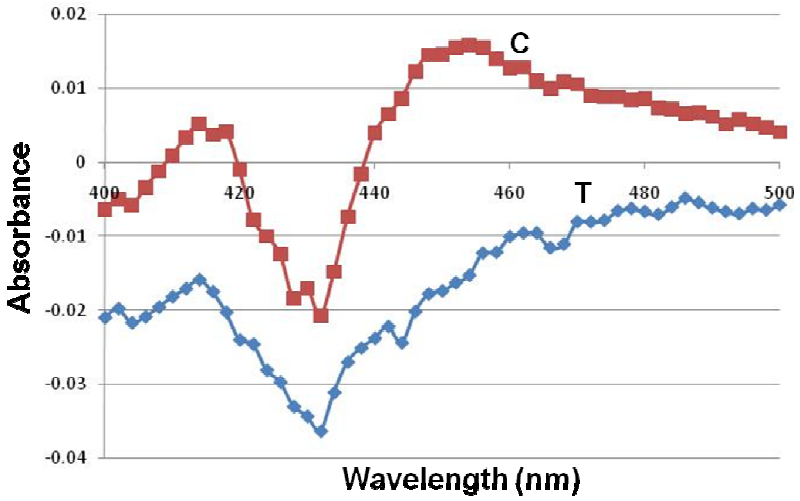



**Figure 4.37: Graph representing the reduced carbon monoxide spectrum (CO-spectrum) obtained using whole cells of transformants of *K. marxianus* UOFS Y1185 transformed with pKM63+CYP102A1.** C – Sample reduced with sodium dithionite only; T – Sample reduced with sodium dithionite and saturated with carbon monoxide.

## 4.12. Biotransformation of HBA by other species with CYP102A1 or CYP505A1 cloned

Since whole-cell activities of CYP102A1 and CYP505A1 were detected in *K. marxianus*, we proceeded to screen other strains for expression of these two enzymes. Four or five transformants proven by PCR to carry chromosomally integrated genes for either CYP102A1 or CYP505A1 were screened for activities by adding 5 mM HBA after 48 h cultivation in YPD at 28°C.

### 4.12.1. Initial screening of *Hansenula polymorpha* transformants with CYP102A1 cloned

Despite the absence of CYP53B1 activity in *H. polymorpha* transformants, we nevertheless attempted expression of CYP102A1 in this organism following the positive results obtained with *K. marxianus* (figure 4.38).

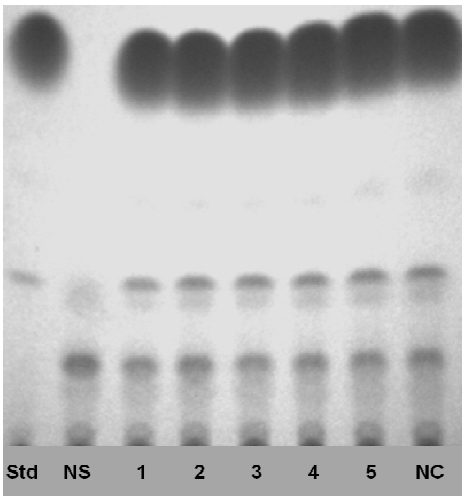



**Figure 4.38: TLC analysis of *H. polymorpha* transformants with CYP102A1 cloned.** Std – HBA substrate standard; NS – test transformant to which substrate was not added; NC – control transformant withno P450 cloned; 1-5 – test transformants in which CYP102A1 was cloned.

Once again there was no difference between transformants into which CYP102A1 was cloned compared to the negative control into which no P450 was cloned. No further attempts for activity were pursued with this yeast.

### 4.12.2. Initial screening of *Saccharomyces cerevisiae* transformants with either CYP102A1 or CYP505A1 cloned

CYP102A1 and CYP505A1 were expressed (separately) in *S. cerevisiae* using the broad range vector. The results for the initial screen are shown in figure 4.39.

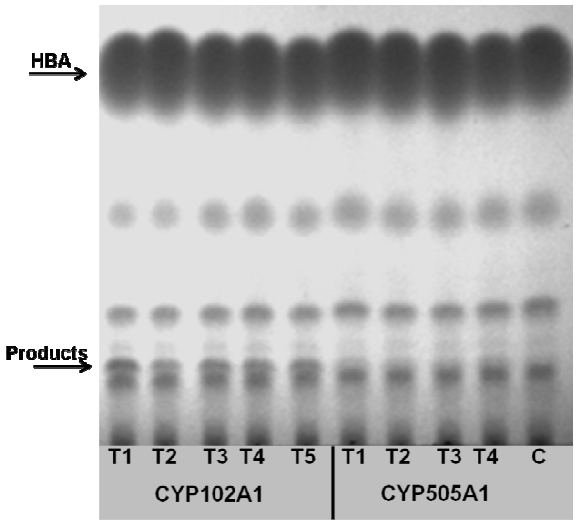



**Figure 4.39: TLC analysis of HBA biotransformation using *S. cerevisiae* transformants with CYP102A1 and CYP505A1 cloned.** T1-5 – transformants with either CYP102A1 or CYP505A1 cloned; C – test transformant with no foreign P450 cloned.

There appears to be an additional spot in the samples from the transformants with CYP102A1 cloned which is absent in the control and CYP505A1 transformants. UV absorbing compounds extracted from the culture broth or present as impurities in HBA made it impossible to decide whether active CYP505A1 was expressed by these *S. cerevisiae* transformants.

### 4.12.3. Initial screening of *Yarrowia lipolytica* transformants with either CYP102A1 or CYP505A1 cloned

*Y. lipolytica* is well known for its efficiency in hydrocarbon degradation, attributable to the organized pathways in the cells involving numerous P450s (Thevenieau *et al*, 2006). Therefore competition for the HBA substrate by wild-type P450s was expected, which form different products from those produced by sub-terminal hydroxylation (Shuping, 2007). The concern was that wild-type activities could efficiently exhaust the substrate before heterologous activity could be detected. To attempt to circumvent this we also tested a *Y. lipolytica* strain with its β-oxidation completely disrupted. Products initially

formed and converted to acids would therefore not be further metabolized by this strain. Unfortunately the strain in which β-oxidation is blocked is derived from strain W29, while the other strain which was tested is derived from strain E150, limiting the direct comparison between the strains to some extent. Wild-type W29 was shown to be less efficient at degrading the initial intact product (4-(5-carboxypentyl)benzoic acid) formed by wild-type activity on HBA (Shuping, 2007).

Comparison of HBA biotransformation by transformants of the two strains with CYP102A1 cloned is shown in figure 4.40.

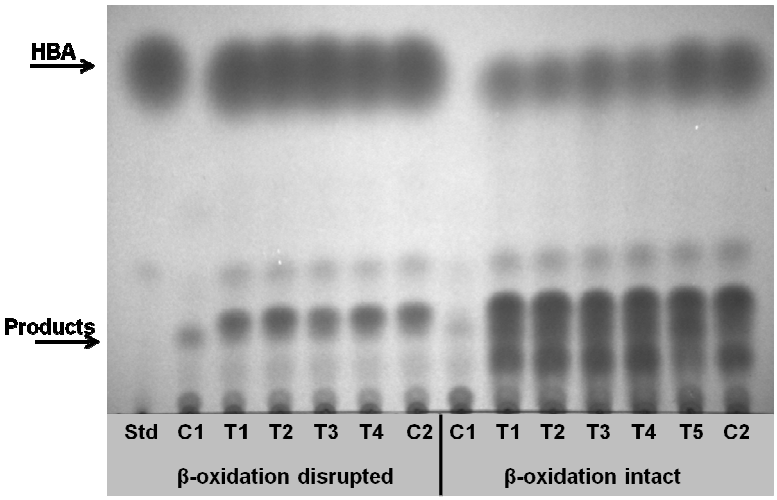



**Figure 4.40: TLC analysis of HBA conversion by transformants of *Y. lipolytica* strains with CYP102A1 cloned.** Std – hexylbenzoic acid (HBA) substrate standard; C1 – test transformants to which substrate was not added; C2 – control transformants with no foreign P450 cloned; T1-5 – test transformants with CYP102A1 cloned.

It is clearly illustrated in figure 4.40 that transformants of the two strains accumulated different products due to wild-type activity. Transformants of *Y. lipolytica* CTY003 rapidly degraded the HBA into three products, while transformant of the β-oxidation disrupted *Y. lipolytica* CTY029 accumulated only the intact dioic acid. Products were identified by comparison with TLC results reported by Van Rooyen (2005) and Shuping (2007). These were however differences between host strains, but no differences

between test and control transformants were observed. It was uncertain if this was due to a lack of CYP102A1 activity, therefore transformants with CYP505A1 cloned were also tested. Results obtained with transformants with CYP505A1 cloned were compared to results obtained with transformants with CYP102A1 cloned (figure 4.41).

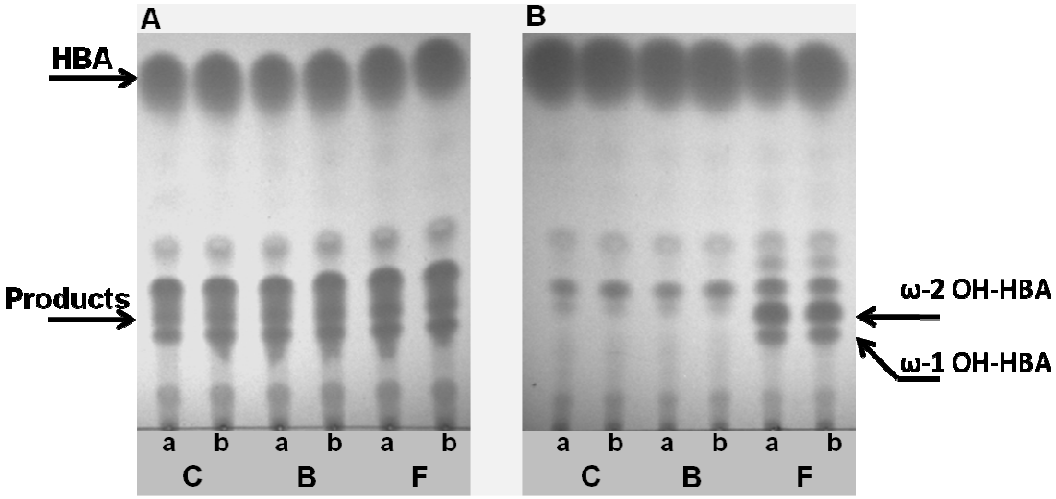



**Figure 4.41: TLC analysis of HBA conversion by transformants of *Y. lipolytica* strains with their β-oxidation pathways either intact (A; CTY003) or disrupted (B; CTY029) with CYP102A1 or CYP505A1 cloned.** C – control transformants not expressing any foreign P450; B – test transformants with CYP102A1 (P450BM3) cloned; F – test transformants with CYP505A1 (P450Foxy) cloned.

Once again transformants of the strain with intact β-oxidation metabolized HBA to the same extent regardless of whether CYP102A1 or CYP505A1 was cloned or not. In the transformants of the strain with disrupted β-oxidation however, we could see clear formation of two main products (indicated on the right hand side of figure 4.41) in samples from transformants with CYP505A1 cloned; which were absent in the control transformants and transformants with CYP102A1 cloned. It is easy to conclude that the lower level of wild type activity in transformants of *Y. lipolytica* CTY029 is due to disruption of the β-oxidation pathway, but it should be mentioned that the parental wild type strain W29 from which strain FT120 has been derived also gave mainly the intact dioic acid during the first 24 h after HBA addition (Shuping, 2007). The limited substrate

conversion by wild-type enzymes of *Y. lipolytica* CTY029 allowed detection of heterologous P450 activity.

### 4.12.4. Screening of *Arxula adeninivorans* transformants with either CYP102A1 or CYP505A1 cloned for biotransformation of HBA

*A. adeninivorans* transformants with CYP102A1 cloned were evaluated for HBA biotransformation (figure 4.42).

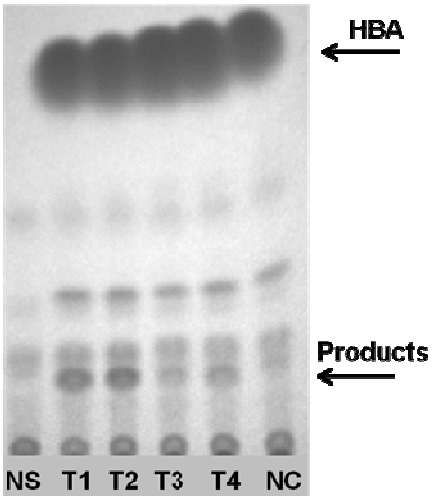



**Figure 4.42: TLC analysis of HBA biotransformation using *A. adeninivorans* transformants with CYP102A1 cloned.** NC – control transformant with no foreign P450 cloned; NS – test transformant to which substrate was not added; T1 – T4 – different test transformants with CYP102A1 cloned.

Unique spots were observed in samples from the test transformants compared to the controls. There was also variation between the test transformants, as was observed for CYP53B1. T1 and T2 have higher activities than the other two, hinting that perhaps two copies compared to one in T3 and 4 are integrated. These results were however not as promising as those observed for CYP53B1 using *A. adeninivorans*.

