ALKANE AND FATTY ACID HYDROXYLATING CYTOCHROME P450 MONOOXYGENASES IN YEASTS

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By

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It is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change.

- Charles Darwin

I'm not afraid to struggle or/and fail, for every start there is a finish line, for every struggle there is a triumph and for every fail there is a lesson learned.

- DSS SHUPING

Success is not final, failure is not fatal: it is the courage to continue that counts.

"Winston Churchill"

Dedication

This thesis is dedicated to my family and relatives, especially my mom Ntombizabantu C. MaSechaba Shuping, who has been the pillar of strength throughout my studies and life, Motsokobi, P. A. for her tremendous undivided support and lastly to my late father Johannes Shuping (1944 -1998).

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The staff in the department, from administrators, academics to cleaning staff

My family and friends

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Abbreviations

3D	Three dimensional
CPR	Cytochrome P450 reductase
CYP	Cytochrome P450
DCA	Dicarboxylic acid
FA	Fatty acid
FAD	Flavin Adenine Dinucleotide
FMN	Flavin mononucleotide
kDa	Kilo-dalton
K_d	Dissociation constant
K_m	Michaelis menten constant
kPa	Kilo-pascal
K_s	Catalytic constant
MPa	Mega-pascals
Mr	Molecular weight
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP+	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
P450	Cytochrome P450 monooxygenase(s)
P450BM3	P450 from *Bacillus megaterium*
P450foxy	P450 from *Fusarium oxysporum*
Tm	Melting temperature
WT	Wild-type
Yl	*Yarrowia lipolytica*

Chapter 1

Introduction to the study

n-Alkane degrading yeasts such as *Yarrowia lipolytica*, *Candida maltosa* and *Candida tropicalis* can use *n*-alkanes and fatty acids as a sole carbon source (Sumita *et al*., 2002). In these yeasts, enzymes in the ω- and β-oxidation pathways metabolize *n*-alkanes and fatty acids to eventually form energy, carbon dioxide and water (Figure 1.1). The first rate-limiting step in *n*-alkane assimilation is the hydroxylation of *n*-alkanes to 1-fatty alcohols by cytochrome P450 monooxygenases belonging to the CYP52 family. These P450s receive reducing equivalents from NADPH *via* P450 reductases (CPRs). Further oxidation of alkan-1-ols yield fatty acids of the corresponding chain length. The resulting fatty acids or fatty acids added as substrates, are hydroxylated a second time at the ω-position by CYP52s. The hydroxy-fatty acids are oxidized to α,ω-dicarboxylic acids. Fatty acids formed from monoterminal oxidation of *n*-alkanes or added as substrates can alternatively act as precursors for lipid biosynthesis or enter the perixosomes. Once in the perixosomes, fatty acids and α,ω-dicarboxylic are activated to corresponding acyl-CoA esters, then metabolized by β-oxidation enzymes to form acetylCoA and eventually carbon dioxide, water and energy (Fickers *et al*., 2005; Scheller *et al*., 1998). Fatty acyl-CoA oxidases, encoded by *POX* genes, catalyze the first rate-limiting step of the β-oxidation pathway. These acyl-CoA oxidases are substrate specific and it has been shown that deletion of certain *POX* genes, depending on the organism, greatly improves accumulation of α,ω-dicarboxylic acids (Wache´*et al*. 2006; Fickers *et al*., 2005; Craft *et al*., 2003; Hara *et al*., 2001; Mobley, 1999).

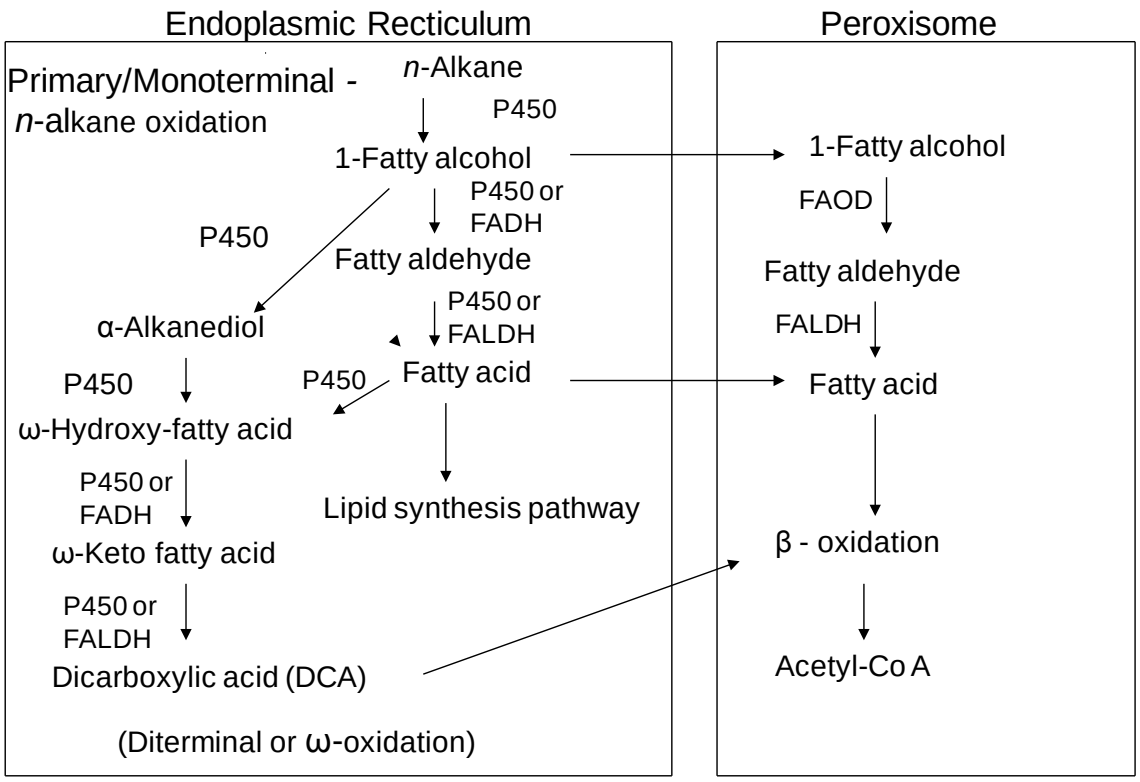

Figure 1.1: *n*-Alkane and fatty acid assimilation in yeast cells. *n*-Alkane assimilation occur via primary oxidation or diterminal oxidation. P450s are responsible for the first rate-limiting step of the hydroxylation of *n*-alkanes to 1-fatty alcohols and fatty acids to ω-hydroxy-fatty acids. The subsequent reactions are also catalyzed by P450s and other enzymes of the ω-oxidation pathways in the endoplasmic recticulum leading to the formation of DCA. Fatty acids and the resulting DCA then undergo β-oxidation in the peroxisome to form energy, carbon dioxide and water (Fickers *et al*., 2005; Scheller *et al*., 1998).

α,ω-Dicarboxylic acids (DCAs) are valuable chemical intermediates used as raw-materials in industrial synthesis of polymers, cosmetics, pharmaceutical products, plastics and lubricants (Wache *et al*., 2006; Liu *et al*., 2004; Craft *et al*., 2003; Arie *et al*., 2000; Mobley, 1999). Traditionally short chain α,ω-dicarboxylic acids are synthesized commercially in large scale using chemical processes. However, α,ω-dicarboxylic acids with chain lengths longer than C_{10} are difficult to produce chemically in large scale. Biocatalytic processes using *n*-alkane utilizing yeasts offer an alternative over chemical processes, because they can produce

long-chain aliphatic α,ω-dicarboxylic acids from substrates such as aliphatic *n*-alkanes and fatty acids (Mobley, 1999). Brassylic acid (1,13-tridecanedioc acid) used as raw-material for production of musk perfume is synthesized industrially from *n*-tridecane by *C*. *maltosa*. Two industrial mutants M2030 and M1210 strains of *C*. *maltosa*, produce up to 125 and 165 gl^{-1} of brassylic acid (Kogure *et al*., 2007). Dodecanedioc acid used for production of nylon and fragrances (Mobley, 1999) is produced from *n*-dodecane by *C*. *tropicalis*. *C*. *tropicalis* mutant strains developed by Picataggio *et al*., (1992) can produce up to 140 g l^{-1} of 1,12-dodecanedioc acid from *n*-dodecane. Strains used for production of α,ω-dicarboxylic acids in industry have always been *Candida* species. To further increase production of α,ω-dicarboxylic acids, the next step is to over-express CYP52s together with the NADPH P450 reductase (Craft *et al*., 2003). DCA production by *n*-alkane degrading yeasts is of great interest because these CYP52s have the highest bioreactor productivities (1.9 g $l^{-1}h^{-1}$) reported thus far for CYP450 processes (Julsing *et al*., 2008).

C. *tropicalis*, *C*. *maltosa, C*. *cloacae* and *C*. *albicans* are well-studied *n*-alkane assimilating yeasts belonging to the genus *Candida* (Cheng *et* al., 2005; Fickers *et al*., 2005; Eschenfeldt *et al*., 2003). Most *Candida* species have the ability to cause diseases, are diploid or partially diploid and their genetic make-up is relatively difficult to manipulate and regulate. Unlike *C*. *tropicalis* and *C*. *maltosa*, *Y*. *lipolytica* is phylogenically distant from the members of the genus *Candida*, is haploid and has a sexual life cycle. Most strains are unable to grow at temperature above 32 ºC. It is a strictly aerobic, non-pathogenic, dimorphic yeast able to grow on substrates rich in proteins as well as hydrophobic substrates. *Y. lipolytica*, due to its specific properties, has been investigated for the production of single cell protein and citric acid, as well as for the production of detergents, lubricants and surfactants from several mono and diterminal oxidation products of *n*-alkanes (Fickers *et al*., 2005).

Various β-oxidation mutants of *Yarrowia lipolytica* have been constructed and evaluated for DCA production. In the work done by Smit *et al*. (2005), single, double (Δ*pox2*, *pox3)*, triple (Δ*pox2*, *pox3*, *pox5)* and quadruple (Δ*pox2*, *pox3*, *pox4*, *pox5)* deletion mutants were used to evaluate the function of these isoenzymes. Only the quadruple deletion mutant accumulated significant amounts of DCAs from *n*-dodecane, *n*-tetradecane and *n*-hexadecane in shake flask experiments. However, DCA concentrations were low compared with concentrations reported for *C*. *tropicalis* with only 8 g l^{-1} dodecanedioc acid, 2.4 g l^{-1} tetradecanedioic acid and 0.7 g l^{-1} hexadecanedioic acid accumulated. At least with their work, Smit *et al*., 2005 showed that *Y*. *lipolytica* mutants can accumulate dioic acids and might be a candidate for further manipulation to develop an industrial strain for DCA production.

In order to improve DCA production in β-oxidation blocked *Yarrowia lipolytica* strains and to evaluate the differences in alkane and fatty acid hydroxylase activity in *Y*. *lipolytica* and *Candida tropicalis*, we decided to (i) compare biotransformation of alkylbenzenes and alkylbenzene derivatives by the wild-type strains, *Y. lipolytica* W29 and *C. tropicalis* ATCC20336, from which mutants had been derived and (ii) clone fatty acid hydroxylase (CYP52) encoding genes from *C. tropicalis* ATCC20336 into β-oxidation disrupted strains of *Y. lipolytica*.

DCA is not accumulated by wild-type strains, since DCA and fatty acids are degraded in the β-oxidation pathway. We decided to use alkylbenzene substrates for the comparison of the alkane hydroxylase activity of the wild-type strains because alkylbenzenes are only partially degraded *via* the β-oxidation pathway as shown in figure 1.2 (Van Rooyen, 2005). From odd chain-length alkylbenzenes, benzoic acid is a major product and phenylacetic acid a minor product. Phenylacetic acid is formed as the only product from even chain-length alkylbezenes. CYP52s are probably also the enzymes responsible for the first rate limiting step of the hydroxylation of alkybenzenes to phenylalkanol. The phenylalkanol and phenylalkanal intermediates have not been observed. The

phenylakanoic acid eventually enters the β-oxidation pathway to be partially degraded to the products. Biotransformation of 4-hexylbenzoic acid and 4-nonyloxybenzoic acid was also used to compare the strains. Intact dicarboxylic acids as well as dicarboxylic acids with the alkyl chains shortened by β-oxidation are accumulated from these substrates (Figure 1.3 and 1.4) (Obiero, 2006, Van Rooyen, 2005).

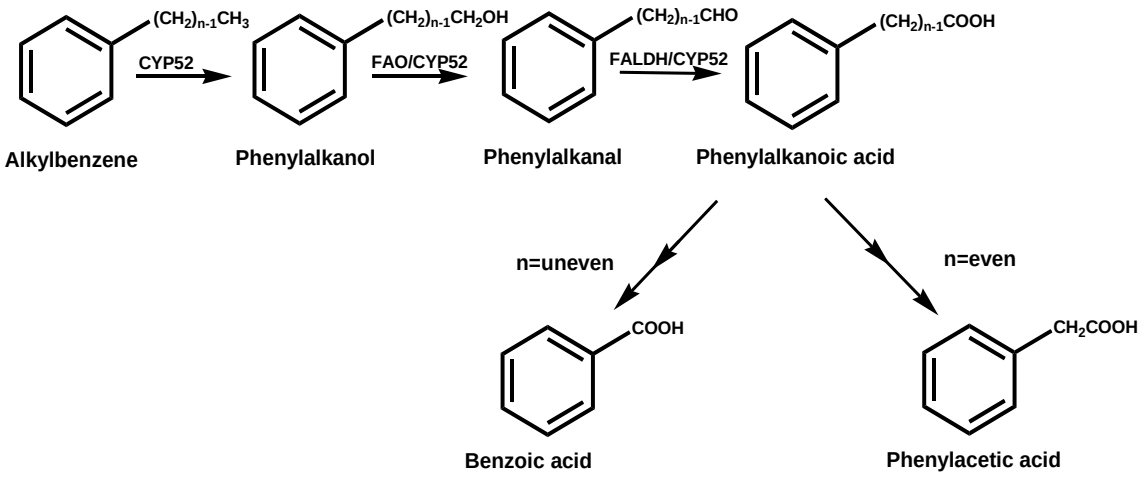

Figure 1.2: Proposed pathway for alkylbenzene degradation in *n*-alkane degrading yeasts (adapted from Van Rooyen, 2005).

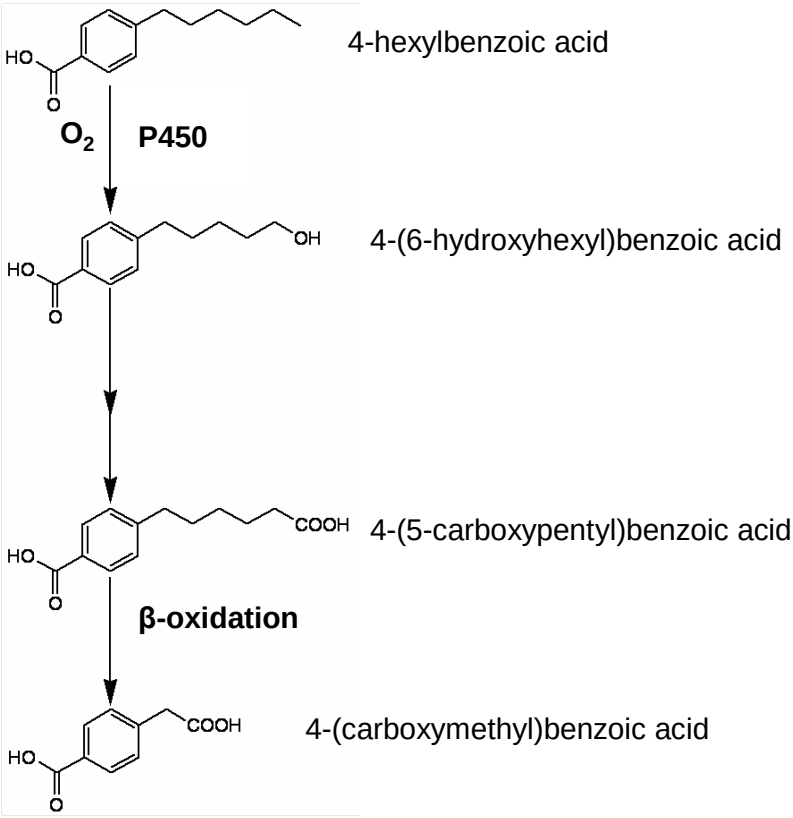

Figure 1.3: Degradation of 4-hexylbenzoic acid by *Yarrowia lipolytica* (Van Rooyen, 2005).

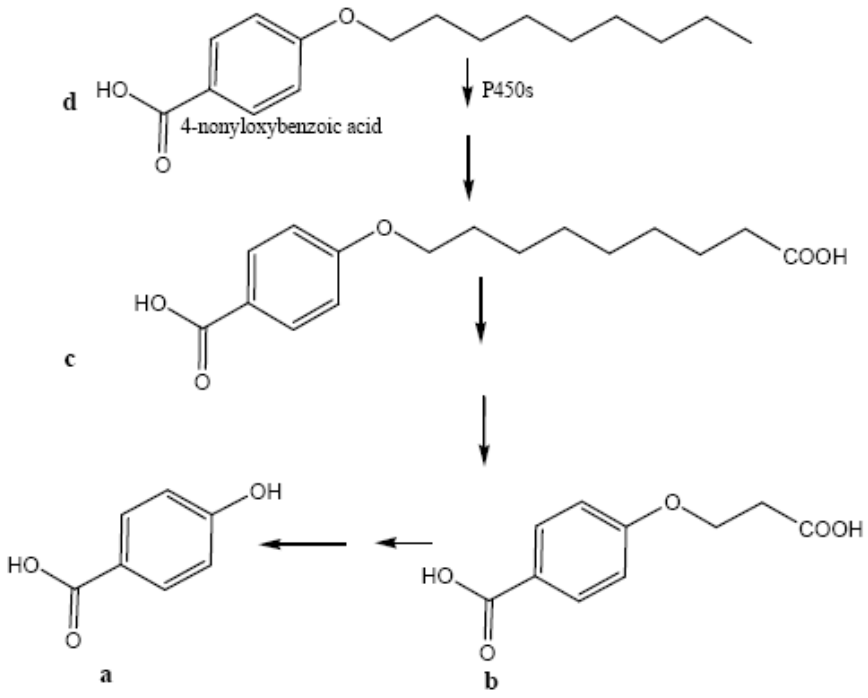

Figure 1.4: Degradation of 4-nonyloxybenzoic acid by *Yarrowia lipolytica*. Three distinct products were observed from the biotransformation of 4-nonyloxybenzoic acid by *Y. lipolytica*, namely product a - *p*-hydroxybenzoic acid, product b the shortened dioic acid 4-(2-carboxyethoxy) benzoic acid and lastly product c an intact dioic acid 4-(8-carboxyoctyloxy) benzoic acid (Obiero, 2006).