After displaying promising activity in one *Y. lipolytica* strain, expression of CYP505A1 was then tested in *A. adeninivorans*. Results of HBA biotransformation by *A. adeninivorans* transformants with CYP505A1 cloned is shown in figure 4.43.

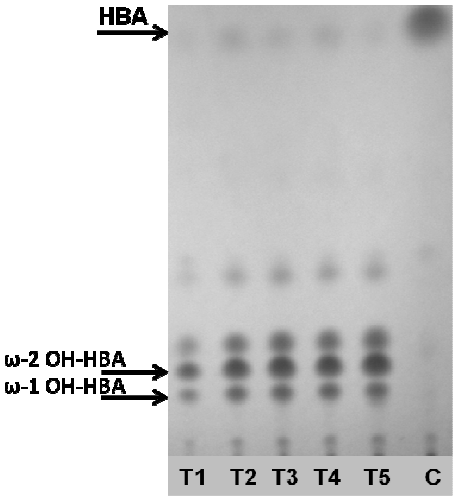



**Figure 4.43: TLC analysis of HBA biotransformation using *A. adeninivorans* transformants with CYP505A1 cloned.** T1-5 – different test transformants with CYP505A1 cloned; C – control transformant with no foreign P450 cloned.

These results were the breakthrough results we had been anticipating for these self-sufficient enzymes. These results were more similar to those obtained for CYP53B1 activity in this host, in that most of the 5 mM HBA had been converted to products by the expressed enzyme.

## 4.13. Time course of HBA biotransformation by *A. adeninivorans* expressing CYP505A1

Since the activity was so high for this enzyme in this host, activity was monitored by sampling at regular intervals over 120 h. Due to the consistency between transformants (another feature shared with CYP53B1 expressed in this host) only two transformants were selected for this analysis.

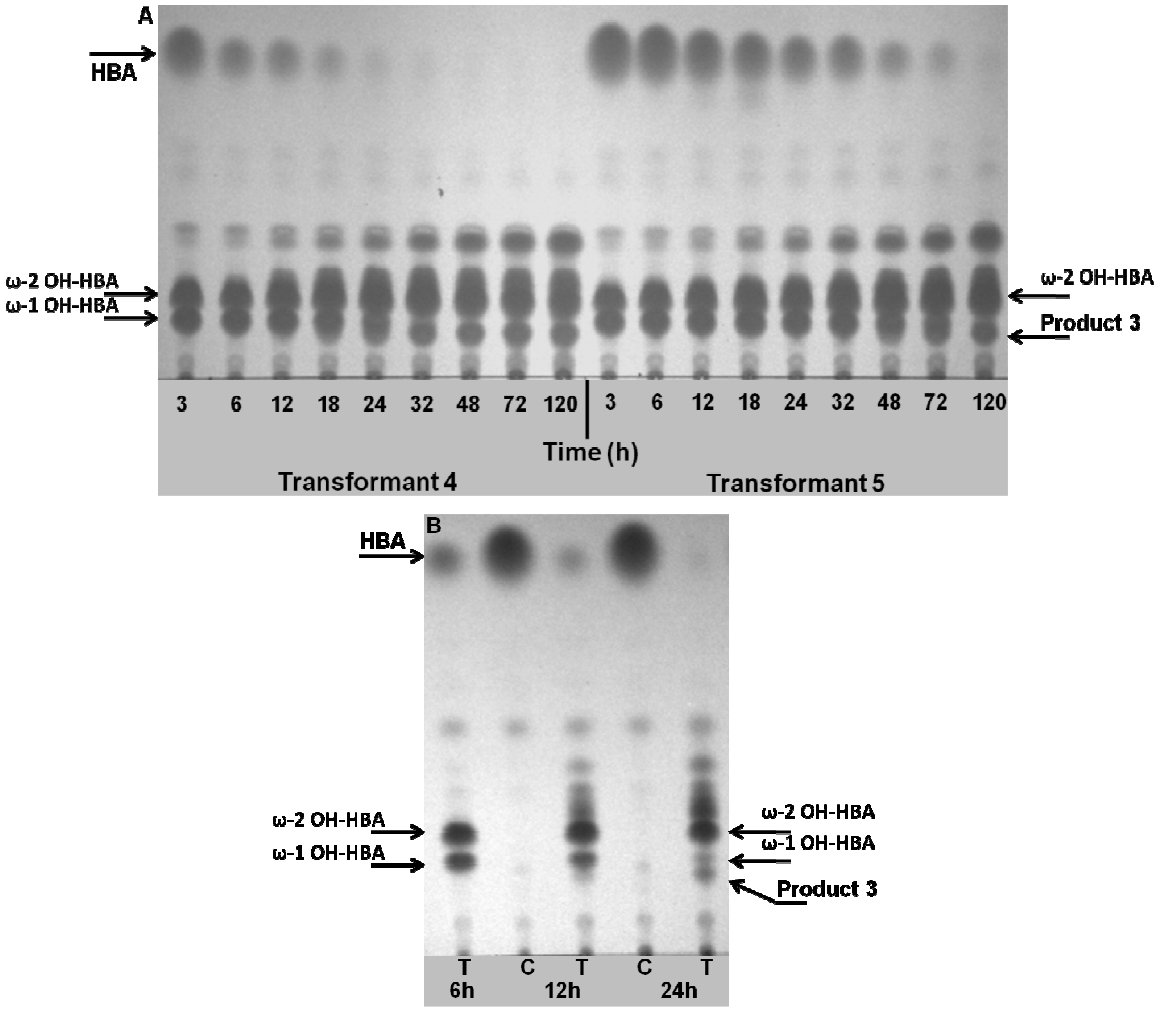



**Figure 4.44: TLC analysis of samples taken at different times during the conversion of 5 mM HBA over time by an *A. adeninivorans* transformant expressing CYP505A1.** Plate A shows conversion by test transformants at various time points until 120 h after HBA addition. Plate B shows comparison of test transformant 4 and control transformant with no foreign P450 cloned at selected time points only. The amount of sample loaded onto the plate in plate B was less than for plate A, to obtain better product resolution.

These results increased our understanding of the product formation from HBA by CYP505A1 expressed by *A. adeninivorans*. It can be observed that, as with CYP505A1 expression in *E. coli*, the same two products which were observed in expression of CYP505A1 in *E. coli* are formed initially, namely ω-2 hydroxyhexylbenzoic acid (ω-2

OH-HBA) and ω-1 hydroxyhexylbenzoic acid (ω-1 OH-HBA). The ratio of ω-2 OH-HBA : ω-1 OH-HBA was confirmed to initially be approximately 1:1, as opposed to the 7.6:1 observed with CYP102A1 (Gudiminchi and Smit, 2011).

Transformant 4 displayed an obviously higher HBA conversion rate than transformant 5. This is similar to the observation with CYP53B1 expressed in *A. adeninivorans*, where we presume that one transformant has two copies of the integrant compared to one copy in the other one. Transformant 4 was used for further experiments.

Using the available GC column and program, we could only detect the ω-2 hydroxyhexylbenzoic acid (ω-2 OH-HBA) product with GC using methylated samples. For GC-MS analysis of selected samples, the samples required methylation and silylation to reduce the retention times of their products, but this was not feasible for large numbers of samples. Therefore for convenience, samples were only methylated and biotransformations were monitored by considering only the ω-2 OH-HBA product, since it remains stable throughout the biotransformations anyway.

The ω-2 OH-HBA forms at an approximate initial rate of 33.4 $\mu mol.h^{-1}$ $g_{DCW}^{-1}$ during the first 3 h and after 6 h the concentration remains stable (figure 4.45). ω-2 OH-HBA accounts for about half of the substrate (2.47 mM). It remains relatively stable throughout the reaction (figure 4.45).

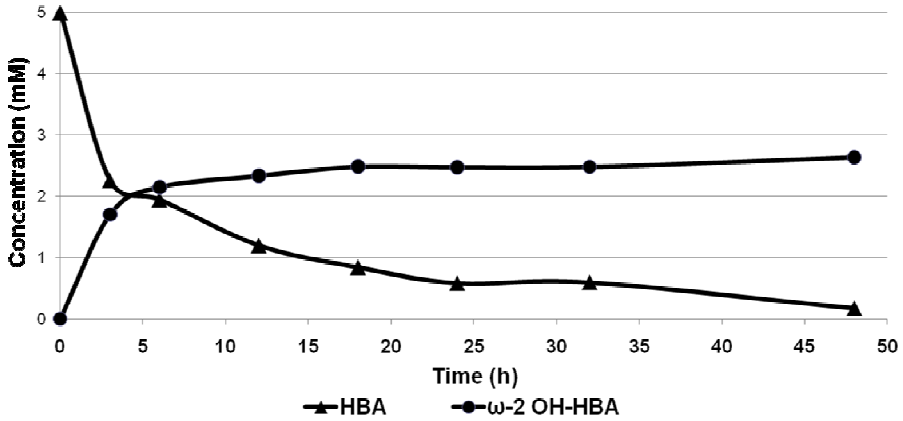



**Figure 4.45: Graph representing conversion of HBA to ω-2 OH-HBA over time by a transformant of *A. adeninivorans* expressing CYP505A1.**

As the substrate approaches depletion however, the ω-1 hydroxyhexylbenzoic acid starts decreasing, while a third product, 'product 3' starts accumulating. This is illustrated more clearly in plate B of figure 4.44. This obviously means that ω-1 hydroxyhexylbenzoic acid is converted to a different product. The fact that this happens as the substrate concentration diminishes, makes it seem like the ω-1 hydroxyhexylbenzoic acid becomes a secondary substrate for the same enzyme under when HBA becomes depleted. Product 3 is most likely a further hydroxylated product of ω-1 hydroxyhexylbenzoic acid, i.e. dihydroxyhexylbenzoic acid, but this needs to be confirmed using GC-MS analysis.

There are also other products which appear above the ω-2 hydroxyhexylbenzoic acid spot on the TLC plate, which also accumulate over time in test transformants. It may be the intact dioic acid which is also produced by *Y. lipolytica* transformants with β-oxidation blocked, regardless of whether a foreign P450 is expressed or not. In *A. adeninivorans* however, the product only accumulates significantly when CYP505A1 is cloned. It is thus probably rather formed by a further reaction on products of HBA catalyzed either by CYP505A1 or perhaps wild-type enzymes.

## 4.14. HBA biotransformations by resting cells of yeasts with either CYP102A1 or CYP505A1 cloned

In most cases two main problems hindered the observation of activities of CYP102A1 and CYP505A1 in most cases, the first of which was the seemingly low activities of expressed P450s. The low activities are not only due to limitations in host expression, but are also due to the fact that, unlike benzoic acid for CYP53B1, 4-hexylbenzoic acid is not a natural or preferred substrate for CYP102A1 or CYP505A1. The second problem was the interference by wild-type P450s. The use of resting cells was considered as a potential solution to both problems.

Firstly, harvesting and resuspension in buffer allows for biomass concentration, which should result in higher activities. Secondly, by using resting cells resuspended in a buffer, protein production after harvesting is not encouraged. Therefore, assuming that the wild-type P450 activities are induced by the added substrate, the wild-type P450 content present in the absence of substrate at the time of harvesting should be low. The use of concentrated resting cell suspensions were therefore intended to simultaneously increase whole cell biocatalyst concentration, while limiting background activities.

### 4.14.1. Preliminary comparison of HBA biotransformations by resting cells of various strains expressing CYP102A1 or CYP505A1

For inter-strain comparisons, transformants of all strains with either CYP102A1 or CYP505A1 cloned were cultured in YPD as before and cells were harvested after 48 h growth and resuspended in phosphate buffer (200 Mm, pH 8; see section 3.10.3) in a 3: 1 buffer: cell pellet ratio (i.e. 3 ml buffer per 1 $g_{WCW}$). While this ratio ensured equal wet cell weight concentrations between species, the same was not true for dry cell weight, as the correlation between wet and dry weights for *A. adeninivorans* (dry cell weights ~4-fold lower than wet cell weights) differed slightly from that of other species (dry cell weights ~5-fold lower than than wet cell weights). As a result *A. adeninivorans* was more concentrated than the other species if dry cell weights are considered.

To further reduce background activity, cultures grown in a chemically defined medium (adapted from Knoll *et al*, 2007) were also tested. Transformants expressing CYP102A1 and CYP505A1 were tested.

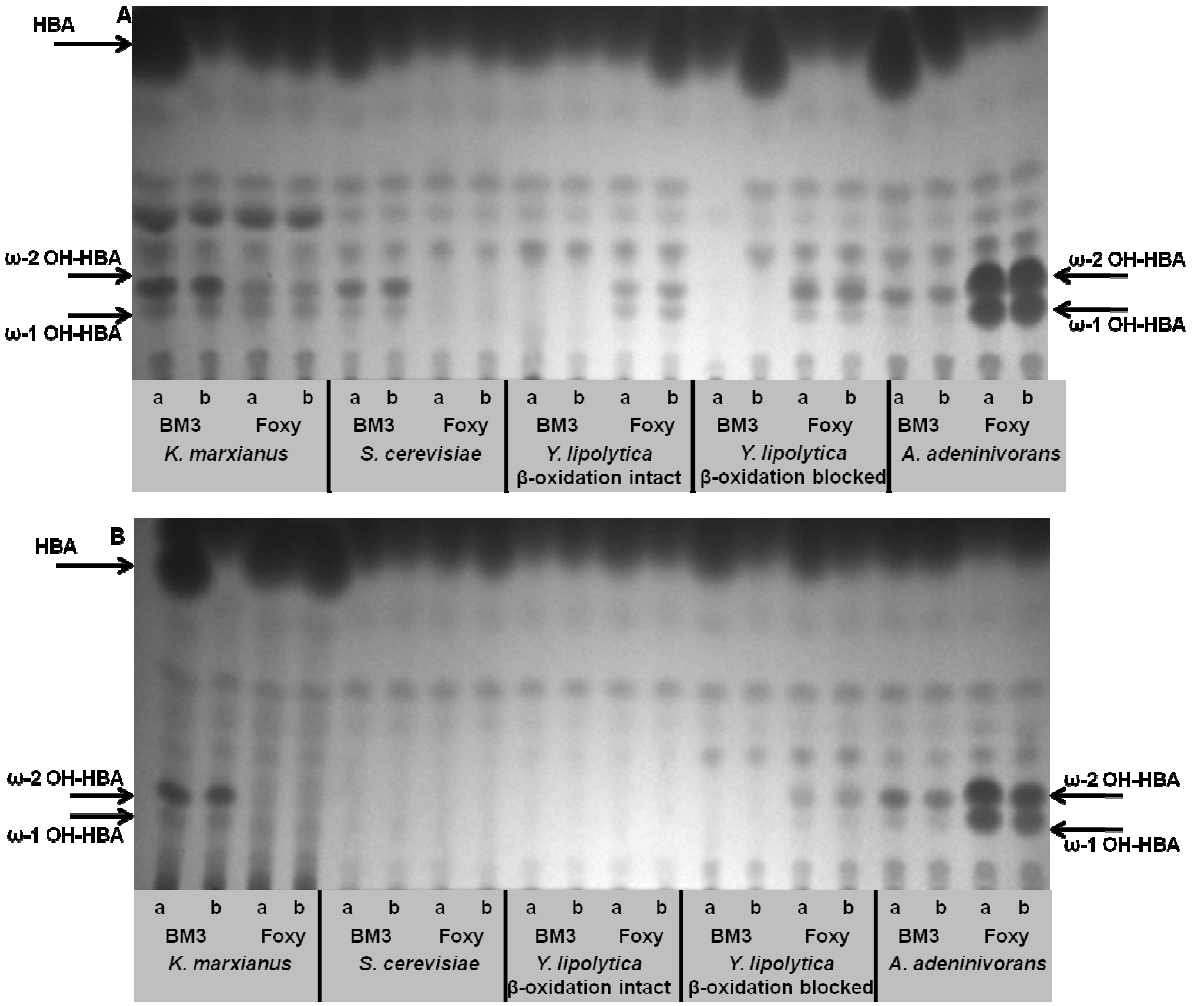



**Figure 4.48: TLC analysis of the comparison of HBA biotransformations by resting cells of various strains with CYP102A1 (BM3) or CYP505A1 (P450Foxy) cloned after growth in YPD (A) or a chemically defined medium (CDM) (B).** 'a' and 'b' are separate assays from the same resting cell resuspension.

From plate A of figure 4.48, it can be observed that when using resting cells from YPD cultures, only transformants of *K. marxianus* and *A. adeninivorans* displayed activity for both P450s. With *S. cerevisiae* transformants only CYP102A1 activity was observable while only CYP505A1 activity was observable for transformants of both of the *Y. lipolytica* strains. It was interesting that with resting cells wild type P450 activity was significantly reduced in transformants of the *Y. lipolytica* CTY003 strain with intact β-oxidation, so that CYP505A1 activity was now observed with transformants of both

strains. The CYP505A1 activity in the transformant of the β-oxidation disrupted strain seemed slightly better than the transformant of the other strain. The best activity was again observed in transformants of *A. adeninivorans* expressing CYP505A1, although the activity was considerably lower than when growing cells were used, especially considering that a lot of HBA remained unhydroxylated, hence further conversion of ω-1 hydroxyhexylbenzoic was not observed. The CYP102A1 activity in *K. marxianus* transformants was also quite promising.

Plate B demonstrates that the chemically defined medium used in this study was very inefficient at promoting P450 expression, as all activities decreased dramatically. Activities for both P450s were still detectable in samples from only *K. marxianus* and *A. adeninivorans* transformants. Other than that, only very little CYP505A1 activity was detected in transformants of the *Y. lipolytica* strain with its β-oxidation blocked, while the other strain of *Y. lipolytica* and the *S. cerevisiae* strain displayed no detectable activity at all.

The chemically defined medium generally led to decreased biomass yields, as can be seen in figure 4.49, with only the β-oxidation blocked derivative of *Y. lipolytica* displaying improved growth in this medium.

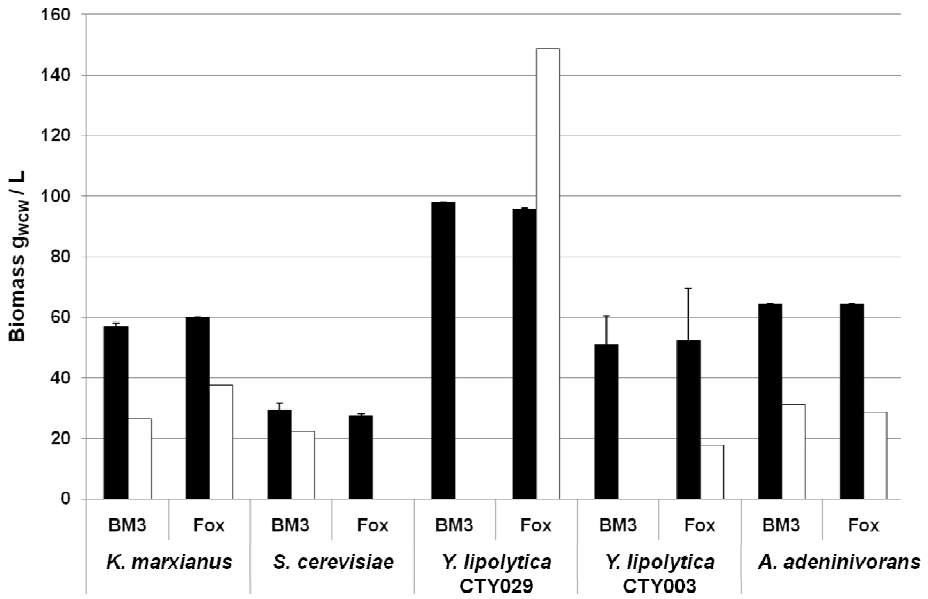



**Figure 4.49: Biomass production by transformants of various yeast strains in YPD medium (black columns) and a chemically defined medium (white columns).** Transformants expressed either CYP102A1(BM3) or CYP505A1 (P450Foxy). Data of YPD cultures derived from two experiments, but the data of the chemically defined medium cultures is derived from only one experiment. Transformants into which empty pKM118 vectors were transformed had comparable growth to transformants with P450s cloned (data not shown).

The chemically defined medium resulted in reduced biomass compared to the YPD, and is therefore not an optimal medium. The fact that the activity from the chemically defined medium in the *Y. lipolytica* strain with its β-oxidation blocked was not better than or equal to the activity obtained from YPD however, suggests that poor growth was not the only reason for decreased activity. The medium was based on a chemically defined medium which was reported to yield high biomass for *A. adeninivorans* in bioreactor studies (Knoll *et al*, 2007), but modified in this study for use in shake flasks. As is evident from the results obtained when using this modified medium, it was not optimal for shake flask experiments during this study.

### 4.14.2. Quantitative analysis of the effects of growth in CDM and addition of 5-ALA on HBA biotransformations by resting cells of various strains expressing CYP102A1 or CYP505A1

From the preliminary results, cases in which enzyme activities were not even observed in samples from YPD cultures were eliminated from quantitative investigations of resting cell activities. Aside from comparing activities from YPD and chemically defined medium, we also investigated the effect of adding the heme precursor 5-aminolevulinic acid (5-ALA) on the enzyme activities. In section 4.5.5 the effect of 5-ALA on activity using growing cells was tested, and found to have no significant effect. Nevertheless, testing 5-ALA addition in this experiment would confirm whether the effects were enzyme specific or not or whether harvesting has an effect. More importantly, we could observe differences in the chemically defined medium (CDM) in the presence of 5-ALA, as the CDM may be limited in terms of requirements for P450 production which could be present in the complex YPD medium.

As described in section 4.13, for convenient GC analysis only the ω-2 OH-HBA product formation was followed, especially since it remains stable throughout the biotransformations. Therefore the results for CYP102A1 are somewhat exaggerated when compared to CYP505A1 results, since (ω-2 OH-HBA) is the main product produced by CYP102A1 but only half of the main product formed by CYP505A1. Nevertheless, it would provide some insight in to the relative activities between species.

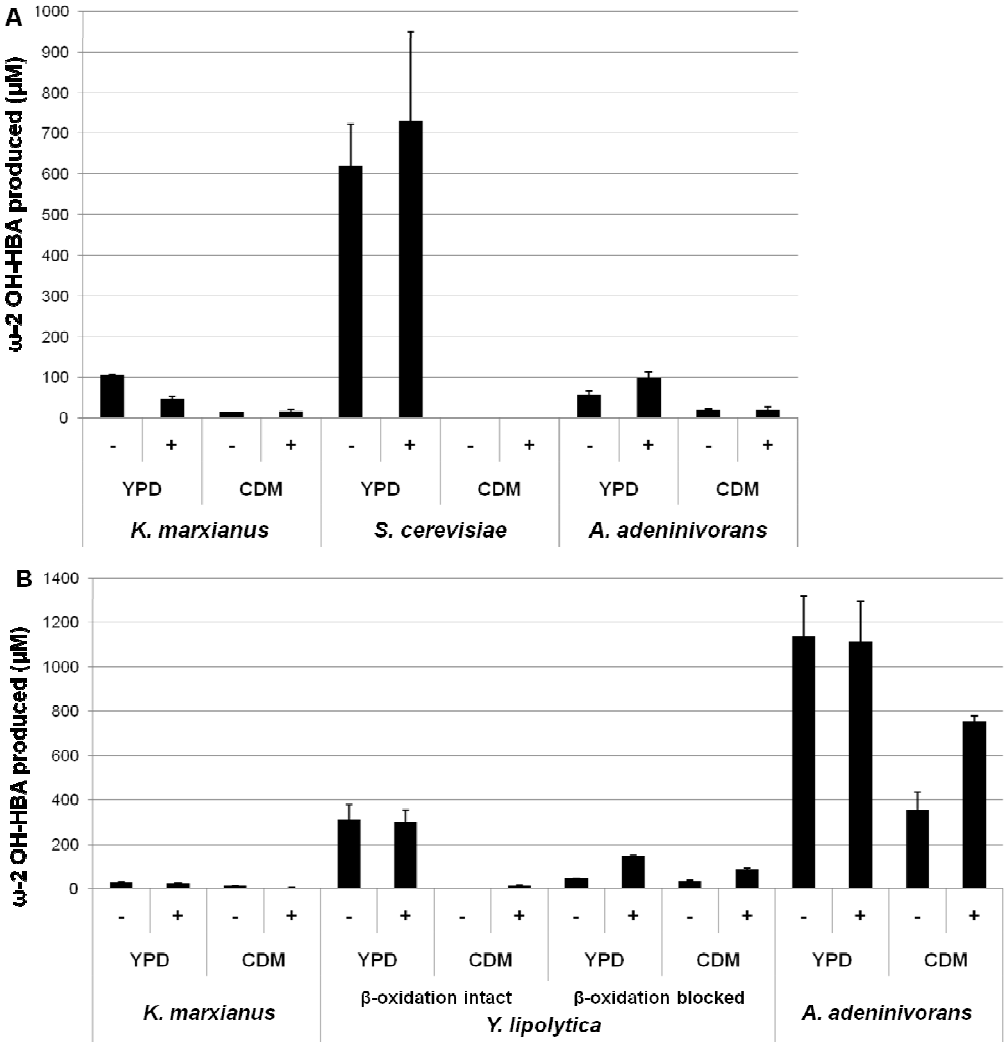



**Figure 4.50: The effect of 5-aminolevulinic acid (5-ALA) on the production of ω-2 OH-HBA by resting cells of transformants expressing CYP102A1 (A) and CYP505A1 (B).** "+" - 5-ALA was added to transformants cultured in YPD or a chemically defined medium (CDM). "-" - no 5-ALA was added.

From the graph of CYP102A1 activity (graph A) it can be seen that the best activity was observed using the transformant of *S. cerevisiae* grown in YPD, and again no activity was observed in CDM for this yeast. The CYP102A1 activities in CDM are also again lower for both *K. marxianus* and *A. adeninivorans* than in YPD. The addition of 5-ALA appeared to reduce the activity of the YPD grown *K. marxianus* transformant by more than half, while nearly doubling the activity of the *A. adeninivorans* transformant. There

was also a slight increase in the activity in the *S. cerevisiae* transformant. Activities remained the same in CDM for all the yeasts.