The CYP52A17 and CYP52A13 P450 monooxygenases have been identified as fatty acid hydroxylases from *Candida tropicalis* ATCC20336. The genes encoding these P450s are strongly expressed when the yeast is induced with emersol 267 and oleic acid and to a lesser extent by *n*-octadecane (Craft *et al*., 2003). CYP52A17 prefers saturated fatty acids of chain length C_{12} to C_{16} with myristic acid (C_{14}) the preferred substrate. In contrast, CYP52A13 hydroxylated saturated fatty acids of the same carbon lengths poorly with low rates. However, the possibilities of these enzymes hydroxylating *n*-alkanes have not been investigated. The two genes were cloned into two β-oxidation blocked *Yarrowia lipolytica* FT-120 strains and bioconversion of *n*-alkanes, fatty acids and 4-hexylbenzoic acid evaluated to determine whether these enzymes accept these

compounds as substrates and whether these enzymes have different activity profiles towards these substrates. It was of particular importance to establish whether any of these enzymes have alkane hydroxylase activity that might be important in DCA production from *n*-alkanes.

Chapter 2

Literature Review

Microbial alkane and fatty acid hydroxylating cytochrome P450 monooxygenases

2.1 Introduction

2.1.1 Cytochrome P450 monooxygenases

Cytochromes P450 monooxygenases constitute an ever-growing superfamily (McLean *et al*., 2005) of heme-thiolate monooxygenases widely distributed in nature and known to give a prominent soret peak at 450 nm, when carbon monoxide is bound to the reduced P450 enzyme (Newcomb and Chandrasena, 2005; Omura, 2005). The P450 superfamily is divided into families, which share ≥40% amino acid sequence identity and subfamilies which share ≥55% amino acid sequence identity (de Groot, 2006). Although the sequence identity between different families is low and in most cases less than 15%, signature motifs keep the tertiary structure of the P450 enzymes conserved (Hannemann *et al*., 2007).

Common features found in a majority of P450 enzymes are known to play an important role in the stability and the function of the protein (Deng *et al*., 2007). The first motif, the heme binding domain Phe-X-X-Gly-X-Arg-X-Cys-X-Gly which is located near the C-terminus is the most conserved motif among P450 enzymes (Werck-Reichhart *et al*., 2000). This motif has the conserved cysteine residue (Figure 2.1) which is found across all P450 enzymes (Deng *et al*., 2007) and which serves as a fifth ligand to the heme iron (Werck-Reichhart *et al*., 2000). The second motif is Glu-X-X-Arg located in the K helix proximal to the heme, is suggested to be crucial in stabilizing the core of the protein (Werck-Reichhart and Feyereisen, 2000). The third motif Ala/Gly-Gly-x-Asp-/Glu-Thr-

Thr/Ser in the I helix is involved in oxygen activation, binding, and transfer of electrons to the heme (Deng *et al*., 2007; Werck-Reichhart *et al*., 2000; Werck-Reichhart and Feyereisen, 2000). Other less conserved regions such as the Pro-Glu/Asp-Arg/His-Phe/Trp and proline-rich regions that anchor the protein to the membrane by forming a hinge near the N-terminus are also found in some P450s (Werck-Reichhart *et al*., 2000). The substrate recognition sites are the most variable and play a huge role in the protein substrate specificity and a P450 enzyme may contain more than one and up to six substrate recognition sites per protein (Werck-Reichhart and Feyereisen, 2000).

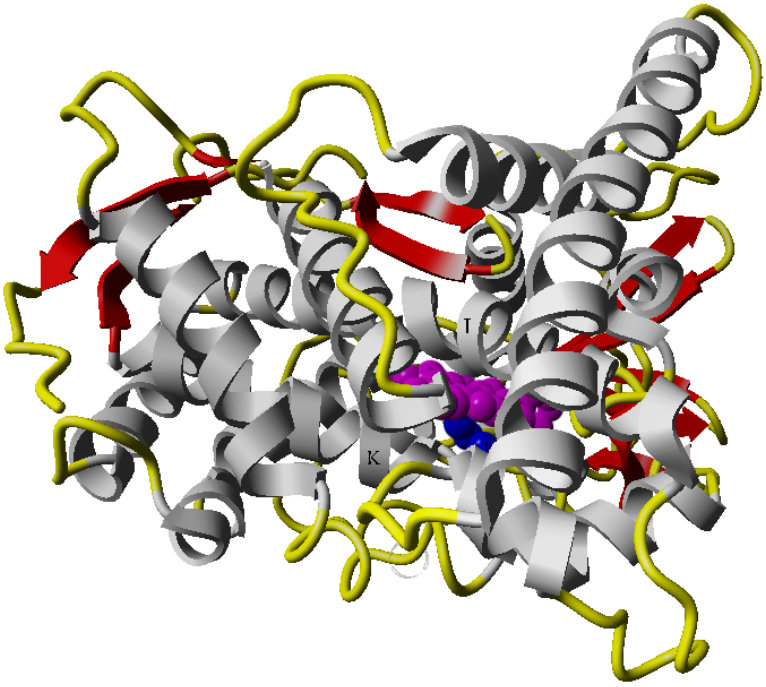

Figure 2.1: The conserved 3D fold of P450s represented by helices (grey), β-sheets (red) and loops (yellow). The axial cysteine (blue) forms a co-ordination to the heme (magenta), which is sandwiched between helices I and K. Molecular visualization was done in Yasara Elmar (Krieger *et al*., 1993), using CYP102A1 (2hpdA.pdb).

The P450 isozymes, also classified as mixed functions oxidases, are extremely diverse (Wiseman and Lewis, 2007). Collectively they catalyze the insertion of

one atom of oxygen regio-selectively and stereo-selectively into over one million chemicals (Shumyantseva *et al*., 2006). Their substrates range in size from ethylene (Mr 28) to more complex substrates such as cyclosporin A (Mr 1201) (Isin and Guengerich, 2007) using either NAD(P)H reducing equivalents or the H_2O_2 shunt pathway.

2.1.2 Reactions

Cytochrome P450s are involved in about 60 distinct types of biotransformation reactions (Shumyantseva *et al*., 2006). The basic reactions catalyzed by P450s often involve hydroxylation, dealkylation (S-, N-, O-), and epoxidation (Isin and Guengerich, 2007). Other reactions as mentioned by Mansuy (2007) include sulphoxidations, isomerization, C-C cleavage, deaminations, desulphurations, dehalogenations, peroxidations, and N-oxide reductions.

A typical P450 hydroxylation is summarized in figure 2.2. The substrate binds to the P450, which displaces H_2O as the sixth ligand of the heme iron and cause a shift from low spin to high spin state. This is also accompanied by an overall protein conformational change that brings the heme iron in close proximity to the reductase and favors the transfer of electrons from electron donor to the heme iron. Electrons are then transported sequentially via NADPH-cytochrome P450 reductase one by one to the heme iron. NAD(P)H + H^+ loses two electrons and two protons as it is oxidized to $NADP^+$. The first electron is used for the reduction of the ferric (Fe^{3+}) heme iron to ferrous (Fe^{2+}) state. This allows molecular oxygen to bind and form the iron-oxygen ($Fe^{2+}OOH$) complex with the addition of a proton and a second donation of an electron from either NA(D)PH cytochrome P450 reductase or cytochrome b5. A second proton causes hemolytic scission of the distal oxygen atom and subsequent formation of H_2O. An unstable ferryl $[FeO]^{3+}$ complex donates its oxygen to the substrate. The hydroxylated product is released and the enzyme returns to its resting state (Isin and Guengerich 2007; Wiseman and Lewis, 2007; Peterson and Graham, 1998).

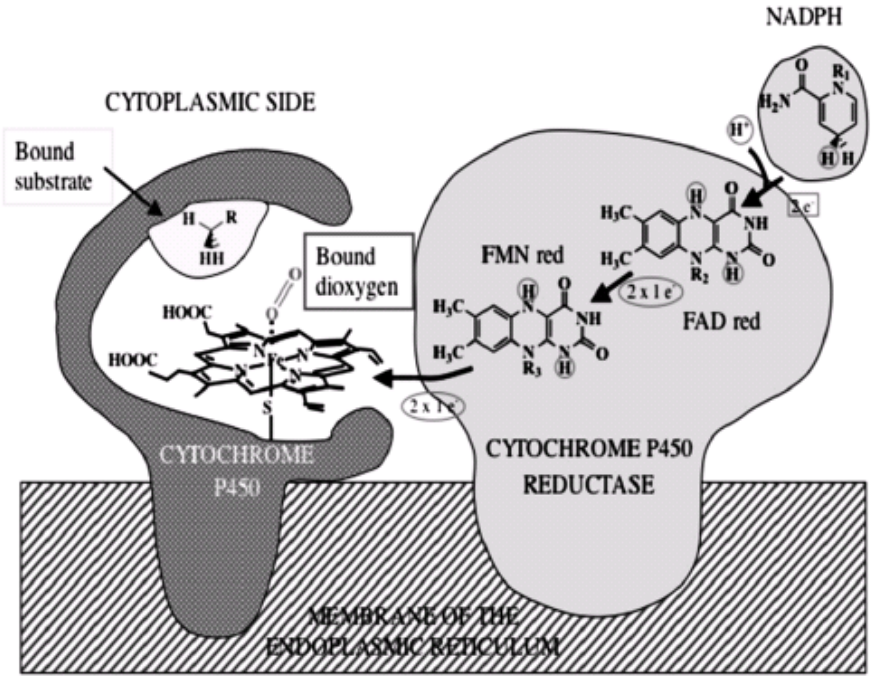

Figure 2.2: Summary of a cytochrome P450 monooxygenase reaction (taken from Ricoux *et al*., 2007).

2.1.3 Cytochrome P450 classes

Prokaryotic P450s are soluble and eukaryotic P450s are associated with the endoplasmic recticulum or inner membranes of the mitochondria. The P450s are classified into ten classes depending on electron transfer mechanism. Alkane and fatty acid hydroxylases belong to classes I, II, IV and VIII (Hannemann *et al*., 2007).

Class I P450s belong to three component systems in which FAD-containing ferrodoxin reductase and an iron-sulfur ferrodoxin transfer electrons from NAD(P)H to the heme catalytic site. They are predominately found in bacteria and mitochondrial membranes of eukaryotes. In bacteria, all components are soluble. However, in the mitochondrial membranes of eukaryotes, the iron-sulphur ferrodoxin protein is the only soluble component.

Class II is the most versatile and largest class. The most common class II P450s are distributed in the microsomal fractions as membrane bound proteins in eukaryotes. These monooxygenases, found in the endoplasmic recticulum of eukaryotes, occur as two integral membrane proteins. A heme containing P450 and a cytochrome P450 reductase containing both FAD and FMN which transport electrons sequentially one by one from NAD(P)H to many of the cytochrome P450 isozymes. In other class II microsomal P450, cytochrome b5 is directly responsible for transferring electrons to cytochrome P450s. In these reactions cytochrome b5 is involved in CPR-independent transfer of both electrons from NADH-cytochrome b5 reductases to P450. It is also involved in the transfer of the second electron to oxyferrous P450 from either NADH or NADPH. Only one class II P450 has been described from bacteria (*Streptomyces carbophilus*). This monooxygenase is soluble and comprises a heme domain CYP105A3 (P450sca) and a separate NADH-dependent P450 reductase containing both FAD and FMN (Hannemann *et al*., 2007).

Class IV comprises a soluble CYP119 (Hannemann *et al*., 2007). This enzyme is one of the P450 enzymes which is apparently reduced by a non-pyridine nucleotide coenzyme (Munro *et al*., 2007). The redox partner mentioned by Nishida and de Montellano (2005) is a 2-oxoacid-ferredoxin oxidoreductase from the archaeon *Sulfolobus tokodaii*. This ferredoxin driven system efficiently reduces CYP119 and supports the hydroxylation of lauric acid.

Class VIII comprises self-sufficient P450s that have diflavin (FAD/FMN) containing reductase components connected to the heme in a single polypeptide chain. These fused P450s have been discovered in various prokaryotes and lower eukaryotes.

2.2 Fatty acid and alkane hydroxylating cytochrome P450 monooxygenases

Fatty acid hydroxylases are classified as in-chain, alpha- and beta or omega-hydroxylases (Figure 2.3) (Benveniste *et al*., 2006). These hydroxylases are ubiquitous in nature (Kahn *et al*., 2001; Zimmerlin *et al*., 1992). The plant fatty acid hydroxylases catalyze the oxidation of saturated and unsaturated fatty acids with high regio- and stereo-specificities at ω, ω-1 or in-chain positions depending on the plant species (Le Bouquin *et al*., 1999; Cabello-Hurtado *et al*., 1998). However, microbial fatty acid hydroxylases have high regio and stereo selectivity and unique properties compared to plant fatty acid hydroxylases (Hannemann *et al*., 2007; Doddapaneni *et al*., 2005; Matsunaga *et al*., 2002). The bacterial fatty acid hydroxylases catalyze the oxidation of in-chain (ω-n, n = 1, 2, 3), as well as alpha- and beta positions of saturated and unsaturated fatty acids with high specificities (Hannemann *et al*., 2007; Matsunaga *et al*., 2002). The interesting thing about some archaeal fatty acids hydroxylases is that they can withstand high temperatures which mesophillic counterparts can not do (Hannemann *et al*., 2007; Nishida and de Montellano 2005). Fungal fatty acid hydroxylases can also attack the in-chain (ω-n, n = 1, 2, 3) position of the fatty acids (Hannemann *et al*., 2007). However, the CYP52s are interesting because they are regio-specific in their oxidative reactions, as they catalyze the oxidation of the ω position (Doddapaneni *et al*., 2005).

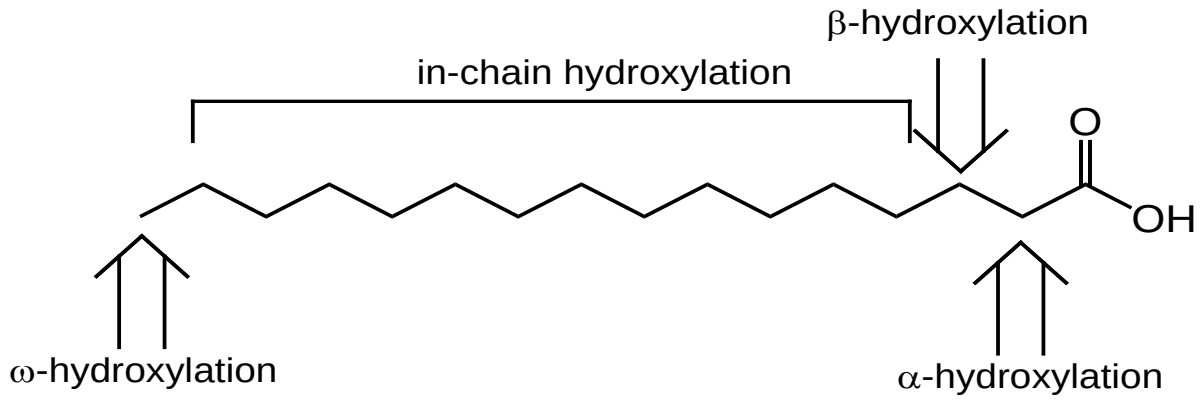

Figure 2.3: Different positions of fatty acid hydroxylation.

Alkane hydroxylases frequently occur in multiple copies in many *n*-alkane degrading microorganisms and exhibit overlapping substrate specificities. Alkane hydroxylases in *n*-alkane degrading microbes play important roles in the degradation of hydrocarbons, treatment of pollutants and in forming useful industrial products (Van Beilen and Funhoff, 2007; Wentzel *et al*., 2007). They catalyze hydroxylation of *n*-alkanes of carbon chain length C_1-C_{44} (Hasanuzzaman *et al*., 2007; Van Beilen and Funhoff, 2007; Wentzel *et al*., 2007).

Not all alkane hydroxylases are P450s; others such as the ALKB like P450s oxidize the majority of medium- and long-chain *n*-alkanes (Rozhkova-Novosad *et al*., 2007). Other families that oxidize *n*-alkanes include the soluble methane monooxygenases (sMMO), toluene monooxygenases (TMO), the particulate methane monooxygenases (pMMO) and napthalene dioxygenase (NDO) (Van Beilen and Funhoff, 2007, Rozhkova-Novosad *et al*., 2007). All these enzyme families like the P450 monooxygenases catalyze the activation of the inert terminal C-H bond of hydrocarbons (Rozhkova-Novosad *et al*., 2007, Van Beilen and Funhoff, 2007; Wentzel *et al*., 2007).

2.2.1 Alpha and beta - fatty acid hydroxylases from bacteria – the CYP152 family

Members of the CYP152 family are self-sufficient heme proteins. Unlike with other P450 monooxygenases, hydrogen peroxide is an oxidant and the shunt pathway is the main route to hydroxylate fatty acids at the α or β position (Matsunaga *et al*., 2002). These enzymes have been isolated from *Sphingomonas paucimobilies* and *Bacillus subtilis* and are called P450SPα and P450BSβ respectively. P450SPα produces only α-OH fatty acids (100%), whereas P450BSβ produces both β-OH (60%) and α-OH fatty acids. These enzymes share 44% amino acids identity (Lee *et al*., 2003, Lee *et al*., 2002). Both the enzymes efficiently introduce an oxygen atom derived from H_2O_2 into

the substrate, and catalytic turnovers of these enzymes are very high (1000 min^{-1}) while their K_m values for H_2O_2 are very low (10^{-2} mM magnitude). They are also unique in their enzymatic mechanism (α-OH, β-OH) compared to P450s which catalyze fatty acid hydroxylation at ω-n (n=1, 2, 3) positions (Matsunaga *et al*., 2002).