From the graph of CYP505A1 activity (graph B) it can be seen that the *K. marxianus* transformant was unaffected by the 5-ALA addition in YPD, while the activity was reduced to nearly nothing in CDM. The addition of 5-ALA to YPD also did not seem to have an effect on activity for the *A. adeninivorans* transformant and the *Y. lipolytica* transformant with intact β-oxidation. The *Y. lipolytica* transformant with β-oxidation blocked however was affected in YPD as well as in CDM, representing the only case in which a positive effect of 5-ALA addition was observed in both media used. The activity increased 3-fold in YPD and 2.5-fold in CDM. In the *A. adeninivorans* transformant cultivated in CDM the activity is nearly doubled in the presence of 5-ALA, while the activity in *Y. lipolytica* cultivated in CDM the activity can only be detected when 5-ALA is added.

The best results overall were obtained with CYP505A1, with transformants of *A. adeninivorans* once again achieving the best results. This transformant probably contains two integrated copies of CYP505A1 (transfomant 4, figure 4.44), but even if one halves the obtained activities of the *A. adeninivorans* transformant they are still higher than any other species, ruling out copy number as the explanation for better activities.

The volumetric yield of ω-2 OH-HBA after 24 h was 1.5 mM, which is less than the 2.5 mM obtained using growing cells after 24 h. Bearing in mind that the resting cell biomass was concentrated compared to the growing cells demonstrates how much better the growing cells performed. This is emphasized if one considers that the specific activity of growing cells after 24 h is 6.1 $\mu mol.h^{-1}$ $g_{DCW}^{-1}$, which is four-fold higher than the 1.5 $\mu mol.h^{-1}$ $g_{DCW}^{-1}$ obtained with resting cells after 24 h. These rates are however probably underestimations of the actual maximum rates, as activities level off much

earlier than 24 h after substrate addition in growing cells, and possibly also in resting cells.

It is important to emphasize that the reproducibility of the assays conducted with resting cells was generally poor, particularly using *S. cerevisiae*. Transformants of the *A. adeninivorans* and the β-oxidation disrupted *Y. lipolytica* strains gave more reproducible results than other strains. Therefore these results should be treated carefully. They do however demonstrate that CYP102A1 and CYP505A1 could be actively produced by the strains, even though only *K. marxianus* and *A. adeninivorans* expressed both P450s efficiently. This was important since activity could not be proven for all yeasts using growing cells.

The results also demonstrated the potential effects of additional 5-ALA on P450 activities in some cases.

### 4.15. Effect of 5-ALA on *Y. lipolytica* CTY029 growing cells in YPD

The results obtained using resting cells demonstrated that 5-ALA may in some cases improve P450 activity. Particularly fascinating results which were obtained with growing cells of *Y. lipolytica* CTY029 transformants (with disrupted β-oxidation) was the color change of the YPD cultures in response to 5-ALA addition (figure 4.51). Similar color changes were observed with transformants with CYP505 cloned as with transformants transformed with empty vector.

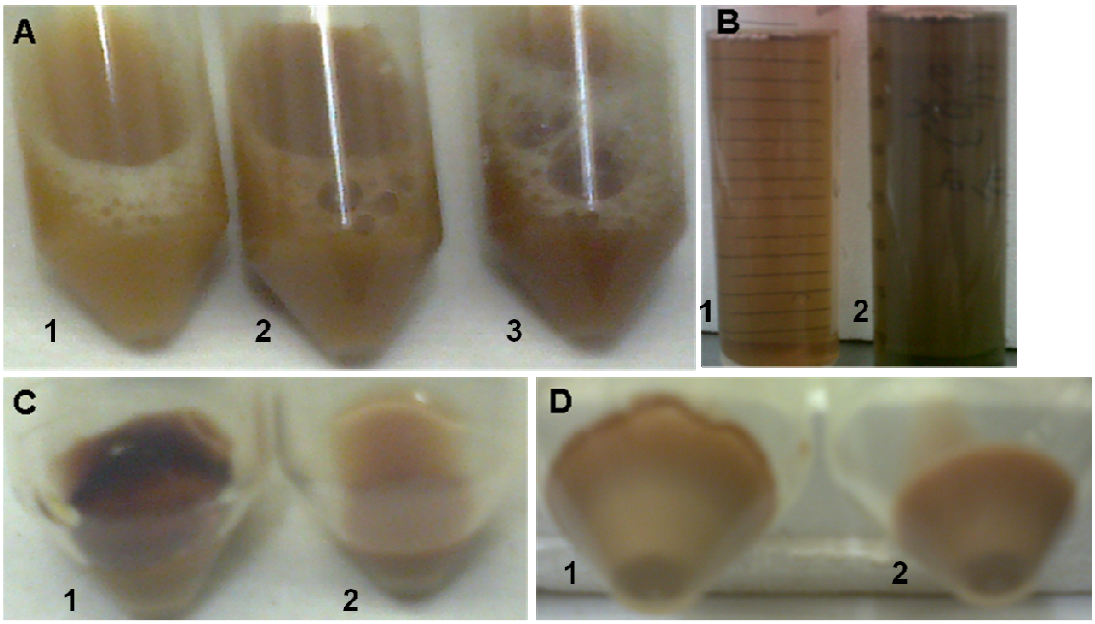



**Figure 4.51: Effect of 5-ALA addition on the color of cultures of a transformant of *Y. lipolytica* CTY029 (β-oxidation disrupted) with CYP505A1 cloned.**

A –(1) culture with only HBA added, (2) culture with 5-ALA and HBA added, (3) culture with only 5-ALA added.

B – supernatant of (1) culture with only HBA added, and (2) culture with HBA and 5-ALA added.

C and D – pellets of (1) culture with only 5-ALA added, and (2) culture with 5-ALA and HBA added.

The colors of the cultures differed subtly (figure 4.51A), as cultures to which 5-ALA were added were somewhat darker with a reddish-brown tint. After harvesting the biomass however, clearer differences were observed between cell pellets and supernatants (figure 4.51B-D). The effect of HBA addition was also particularly interesting. The supernatant of the culture with 5-ALA added was notably different from that of the culture without its addition (figure 4.51B). The harvested cells displayed a distinctly red color when 5-ALA had been added, assumed to be excess heme produced using the heme precursor. More revealing was that in cases where 5-ALA and the substrate HBA were added, the red color was located integrally in the cell pellet, while when 5-ALA was added in the absence of HBA, the red color accumulated predominantly on top of the

pellet (figure 4.51C, D). This indicated that while this strain is obviously efficient at producing heme when 5-ALA is supplied, the heme production is excessive for the regular cellular requirements, and excess heme is exported from the cells. When HBA is present production of wild-type P450s is activated, causing a higher demand for heme and hence made available, which is also beneficial for the production of the cloned CYP505A1, hence the improved activity observed upon 5-ALA addition. Based on these results in strains like the β-oxidation blocked *Y. lipolytica* strain it may be worthwhile to coexpress a gene like *hemA* to increase heme production in such a strain.

## 4.16. Further investigation of activity in an *A. adeninivorans* transformant expressing CYP505A1

Since it was clear that the best activity was achieved with *A. adeninivorans* expressing CYP505A1, especially when using growing cells, further work was done with these transformants was planned to attempt to improve the activity further.

### 4.16.1. Increasing substrate (HBA) concentration

The substrate concentration was increased in a similar way that yielded good results with CYP53B1 in this host. Firstly the concentration of HBA added at 48 h growth was raised from the 5 mM used in the previous sections to concentrations of 7.5 mM and 10 mM (figure 4.52).

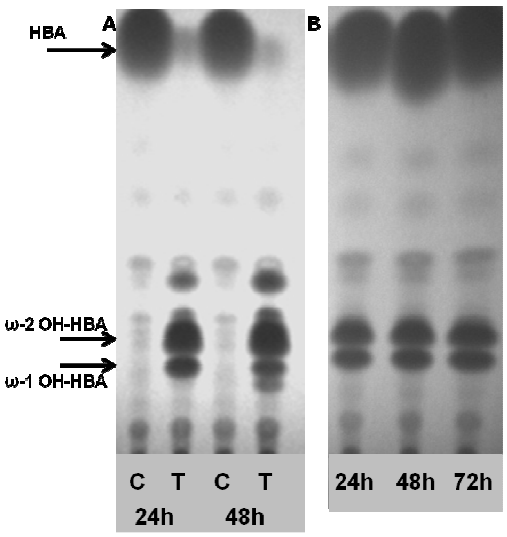



**Figure 4.52: TLC analysis of HBA biotransformation by a transformant of *A. adeninivorans* expressing CYP505A1, with HBA concentrations increased to 7.5 mM (A) and 10 mM (B) by a transformant of *A. adeninivorans* expressing CYP505A1.** C – transformant not expressing any foreign P450; T – transformant expressing CYP505A1. Sampling times are indicated in each case.

When 7.5 mM HBA was added biotransformation results were similar to when 5 mM was used except that more ω-1 OH-HBA remained after 24 h biotransformation. The 10 mM HBA seemed to already be over the threshold of the conversion capability of CYP505A1 in *A. adeninivorans* under these conditions, as only the first two products (ω-2 hydroxyhexylbenzoic acid and ω-1 hydroxyhexylbenzoic acid) were produced, and most of the substrate still remained unconverted by 72 h after substrate addition.

It is possible that since the hexylbenzoic acid (HBA) is not a natural substrate for this enzyme, unlike benzoic acid (BA) for CYP53B1, it has some toxic affects to the cells and / or the enzyme. A decrease in active CYP102A1 in *E. coli* was also observed after 19 h in the presence of 5 mM HBA, compared to 4 h (Gudiminchi and Smit, 2011).
In case the lack of conversion of the higher 10 mM HBA concentration was due to decreased pH, the biotransformation using 10 mM HBA was repeated this time using buffered medium. Phosphate buffer was added at concentrations of 50, 100 and 200 mM to the YPD, compared to non-buffered YPD (figure 4.53).

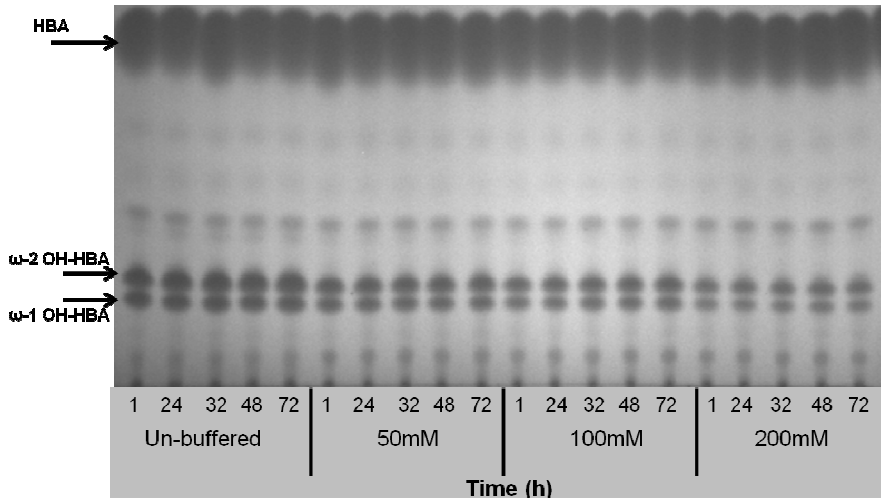



**Figure 4.53: TLC analysis showing the effect of buffer concentration on the biotransformation of 10 mM HBA by a transformant of *A. adeninivorans* expressing CYP505A1.**

Figure 4.53 demonstrates that higher buffer concentrations in fact decreased conversion of the substrate, rather than increase it. The increased buffer concentration possibly increases membrane permeability, which could lead to increased exposure of the enzyme to deactivating levels of substrate; or alternatively loss of cell viability (Tsotsou *et al*., 2002).

The HBA was then fed to the cultures in two 5 mM aliquots at 24 h intervals. Plate A of figure 4.54 demonstrates that after the second addition, not much further conversion took place, indicating that although the substrate showed good conversion initially, it lost activity at some point before the second addition.

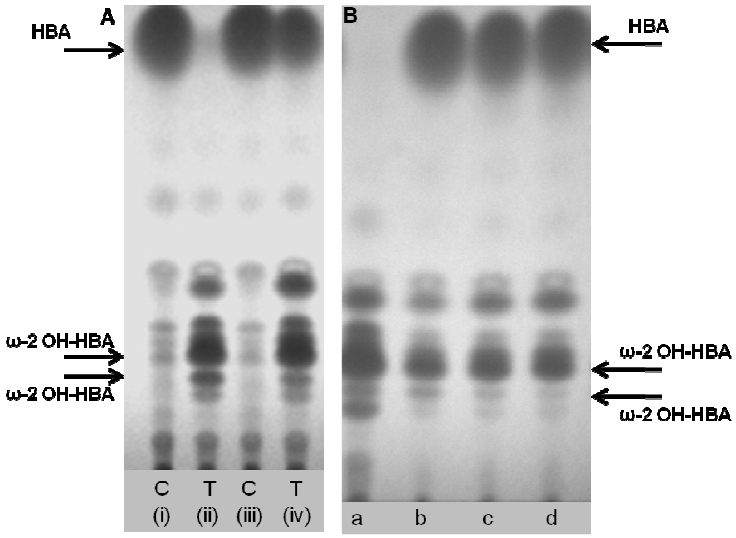



**Figure 4.54: TLC analysis of feeding strategies during HBA biotransformation by a transformant of *A. adeninivorans* expressing CYP505A1.**

Plate A: C – control strain expressing no foreign P450; T – test transformant expressing CYP505A1. (i) and (iii) – 24 h and 48h after addition of 5 mM HBA to control, respectively; (ii) – 24 h after addition of initial 5 mM HBA to test transformant, point of second 5 mM addition; (iv) – 24 h after second addition to test transformant.