Torres *et al*. (2007) showed that the bacterial cytochrome P450BSβ is also capable of catalyzing the oxidation of azulene, anthracene, and 9-methyl-anthracene, using different peroxides as electron acceptors. In addition, this bacterial cytochrome P450 showed even higher catalytic activities than other bacterial cytochromes P450 such as P450cam and P450BM3.

Modeling studies have shown that Pro243 and Arg242 appear to be critical residues for the reaction and might also be involved in the proton delivery (Matsunaga *et al*., 2001). The crystal structure of P450BSβ (Lee *et al*., 2003) has also shed some light on these key residues. For example, in this enzyme the I helix is distorted by Pro243, which is located at the sixth coordination position, normally occupied by water, on the heme iron. The Arg242 residue stabilizes the fatty acids (i.e. palmitic acid) by forming hydrophobic and electrostatic interactions with the substrates. Other key residue such as Phe289 also makes hydrophobic contacts with palmitic acid. The side chains of Asn239, Arg242 and Gln85 create a polar environment to accommodate the fatty acid carboxylate and the H_2O_2 substrate (Munro *et al*., 2006).

2.2.2 Sub-terminal fatty acid hydroxylases from archeae, bacteria and fungi

2.2.2.1 The CYP119 family

The number of known thermophilic cytochrome P450s is small. Only three such enzymes have been purified, crystallized and their structures determined. The first and most extensively studied of these three enzymes is CYP119, from

Sulfolobus solfataricus. This organism is a sulphur autotroph, which can withstand high temperatures ranging between 78 and 86 °C and acidic pH values between 3 and 4 (Nishida and de Montellano, 2005; Koo *et al*., 2000)

CYP119 is a highly thermostable P450 enzyme (T_M = 91 °C) which belongs to class IV. Homology modeling and crystal structures indicate that there are three factors contributing to CYP119 thermal stability. That is (a) a higher density of salt bridges, (b) a relatively low density of alanines coupled with a high incidence of isoleucines in the interior of the protein, resulting in better side-chain packing, and (c) the presence of extended aromatic clusters that are not present in mesophilic P450 structures (Hannemann *et al*., 2007). With 368 residues compared to P450cam (414 residues) and P450eryF (403 residues), CYP119 is relatively smaller than known mesophilic enzymes. Moreover mesophilic enzymes denature irreversibly at high pressure (130 MPa) (1 MPa = 9.872 atm) while CYP119 can withstand pressures up to 200 MPa without converting to the inactive P420 form (Hannemann *et al*., 2007; Nishida and de Montellano, 2005)..

CYP119 hydroxylates fatty acids at the ω-1 position. Fatty acids are not only good substrates but also excellent ligands for CYP119. For example, lauric acid binds with K_s = 1.2 μM a value comparable to the binding of myristic acid, palmitic acid, and stearic acids. The 10-carbon capric acid binds somewhat less tightly (K_s = 28 μM), as does the 20-carbon arachidonic acid (K_s = 5 μM). All these fatty acids bind much more tightly than styrene, which binds with K_s = 530 μM. This enzyme can also catalyze the hydroxylation of other ω-n positions of the fatty acids. This was shown using CYP119:putidaredoxin:putidaredoxin reductase, single (D77R and T214V) and double (D77R/T214V) mutants to hydroxylate lauric acid. Analysis of the hydroxylated products formed at room temperature revealed that although oxidation at ω-1 was the favored reaction, other position were hydroxylated as follows ω-2, ω- (n > 3), and ω-3 (Nishida and de Montellano, 2005).

Lastly, thermophilic enzymes are of potential interest from several points of view. Elucidation of the features that convey thermostability could lead to the modification of mesophilic proteins to convert them into more stable biocatalysts. The structural stability offered by thermophilic P450 enzymes if exploited can allow mesophilic counterparts to carry out difficult mechanistic investigations. Most importantly, thermophilic P450 enzymes have a rich potential utility as catalysts in industrial settings (Nishida and de Montellano, 2005).

2.2.2.2 CYP102A1- P450 BM-3

CYP102A1 also known as P450BM-3, a fatty acid hydroxylase from a soil bacterium *Bacillus megaterium,* is one of the most extensively studied P450s and is widely used to understand the structure and mechanisms of cytochrome P450 enzymes. P450BM-3 is a water soluble, catalytically self-sufficient flavocytochrome belonging to class VIII. It contains a P450 heme domain and an NADPH-dependent diflavin reductase domain in a single 119 kDa polypeptide chain (Wiseman and Lewis, 2007; Hannemann *et al*., 2007; Huang *et al*., 2007; Wanatabe *et al*., 2007; Yun *et al*., 2007; Appel *et al.,* 2001; Okita and Okita, 2001). This enzyme catalyzes the NADPH-dependent hydroxylation of several medium and long-chain saturated fatty acids C_{12}-C_{22} and various unsaturated and polysaturated fatty acids at the ω–1 through ω–3 position (Hannemann *et al*., 2007; Hilker *et al*., 2007; Munro e*t al*., 2007; Girvan *et al*., 2006). However, CYP102A1 has also been reported to catalyze reactions of drugs which are typical substrates for mammalian P450s (CYP 2E1, 2D6, 1A2 and 3A4) at comparable or higher rates (Yun e*t al*., 2007). The reactions included hydroxylation of chlorzoxazone, aniline and *p*-nitrophenol, N-dealkylation of propranolol and dehydrogenation of nifedipine (Di Nardo *et al*., 2007). The highest catalytic activity recorded at ~17 000 turnovers min^{-1} (K_s ~ 285 s^{-1}, based on NADPH oxidation) has been reported for the epoxidation of arachidonic acid catalyzed by CYP102A1 (Julsing *et al*., 2008; Hilker *et al*., 2007; Munro e*t al*., 2006; Girvan *et al*., 2006; Neeli *et al*., 2005; Munro e*t al*., 2002).

X-ray structures, modeling and mutation studies have indicated the role of specific amino acids in the structure and mechanism of CYP102A1 (Figure 2.4). Arg47 and Tyr51 are found at the entrance of the active site which serve as anchoring site for the carboxylate group of the fatty acids (Feenstra *et al*., 2007, Kitazume *et al*., 2002) and thus enhance binding of the fatty acids (Chowdhary *et al*., 2008; Munro *et al*., 2002). These amino acids play a critical role in interacting with the carboxy group of the long chain fatty acids (Munro *et al*., 2002) as the substrates enter the active site of P450BM3 (Girvan *et al*., 2006). The positively charged Arg47 interacts with the negatively charged carboxylate anion of the long chain fatty acids (Chowdhary *et al*., 2008) forming a salt bridge, while the Tyr51 forms a hydrogen bond with the carboxylate (Feenstra *et al*., 2007). Removal of the two residues at the entrance to the active site diminishes catalytic efficiency with all substrates of chain length > C_6 (Girvan *et al*., 2006). Mutation studies have also revealed that Phe42 forms a hydrophobic 'lid' over the mouth of the substrate channel (Hilker *et al*., 2007; Munro *et al*., 2002). A point mutant of Phe42Ala showed that this residue is important catalytically, with large increases in the K_d and K_m values of substrates. Phe87 is responsible for regio-specificity (Munro *et al*., 2002). This residue plays a determining role in the binding modes of the substrates (Feenstra *et al*., 2007) and is critical to high rate substrate turnover as it lies above the heme plane and near the O_2 binding site (Hilker *et al*., 2007). A mutation of Phe87Val possesses a stronger binding for both arachidonic acid and palmitoleic acid ((*Z*)-9-hexadecenoic acid) compared to the wild-type P450BM3 (Hilker *et al*., 2007). It has also been shown that Phe87 protects the terminal methyl of the fatty acid from oxidative attack by compound I, resulting in hydrophobic interactions shifting the substrates away from hydroxylation at the ω-methyl group of the long chain fatty acid (Feenstra *et al*., 2007; Warman *et al*., 2005; Munro *et al*., 2002). This residue can be a target for ω-methyl oxygenation (Warman *et al*., 2005) and mutation of Phe87Ala has increased peroxygenase activity (Otey *et al*., 2004). Phe393 residue heavily influences the electronic nature of the heme (Clark *et al*., 2006) influencing the equilibrium between the rate of heme reduction and the rate at which the ferrous

heme can bind and thus reduce oxygen (Girvan *et al*., 2006). Mutation studies of Ala264Glu have supported the idea that these ‘’substrate-free‘’ and “substrate-bound” conformations co-exist in equilibrium in solution in the absence of substrate. Mutants of Ala82Phe and Ala82Trp bind fatty acids more tightly compared to the wild-type, and show increased catalytic efficiency. These mutants are suggested to bind small molecules more tightly because of their ability to oxidize indole (Huang *et al*., 2007). The Leu181Lys and the double mutant Leu75Thr/Leu181Lys variants considerably promoted catalysis of short chain fatty acids and gave improved turnover with hexanoic acid, butanoic acid and octanoic acid (Feenstra *et al*., 2007; Girvan *et al*., 2006).

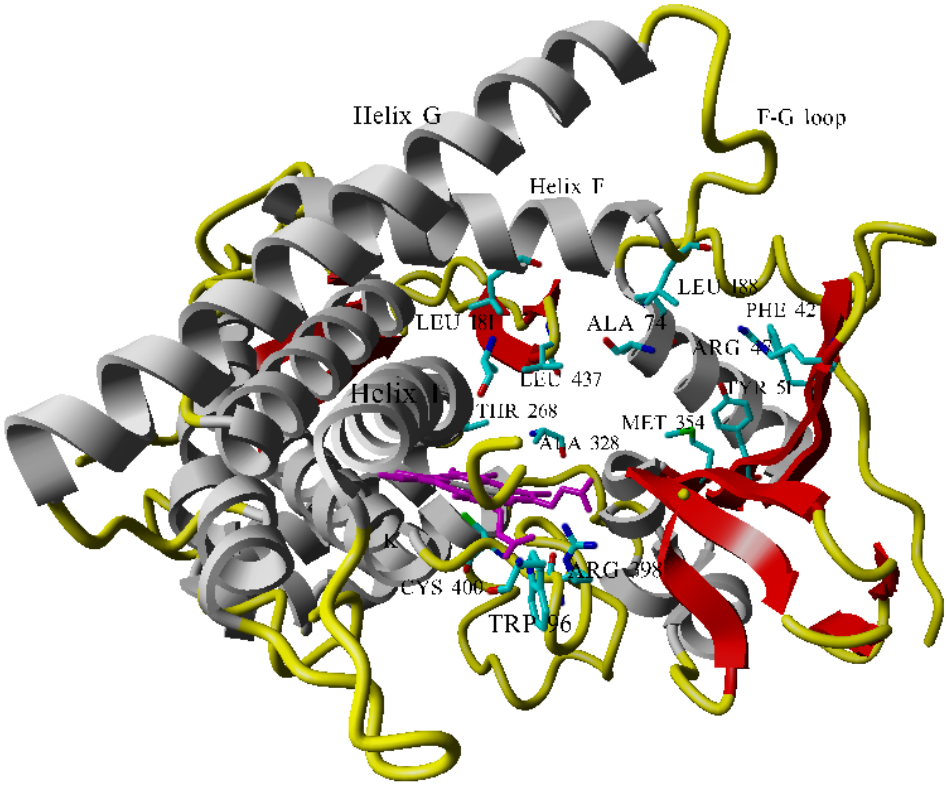

Figure 2.4: The crystal structure of CYP102A1. The amino acids that play an important role in the function and mechanism of CYP102A1 within the active site are shown as well as the heme group (displayed in magenta). These amino acids have been the targets in many studies of the structure and function of CYP102A1. Cytochrome P450 3D fold, generated by YASARA program (Krieger *et al*., 1993), PDB coordinates 1BVY.

2.2.2.3 The CYP505 family

Cytochrome P450foxy (CYP505A1) with MW 118 kDa is a self-sufficient P450 from *Fusarium oxysporum*. It also belongs to class VIII, and the P450 domain shares 40.6% amino acid similarity (homology) with the P450 domain of BM3, while the reductase domains share 35.3% amino acid similarity (Doddapaneni *et al*., 2005; Shoun and Takaya, 2002). The substrate specificity of P450foxy is towards saturated fatty acids with a shorter chain length (C_9–C_{18}) (Figure 2.5) and as with BM3 hydroxylation occurs from the ω-1 to ω-3 positions (Hannemann *et al*., 2007; Kitazume *et al*., 2002). Hydroxylase activity is much lower than with P450BM3 at 1800 min^{-1} and tridecanoic acid is the preferred substrate (Girvan *et al*., 2006; Kitazume *et al*., 2002).

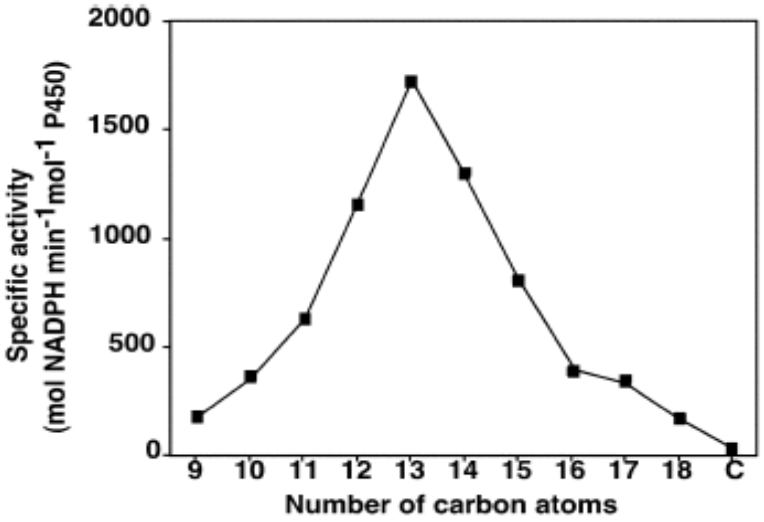

Figure 2.5: Substrate specificity of P450foxy toward saturated fatty acids (Kitazume *et al*., 2002).

Alignment of the amino acid sequence of P450BM3 with P450foxy has revealed that the differences we see in substrate preference are due to the different amino acids these enzymes possess at the mouth of the active site (Figure 2.6). In P450BM3 Phe42 serves as a hydrophobic lid that excludes solvent water to

strengthen the electrostatic interaction of Arg47 and the substrates. Arg47 and Tyr51 are found at the entrance and bind the carboxy group of long chain fatty acid. These residues in P450foxy are replaced by Leu43, Lys48, and Phe52, respectively. However, other key amino acid residues such as Leu75, Phe87, Leu181, Ile263, and Leu437 which are inside the pocket of P450BM3 are also the same in P450foxy. P450foxy is more hydrophobic at the entrance of the active site and this is increased as a result of the substitution of Phe with Leu and Tyr with Phe. As a result of this substitution it is impossible to have the hydrogen bonding that supports the electrostatic interaction of the positive charge (Lys48 in case of P450foxy) with the carboxylate. Therefore the interaction of the fatty acid carboxy group with the amino acid residues at the entrance is weaker in P450foxy than in P450BM3. Also, the more hydrophobic environment around the entrance of P450foxy would permit another fatty acid molecule to partially penetrate the channel from its aliphatic head after the first molecule has already occupied the channel. As a result of this, hydroxylation of short chain fatty acid is preferred while hydroxylation of longer chain fatty acids is inhibited in P450foxy (Kitazume *et al*., 2002).

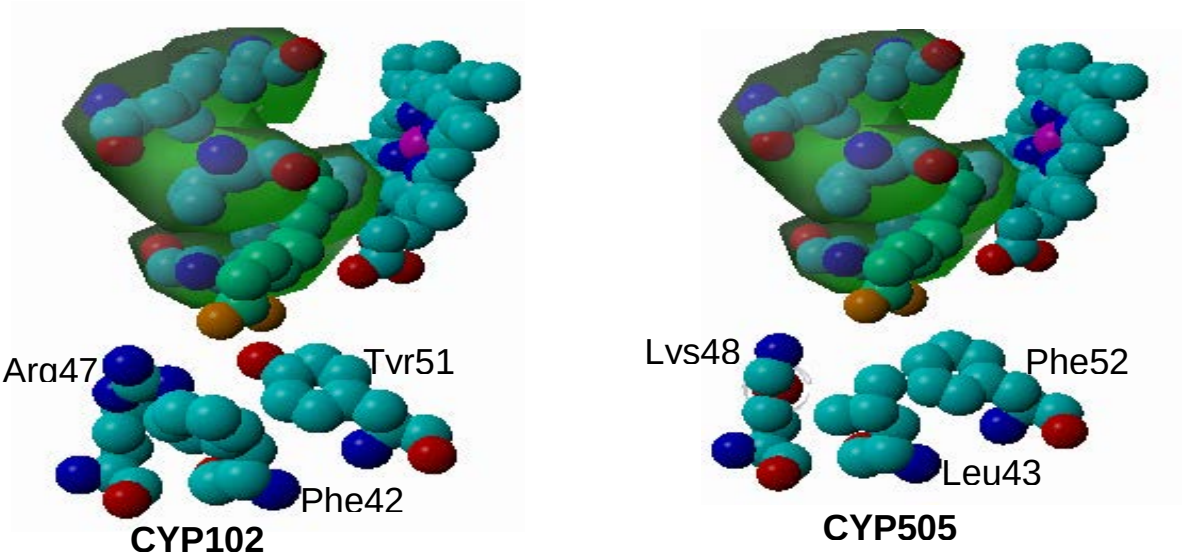

Figure 2.6: Structure of CYP102A1 WT and of a model of CYP102A1 with Phe42, Arg47 and Tyr51 mutated *in silico* to the residues present in CYP505A1 i.e. Leu43, Lys48 and Phe 52.

2.2.3 Terminal alkane hydroxylases from bacteria – the CYP153 family

The CYP153 family belongs to class I. Members of this family are known to catalyze monoterminal oxidation of aliphatic, alicyclic, and alkyl-substituted compounds with high regio- and stereo-selectivity (Funhoff *et al*., 2007). *In-silico* docking of *n*-alkanes in a homology model of CYP153A6 showed that *n*-alkanes were positioned such that hydroxylation would take place at the terminal methyl groups (Van Beilen and Funhoff, 2007). CYP153A1 from *Acinetobacter* sp. EB104 was the first CYP153 found in bacteria. The *Acinetobacter* sp. EB104 could grow in the presence of *n*-alkanes (*n*-hexane to *n*-hexadecane), biphenyl, indene and phenanthrene. CYP153s are the only soluble CYP enzymes capable of terminal aliphatic *n*-alkane hydroxylation, displaying high regio-specificity and high activity compared with other P450 enzymes. *In vivo* CYP153s hydroxylate alkanes ranging from C_5 (*n*-pentane) to C_{10} (*n*-decane) (Funhoff *et al*., 2007).

In addition to hydroxylation activity, all heterogously expressed CYP153 enzymes also catalyze epoxidation. These enzymes catalyze enantio-selective epoxidation, a major challenge in synthetic chemistry (Funhoff *et al*., 2007). Fujii *et al*. (2006) cloned CYP153 from *Acinetobacter* sp. OC4 into *Escherichia coli* and showed that this P450 enzyme can also convert *n*-alkanes or alkanols to α,ω-alkanediols. This was the first report on bio-production of α,ω-alkanediols from *n*-alkanes or alkanols by a bacterial enzyme.

Based on homology modeling studies almost all presumed key amino acid residues in the active site of CYP153s are conserved, except residues 98, 99, 101, 195, 250, 251 and 407 in CYP153A6, suggesting that these residues do not play a role in substrate preference. CYP153A11 may prefer cyclic compounds due to the presence at the entrance of the active site of a Leu-residue (position 411), instead of the bulky Phe-residue, present in all other CYP153s (Funhoff *et al*., 2007).

2.2.4 Terminal alkane and fatty acid hydroxylases from fungi – the CYP52 family

Several yeasts produce multiple alk isozymes encoded by multiple *alk* genes. The alk isozymes belong to the CYP52 family (Van Beilen and Funhoff, 2007). These yeasts have the ability to use alkanes and fatty acids as carbon source. In these yeasts, enzymes in the ω- and β-oxidation pathways metabolize *n*-alkanes and fatty acids. The CYP52 enzymes accompanied by NADPH cytochrome P450 reductases (CPR) are responsible for the first rate-limiting step in degradation of *n*-alkanes and hydroxylation of fatty acids (Craft *et al*., 2003; Zimmer *et al*., 1996).

The CYP52 enzymes catalyze terminal hydroxylation of *n*-alkanes and ω-hydroxylation of fatty acids (Doddapaneni *et al*., 2005). Expression of most of the CYP52 genes is induced by long-chain aliphatic hydrocarbons such as *n*-alkanes, alkenes, fatty alcohols, fatty acids, cycloalkanes and alkylbenzenes. The degree of induction varies from gene to gene and depends on the chemical structure of the inducer (Scheller *et al*., 1996).

Some CYP52s efficiently catalyze the complete oxidation of mono and diterminal hydroxylated products of *n*-alkanes and fatty acids yielding α,ω-dicarboxylic acids (Fickers *et al*., 2005; Eschenfeldt *et al*., 2003; Scheller *et al*., 1998). Such examples were shown with CYP52A3 (P450ALK1A) from *Candida maltosa* using *n*-alkanes and fatty acids (Scheller *et al*., 1998) and with CYP52A13 and CYP52A17 from *Candida tropicalis* using fatty acids of various chain lengths as substrates (Eschenfeldt *et al*., 2003). This characteristic, allow by-passing of the peroxisomal fatty alcohol oxidases and fatty aldehyde dehydrogenases in producing fatty acids and/or dicarboxylic acids for β-oxidation (Chapter 1, Figure 1.1) (Scheller *et al*., 1998).

The CYP52 *alk* genes occur in multiple copies and their numbers differs depending on the organism (Kogure *et al*., 2005; Craft *et al*., 2003; Iida *et al*., 2000). *C*. *maltosa* has eight structurally related *alk* genes designated *ALK1* to *ALK8*. These genes occur in two allelic variants and their expression is repressed by glucose, depressed by glycerol and induced by *n*-alkanes (Kogure *et al*., 2005). Zimmer *et al*. (1996), were able to show the function of four of the eight P450alk enzymes (ALK1-3 and ALK5) in the degradation of *n*-alkanes and fatty acids. For example P450ALK1A (CYP52A3) is the major alkane hydroxylase (Figure 2.7), as it oxidized *n*-alkanes more efficiently than any of the other CYP52 enzymes, and activity towards fatty acids was relatively poor. P450ALK2A (CYP52A5) oxidized all the substrates efficiently almost at the same rate exhibiting broad substrate specificity. P450ALK3A (CYP52A4) is an efficient *n*-alkane hydroxylase, and had relatively low activity towards palmitic acid and oleic acid. However, myristic acid was converted with high efficiency. P450ALK5A (CYP52A9) is a fatty acid hydroxylase as it had relatively poor activity towards *n*-alkanes. Only four of these genes are highly inducible by *n*-alkanes (Zimmer *et al*., 1996), namely *ALK1*, *ALK2*, *ALK3* and *ALK5* (Cheng *et al*., 2005; Kogure *et al*., 2005). Complete disruption of these genes lead to the failure of this organism to grow on *n*-alkanes (Zimmer *et al*., 1996). *ALK2* is also inducible by peroxisome proliferators such as clofibrate, which are structurally unrelated to long-chain hydrocarbons (Kogure *et al*., 2005).

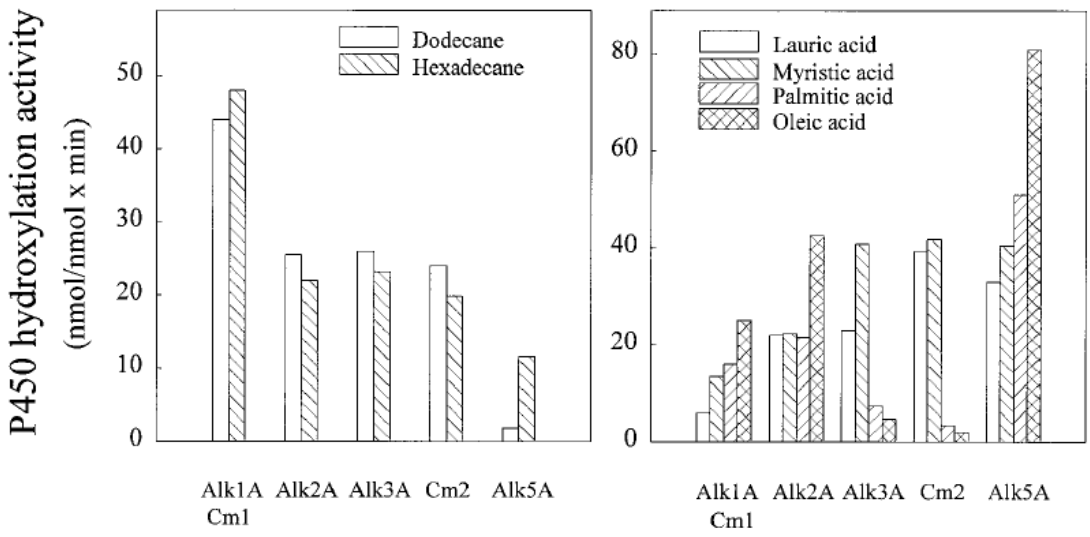

Figure 2.7: Substrate turnover rates of the major *n*-alkane-inducible *C. maltosa* P450 forms. P450Cm1 and P450Cm2 differ at 1 and 7 amino acid positions, from ALK1A and ALK3A respectively (Zimmer *et al*., 1996).

Yarrowia lipolytica has twelve *CYP52* genes (Fickers *et al*., 2005). Only *ALK1* and *ALK2* are essential for growth on *n*-alkanes, because double deletion of the genes resulted in poor growth on *n*-alkanes. *ALK1* is actually important for growth on short chain *n*-alkanes (*n*-decane), because disruption of the gene resulted in poor growth on *n*-decane. The *ALK2* gene is important for growth on long chain *n*-alkanes since a further disruption resulted in poor growth on *n*-hexadecane (Iida *et al*., 2000). Single disruptions of *ALK2*, *ALK3*, *ALK4* or *ALK 6* did not affect growth on *n*-alkanes. *ALK3*, *ALK5* and *ALK7* are fatty acid hydroxylases. No ω-hydroxylase activity was detected with *ALK1*, *ALK2*, *ALK4* or *ALK6*. The involvement of the enzymes in over-oxidation of *n*-alkanes and fatty acids remains to be clarified (Fickers *et al*., 2005). Other ALKs might catalyze hydroxylation of longer chain *n*-alkanes or chains of other hydrocarbons (Iida *et al*., 2000). The *Y. lipolytica CYP52* genes are induced by *n*-alkanes and fatty acids, de-repressed by glucose and repressed by glycerol as shown in figure 2.8 (Iida *et al*., 2000). The other four genes (*ALK9* to *ALK12*), are not well documented in their oxidation reactions, but these genes present higher homology to *ALK1-ALK3* (Fickers *et al*., 2005).

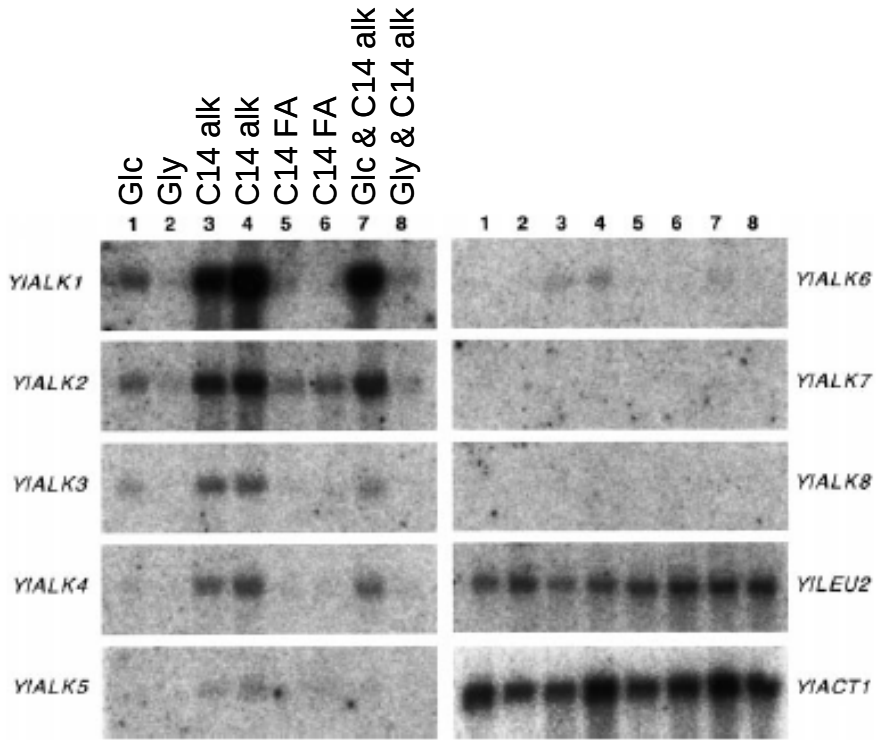

Figure 2.8: Northern hybridization analysis of *YlALK* genes. Lane 1 -glucose (Glc), lane 2 - glycerol (Gly), lane 3 & 4 - tetradecane (C14 alk), lane 5 & 6 - myrisitc acid (C14 FA), lane 7 - Glucose (Glc) + C14 FA, lane 8 - Glycerol (Gly) + tetradecane (C14 alk) were used as carbon sources. Probes for YlLEU2 and YlACT1 were used as controls (Iida *et al*., 2000).

Candida tropicalis ATCC20336 has ten CYP52 genes; eight occur as allelic variants, only four (*CYP52A13*, *CYP52A14* and *CYP52A17*, *CYP52A18*) are strongly induced by commercial feedstock Emersol 267 (Emersol 267 had the following fatty acid composition: 2.4% $C_{14:0}$, 0.7% $C_{14:1}$, 4.6% $C_{16:0}$, 5.7% $C_{16:1}$, 5.7% $C_{17:1}$, 1.0% $C_{18:0}$, 69.9% $C_{18:1}$, 8.8% $C_{18:2}$, 0.3% $C_{18:3}$, and 0.9% $C_{20:1}$) and oleic acid (Craft *et al*., 2003). Activity assays with recombinant *CYP52A13* expressed in *Sf9* insect cells indicated that CYP52A13 favor long chain fatty acids, especially oleic acid. In contrast, these assays indicated that CYP52A17 preferred short chain fatty acid of chain length C_{12} to C_{16} with highest turnovers for myristic acid (Figure 2.9). The CYP52A17 enzyme over-oxidized the fatty acids. However, the oxidation of *n*-alkanes was not tested (Eschenfeldt *et al*., 2003). In *C*. *tropicalis* limited oxygen supply below partial pressure 4 kPa (ca.

20% of air saturation) promotes the biosynthesis of the P450 monooxygenases. A further decrease in oxygen level, increases cellular cytochrome P-450 content from 35 to 80 pmol (mg of dry cell weight)$^{-1}$ (Kappeli, 1986).

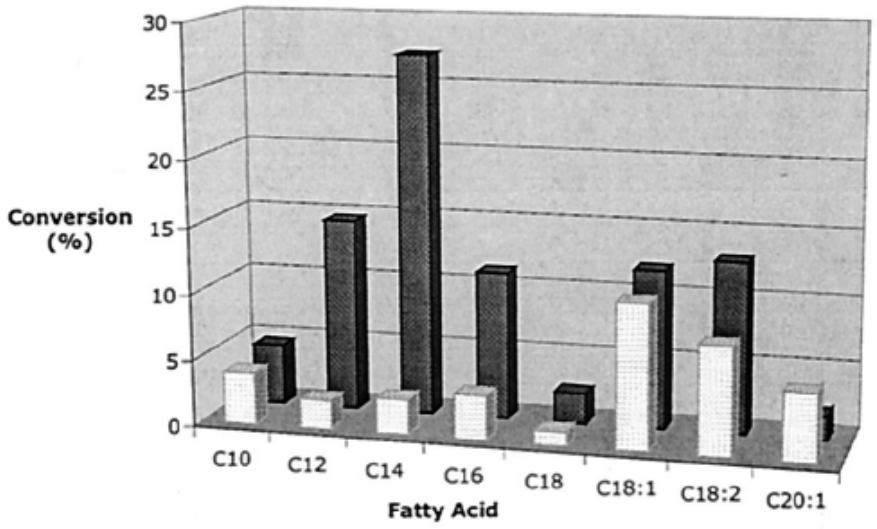

Figure 2.9: Oxidation of fatty acids by CYP52A13 (white columns) and CYP52A17 (black columns). The total percentage of fatty acids converted to the corresponding ω-hydroxy fatty acid and dicarboxylic acid by 60 µl of *Sf9* insect cell microsomes containing expressed CYP52A13 and CYP52A17 (Eschenfeldt *et al.,* 2003).

The CYP52s have wide substrate specificities towards *n*-alkanes and fatty acids as indicated in table 2.1 (Fickers *et al*., 2005; Eschenfeldt *et al.,* 2003; Zimmer *et al*., 1996). Their ability to attack the terminal positions of *n*-alkanes and fatty acids has been exploited in industry for the formation of α,ω-dicarboxylic acids (Kogure *et al*., 2007; Mobley, 1999). In these processes, mutants which are β-oxidation blocked (*C*. *maltosa* and *C*. *tropicalis*) are used as whole cell biocatalysts (Julsing *et al*., 2008; Kogure *et al*., 2007; Mobley, 1999). These mutants have been reported to accumulate concentrations of above 100 g l^{-1} DCA (Julsing *et al*., 2008; Mobley, 1999; Picataggio *et al*., 1992) and the CYP52s have the highest bioreactor productivities for CYP450 processes (Julsing *et al*., 2008). Substrate specificities of the enzymes are used as bench marks for the production of α,ω-dicarboxylic acids of various chain length. For example

brassylic acid is produced from *n*-tridecane using mutant strains of *Candida maltosa* (Kogure *et al*., 2007) and dodecanedioc acid which is used for the synthesis of nylon is produced from *n*-dodecane (Mobley, 1999) using a *Candida tropicalis* mutant strain.

There are no X-ray structures for CYP52s. Therefore, it was difficult to discuss about the role of amino acids in enzyme mechanisms. However, a lot of work has been done on induction and enzyme assays of CYP52s using *n*-alkanes and fatty acids (Kim *et al*., 2007; Craft *et al*., 2003; Eschenfeldt *et al*., 2003; Iida *et al*., 2000; Zimmer *et al*., 1996). Until recently these enzymes were only expressed in *Saccharomyces cerevisiae* (Scheller *et al*., 1998; Scheller *et al*., 1996; Zimmer *et al*., 1996) and insects cells (Eschenfeldt *et al*., 2003). Recently CYP52A21 from *C. albicans* was expressed in *E. coli* – paving the way for crystallization and X-ray structure determinations (Kim *et al*., 2007). The protein was targeted to the membranes and was solubilized (using surfactant) before it was tested on fatty acids. Some mammalian P450s were crystallized after expression in membranes of *E. coli* and solubilization with surfactant (Kolar *et al*., 2007).

Table 2.1: Summary of the substrates specificities of the CYP52s from yeasts

Yeast	CYP52s	Substrates specificities	Substrates preferred **
C. maltosa			
	ALK1	Alkane hydroxylase	C_{12} & C_{16} (Zimmer *et al*., 1996).
	ALK2	Alkane and fatty hydroxylase	C_{12} & C_{16} & C_{12}FA, C_{14}FA, C_{16}FA & $C_{18:1}$FA (Zimmer *et al*., 1996).
	ALK3	Alkane and fatty hydroxylase	$C_{12,}$ $C_{16,}$ C_{14}FA & C_{16}FA (Zimmer *et al*., 1996).
	ALK5	Fatty acid hydroxylase	C_{12}FA, C_{14}FA, C_{16}FA & $C_{18:1}$FA (Zimmer *et al*., 1996).
Y. lipolytica			
	ALK1	Alkane hydroxylase	C_{10} (Fickers *et al*., 2005, Iida *et al*., 2000).
	ALK2	Alkane hydroxylase	C_{16} (Fickers *et al*., 2005, Iida *et al*., 2000).
	ALK3, ALK5 and ALK7	Fatty acid hydroxylase	C_{12}FA (Fickers *et al*., 2005).
C. tropicalis			
	CYP52A13	Fatty acid hydroxylase	$C_{18:1}$FA, $C_{18:2}$FA, $C_{20:1}$FA (Eschenfeldt *et al.,* 2003).
	CYP52A17	Fatty acid hydroxylase	C_{12} to C_{16} (Eschenfeldt *et al.,* 2003).
C. albicans			
	CYP52A21	Fatty acid hydroxylase	C_{12}FA, C_{14}FA, C_{16}FA (Kim *et al*., 2007).