Plate B: 'a' – 24 h after initial 5 mM addition of HBA, point of cell harvesting, resuspension in buffer, and re-addition of 5 mM HBA; 'b','c' and 'd' are 24, 48 and 72 h after substrate addition to resupended cells.

In plate B of figure 4.54, cells were harvested, removing excess substrate and products, and resuspended in 0.9% NaCl solution (since phosphate buffer decreased activity), before re-addition of 5 mM HBA. Even after excess substrate and products were removed, activity was still poor for the second addition. This observation could mean that the initial substrate damages the enzyme in an irreversible way, or that one of the products formed irreversibly binds to the active site, causing activity loss.

To test if it was not a question of the enzyme simply losing activity over time, 5 mM HBA was added 24 h later than usual, i.e. at 72 h of cultivation instead of 48 h (figure 4.55).

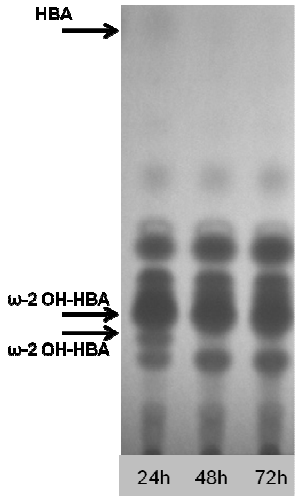



**Figure 4.55: TLC analysis of HBA conversion by 72 h old YPD cultures of *A. adeninivorans* expressing CYP505A1.** Time points indicated represent times after the addition of 5 mM HBA.

The nearly complete substrate conversion, product distribution and formation rate resembled those observed when substrate was added at 48 h, demonstrating that adding the substrate after 72 h growth had no significant effect. This means that the enzyme remains relatively stable in the absence of substrate.

The other possible explanation for the loss of activity in the presence of substrate is that the enzyme stability is due to a protective effect of the cells, and that higher concentrations of HBA result in partial cell lysis, exposing the enzyme to detrimental environmental factors, for example extracellular proteases.

### 4.16.2. Effect of 1, 10-phenanthroline on CYP505A1 activity

Although the 1, 10-phenanthroline did not positively affect the activity of CYP53B1 (section 4.9), it was also added to cultures of *A. adeninivorans* transformants expressing CYP505A1 to test if it could indirectly improve detection of heterologous activity by decreasing background activities.

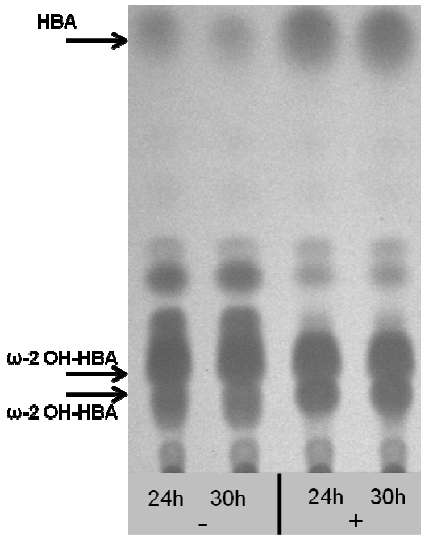



**Figure 4.56: TLC analysis of the effect of addition of 1, 10-phenanthroline on HBA conversion by a transformant of *A. adeninivorans* expressing CYP505A1.** '-' – indicates no added 1, 10-phenanthroline; '+' – indicates where 1, 10-phenanthroline was added. Time intervals after substrate addition are given.

As was observed with CYP53B1, the 1, 10-phenanthroline in fact had an inhibitory effect on the substrate conversion. As was discussed in that case, the negative effect is probably due to inhibitory effects on other cellular components indirectly required for CYP505A1 activity, such as dehydrogenases involved in NADPH regeneration.

### 4.17. Expression of N-terminally His-tagged CYP505A1 in *A. adeninivorans*

CYP505A1 was amplified from pET28b+CYP505A1 to include the N-terminal His-tag from the vector. The amplicon was cloned into pKM173 and pKM177 for expression in *A. adeninivorans.* If His-tagged CYP505A1 could be successfully expressed in the yeast, Western blot analysis can in future be used to compare CYP505A1 production in the different yeasts. Using pKM173 and pKM177 allows us the option of cloning a double-expression cassette for CYP505A1 to be integrated into the host chromosome, similar to what was done for the coexpression of P450s with CPRs.

### 4.17.1. Effect of doubling the CYP505A1 expression cassette integrated into the *A. adeninivorans* genome

Theoretically, targeting rDNA for integration could potentially lead to large amounts of integrations initially, with the integration number stabilizing at a number sufficient to confer antibiotic resistance to the host. As previously mentioned in this chapter, the copy numbers of the genes were not determined, but for *A. adeninivorans* at least are expected to be relatively low. Through use of a deficient marker the copy numbers can be increased, but such markers were not used during this study.

By using pKM173 and pKM177 used for coexpression of P450s and CPRs, we can instead introduce duplicate copies of the CYP505A1 expression cassette in order to potentially increase gene dosage. It is not guaranteed that the integration events would be equal in cases where the plasmid carries two copies of the expression cassette compared to only one copy. However, assuming that the integration events do take place equally, then the expression cassette and hence the gene of interest should be doubled in cases where the plasmid contains two copies.

Single and double-cassette transformants were prepared for CYP505A1 and His-CYP505A1 during fresh transformations, in order to eliminate differences attributable to different rounds of transformations.

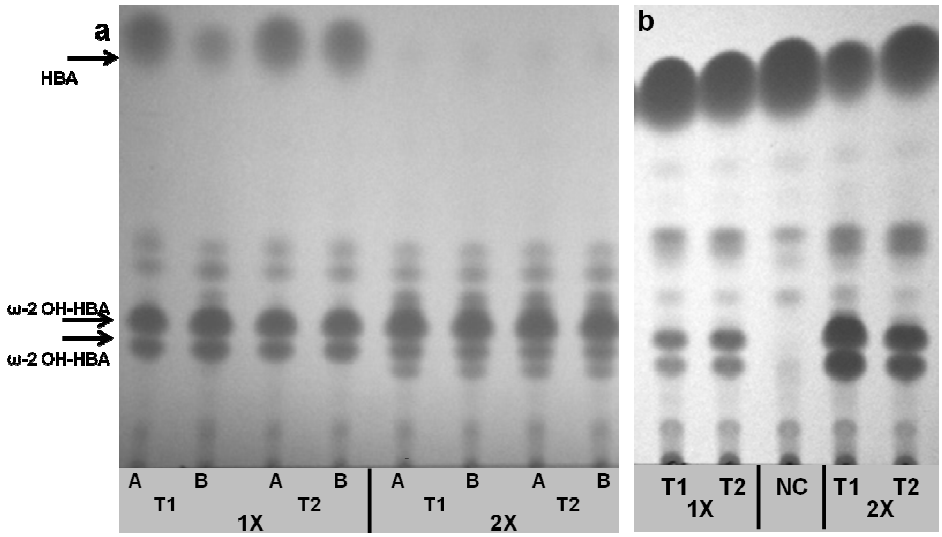



**Figure 4.57: TLC analysis of the differences in CYP505A1 activity between transformants of *A. adeninivorans* transformed with a single copy expression cassette (1X) compared to those transformed with a double copy cassette (2X). results for both CYP505A1 (A) and His-CYP505A1 (B) are shown.** T1 – T2 are separate transformants, A and B are samples from different cultures of the same transformant.

Transformants with the double CYP505A1 expression cassette displayed higher activities than the activities of transformants with the single cassette. The further conversion of ω-1 OH-HBA was in progress and the substrate was basically completely consumed. The improvement indicates that higher amounts of the enzyme may be present, most likely due to the higher gene copy number. The activities of the single expression cassette transformants were however lower than those previously obtained (figure 4.43). One transformant from the first round of transformations had double the activity of another transformant, probably indicating 2 copies compared to one (figure 4.44). The new single-cassette transformants (figure 4.57) therefore also probably have one copy integrated, hence the lower activities than observed in the double cassette transformants.

The N-terminal His-tag decreases the activities of CYP505A1, and the double-cassette was required to give comparable activities to single cassettes of the wild-type CYP505A1. The reasons for this decrease are uncertain at this point.

### 4.18. Sub-cellular localization of CYP505A1 expressed by *A. adeninivorans*

CYP505A1 was demonstrated to be loosely membrane bound in its natural host *Fusarium oxysporum*, but when it was expressed in *S. cerevisiae* it was found primarily in the soluble fraction (Kitazume *et al*, 2000). Presumably some mechanism for binding the enzyme to the membrane in the fungal host is not present in *S. cerevisiae*. To test whether this was also the case after expression by *A. adeninivorans* as well, activity analysis of sub-cellular fractions was carried out. Cells were disrupted using a cell disrupter from Constant Cell Disruption Systems, and the outer membranes and non-lysed cells were separated by centrifugation ('debris' fraction). The resultant supernatant was ultracentrifuged (12000 g for 30 minutes) to pellet the mitochondrial fraction, and the resultant supernatant was subjected to further ultracentrifugation (87000 g for 2 hours) to separate the microsomal and cytosolic (soluble) fractions. Activity assays were performed for each fraction (figure 4.58).

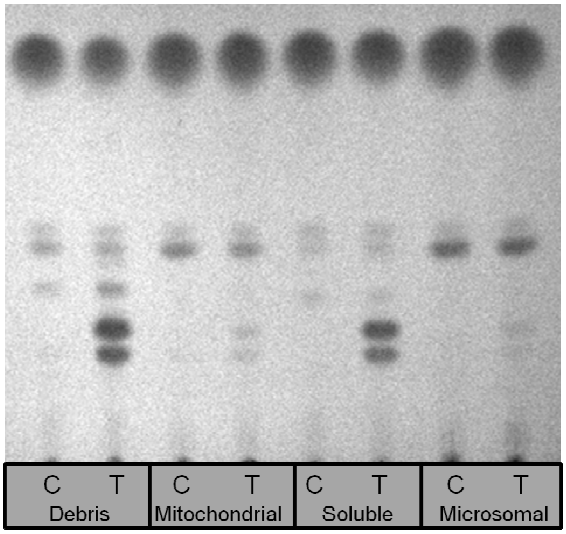



**Figure 4.58: TLC analysis of CYP505A1 activity in different cellular fractions of *A. adeninivorans*.** C – control transformant with no foreign P450 expressed; T – test transformant expressing CYP505A1.

It is not surprising that the ‘debris’ fraction displayed high activity, as it also includes non- or partially lysed cells. Among the sub-cellular fractions however, the activity is definitely clearest in the soluble fraction, which is in agreement with the results obtained during expression by *S. cerevisiae* (Kitazume *et al*, 2000). We can clearly observe more wild-type activity in the microsomal fraction, where wild-type P450s are located.

Attempts to reproduce assays with cell-free extracts were largely unsuccessful, despite the addition of protease inhibitors. This was quite unfortunate, as comparisons of cell-free activities of the different strains may have given more insight into the lack of whole-cell activities observed in some cases.

# Chapter 5: General discussion

Cytochrome P450 monooxygenases (P450s, CYPs) are promising enzymes for a variety of applications, particularly in the fields of drug design and metabolism, fine chemical synthesis, and bioremediation (Kumar, 2010). However, so far the only realistic way to apply them to large-scale reactions is by using whole cell biocatalysis, due to enzyme instability, requirement for regeneration of expensive cofactors, and, in many cases, co-protein dependence (O' Reilly *et al*, 2011; Gillam, 2007; Urlacher and Girhard, 2011). Therefore the identification of appropriate hosts for heterologous expression of P450s is important.

*Escherichia coli* has several shortcomings in the expression of especially eukaryotic P450s and functioning as a whole cell biocatalyst (O' Reilly *et al*, 2011; Urlacher and Girhard, 2011). Therefore yeasts, as unicellular eukaryotes, are obvious alternatives for eukaryotic P450 expression and potential whole cell biocatalysis. Reports on whole cell biocatalysis using yeasts expressing P450s have however also been limited thus far (Zollner *et al*, 2010). Selecting an appropriate host is therefore challenging, especially since expression systems have not been optimized for many yeasts; and in the cases where they have, the species-specific vectors differ in factors such as promoter strength and induction / cultivation conditions, complicating comparisons. A common vector which is applicable to multiple yeasts under similar conditions is necessary to make valid inter-species comparisons, without the bias encountered when using optimised host-vector combinations. Such a vector was constructed in our group (Albertyn *et al*, 2011), and is similar to the CoMed system (Steinborn *et al*, 2006). Our broad-range vector contained targets for rDNA integration in host chromosomal DNA; a hygromycin resistance marker for transformant selection; and a translational elongation factor (TEF) promoter to regulate constitutive expression.