*Only CYP52s which are characterized to oxidize *n*-alkanes and fatty acids are indicated.** C_{10} – *n*-decane, C_{12} – *n*-dodecane, C_{16} – *n*-hexadecane, C_{12}FA – lauric acid, C_{14}FA – myristic acid, C_{16}FA – palmitic acid, $C_{18:1}$FA – oleic acid, $C_{18:2}$FA – linoleic acid and $C_{20:1}$FA – eicosenoic acid.

2.3 Conclusion

Microbial cytochrome P450 alkane and fatty acid hydroxylases are diverse in their reaction mechanisms and have unique properties. The bacterial P450s are soluble and catalyze the hydroxylation of *n*-alkanes and fatty acids and epoxidation of derivatives with double bonds. CYP102A1 is a self-sufficient monooxygenase. It has the highest catalytic efficiency reported at ~17 000 turnovers min^{-1} (K_S ~ 285 s^{-1}, based on NADPH oxidation) for epoxidation of arachidonic acid, and also catalyzes sub-terminal hydroxylation (ω-n, n = 1, 2, 3) of long-chain fatty acids. CYP152 is different from other P450s because it is a self-sufficient peroxygenase and catalyzes α- and β-hydroxylation of fatty acids with high regio-specificity. CYP153 is the only bacterial P450 that is an alkane hydroxylase. It catalyzes the terminal oxidation of *n*-alkanes, and epoxidation of alkenes. No bacterial enzymes have been reported that catalyze terminal hydroxylation of fatty acids. Archaeal CYP119 fatty acid hydroxylase is also soluble, catalyzes sub-terminal hydroxylation of fatty acids (ω-n, n = 1, 2, 3) and can withstand high temperatures which mesophilic counterparts can not do.

The eukaryotic alkane and fatty acid hydroxylases are membrane bound proteins. Fungal CYP505A1 is a self-sufficient fatty acid hydroxylase which can also attack the in-chain position of the fatty acids (ω-n, n = 1, 2, 3). The CYP52s are unique in that they catalyze terminal hydroxylation of both *n*-alkanes and fatty acids. Unlike other P450 enzyme systems this family include both alkane and fatty acid hydroxylases with broad range substrate specificities which can catalyze complete oxidation of alkanes and fatty acid to α,ω-dicarboxylic acids. This intrinsic property is used in industry to produce valuable long chain α,ω-dicarboxylic acids which are the starting material for the synthesis of a number of valuable products. This is in fact the only P450 based process with high enough productivities for commercial production of fine chemicals.

Only three fatty acid hydroxylases have been crystallized, CYP102A1, CYP119 and P450BSβ. Crystal structures, modeling and mutation studies have shed some light on enzyme mechanisms. However, no X-ray structures are available for a terminal hydroxylase or an alkane hydroxylase. Given the importance of the CYP52s this is an important problem that should be addressed in future.

Chapter 3

Alkane and fatty acid hydroxylase activity in wild type *Y. lipolytica* and *C. tropicalis* strains probed with alkylbenzenes and alkylbenzoic acids

3.1 Introduction

Aromatic hydrocarbons are not only valuable compounds that are extensively used in industry (Prenafeta-Boldu et al., 2001; Tegeris and Balster, 1994) for the synthesis of products such as fragrances (Fortineau, 2004) and medicines (Craker, 2007), but are also important contributors to environmental contamination (Andreoni and Gianfreda, 2007; Barbosa *et al*., 2007; Phale *et al*., 2007) and diseases (Heider *et al*., 1999; Tegeris and Balster, 1994; Phale *et al*., 2007). *n*-Alkane degrading yeasts use monooxygenases designated CYP52s to oxidize alkylbenzenes (Scheller *et al*., 1996). Alkylbenzenes undergo ω- and β-oxidation in yeasts (Van Rooyen, 2005) and this results in the formation of phenylacetic acid as the only product in the case of alkyl chains with even chain lengths or benzoic acid as a major product and phenylacetic acid as the minor product in the case of alkyl chains with odd chain lengths (Van Rooyen, 2005, Hou *et al*., 1994). Substituted alkylbenzenes such as 4-hexylbenzoic acid and 4-nonyloxybenzoic acid give dioic acid products, also resulting from the hydroxylation of a terminal methyl group (Chapter 1, Figures 1.3 and 1.4), while *p*-cymene and 4-ethyltoluene are hydroxylated at the methyl substituent (Figure 3.1).

Because products are accumulated from alkylbenzenes and their derivatives, while *n*-alkanes are completely degraded, we used different alkylbenzenes, hexylbenzoic acid and nonyloxybenzoic acid to compare the hydroxylation ability and substrate specificity of the wild-type strains *Yarrowia lipolytica* W29 and

Candida tropicalis ATCC20336. These two strains are the parental strains of the dicarboxylic acid producing strains that are used in our laboratory.

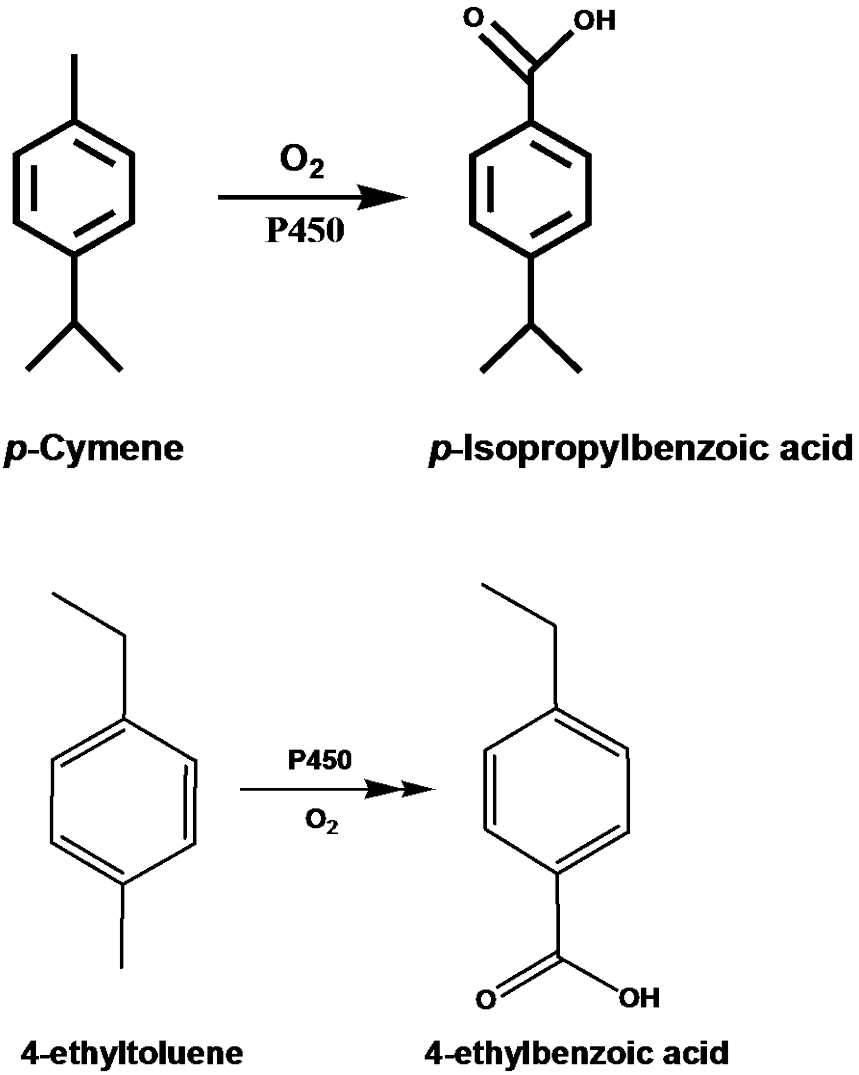

Figure 3.1: A typical P450 hydroxylation reaction of *p*-cymene to *p*-isopropylbenzoic acid and 4-ethyltoluene to 4-ethylbenzoic acid.

3.2 Material and methods

3.2 Part A: General methods

3.2.1 Growth Media

LN broth contained (per liter distilled water): 40 g glucose, 10 g tryptone and 10 g yeast nitrogen base (YNB) containing amino acids and ammonium phosphate.

YP_2D_2 broth contained (per liter distilled water): 10 g yeast extracts (Merck), 20 g peptone (Merck) and 20 g glucose.

3.2.1 Microorganisms

The yeast strains used in this study were *Yarrowia lipolytica* W29 and *Candida tropicalis* ATCC20336. All strains were stored in LN broth containing glycerol (7% v/v) under liquid N_2 in the MIRCEN yeast culture collection of the University of the Free State, South Africa. Strains were revived on YP_2D_2 agar.

3.2.2 Growth conditions

Cultivation in liquid media was, unless stated otherwise, performed in 25 ml YP_2D_2 broth in 250 ml Erlenmeyer flasks for pre-cultures and 50 ml YP_2D_2 broth in 500 ml Erlenmeyer flasks in the case of main cultures, on a rotary shaker at 180 rpm at 30 °C. Shake-flasks were inoculated with 10% v/v of YP_2D_2 pre-cultures grown for 24 h.

3.2.3 Turbidemetric measurements

Culture samples (200 µl) were suitably diluted (depending on the turbidity) before transfer to a microtitre plate. Optical densities (ODs) were measured at 620 nm using a Labsystems iEMS reader MF (Thermo BioAnalysis Company, Helsinki Finland).

3.2.4 Dry weight measurements

Cyclohexane (2 ml) and 5 M NaOH (400 µl) were added to samples (4 ml) in test tubes, vortexed for 5 min and then filtered under vacuum through dried and pre-weighed glass fiber filters (GF52 47MM BX200; Schleicher and Schuell and pore size 1.2 µm). The biomass on the filter was washed with a mixture of distilled water (4 ml), cyclohexane (2 ml) and 5 M NaOH (400 µl) followed by washing with distilled water (26 ml). Biomass on filters were dried overnight in an oven (100 °C), and then cooled in a desiccator before it was weighed (Smit *et al*., 2005).

3.2.5 Extraction and analysis

Samples (500 µl) were taken at regular intervals and acidified to a pH 3 of 200 µl 1 M HCl. The samples were extracted once with 600 µl ethyl acetate (Fluka) containing myristic acid (0.1% w/v) (The British Drughouses) as internal standard and the phases separated by centrifugation (10 000 x *g* for 10 min). Samples of the extracts (50 µl) were transferred to gas chromatography vials and methylated with trimethylsulfonium hydroxide (50 µl) (Smit *et al*., 2005).

Gas Chromatography (GC) analysis of methylated samples was carried out on a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a CP-Wax CB column (Chrompack) measuring 30 m x 0.53 mm x 1 µm. GC conditions were as follows: initial oven temperature was 120 °C held for 5 minutes, increasing at 10 °C min^{-1} to a final temperature of 250 °C held for 12 minutes. Flow through the column was at 6 ml min^{-1} hydrogen

with a split ratio of 1:50. The temperature of the flame ionisation detector (FID) was 280 °C. The sample volume was also 1 µl.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of methylated samples was carried out on Finnigan Trace Ultra Gas Chromatograph coupled to a Finnigan DSQ mass analyzer. The GC was equipped with HP 5 (Hewlett Packard) column measuring 60 m x 0.32 mm x 0.25 µm with Helium (He) as a carrier gas at flow rate of 1 ml min^{-1} and a split ratio of 1:40. The inlet temperature was 235 °C. The initial oven temperature was 70 °C held for 3 minutes, increased by 10 °C per minute until a final oven temperature of 300 °C was reached and held for another 20 minutes. The sample volume was also 1 µl.

For Thin Layer Chromatography (TLC) analysis samples (10 µl) were spotted onto Alugram Sil G/UV_{245} TLC plates (Machery-Nagel) containing a fluorescence indicator. Plates were developed using a mobile phase consisting of di-*n*-butyl-ether (Merck), formic acid (Merck) and water in a 90:7:3 ratio. Plates were visualized under short wavelength UV-light.

3.3 Part B: Experiments

3.3.1 Biotransformation of alkylbenzenes by *Yarrowia lipolytica* W29

To evaluate biotransformation of alkylbenzenes, main cultures of *Y*arrowia *lipolytica* W29 were grown for 12 h under standard conditions before 30 mM substrate (butylbenzene, hexylbenzene, nonylbenzene, or *p*-cymene) was added. The optical densities were measured every 12 h for 48 h and then every 24 h. Biomass determinations were done at 48 h and 72 h growth. Samples were taken at regular intervals and analyzed using gas chromatography.

3.3.2 The effect of inducers and co-substrate on the biotransformation of alkylbenzenes by *Yarrowia lipolytica* W29

To test the effect of inducers/co-substrate on the biotransformation of alkylbenzenes two sets of experiments were done.

Main cultures of *Y*. *lipolytica* W29 were grown for 24 h before *n*-dodecane (1% v/v); *n*-tretradecane (1% v/v), oleic acid (1% v/v) and glucose (1% v/v) were added as inducers/co-substrates. To avoid possible toxicity, 30 mM alkylbenzene (Table 3.1) was added 24 h after induction and a further 30 mM added 12 h after substrate addition. Biomass determinations were done after 48 h of growth. Samples were taken at regular intervals and analysed for product formation.

To test nonylbenzene as an inducer of CYP52 genes, *Y*. *lipolytica* W29 main-cultures were grown for 24 h before glucose (1% v/v) was added as a co-substrate. After 36 h growth, 60 mM nonylbenzene or 30 mM nonylbenzene together with 30 mM alkylbenzene(s) (hexylbenzenes, octylbenzenes, decylbenzenes, p-cymene and limonene respectively) were added as substrates respectively. Biomass determinations were done after 36 h of growth. Samples were taken at regular intervals and analysed for product formation.

3.3.3 The effect of inducers and co-substrate on the biotransformation of nonylbenzene and hexylbenzenes by *Candida tropicalis* ATCC20336

To detect the effect of inducers/co-substrate on the biotransformation of nonylbenzene, *Candida tropicalis* ATCC20336 was cultured under standard conditions. The main-cultures were grown for 18 h before *n*-dodecane (1% v/v); oleic acid (1% v/v) and glucose (1% v/v) were added as inducers/co-substrates. After a further 18 h growth nonylbenzene and hexylbenzene (60 mM) were added as substrates. Biomass determinations were done just before substrate addition (i.e. 36 h growth). Samples were taken at regular intervals for GC analysis.

3.3.4. Biotransformation of 4-hexylbenzoic and 4-nonyloxybenzoic acid with or without an inducer by *Yarrowia lipolytica* W29 and *Candida tropicalis* ATCC20336

Yeast strains were grown under standard conditions. Oleic acid was added as an inducer to main cultures grown for 24 h. 4-Hexylbenzoic acid (0.1% w/v) and 4-nonyloxybenzoic acid (0.1% w/v) were added as substrates 24 h later. When no inducer was added, substrates were added after 24 h growth. Samples were taken every 12 h for 48 h and analyzed with thin layer chromatography.

3.4 Results and Discussion

Aromatic hydrocarbons including alkylbenzene are considered less degradable compared to aliphatic hydrocarbons (*n*-alkanes) (Atlas, 1981; Atlas, 1978). Hydrocarbons are generally removed from crude oils in a ‘‘quasi-stepwise’’ process, roughly in the order of *n*-alkanes > alkylcyclohexanes > alkylbenzenes > acyclic isoprenoids > alkylnaphthalenes > bicyclic alkanes > alkylphenanthrenes > steranes > hopanes (George *et al*., 2002). This might explain why most of the published work on hydrocarbon degradation by yeasts, has concentrated more on metabolism of *n*-alkanes (Van der Klei and Veenhuis, 2006; Wache *et al*., 2006; Fickers *et al*., 2005). However, in our lab much work has been done on the bioconversion of alkylbenzenes, alkylbenzoic acids and alkoxybenzoic acids using *Yarrowia lipolytica* strains. In this study we compared the ability of *Candida tropicalis* ATCC20336 and *Y*. *lipolytica* W29 wild-type strains to degrade these aromatic compounds to aromatic acid products.

3.4.1 Biotransformation of alkylbenzenes by *Yarrowia lipolytica* W29

Y. *lipolytica* W29 only oxidized hexylbenzene and nonylbenzene but not butylbenzene or *p*-cymene. The experiment was therefore repeated with hexylbenzene and nonylbenzene. Biotransformation of nonylbenzene and

hexylbenzene yielded benzoic acid and phenylacetic acid as the major products, respectively (Figure 3.2). Although benzoic acid formation in the two independent experiments was different (16.6 mM and 11.4 mM after 72 h), it was evident that nonylbenzene oxidation to benzoic acid was the favored reaction.

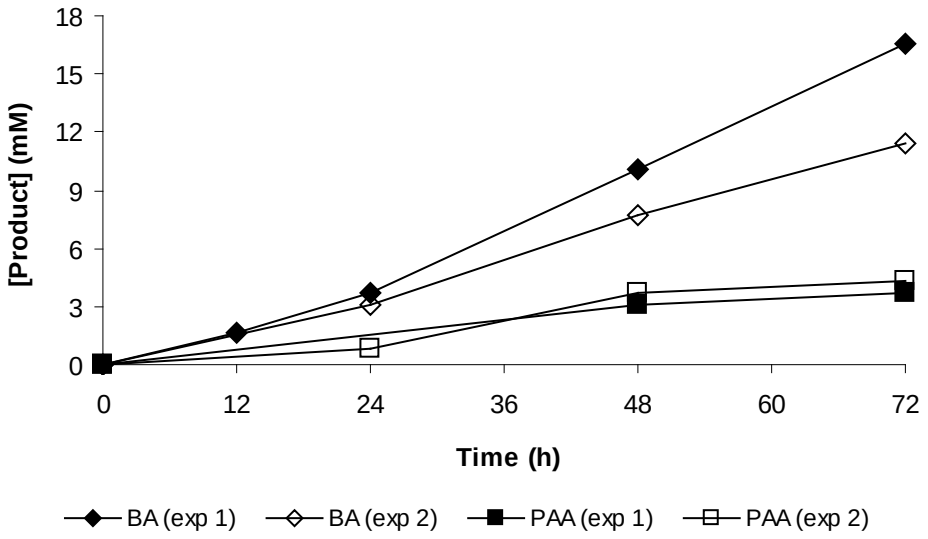

Figure 3.2: Biotransformation of hexylbenzene and nonylbenzene by *Yarrowia lipolytica* W29. Benzoic acid (BA) (open and closed diamonds) is a major product of nonylbenzene and phenylacetic acid (PAA) (open and closed squares) is a major product of hexylbenzene. The graph represents two independent experiments. Substrates (30 mM alkylbenzene) were added at 12 h growth. Time 0 h represents the time at which the substrates were added.

Nonylbenzene was apparently the only of these alkylbenzenes that was not toxic to *Y. lipolytica* W29, since biomass production after 48 h (8.3 g dry weight L^{-1}) was comparable to the control (7.6 g dry weight L^{-1}) (Figure 3.3 (A)). In the case of the shorter chain alkylbenzenes biomass production after 48 h and 72 h ranged between 3.5 and 5 g dry weight L^{-1}. It was not clear why the control strain continued to produce biomass up to 72 h growth, while it did not happen in the presence of nonylbenzene. Work done in our laboratory had indicated that all the glucose is consumed by *Y. lipolytica* within 48 h when YP_2D_2 medium is used (Ramorobi, 2008).

In the first experiment growth of *Yarrowia lipolytica* was also followed by measuring optical density (OD) at 620 nm (Figure 3.4 (B)). Before the substrates were added, after just 12 h growth, the optical densities were low. Twelve hours later the strain seemed to adapt to only nonylbenzene and hexylbenzene when compared to the control. In fact, in the presence of butylbenzene and *p*-cymene the optical densities did not increase. When comparing optical densities with biomass measured after 48 and 72 h growth, there are notable discrepancies. Biomass at 48 h was almost the same for the control and the culture grown in the presence of nonylbenzene, however, the OD of the nonylbenzene culture was almost double that of the control. This might be because emulsification of the nonylbenzene by the yeast contributed to the OD. This also happened with hexylbenzene, where ODs were higher than for the control, although biomass was lower and with *p*-cymene and butylbenzene, where ODs at 48 h were almost the same as for the control, although the biomass was significantly lower. The increase in biomass of the control culture at 72 h is, however, reflected by the OD measurement, indicating that OD measurements in the absence of hydrocarbon substrates is reliable. Because hydrocarbons interfere with OD measurements, OD measurements were not used in the rest of the study.

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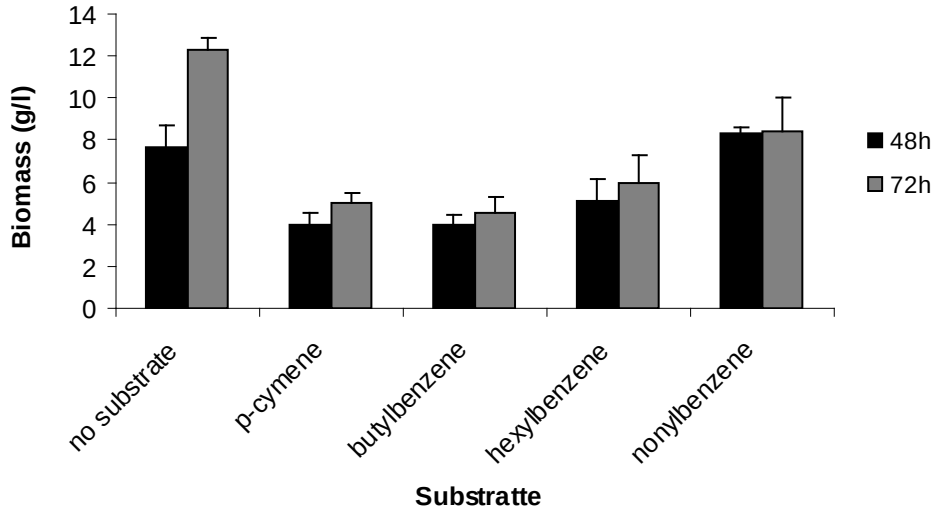

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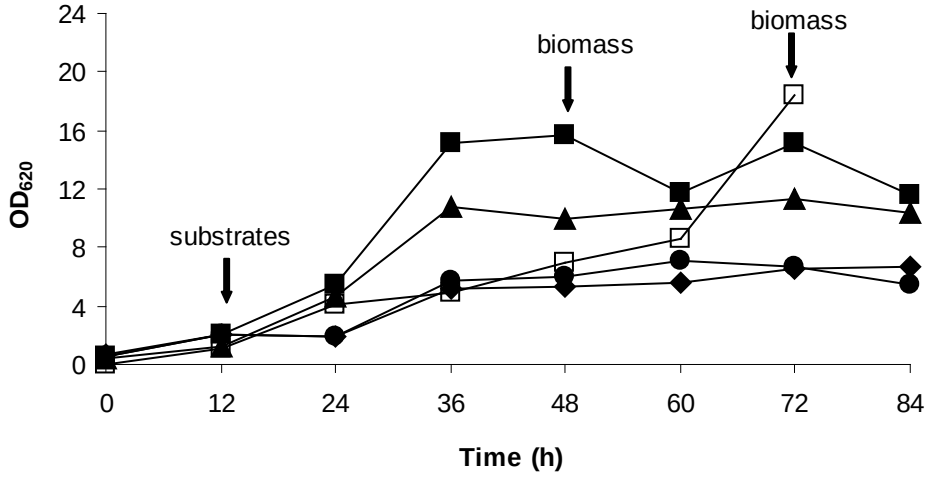


Figure 3.3: Effect of alkylbenzenes on biomass production and growth of *Yarrowia lipolytica* W29 wild-type strain. (A) Biomass determinations were done in triplicates after 48 h and 72 h growth. The substrates were added after 12 h growth. (B) The control (□) had no substrates. The substrates, *p*-cymene (●), butylbenzene (◆), hexylbenzene (▲) and nonylbenzene (■), were added after 12 h of growth.

Butylbenzene has also been reported to be toxic to strains derived from *Yarrowia lipolytica* E150 (Van Rooyen, 2005). Although we did not trace any product in the

biotransformation of *p*-cymene, some researchers have reported a *pseudomonad* strain that is able to grow on *p*-cymene as a carbon source (Duetz *et al*., 2003). In this experiment lack of biotransformation of *p*-cymene and butylbenzene can probably be attributed to toxicity to *Y*. *lipolytica* W29 cells (Figure 3.3 (B)), when the substrates were added so early (i.e. after 12 h growth).

3.4.2 The effect of inducers and co-substrate on the biotransformation of alkylbenzenes by *Yarrowia lipolytica* W29

In the literature there is evidence that degradation of aromatic hydrocarbons can be enhanced by the presence of substrates such as *n*-alkanes and fatty acids through co-oxidation or co-metabolism. This is done by growing microorganisms first on *n*-alkanes and then adding alkylbenzenes (Koma *et al*., 2003; Atlas, 1981; Horvath, 1972; Douros and Frankanfeld, 1968). It is not always clear whether these substrates served only as co-substrates providing energy for growth and the degradation of the aromatic hydrocarbons or also as inducers of the enzymes responsible for the degradation of the aromatic hydrocarbons (Koma *et al*., 2003; Iida *et al*., 2000; Craft *et al*., 2003; Douros and Frankenfeld, 1968). Yeast CYP52s have been shown to be induced by *n*-alkanes, fatty acids and alkylbenzenes (Scheller *et al*., 1996). Therefore the effect of different inducers/co-substrates on the biotransformation of various alkylbenzenes by *Yarrowia lipolytica* W29 was investigated.

The results obtained for the biotransformation of alkylbenzenes by *Y*. *lipolytica* W29 when *n*-dodecane, *n*-tetradecane and oleic acid were added as co-substrates and inducers of *CYP52* genes and glucose as a co-substrate are summarized in table 3.1. It has been shown by others (Gumede, personal communication) that the additional glucose disappears within 24 h. The results summarized in table 3.1 shows that nonylbenzene and hexylbenzene hydroxylation did not require prior induction by *n*-alkanes or oleic acid. 4-Ethyltoluene was only degraded to ethylbenzoic acid when *n*-tetradecane was

used as an inducer. Nonylbenzene was the best substrate because on average, more than 10 mM benzoic acid was produced from it, except when *n*-tetradecane was used as an inducer. Less than 5 mM of acid products were formed from hexylbenzene and 4-ethyltoluene. Hexylbenzene seemed to be a poor substrate because of low product formation and no product was observed when tetradecane was used as an inducer. However, it should be noted that derivatives of the E150 strains of *Y. lipolytica* have been reported to give almost complete conversion of hexylbenzene to phenylacetic acid (Ramorobi, 2008). These strains in fact preferred hexylbenzene over nonylbenzene. The oxidation of other alkylbenzenes by *Y. lipolytica* W29 could not be induced by *n*-alkanes or oleic acid. Glucose is not an inducer of *CYP52* genes but rather a readily available carbon and energy source. It is also not a repressor of *Y*. *lipolytica* CYP 52 genes (Iida *et al*., 2000). Thus the ability of different alkylbenzenes to induce uptake systems and CYP52s for their degradation by *Y. lipolytica* W29 was evaluated. It is difficult to explain why *Y. lipolytica* W29 could only accept nonylbenzene and hexylbenzene as substrates but failed to oxidize other alkylbenzenes even when *n*-tetradecane and oleic acid were used as inducers, because our experiments were limited to whole cell biotransformation. However, lack of oxidation or degradation of alkylbenzenes with alkyl chains of less than five carbons has been reported (Kennes and Veiga, 2004; Douros and Frankanfeld, 1968). In this respect it is again of interest that Ramorobi (2008) occasionally obtained conversion of butylbenzene by the E150 derivatives, although conversions were low.

Table 3.1: Co-oxidation of alkylbenzenes with co-substrate/inducers by *Yarrowia lipolytica* W29

Co-substrate/Inducer **Substrates**	**Glucose**	***n*-Dodecane**	***n*-Tetradecane**	**Oleic acid**
Ethylbenzene	-	nd	-	-
Propylbenzene	-	nd	-	-
Butylbenzene	-	nd	-	-
sec-Butylbenzene	-	nd	-	-
Pentylbenzene	-	nd	-	-
Hexlbenzene	+	+	-	+
Heptylbenzene	-	nd	-	-
Octylbenzene	-	nd	-	-
Nonylbenzene	+++	+++	++	+++
Decylbenzene	-	nd	-	-
p-Cymene	-	nd	-	-
4-Ethyltoluene	-	nd	+	-
tert-Butyltoluene	-	nd	-	-

Symbols: nd-not done, - no product formed, + little product formed (less than 5 mM), ++ product formation is between 5 mM-10 mM, +++ product formation more than 10 mM. Co-substrates or an inducer was added after 24 h growth and substrates added 24 h later. Cultures were incubated at 30 ^{o}C on a rotary shaker.

Figure 3.4 summarizes the results obtained for the biotransformation of nonylbenzene. High product formation and biomass (16.3 mM and 18 g l^{-1} +/-1) was obtained when oleic acid was used as an inducer; although the highest specific rates were obtained when *n*-dodecane was used as an inducer of the *CYP52* genes (18 µmol $h^{-1}g^{-1}$ dry weight). Experiments with glucose and *n*-tetradecane were repeated and give an indication that the variation between experiments can be quite big, although the trend was still the same.

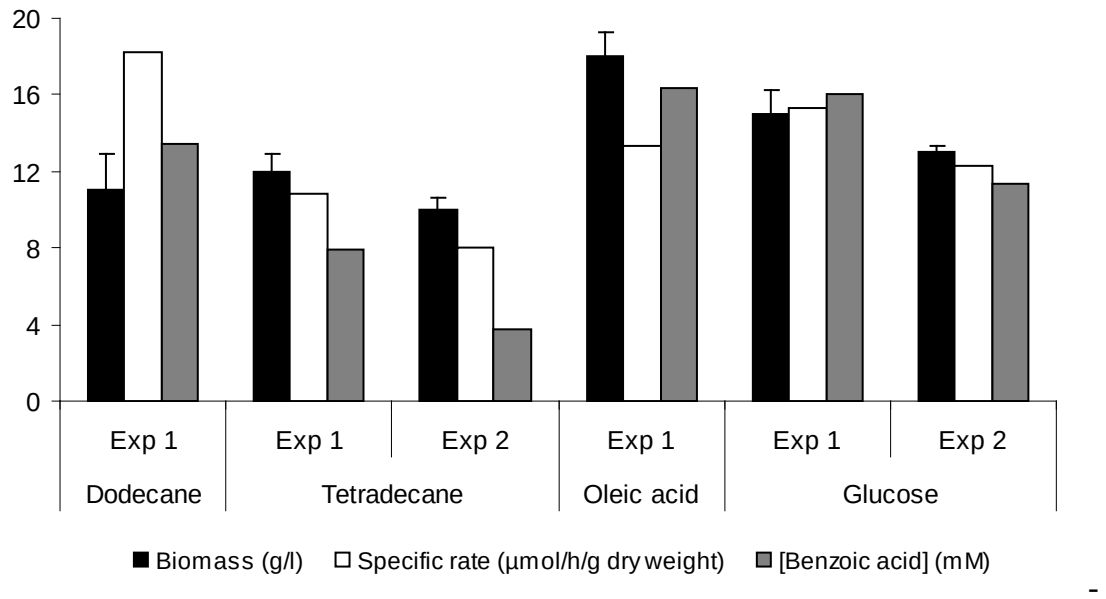


Figure 3.4: The effect of *n*-dodecane (C_{12}), *n*-tetradecane (C_{14}), oleic acid and glucose on biotransformation of nonylbenzene by *Yarrowia lipolytica* W29. Biomass determinations were done in triplicate after 48 h growth (just before substrate addition) and benzoic acid concentrations were determined 72 h after substrate addition. The rates were calculated using the best fit for the 0 h, 24 h, 36 h, 48 h and 72 h data points. The biomass at 48 h growth was used to determine the specific rates.

Because we obtained significant biotransformation of nonylbenzene with or without addition of inducers (Table 3.1 and figure 3.4) we decided to look at the possibility of using nonylbenzene as an inducer for the biotransformation of hexylbenzene, octylbenzene, decylbenzene, *p*-cymene and 4-ethytoluene. In this experiment glucose (0.5%) was added as a co-substrate after 24 h growth, while nonylbenzene and the other alkylbenzenes were added 12 h later. Nonylbenzene did not induce the oxidation of even chain length alkylbenzenes, since no phenylacetic acid was formed from any of these substrates (Table 3.2). These alkylbenzenes in fact had a negative impact on the oxidation of nonylbenzene, because there was also no benzoic acid formed from nonylbenzene in the presence of these compounds. Oxidation of *p*-cymene to *p*-isopropylbenzoic acid (2.3 mM) was observed (Figure 3.5), although *p*-cymene also hindered production of benzoic acid (13.1 mM after 72 h) from

nonylbenzene when compared to the control (20.6 mM after 72 h) (Figure 3.6). The oxidation of 4-ethyltoluene to 4-ethylbenzoic acid (0.7 mM after 72 h) in the presence of nonylbenzene was also observed (Figure 3.5). Product formation was low compared to when *n*-tetradecane was used as an inducer (1.5 mM after 72 h, results not shown). However, oxidation of 4-ethyltoluene to 4-ethylbenzoic acid did not hinder benzoic acid production (21.1 mM after 72 h) from nonylbenzene.

Table 3.2: Biotransformation of other alkylbenzenes in the presence of nonylbenzene by *Yarrowia lipolytica* W29. Glucose was added as a co-substrate after 24 h of growth, 12 h later 30 mM nonylbenzene with 30 mM alkylbenzene were added as substrates. One flask with 60 mM nonylbenzene was kept as control.

Substrates	**Product formation (from other alkylbenzenes)**	**Product formation (from nonylbenzene)**
Hexylbenzene	-	-
Octyllbenzene	-	-
Nonylbenzene	NA	+++
Decylbenzene	-	-
p-Cymene	+	+++
4-Ethyltoluene	+	+++

Symbols: - no product formed, + little product formed (less than 5 mM), ++ product formation is between 5 mM-10 mM, +++ product formation more than 10mM, NA-not applicable. Cultures were incubated at 30 °C on a rotary shaker.

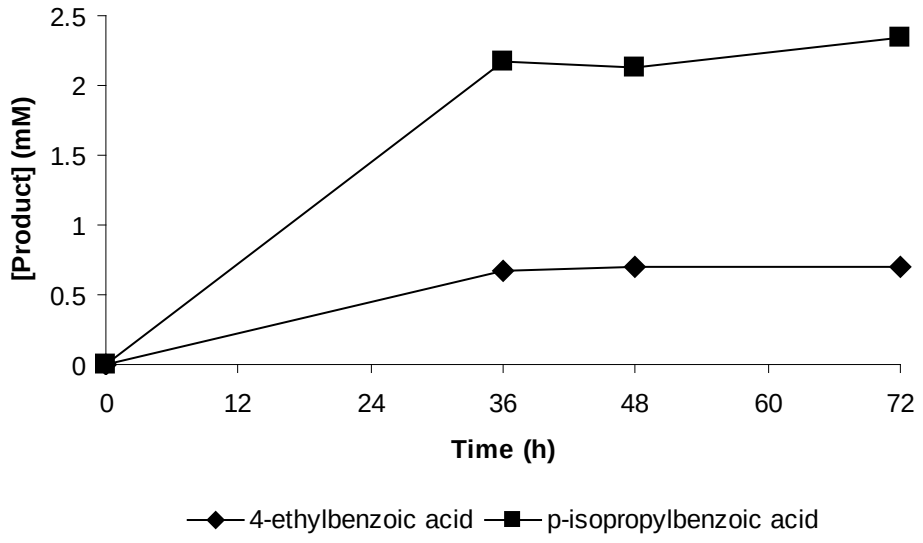


Figure 3.5: Biotransformation of 4-ethyltoluene and *p*-cymene by *Yarrowia lipolytica* W29 with nonylbenzene added as an inducer. 4-Ethylbenzoic acid (♦) was formed from 4-ethyltoluene and *p*-isopropylbenzoic acid (■) from *p*-cymene.