In this study we used this broad-range vector to investigate heterologous expression of (predominantly eukaryotic) P450s in different yeasts, and studied the whole-cell

biocatalytic capabilities of the recombinant strains. This is to our knowledge the first use of a common vector system to compare different yeasts for these purposes. We compared *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Hansenula polymorpha*, *Yarrowia lipolytica* and *Arxula adeninivorans*. Expression of eukaryotic P450s has been reported in *S. cerevisiae* and *Y. lipolytica* before, but not (to the best of our knowledge) in the other three.

To validate the efficiency of the vector system and hosts, we used four reporter P450s, which are two self-sufficient P450s, namely CYP102A1 (prokaryotic) and CYP505A1 (eukaryotic); and two class II P450s, namely CYP53B1 and CYP557A1 (both eukaryotic). The self-sufficient P450s are both sub-terminal fatty acid hydroxylases. 4-Hexylbenzoic acid (HBA) had previously been proven to be a non-natural substrate for CYP102A1 (Gudiminchi and Smit, 2010), and was shown in this study to also be a substrate of CYP505A1. It was therefore used to assay for activities of both self-sufficient P450s investigated in this study.

CYP53B1 is a benzoate-*para*-hydroxylase, and could be assayed using its natural substrate benzoic acid (BA). CYP557A1 is a putative alkane and fatty acid hydroxylase, for which the preferred substrates have not yet been established. Hexylbenzoic acid, three alkylbenzenes (hexylbenzene, nonylbenzene and *p*-cymene) and an aliphatic monoterpene (R-(+)-limonene) were used to screen for activity of this enzyme.

The constitutive promoter in the broad range vector allowed expression in all of the yeasts tested by growth for the same duration, in the same medium, and at the same temperature. Substrates were added to 48 h cultures, by which stage all of the tested yeasts were in stationary phase. Samples taken after biotransformation periods were analysed using thin-layer chromatography (TLC) and gas chromatography (GC).

**General success of the broad-range vector in various yeasts**

Using CYP53B1 we demonstrated successful heterologous P450 expression using the broad range vector and whole-cell biocatalytic activity in all of the hosts tested except *H. polymorpha*. This was, to the best of our knowledge, the first reports of P450 expression in *A. adeninivorans*, which displayed the best activities, as well as *K. marxianus*, which had the worst.

In the case of the self-sufficient P450s both *K. marxianus* and *A. adeninivorans* expressed both CYP102A1 and CYP505A1 successfully, *S. cerevisiae* only expressed CYP102A1 and *Y. lipolytica* only expressed CYP505A1. The best CYP505A1 activity by far was exhibited by *A. adeninivorans*. *S. cerevisiae* had comparable CYP102A1 activity to *A. adeninivorans*, while *K. marxianus* generally seemed better, although in one experiment *S. cerevisiae* gave much higher CYP102A1 activity than either *K. marxianus* or *A. adeninivorans.* As in the case of CYP53B1 *H. polymorpha* did not exhibit any whole cell activity when transformed with CYP102A1, and was therefore not tested for CYP505A1 activity.

CYP557A1 was the only selected reporter gene for which activity could not be absolutely demonstrated during this study. Transformants from neither *K. marxianus* nor *A. adeninivorans* into which CYP557A1 was cloned could give conclusively better activities than transformants into which no P450 was cloned. CYP557A1 is a putative fatty-acid and alkane hydroxylase based on amino acid sequence analysis, but its activity has never been unquestionably demonstrated thus far.

At this point it is not clear whether the enzyme is not functionally produced or if inappropriate substrates have been used for assaying, but since CYP53B1 worked well in all tested hosts (except *H. p*olymorpha) it is unlikely to be the hosts themselves. Therefore the problem was more likely related to the substrates used. Unlike the benzoic acid for CYP53B1, there is a high level of competition for the substrates used to

test CYP557A1 by the native P450s of *Y. lipolytica* and specifically *A. adeninivorans* (which performed the best for CYP53B1 and CYP505A1); for example the activity of wild-type *A. adeninivorans* to convert limonene to perillic acid (Van Rensburg *et al*, 1997). This presents a massive challenge when trying to identify activity of the cloned P450.

CYP557A1 requires further investigation, preferably without background activities, possibly using expression in *E. coli* and / or possibly purification of the enzyme and a CPR. These approaches fell outside of the immediate scope of this work however, and were hence not pursued.

**Copy number of cloned P450s**

Taking specifically CYP53B1 activities into account, considerable variation was encountered between hosts and in some cases between transformants. It is possible that different transformants may have different copy numbers, which is the reason why multiple transformants were screened in each case. Exact copy numbers were not determined as suitable markers for quantitative PCR for each species were not available (Terentiev *et al*., 2004b). Aside from being time consuming and potentially arduous work, it was not considered to fall directly in the scope of this project. Therefore, the most promising transformants from each species were selected during the initial screens for direct comparisons under an established set of conditions.

Copy number is however not considered to be the reason for the superior activities observed with *A. adeninivorans* transformants. One can clearly observe a nearly two-fold difference in activity between the two transformants of *A. adeninivorans* transformed with pKM177+CYP53B1, the biggest difference between transformants of any given host transformed with this plasmid (figure 4.11, chapter 4). A similar occurrence was observed when *A. adeninivorans* expressed CYP505A1, as one transformant had half of the activity of another one. These differences suggests that one

transformant in each case has double the copy number of the other. Since it is more likely that two randomly selected transformants will contain 1 and 2 integrated copies than for example 3 and 6, we can assume that copy numbers of 1 and 2 are present. Similarly, in a study in which a hygromycin resistance marker was expressed under the control of the *A. adeninivorans* TEF promoter and was also targeted to rDNA for integration, 1-3 stably integrated copies were obtained in *A. adeninivorans* (Rosel and Kunze, 1998). Since the other yeasts cannot have less than 1 integrated copy if they display cloned P450 activity and hygromycin resistance, copy number is ruled out as a cause for the better activities observed with *A. adeninivorans*. In fact it is quite likely that some transformants of *S. cerevisiae* and *Y. lipolytica* contained 2 or 3 copies of the vector, since two transformants transformed with pKM173+CYP53B1+UmCPR with 2 or 3 times higher activities than the minimum could in each case be selected from TLC (figure 4.6, chapter 4).

Constructs were prepared containing sequences for N-terminally polyhistidine-tagged versions of both CYP102A1 and CYP505A1. The construct containing His-CYP102A1 has been cloned into all of the selected yeast strains, while the His-CYP505A1-containing construct has so far been cloned into *A. adeninivorans* and can be likewise cloned into other species. This will allow future determination of the resultant His-tagged protein levels using Western blot analysis with anti-His antibodies. This will facilitate correlations between relative amounts of expressed P450s and observed activities between species.

***H. polymorpha***

No activity was detected when *H. polymorpha* transformants were tested for activity of cloned CYP53B1 and CYP102A1, and thus this yeast was not tested for activity of CYP557A1 or CYP505A1. The lack of activity in *H. polymorpha* transformants is puzzling. It may be that the strain used for this study is not as useful for heterologous expression as the reported strains, as considerable variation in properties between

different *H. polymorpha* strains have been reported (Kang and Gellissen, 2005). In preliminary work in which xylanase from *Thermomyces lanigunosis* and vannilyl alcohol oxidase (VAO) from *Penicillium simplicissimum* were expressed in this strain, Dr. M. Labuschagne also obtained poor results, although it performed equally or better than the *S. cerevisiae* strain tested (Albertyn *et al*, 2011). That was however a different *S. cerevisiae* strain from the one used in this study, complicating comparisons. One could directly compare the *S. cerevisiae* and *H. polymorpha* strains used in this study for the expression of other reporter enzymes.

One could alternatively test CYP53B1 expression using our vector in *H. polymorpha* strains in which successful heterologous expression of other proteins has been reported, which would address the minor possibility that *H. polymorpha* is just not a good host for cytochrome P450s. This is however also quite unlikely, considering that the related methylotrophic yeast *Pichia pastoris* has been frequently used for heterologous P450 expression (see chapter 1). Possibilities that the problems are associated with transcriptional or translational factors, such as codon bias are also considered unlikely since they do not seem to affect the other yeasts tested as drastically.

Since P450s have not previously been heterologous expressed in *H. polymorpha* it is difficult to predict whether constitutive expression of cloned P450s could have any negative physiological effects on the *H. polymorpha* cells, although this is not very likely, since it did not affect the other tested yeasts. Foreign protein production by *H. polymorpha* was equal to or better than by *S. cerevisiae*, but worse than *A. adeninivorans*, when the CoMed system was used for the expression of green fluorescence protein (GFP) (Terentiev *et al*., 2004b) and interleukin-6 (IL6) (Boer *et al*, 2007). Dr. Labuschagne also confirmed that the TEF promoter indeed works in this yeast, since transformation efficiencies similar to or better than for the other yeasts were obtained when the *Y. lipolytica, A. adeninivorans and S. cerevisiae* TEF promoters were used to drive expression of the hygromycin resistance gene used for selection of

transformants (Albertyn *et al*, 2011). Most of the promising results of heterologous expression obtained using *H. polymorpha* have however been reported using strongly inducible promoters (van Dijk R *et al*., 2000), although the constitutive GAP promoter has also been successfully applied (Heo *et al*, 2003). This strain could therefore be tested for CYP53B1 expression using a vector more suited to *H. polymorpha* with inducible promoters to see if expression improves.

***K. marxianus***

The results obtained with *K. marxianus* in this study are also regarded as tentative. Preliminary work demonstrated that this yeast (as well as *K. lactis*) had far greater integrant instability than the other tested species (Albertyn *et al*, 2011). Likewise, we initially obtained quite promising activities and a whole-cell CO-difference spectrum with CYP102A1 expressed by this organism, but both decreased over time. Therefore results obtained in this study are relevant in terms of the long-term use of this strain, but are probably underestimations of the activities achievable using fresh transformants. This may be due to competition between the homologous recombination (HR) and non-homologous end-joining (NHEJ) mechanisms within the cell for the integration cassette. Studies showed that the deletion of either component of the Ku70p/Ku80p heterodimer decreases efficiency of the NHEJ while improving HR efficiency, since the Rad52 family of enzymes do not have competition for DNA end binding (Kooistra *et al*, 2004; Abdel-Banat *et al*, 2009). The ploidy of the strain used is also very important, as haploid strains are generally more amenable to genetic manipulation than diploid strains (Lane and Morrissey, 2010).

The instability of integrants is in itself obviously a negative feature for a host intended for industrial application, therefore only strains which have genetic modifications increasing integrant stability can be realistically considered. Nevertheless, we did prove the capability of this yeast to heterologously express P450s and act as a whole cell

biocatalyst. This is to our knowledge the first such report and means that this organism may yet become more relevant to future P450 work.

**Activity of self-sufficient P450s**

When CYP53B1 was cloned product formation could be observed in TLC assays of extracts from all the yeasts (except *H. polymorpha*), when biotransformations were carried out with stationary phase growing cells. This was however not generally the case for the self-sufficient P450s, as activities of transformants using growing cells were often too low to unambiguously detect the formation of products above the noise created by impurities present in HBA and/or extracted from the culture broth. Additionally product formation due to hydroxylation and degradation by wild-type enzymes of *Y. lipolytica* E150 transformants also prevented detection of products formed by the cloned P450s when stationary phase cultures were used. The use of concentrated resting cell suspensions resulted in more easily detectable product formation with *S. cerevisiae* (CYP102A1 only) and *K. marxianus* (CYP102A1 and CYP505A1) and abolished the wild-type conversion of HBA by *Y. lipolytica* E150 transformants so that CYP505A1 activity could be observed with transformants of both *Y. lipolytica* E150 and CTY029. The use of resting cells, however, reduced HBA conversion by *A. adeninivorans* transformants with CYP505 cloned. Even when using resting cells we could not detect activities for both enzymes in all hosts. Only *K. marxianus* and *A. adeninivorans* demonstrated activity for both, while *S. cerevisiae* still only displayed CYP102A1 activity, while the two *Y. lipolytica* strains still only displayed CYP505A1 activity. Despite the fact that the resting cell specific activity of *A. adeninivorans* was approximately 4-fold lower than that obtained using growing cells, resting cells of *A. adeninivorans* still exhibited higher activities than the other species.

The two products produced by HBA conversion by whole cells of *E. coli* expressing CYP505A1, were the same as the products formed by CYP102A1, namely the sub-terminally hydroxylated products ω-1 hydroxyhexylbenzoic acid (ω-1 OH-HBA) and ω-2

hydroxyhexylbenzoic acid (ω-2 OH-HBA) (figure 1.12); although in different ratios. With CYP102A1 the ω-2 OH-HBA: ω-1 OH-HBA ratio is 7:1 (Gudiminchi and Smit, 2011); while with CYP505A1 this ratio is initially 1:1. When the HBA is nearly completely consumed by CYP505A1, the ω-1 OH-HBA product concentration starts decreasing, with concomitant production of other products, presumably further hydroxylated products of ω-1 OH-HBA such as di-hydroxyhexylbenzoic acid. Meanwhile, a constant level of ω-2 OH-HBA is maintained. Such further hydroxylations and / or oxidations were not observed during HBA conversion by CYP102A1 or CYP505A1 expressed in *E. coli* (Gudiminchi and Smit, 2011); nor by CYP102A1 cloned in *A. adeninivorans*.

Although CYP102A1 in *E.* coli completely converted the substrate, these additional products were never observed. For CYP102A1 in *A. adeninivorans* and CYP505A1 in *E. coli* however, HBA was never completely degraded. It is therefore unclear at this stage whether CYP505A1 is accepting ω-1 OH-HBA as a secondary substrate once HBA is nearly depleted, or whether the later activities are catalysed by wild-type enzymes of the yeast. It is probably a combination of both.

*K. marxianus* and *S. cerevisiae* appeared to express the bacterial enzyme CYP102A1 somewhat better than *A. adeninivorans*, although still poorly compared to *E. coli*. These observations may have something to do with the fact that *K. marxianus* and *S. cerevisiae* are phylogenetically more closely related, while being more distant from *Y. lipolytica* and *A. Adeninivorans* (Albertyn *et al*, 2011*;* Lane and Morrissey, 2010). In fact *A. adeninivorans* is more closely related to *K. marxianus* and *S. cerevisiae* than *Y. lipolytica*, perhaps explaining why CYP102A1 activity was observed with these three species but not with *Y. lipolytica* (Albertyn *et al*, 2011*;* Lane and Morrissey, 2010).

While the CYP102A1 activities in *K. marxianus* and *S. cerevisiae* were significantly better than the other hosts tested, more bacterial P450s would need to be expressed in the different hosts before conclusions about bacterial P450 expression in general can be made. In this case our focus was on eukaryotic P450s, and CYP102A1 was only

included, because it is known as a stable and highly active self-sufficient P450. The expression of CYP102A1 in *E. coli* is still far better and this study did not specifically aim to improve on that system (Gudiminchi and Smit, 2010; Schneider *et al*, 1998).

The differences, in biomass production and HBA conversions by transformants of the two *Y. lipolytica* strains were likely due to differences between parental strains of E150 and FT120. The FT120 derivative demonstrated similar activities whether growing or resting cells were used. The E150 derivative on the other hand only demonstrated CYP505A1 activities in resting cells, as wild-type enzymes out-competed CYP505A1 in growing cells of this strain. A FT120 predecessor with its β-oxidation pathway intact could be directly compared to the strains used in this study for its natural activity and activity of heterologously expressed P450s toward HBA, to understand how much of what we observed is due to the differences in β-oxidation pathways and how much is due to characteristics of the parental strain.

**Effect of cytochrome P450 reductase (CPR) partners**

We demonstrated that the natural CPRs in all the tested yeasts (except *H. polymorpha*) were able to facilitate detectable CYP53B1 activity. Various reports have demonstrated that heterologous P450 activities can be further enhanced by overexpression of wild-type CPRs or coexpression of the natural CPR partner of the heterologous P450 (Schiffler *et al*, 2004; Nthangeni *et al*, 2004).

Intermixing of eukaryotic CPRs and P450s has not been quite as bold, with perhaps the exception of activities in membrane fractions of human P450s being reconstituted using purified rat CPR (Kim *et al*, 2008; Imaoka *et al*, 1996). For this study, we coexpressed CPRs from *Yarrowia lipolyica* (YlCPR), *Rhodotorula minuta* (RmCPR) and *Ustilago maydis* (UmCPR) in different yeast species to test their efficiencies in enhancing heterologous CYP53B1 activity in the different hosts. RmCPR is a natural CPR partner of CYP53B1, while coexpression of YlCPR in *Y. lipolytica* is in fact overexpression of

the native CPR. In other cases however, YlCPR was foreign to both the host and the CYP53B1. In all cases UmCPR was foreign to both host and CYP53B1, although it is also of basidiomycetous origin like RmCPR and CYP53B1. Our approach of comparing these three different CPRs for enhancement of activity of a P450 was therefore quite novel.

The first surprising result was by how much the various reductases differed in improving CYP53B1 activity. These differences were most prominent during expression in *A. adeninivorans*, therefore they were investigated further using this host. The basidiomycetous RmCPR and UmCPR improved CYP53B1 activity much more than the YlCPR. This could be because CYP53B1, RmCPR and UmCPR originate from the basidiomycetes, but on the other hand the YlCPR is ascomycetous, like the hosts used in this study. The real surprise however was that the UmCPR improved CYP53B1 activity even more dramatically than its natural partner RmCPR. The activities obtained with other *A. adeninivorans* transformants were only 23% (no cloned CPR), 13% (YlCPR) and 47% (RmCPR) that of the UmCPR activity. One explanation for the observation that YlCPR seemed to actually reduce the activity, is that the YlCPR is less compatible with CYP53B1 or has lower activity than the wild-type *A. adeninivorans* CPR (AaCPR) and that heterologous expression of the YlCPR either reduces expression levels of the AaCPR or displaces AaCPR from CYP53B1.

The enhancement of CYP53B1 activity by UmCPR was however not equal in all species. The improvement was better in *Y. lipolytica* (15-fold higher volumetric yield, 11.5-fold higher specific activity) than in *A. adeninivorans* (5-fold higher volumetric yield and specific activity) supporting perhaps the notion that activity or compatibility of YlCPR with CYP53B1 is poor. By comparison UmCPR barely improved CYP53B1 activities in *S. cerevisiae* and *K. marxianus*. These results, like the expression of CYP102A1 and CYP505A1, seem to group *S. cerevisiae* and *K. marxianus* separately from *A. adeninivorans* and especially *Y. lipolytica*.

Another fascinating observation was made during investigation of CYP557A1 activities. Although CYP557A1 activity could not be conclusively proven, a definite improvement in limonene hydroxylation by *A. adeninivorans* occurred when UmCPR or RmCPR was expressed, either alone or with CYP557A1. This is strong evidence that these CPRs are in fact enhancing the wild-type *A. adeninivorans* P450s, and even in this case UmCPR improved limonene hydroxylation more than RmCPR. This observation strengthens the suggestion from the CYP53B1-CPR coexpressions that the UmCPR is in fact superior to the other two.

The basidiomycetous CPRs may have higher activities or better interactions with P450s than YlCPR. If the demand for CPR activity is high due to high amounts of P450s, it is possible that the CPR may be more active. Fungi can have very high amounts of P450s, as in the cases of *Aspergillus oryzae* (155 P450 genes) or *Postia placenta* (250 P450 genes), while *Y lipolytica* has 17 P450s (Hedeler *et al.*, 2007; Floudas *et al*., 2012). Searching the *Ustilago maydis* Database of the Munich information centre for protein sequences (MIPS) with 'cytochrome P450' as the query results in 17 hits, 2 of which are labelled as cytochrome P450s while the rest are putative P450s **(http://mips.helmholtz-muenchen.de/genre/proj/ustilago/listSearch.html).** A BLASTp search of the *U. maydis* (strain 521) genome on the NCBI database, using a P450 sequence such as that of the CYP53B1 used in this study as a query, delivered sequences of 20 putative cytochrome P450s (http://www.ncbi.nlm.nih.gov//blast/). Searches for P450s in the genomes of basidiomycetous yeasts such as *Rhodotorula glutinis* and *Cryptococcus neoformans* however delivered only 5 P450s for each yeast, indicating that *Rhodorula minuta* probably also have such a relatively small number of P450s (i.e. less than *Y. lipolytica*). This result then makes the superior activity of transformants with the RmCPR when compared with ones with the YlCPR difficult to explain.

The relevant specific activity of each CPR can be determined by monitoring of cytochrome *c* reduction (Narhi and Fulco, 1986). This would determine to what extent

the CPR activities themselves differ. It would be better to first express the CPRs in *E. coli* and perhaps purify them before conducting such assays, to eliminate background activity interference. The specific interactions between the CPR and the P450s can be investigated by their coexpression in *E. coli* using the pETDuet vector system, provided that all relevant proteins are produced and correctly folded; or by reconstitution of P450 fractions with the purified reductases.

The complexity of P450-CPR interaction is demonstrated by an interesting report on a study on CYP53A15 and two natural CPR partners from *Cochliobolus lunatus* (Lah *et al.*, 2011). It was demonstrated that although both CPRs interact with CYP53A15, they appear to do so differently and possibly for different physiological reasons. The CPR1 is constitutively expressed and is essential for normal growth of the organism. CPR1 co-operates with CYP53A15 preferably for primary metabolism of benzoic acid. CPR2 appears to be involved in more specialized xenobiotic metabolism, as it is inducible by other substrates such as isoeugenol and 3-methoxybenzoic acid, while CPR1 is not. The striking observation however was that activity of one P450 can be manipulated by the two CPRs, where presumably by interacting differently with CYP53A15, different products were produced by the same P450. For example CPR1 causes CYP53A15 to produce the monohydroxylated *p*-hydroxybenzoic acid (pHBA), but CPR2 causes the dihydroxylation to 3,4-dihydroxybenzoic acid, a compound which is primed for ring cleavage as an early step in the degradation pathway of aromatic compounds (Lah *et al*, 2011).

**Comparison of most promising activities with activities reported in the literature**

The best activities obtained in this study were obtained with *A. adeninivorans* transformants expressing either CYP505A1 or coexpressing CYP53B1 and UmCPR. We compared our results to some high activity levels reported for whole cell P450 activities in table 1. Although our activities with CYP102A1 were poor, our system generally compares very favourably, as only three of the reports really compete with our

results with eukaryotic P450s. Two of these are expressions of CYP102A1 in *E. coli*, which is a combination known to give high activities (Schneider *et al*, 1998; Gudiminchi and Smit, 2010), while the other was a cinnamate hydroxylase developed as a short-duration assay in *S. cerevisiae*, and can therefore not be compared for maximum productivities (Chen and Morgan, 2006).

We reached by far the highest product yield, using *A. adeninivorans* coexpressing CYP53B1 and UmCPR. The closest maximum yield to that was also obtained using CYP53B1. In that study however, CYP53B1 and YlCPR were coexpressed in *Y. lipolytica* under strong inducible promoters (therefore it was actually overexpression of the native *Y. lipolytica* CPR). The biotransformations were performed under optimized conditions in shake flasks in which continuous addition of inducer maintained the initial rate of 5 $\mu mol.h^{-1}\ g_{DCW}^{-1}$ for up to 200 h. The maximum yield obtained after 200 h was 11.5 mM of pHBA, which was approximately a 30% conversion of the 41 mM BA which was added (Shiningavamwe *et al*, 2005). We obtained 100% conversion of 15 mM BA to pHBA in 120 h, under non-optimised shake flask conditions using *A. adeninivorans* carrying presumably only two copies of each, UmCPR and CYP53B1.

The promising results obtained in this study using *A. adeninivorans* are even more intriguing when one considers that not one component of our broad range vector system originated from *A. adeninivorans*, therefore it was theoretically the least favoured of the represented yeasts to succeed.

**Table 1: Comparison of the most promising data obtained during this study with some of the most promising whole-cell data P450 from literature.**

| Species | Cloned P450 | Substrate | Initial rate | | | Maximum | | | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | | Specific ($nmol.min^{-1}.g_{DCW}^{-1}$) | Volumetric (μM/min) | Duration (h) | Yield (mM) | Duration (h) | Total production rate (μM/h) | |
| *E. coli* | CYP102A1 | Pentadecanoic acid | 5000 | 10 | 0.5 | 1.85 | 40 | 46 | Schneider *et al*, 1998 |
| | | | | | | 1.2 | 10 | 120 | |
| | | 4-Hexylbenzoic acid | 160 | 32 | 1 | 4.3 | 4 | 1080 | Gudiminchi and Smit, 2010 |
| *S. cerevisiae* | Cinnamate 4-hydroxylase | Cinnamic acid | 3600 | 4.9 | 0.17 | n.a.[a] | - | - | Chen and Morgan, 2006 |
| | CYP102A1 | 4-Hexylbenzoic acid | 83 | 0.57 | 24 | 0.82 | 24 | 34.2 | This study |
| *Y. lipolytica* | CYP53B1 | Benzoic acid | 80 | 0.844 | 42 / 200[b] | 11.6 | 200 | 58 | Shiningavamwe *et al*, 2006 |
| *S. pombe* | CYP509C12 | Progesterone | 3.88 | 1.4 | 1 | 0.3 | 48 | 6.3 | Petrič *et al*., 2010 |
| | | | | | | 0.3 | 6 | 50 | |
| | CYP2C9 | Diclofenac | 39 | 0.313 | 24 | 1.51 | 72 | 21 | Dragan *et al*, 2011 |
| *A. adeninivorans* | CYP505A1 | 4-Hexylbenzoic acid | 1110 | 18.9 | 3 | 4.94 | 24 | 210 | This study |
| | CYP53B1 | Benzoic acid | 260 | 4.33 | 3 | 15 | 120 | 125 | |

[a] Not applicable as experiment was set up for screen rather than production;

[b] Rate was maintained up to 200 h by regular supplementation with the inducer.