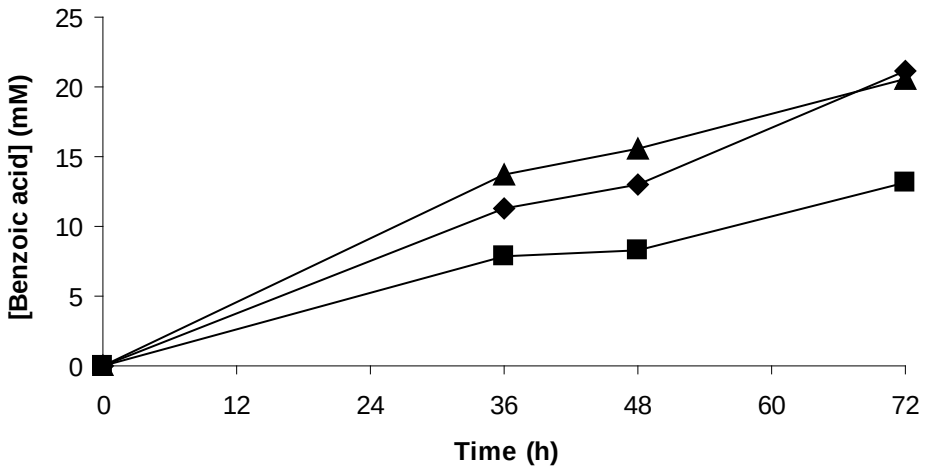


Figure 3.6: Biotransformation of nonylbenzene (30 mM) by *Yarrowia lipolytica* W29 in the presence of 4-ethyltoluene (30 mM) (◆) and *p*-cymene (30 mM) (■). The control (▲) had 60 mM nonylbenzene.

3.4.3 The effect of inducers and co-substrate on the biotransformation of nonylbenzene and hexylbenzene by *Candida tropicalis* ATCC20336

Candida tropicalis ATCC20962 is a well established industrial strain and can produce over 100 g l^{-1} DCA within 114 h (Wache *et al*., 2006). For comparative studies, the parental strain *C. tropicalis* ATCC20336 was investigated for biotransformation of nonylbenzene and hexylbenzene. The organism was grown in YP_2D_2 broth. A different approach was taken where the cells were induced after 18 h growth and the substrate added after 36 h growth. The time of substrate addition was set at 36 h, because in the final experiments with *Y. lipolytca*, substrate addition after 36 h growth had given the best conversions. However, it was decided to add the inducers after 18 h rather than 24 h to allow more time for induction. Biomass determinations were done at 36 h just before the substrates were added.

The highest product formation (Figure 3.7) and specific rates for nonylbenzene biotransformation (Figure 3.8) was obtained after induction with 1% (v/v) dodecane (40 mM benzoic acid and 56 µmol $h^{-1}g^{-1}$ dry weight respectively) followed by induction with 1% (v/v) oleic acid (27 mM and 45 µmol $h^{-1}g^{-1}$ dry weight respectively). In fact the presence of inducers significantly improved biotransformation of nonylbenzene over time (Figure 3.7).

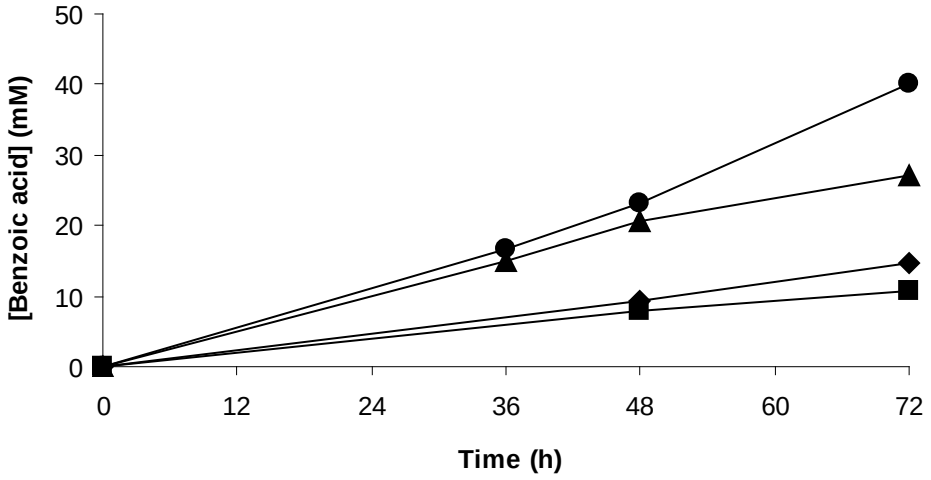


Figure 3.7: Biotransformation of nonylbenzene (60 mM) with or without the co-substrates/inducer by *Candida tropicalis* ATCC20336. One culture received no additional co-substrate/ inducer (◆), while additional glucose (■), *n*-dodecane (●) and oleic acid (▲) were added as co-substrates/inducers to the others after 18 h growth. The substrates were added 18 h later.

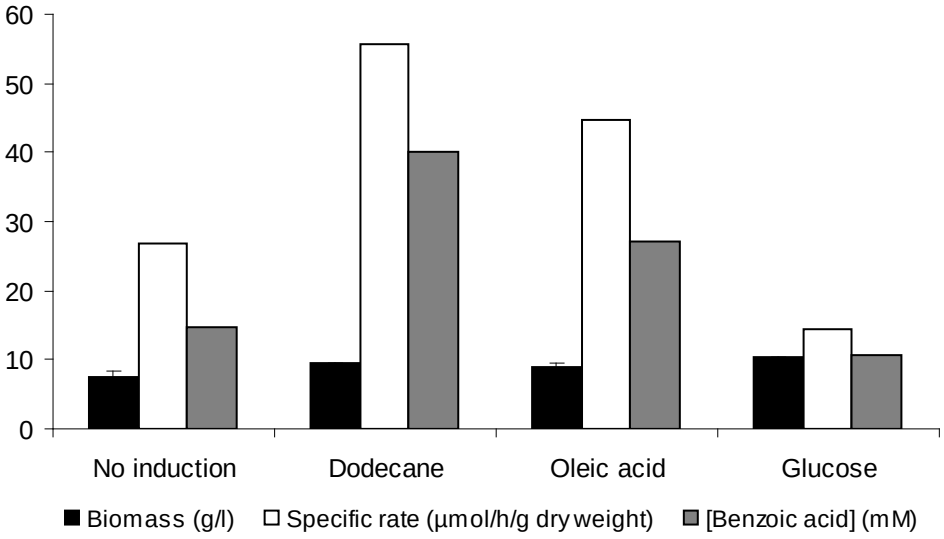


Figure 3.8: The effect of *n*-dodecane (C12), oleic acid and glucose on the biotransformation of nonylbenzene by *Candida tropicalis* ATCC20336. Biomass determinations were done in triplicate after 36 h growth just before substrate addition and benzoic acid concentration was determined 72 h after substrate addition. The rates were calculated using the best fit for the 0 h, 24 h, 36 h, and 48 h and 72 h data points.

For hexylbenzene biotransformation (Figure 3.9), the highest product formation was also obtained after induction with 1% (v/v) *n*-dodecane (32 mM), followed by glucose as a co-substrate (31 mM). Induction with oleic acid (1% v/v) gave the lowest conversion, although more phenylacetic acid (20 mM) was formed than ever obtained with *Y. lipolytica* W29. The biomass before substrate addition was almost the same (ca. 10 g dry weight L^{-1}) for all the *C. tropicalis* ATCC20336 cultures, showing that the inducers/co-substrate did not have an effect on the biomass.

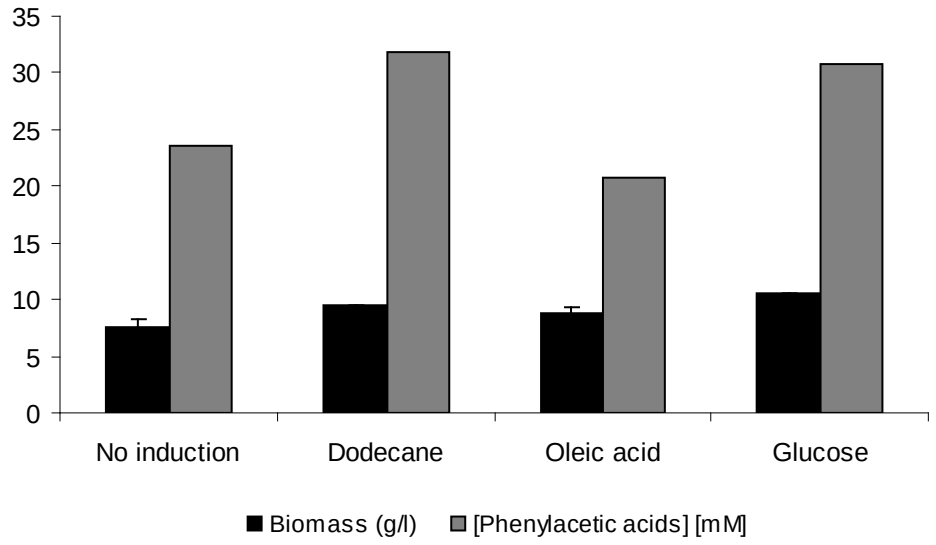


Figure 3.9: The effect of dodecane (C12), oleic acid and glucose on the biotransformation of hexylbenzene by *Candida tropicalis* ATCC20336. Biomass determination was done after 36 h before substrate addition and benzoic acid concentration was determined after 72 h of substrate addition.

3.4.4. Biotransformation of hexylbenzoic acid and nonyloxybenzoic acid with or without an inducer by *Yarrowia lipolytica* W29 and *Candida tropicalis* ATCC20336

Previously, Van Rooyen (2005) and Obiero (2006) in our laboratory showed that when an alkyl chain is attached to benzoic acid (i.e. 4-hexylbenzoic acid or 4-nonyloxybenzoic acid) oxidation of the alkyl chain can occur. Van Rooyen (2005)

reported oxidation of hexylbenzoic acid to 4-(5-carboxypentyl) benzoic acid (intact product) as well as degradation to 4-(carboxymethyl) benzoic acid by strains derived from *Yarrowia lipolytica* W29. The identity of these products was determined by GC-MS analysis. 4-Hexylbenzoic acid is thus firstly hydroxylated at the terminal position, most likely by CYP52 enzymes, to form 4-(6-hydroxyhexyl) benzoic acid. Other enzymes oxidize the hydroxylated product to 4-(5-carboxypentyl) benzoic acid (intact product). The resulting 4-(5-caboxypentyl) benzoic acid then enters β-oxidation to be degraded to 4-(carboxymethyl) benzoic acid (degraded product) (Chapter 1, Figure 1.3). By comparing TLCs from this experiment (Figure 3.10) with ones reported by Van Rooyen (2005) it was concluded that the biotransformation of 4-hexylbenzoic acid by *Y*. *lipolytica* W29 and *C. tropicalis* ATCC20336 in both cases, yielded 4-(5 caboxypentyl) benzoic acid (intact product) as the major product. This result was confirmed with GC-MS analysis (Figure 3.11). After 48 h biotransformation *Y. lipolytica* had produced small amounts of the degraded dioic acid (4-(carboxymethyl) benzoic acid), while *C. tropicalis* did not significantly degrade the initial dioic acid product. An important observation was that *Y. lipolytica* W29 only oxidized 4-hexylbenzoic acid when oleic acid was not added as inducer (Figure 3.10). In contrast, *C. tropicalis* ATCC20336 only formed the product when the organism was induced with oleic acid (Figure 3.10).

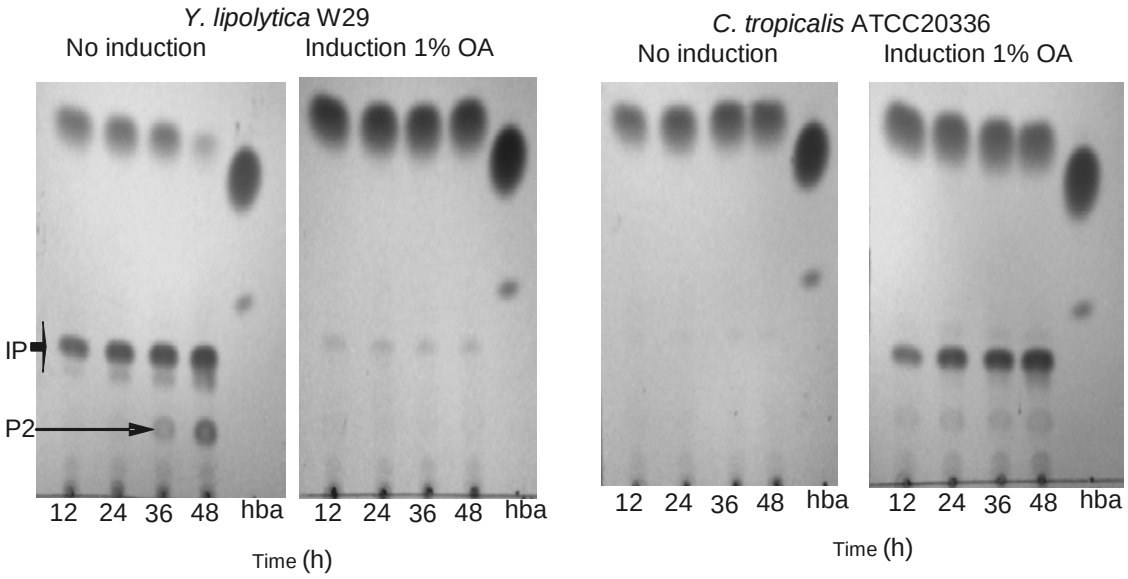
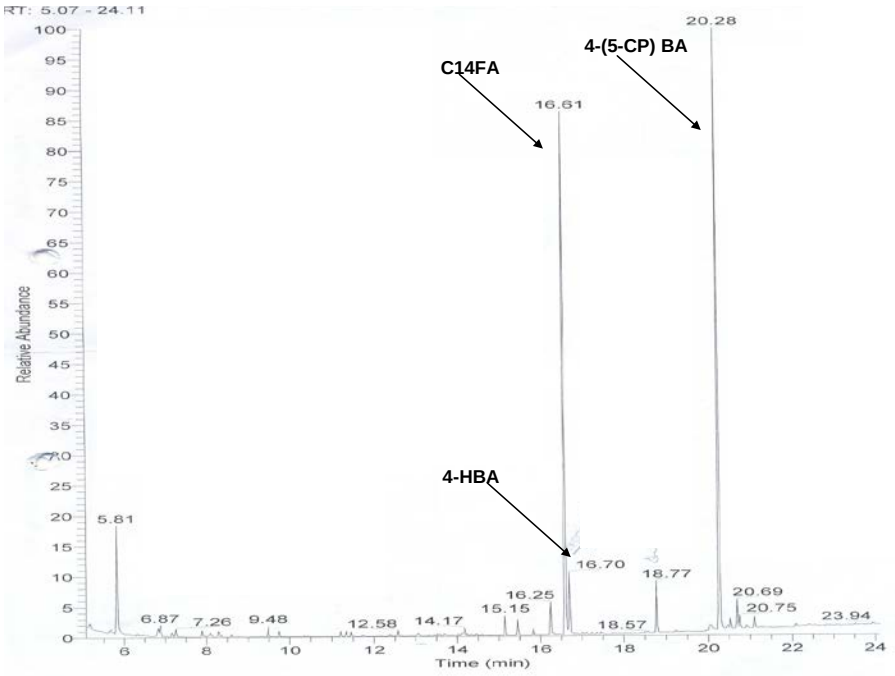


Figure 3.10: Biotransformation of 4-hexylbenzoic acid (4-HBA) by *Yarrowia lipolytica* W29 and *Candida tropicalis* ATCC20336. The product labelled IP was identified as 4-(5-caboxypentyl) benzoic acid (intact product) (IP), while P2 was identified as 4-(carboxymethyl) benzoic acid. One set of flasks was induced with 1% (v/v) oleic acid and the other not induced.

A



B

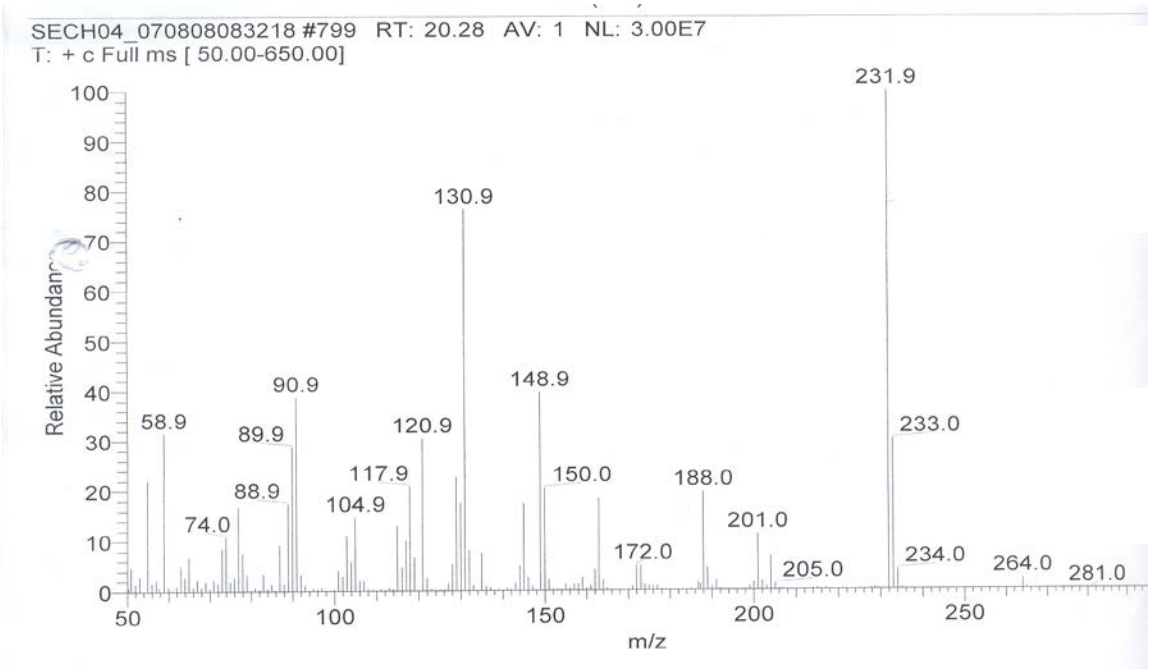


Figure 3.11: GC-MS of biotransformation intermediates on 4-hexylbenzoic acid (4-HBA) by *Yarrowia lipolytica* W29 wild-type strain after 72 h of conversion phase. (A) The GC-chromatogram indicating the substrate (4-HBA), product 4-(5-caboxypentyl) benzoic acid (4-(5-CP) BA) and the internal standard myristic acid (C14FA), (B) mass spectrum of the major product 4-(5-caboxypentyl) benzoic acid .