# Chapter 6: Concluding remarks and future research

The main aim of this study was to use a broad-range vector system to compare heterologous P450 expression in various yeast species under the same conditions. More specifically, we required a host which could facilitate whole-cell biocatalysis, since this is currently the only feasible approach for large-scale applications of these enzymes. Eukaryotic P450s were specifically targeted, since they have received limited attention, other than drug metabolic studies using mammalian P450s. In addition, *E. coli* is limited in its capacity to produce eukaryotic P450s and to facilitate whole-cell biocatalysis. Some of the possible routes which can now be followed based on observations made during this study will be briefly discussed.

We obtained very promising results during this study which confirmed that our broad-range vector is applicable to inter-species comparisons of heterologous protein expression. For our purposes, it particularly represents a means for screening various yeasts for their ability to act as heterologous P450-expressing whole-cell biocatalysts. Variations of the vector allowed simultaneous integration of individual expression cassettes for P450s and their CPR partners, as was done for CYP53B1 and CYP557A1 in combination with various CPRs during this study. Alternatively, they can be used for simultaneous integration of two expression cassettes for the same gene for increased gene dosage, as proven for CYP505A1 in this study.

These vectors could be used in future to test P450 expression and whole-cell biocatalytic abilities in even more species. For example *Schizosaccharomyces pombe* could be of interest due to its frequent use in human P450 research (Dragan *et al*, 2011; (Drăgan *et al.*, 2005). Alternatively different strains of particular yeasts can be tested, as for example considerable physiological variations between *K. marxianus* strains have been reported (Rocha *et al*, 2011). Additionally, considerable variation in activity of a β-galactosidase from *Saccharomycopsis fibuligera* was observed when the gene was expressed in various industrial strains of *S. cerevisiae* (Gurgu *et al*, 2011). It could

therefore be interesting for example to compare heterologous expression in the *A. adeninivorans* strain used in this study to the commonly used LS3 strain (Boer *et al*, 2005) or different strains of *H. polymorpha*. The targeted recombinant proteins could include, but need not be limited to, P450s.

Applying the broad-range vector to identifying other hosts can be done to search for hosts with possibly even better activities than those of *A. adeninivorans* displayed in this study. Otherwise it can be used to identify hosts which are better suited to certain applications, thereby expanding the heterologous expression toolbox to include hosts adapted for different conditions such as temperature, salinity, osmolarity, etc.

The broad-range vector can particularly be used in combination with CYP53B1 for screening of other hosts, as this study has once again proven it to be a useful reporter enzyme. Benzoic acid (R496,58 / 500 g) is relatively inexpensive compared to commonly used substrates for (especially human) P450 assays, such as derivatives of coumarin, for example 7-ethoxycoumarin (R2067,75 / g); resorufin, for example benzyloxyresorufin (R1836,22 / 5 mg) and ethoxyresorufin (R723,31 / mg); and naphthalene, for example 1-methoxynaphthalene (R534,90 / 50 g) (O' Reilly *et al*, 2011; prices according to www.sigmaaldrich.com on 25/06/2012). This is therefore also useful for scaling-up studies using promising transformants such as those obtained for *A. adeninivorans*.

On a similar note, 4-hexylbenzoic acid (HBA) has now been demonstrated as a non-natural substrate for both sub-terminal fatty-acid hydroxylases CYP102A1 and CYP505A1, and can therefore be used for screening of other similar enzymes or mutants of CYP102A1 and CYP505A1. Although it is relatively more expensive than benzoic acid (HBA was R549,28 / g at www.sigmaaldrich.com on 25/06/2012), the substrates commonly used to screen for these types of enzymes, the *p*-nitrophenoxy carboxcylic acids (pNCAs), are not available from regular suppliers of P450 substrates, and therefore often require chemical synthesis prior to use (Schwaneberg *et al*, 2001).

The terminal hydroxylation of HBA by the wild type P450s in alkane degrading yeasts such as *Y. lipolytica* and *A. adeninivorans* further demonstrates the usefulness of HBA as a mimic for studying fatty acid hydroxylation by different types of P450s.

It could be tempting to instead use CYP505A1 as a reporter enzyme for further screening of yeasts using the broad-range vector, since it does not require coexpression of a CPR. We could not however observe any activity of this enzyme in *S. cerevisiae*, or in growing cells of *Y. lipolytica* which had strong wild-type activities towards the substrate employed. It may therefore be better to use CYP505A1 as a secondary reporter enzyme.

Most of the emphasis of this study was on the expression of basidiomycetous and ascomycetous P450s and CPRs. Heterologous expression of fungal / yeast P450s is a relatively neglected area of research, as most work on eukaryotic P450s is devoted to mammalian P450s for drug research, and also to a lesser extent plant P450s (Zollner *et al*, 2010; Gillam, 2007; Kitaoka *et al*., 2011). Some fungi have P450 complements (CYPomes) consisting of hundreds of P450s, and therefore represent enormous potential sources of P450s with attractive activities or other characteristics (Ide *et al*, 2012; Nazir *et al*, 2011; Floudas *et al.*, 2012). More self-sufficient eukaryotic P450s are being discovered in yeasts and fungi, which could be tested in a system such as this one for interesting properties (for example Doddapaneni *et al*, 2005).

This further validates the relevance of the reporter enzymes used in this study. *A. adeninivorans* showed particular promise during expression of both ascomycetous and basidiomycetous P450s; therefore it is a candidate for cloning of libraries of fungal P450s into this organism, as was done using *S. cerevisiae* (Ide *et al*, 2012; Nazir *et al*, 2011). Whole-cells could then be conveniently screened for cloned P450 activities.

Alternatively, other more specific P450s can be expressed in *A. adeninivorans* such as human P450s which are of such high interest for drug design studies. We demonstrated

in this study that high levels of whole-cell activity can be attained using transformants of this organism expressing P450s. Therefore it can increase the throughput of human P450 activity screens as assays can be applied to whole cells, thereby dramatically decreasing the time and effort required for fractionation steps, while also most likely increasing the P450 activities (Zollner *et al*, 2010).

Another possibility would be to further optimize the activities in *A. adeninivorans*. This could be done by using a vector better suited to *A. adeninivorans*, although promoters as appealing as the alcohol oxidase promoters of *P. pastoris* and *H. polymorpha* have not been described for this yeast so far. Further process optimizations can also be pursued such as defining the most appropriate parameters for activity in this host.

One of the most fascinating observations in our study was the dramatically different effects elicited by the different cytochrome P450 reductases (CPRs) when coexpressed with CYP53B1, especially in *A. adeninivorans*. The CPR from *Ustilago maydis* (UmCPR) supported CYP53B1 activity far more efficiently than CPRs from *Rhodotorula minuta* (RmCPR) and *Yarrowia lipolytica* (YlCPR), although RmCPR was also reasonably efficient. It was particularly surprising because RmCPR originates from the same host as CYP53B1 and is presumably its natural partner, yet UmCPR improved CYP53B1 activity more than RmCPR did. Rather remarkably, RmCPR and especially UmCPR also seemed to increase the activities of the *A. adeninivorans* wild-type P450s towards limonene. Coexpression of UmCPR with CYP53B1 had an even larger effect in *Y. lipolytica* than it did in *A. adeninivorans.*

It is unclear whether the differences are attributable to the CPRs having higher activities, better interaction with the P450, or being more actively formed by these hosts. The reasons still need to be resolved experimentally. Eventually however, more fungal CPRs could therefore also be tested for improvement of CYP53B1 activity in *A. adeninivorans* and / or *Y. lipolytica*, to see how they compare to the impressive UmCPR in terms of activity and product distribution. Since Lah *et al* (2011) reported that different

products were produced from the same P450 (CYP53A13) in combination with different CPRs, other substrates could also be tested with CYP53B1 with these CPRs to test differences in substrate specificity and products formed (Lah *et al*, 2011).

The reporter enzymes used in this study can also be improved. Similar mutations as those performed on CYP102A1 could be applied to CYP505A1 to allow it to act on other substrates which provide higher value products; or to transform it to an alkane and or fatty acid terminal hydroxylase (Whitehouse *et al*, 2011). This would add to the value of such a whole cell biotransformation system, whether for bioconversion of substrates to value-added products; drug studies; or bioremediation (Kumar, 2011). The class II P450s and CPRs tested in this study were inserted in the coexpression vectors in an order which could allow fusion of the genes to be constructed by PCR, with the P450 component at the N-terminal, and the CPR component at the C-terminal. If the fusions are correctly constructed, they should result in further improvements of the P450 activity over those observed during coexpression (Hayashi *et al*, 2000).

This study has given some insight into possibilities for further applications in the somewhat under-developed field of eukaryotic P450 research, and will hopefully catalyze further research and improvements in this field.

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## Summary

Cytochrome P450 monooxygenases (P450) are a diverse, ubiquitous family of important enzymes which catalyze a variety of activities. Their outstanding abilities to hydroxylate non-activated hydrocarbons make them attractive enzymes for applications in the fields of chemical synthesis and bioremediation. Large scale applications of P450s are however largely limited by: (i) their requirement for expensive cofactors; (ii) dependence on co-proteins; and (iii) limited enzyme stability.

These problems are largely circumvented by using whole-cell biocatalysis. Therefore the identification of appropriate hosts for heterologous expression is important. *E. coli* has several limitations in its ability to express eukaryotic P450s, therefore yeasts are attractive alternatives, but have scarcely been used for whole-cell applications (Zollner *et al*, 2010).

The aim of this study was therefore to investigate several ascomycetous yeasts for their potential as P450-expressing whole-cell biocatalysts. To perform parallel, unbiased comparisons, the vector, cultivation conditions and assay conditions needed to be consistent between the different yeasts. This was facilitated by a broad-range vector system designed in our group, which allowed genomic chromosomal integration of foreign DNA at the 18S rDNA regions, and expression using a constitutive TEF promoter. Cultures of different yeasts were grown for the same duration at the same temperature in a medium common among strains, prior to activity assays.

Using CYP53B1 as a reporter enzyme we demonstrated whole-cell activity for all of the yeasts tested, except for *H. polymorpha*. The native reductase systems allowed detectable activities of CYP53B1 in all of the other yeasts, with the highest activity (2.3 $\mu mol.h^{-1}$ $.g_{DCW}^{-1}$) found in *A. adeninivorans.* Coexpression of cytochrome P450 reductases (CPR) from *Yarrowia lipolytica* (YlCPR), *Rhodotorula minuta* (RmCPR) and *Ustilago maydis* (UmCPR) all led to improvements of the CYP53B1 activities, with the

highest activity (11.5 µmol.$h^{-1}$.$g_{DCW}^{-1}$) obtained with CYP53B1 and UmCPR coexpressed in *A. adeninivorans*. The effects of the cloned UmCPR also differed between the hosts, with the biggest improvements observed in *A. adeninivorans* and *Y. lipolytica*. RmCPR and UmCPR improved the CYP53B1 activity more dramatically than YlCPR, possibly because CYP53B1, RmCPR and UmCPR are all of basidiomycetous origin.

The CYP53B1 activity achieved in this study was considerably better than results obtained previously in our group. In the previous study, a *Y. lipolytica* strain with multiple copies of CYP53B1 and an additional copy of YlCPR both under the regulation of strong inducible promoters was used, under optimised and constant induction conditions. (Shiningavamwe *et al*, 2006). The activity obtained in this study on the other hand was achieved using an *A. adeninivorans* strain carrying presumably only one or two copies of CYP53B1 and UmCPR, expressed under the control of a constitutive promoter, and under non-optimised conditions.

Activity of the self-sufficient P450s CYP102A1 and CYP505A1 was assayed using hexylbenzoic acid (HBA) as a non-natural substrate, since wild-type β-oxidation pathways would not permit the use of fatty acids as substrates. HBA is hydroxylated at the ω-1 and ω-2 positions of the alkyl chain by both CYP102A1 and CYP505A1 during this study. Better expression of the bacterial CYP102A1 was obtained using *K. marxianus* and *S. cerevisiae*, than in *A. adeninivorans;* whereas expression of the eukaryotic CYP505A1 was better in *Y. lipolytica* and especially *A. adeninivorans*. The best activity observed with a self-sufficient P450 was obtained once more with *A. adeninivorans* expressing CYP505A1 (33 µmol.$h^{-1}$ $g_{DCW}^{-1}$).

Overall, the vector system allowed successful expression of P450s and CPRs from bacterial, ascomycetous and basidiomycetous fungal origin, in multiple ascomycetous yeast hosts. The differential effects of different CPRs on a class II P450 were demonstrated, and the UmCPR was in this case found to be an excellent P450

reductase. We report heterologous P450 expression in *A. adeninivorans* for the first time, and it proved to be, of the yeasts tested, the host with the highest potential for efficient P450 expression and whole cell biocatalysis.

The findings of this study provide insight for the improvement of the field of eukaryotic P450 research and particularly whole-cell biocatalysis, and could potentially assist in enhancing the applications of these promising enzymes.

**Key words:** Cytochrome P450 monooxygenases; heterologous expression, ascomycetous yeasts, broad-range expression vector, cytochrome P450 reductase, coexpression, self-sufficient P450, *Arxula adeninivorans*, whole cell biocatalysis