4-Nonyloxybenzoic acid has a long alkyl chain attached to *p*-hydroxybenzoic acid. Obiero (2006) reported that oxidation of 4-nonyloxybenzoic acid by *Yarrowia lipolytica* W29 gave three distinct products. These three products were identified as intact dioic acid 4-(8-carboxyoctyloxy) benzoic acid, the shortened dioic acid 4-(2-carboxyethoxy) benzoic acid and *p*-hydroxybenzoic acid. The CYP52 enzymes in *Y. lipolytica* are most likely also responsible for the first step of terminal hydroxylation of 4-nonyloxybenzoic acid and oxidation by the CYP52s or other enzymes lead to the formation 4-(8-carboxyoctyloxy) benzoic acid and subsequent formation of 4-(2-carboxyethoxy) benzoic acid. The 4-(2-carboxyethoxy) benzoic acid is further degraded to *p*-hydroxybenzoic acid (Chapter 1, Figure 1.4).

In the present study the biotransformation of 4-nonyloxybenzoic acid by *Y. lipolytica* W29 yielded three products (Figure 3.12), as expected and reported by Obiero (2006). *C. tropicalis* ATCC20336 also oxidized 4-nonyloxybenzoic acid to 4-(8-carboxyoctyloxy) benzoic acid, 4-(2-carboxyethoxy) benzoic acid and *p*-hydroxybenzoic acid (Figure 3.12). With both yeasts the oxidation of the substrate occurred only when oleic acid was used as an inducer. The 4-nonyloxybenzoic acid was already depleted after 12 h in the case of *C. tropicalis* ATCC20336 compared to 24 h in the case of *Y. lipolytica* W29.

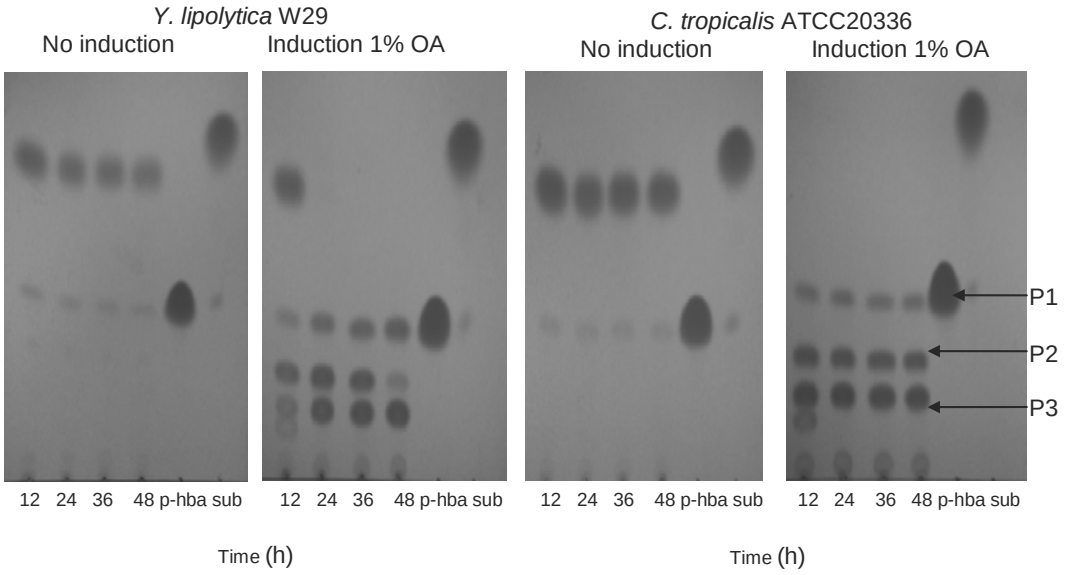


Figure 3.12: Biotransformation of 4-nonyloxybenzoic acid (0.1 % w/v) by *Y. lipolytica* W29 and *Candida tropicalis* ATCC20336. Three distinct products were formed, P1 *p*-hydroxybenzoic acid, P2 the shortened dioic acid 4-(2-carboxyethoxy) benzoic acid and lastly P3 an intact dioic acid 4-(8-carboxyoctyloxy) benzoic acid. One set of flasks was induced with 1% (v/v) oleic acid and the other not induced.

3.5 Conclusion and future prospect

Candida tropicalis and *Yarrowia lipolytica* are different in their morphology, phylogeny, behaviour and most importantly their genetic make-up (Cheng *et al*., 2005; Fickers *et al*., 2005; Iida *et al*., 2000; Craft *et al*., 2003). *Y. lipolytica* strains have 12 *CYP52* genes (Iida *et al*., 2000) and *C*. *tropicalis* ATCC20336 and its derivatives have 10 distinct *CYP52* genes (Craft *et al*., 2003). *n*-Alkane degrading yeast such as *C*. *tropicalis, C*. *cloacae* and *C*. *maltosa* are organisms of considerable commercial importance (Cheng *et al*., 2005). One of the *C*. *tropicalis* ATCC20336 derivatives has been reported to produce over 100 g l^{-1} DCA (Wache *et al*., 2006) within 114 h. To understand why *C*. *tropicalis* mutant strains perform better than mutant *Y*. *lipolytica* strains (Smit *et al*., 2005) it was important to compare the alkane hydroxylase activity of the corresponding wild-type strains of *C*. *tropicalis* and *Y*. *lipolytica*. For comparison alkylbenzenes, 4-hexylbenzoic acid and 4-nonyloxybenzoic acid were used as substrates because their products can be accumulated by the wild-type strains while *n*-alkanes and fatty acids are completely degraded by these organisms.

The *Y*. *lipolytica* W29 wild-type strain did not need inducers of *CYP52* genes like *n*-alkanes and oleic acids to improve biotransformation of nonylbenzene and hexylbenzene or other alkylbenzenes. *Y*. *lipolytica* W29 gave the highest benzoic acid production from nonylbenzene when the cultures were not induced with *n*-alkanes or oleic acid. This was also observed even when the substrate was added at earlier time just after 12 h of growth. The final benzoic acid product formed by *Y. lipolytica* W29 at 72 h after substrate addition, was not influenced by when the substrate was added, as the final product formed from biotransformation of nonylbenzene, was comparable regardless on whether the substrate was added after 12 h, 24 or 36 h growth. In the case of *C*. *tropicalis* ATCC20336, dodecane and oleic acid significantly improved biotransformation of nonylbenzene. *n*-Dodecane improved production by 73% to give benzoic acid production of 40 mM (highest) at a specific rate of 45 $\mu molh^{-1}g^{-1}$ dry weight

(highest) and substrate conversion of 67%. These were notable results since we could not obtain any comparable results with *Y. lipolytica* W29. The biotransformation of hexylbenzene by *C*. *tropicalis* ATCC20336 was improved compared to the biotransformation of hexylbenzene by *Y. lipolytica* W29 which yielded in all experiments less than 5 mM phenylacetic acid. *n*-Dodecane again significantly improved biotransformation of hexylbenzene to phenylacetic acid (31 mM) by *C*. *tropicalis* ATCC20336. The least production (20 mM phenylacetic acid) was obtained when the organism was induced with 1% (v/v) oleic acid. When the *C*. *tropicalis* ATCC20336 cultures, were not induced, 23 mM phenylacetic acid was produced. Nonylbenzene was the only substrate to be significantly oxidized by *Y. lipolytica* W29 while both hexylbenzene and nonylbenzene were better oxidized by *C*. *tropicalis* ATCC20336.

Nonylbenzene as substrate induced the CYP52 enzymes from *Y*. *lipolytica* W29 and *C*. *tropicalis* ATCC20336 as it was significantly degraded to benzoic acid even when no other inducers were added. Hexylbenzene also induced the CYP52 enzymes as it was significantly degraded to phenylacetic acid by *C*. *tropicalis* ATCC20336. The observation that *C. tropicalis* ATCC20336 significantly oxidized hexylbenzene, raised the possibility that this strain might oxidize the short chain alkylbenzenes, which *Y. lipolytica* W29 failed to oxidize (Table 3.1). *Y. lipolytica* W29 only converted *p*-cymene to *p*-isopropylbenzoic acid and 4-ethyltoluene to 4-ethyllbenzoic acid when nonylbenzene was also present. *C. tropicalis* can in future be tested for the biotransformation of the *p*-cymene and 4-ethyltoluene. In future microsomal fractions should also be prepared of cultures used for biotransformation experiments to determine P450 content and CPR activity as well as to compare hydroxylase activity towards different chain length alkanes, fatty acids and alkylbenzenes.

In the case of 4-nonyloxybenzoic acid both the organisms oxidized the substrate only in the presence of oleic acid to form the same three products. Oxidation of 4-hexylbenzoic acid by *Y*. *lipolytica* W29 occurred in the absence of oleic acid but

not in its presence, while oxidation of 4-hexylbenzoic acid by *C*. *tropicalis* ATCC20336 occurred in the presence of oleic acid. This raised the possibility that 4-hexylbenzoic acid might be used as marker substrate to monitor expression of *C. tropicalis* ATCC20336 P450s in *Y. lipolytica* strains. We thought it would be a good idea to clone the *CYP52A13* and *CYP52A17* genes from *C. tropicalis* into β-oxidation disrupted strains of *Y. lipolytica* under the *pPOX2* promoter that is induced by oleic acid. According to Craft *et al*., 2003 *CYP52A13* and *CYP52A17* are strongly induced by oleic acid. Thus the expression of these genes were to be monitored using oleic acid induced *Y. lipolytica* cultures and 4-hexylbenzoic acid as a substrate. It was hoped that these experiments might shed some light on the role of these enzymes in the ability of *C. tropicalis* ATCC20962 to produce over 100 g l^{-1} DCA and that it might improve DCA production by *Y. lipolytica*.

Chapter 4 Cloning and functional expression of *CYP52A13* and *CYP52A17* from *C. tropicalis* ATCC20336 in *Y. lipolytica* FT120 strains

Candida tropicalis ATCC20336 and its derivatives contain ten unique cytochrome P450 monooxygenases (Eschenfeldt *et al*., 2003). The derivative β-oxidation blocked strains have been reported to accumulate concentrations of above 100 g l^{-1} DCA within 114 h and bioreactor productivities of up to 1.9 g $l^{-1}h^{-1}$, which make these the CYP450 processes with the highest productivities (Julsing *et al*., 2007; Mobley, 1999, Picataggio *et al*., 1992). In a study done by Craft *et al*. (2003), four of these *CYP52*s (two allelic pairs *CYP52A13* & *CYP52A14* and *CYP52A17* & *CYP52A18*) were highly induced by emersol 267 (a mixture of fatty acids, containing mainly oleic acid), pure oleic acid and pure *n*-octadecane. In another study done by Eschenfeldt *et al*. (2003), CYP52A13 and CYP52A17 were expressed in *Sf9* insect cells and microsomal fractions were used to observe relative activities towards fatty acids of different chain lengths. CYP52A17 and CY52A13 were found to oxidize oleic acid at almost the same rate. CYP52A17 however showed higher activity towards shorter chain saturated fatty acids (chain length C_{10} to C_{16}), displaying highest activity toward myristic acid. In contrast, CYP52A13 oxidized saturated fatty acids of chain length C_{10} to C_{16} more slowly. However, activity towards *n*-alkanes was not investigated in these previous studies. The aims of the study described in this chapter were to clone *CYP52A13* and *CYP52A17* into β-oxidation blocked *Y. lipoytica* strains and to evaluate the effect on dioic acid production from *n*-alkanes, fatty acids and 4-hexylbenzoic acid.

4.1 Materials and methods

4.1 Part A Molecular biology

4.1.1 Strains, vectors and media

The yeasts strains used in this study are summarized in table 4.1. All strains were stored in LN broth containing glycerol (7% v/v) under liquid N2 in the MIRCEN yeast culture collection of the University of the Free State, South Africa.

LN broth contained (per liter distilled water): 40 g glucose, 10 g tryptone and 10 g yeast nitrogen base (YNB) (containing amino acids and ammonium phosphate)

Escherichia coli XL10 gold (TetrD(*mcrA*)*183* D(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*q*Z*D*M15* Tn*10* (Tetr) Amy Camr] was used for plasmid preparation. *E. coli* carrying plasmid was grown in Luria-Bertani (LB) broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl] supplemented with 100 µg ml^{-1} ampicillin or 50 µg ml^{-1} kanamycin]. The cloning of PCR products was performed using pGem-T Easy vector (Promega). Expression vector JMP62 (Figure 4.1) and the β-oxidation blocked strain *Y. lipoytica* FT120, the parental strain of the strains used in this study, were generous gifts from Dr. J.-M Nicaud of the Laboratoire de Microbiologie et Génétique Moléculaire, INRA CNRS INAP-G, France.

Table 4.1: Summary of the strains used

Strains	Properties
CTY029 (control strain) [1]	Leu+, Ura+, ΔPOX1-6, pPOX2-ylCPR
CTY026 (control strain) [1]	Leu+, Ura+, ΔPOX1-6, pPOX2-ylCPR, pICLylCPR
CTY022 (parental strain for cloning)[1]	Leu+, Ura-, ΔPOX1-6, pPOX2-ylCPR
CTY021 (parental strain for cloning)[1]	Leu+, Ura-, ΔPOX1-6, pPOX2-ylCPR, pICL-ylCPR

1. Strains were constructed by Chris Theron in the laboratory of Prof. J. Albertyn in the Department of Microbial, Biochemical and Food biotechnology, University of the Free State, P.O Box 339, Bloemfontein, 9300, South Africa.

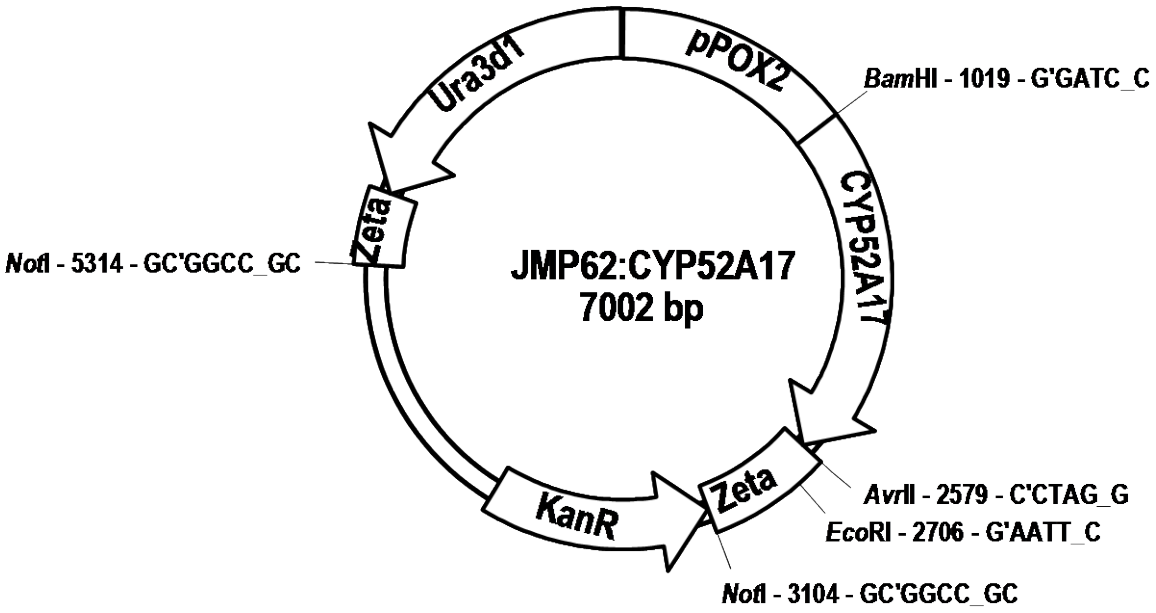


Figure 4.1: Map of JMp62 vector containing CYP52A17 created with the pDRAW32 version 1.1.93 (ACACLONE). The genes are cloned downstream of the *pPOX2* promoter using *Bahm* HI and *Avr*II restriction sites. The *Not*I restriction enzyme was used to remove the bacterial cassette.

4.1.2 Genomic DNA isolation

Yeast cultures were grown in YPD broth (10 ml) in 100 ml flasks at 30 °C on a rotary shaker for 24 h. Cells were transferred to 2 ml eppendorf tubes, centrifuged for 1 minute at 13 200 rpm and the supernatant removed by

aspiration. The pellet was resuspended in 500 µl lysis buffer [100 mM Tris-HCl (pH8.0); 50 mM EDTA; 1% SDS] by vortexing. Acid-washed glass beads (400-500 microns) were added to about 2 mm below the meniscus of the suspension followed by vigorous vortexing for 4 min and immediate cooling on ice for 5 min. The supernatant was transferred to a new tube, 275 µl ammmonium acetate (7 M, pH 7.0) was added to the liquid, vortexed for 30 s and incubated for 5 min at 65 °C followed by cooling on ice for 5 min. Chloroform (500 µl) was added to the solution, the mixture was vortexed briefly and then centrifuged at 10 000 x g for 5 min at 4 °C. The supernatant was transferred to a new 2 ml eppendorf tube, isopropanol (1 ml) added, the tube gently inverted (6 times) and incubated at -20 °C for 30 min. The mixtures were centrifuged at 10 000 x *g* for 5 min at 4 °C. The pellet was washed with 70% ethanol and centrifuged at 10 000 x *g* for 5 min at 4 ºC. The pellet was dried and dissolved in 50 µl 1xTE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) + RNase, vortexed for 30 s, incubated for 1 hour at 37 °C and vortexed again (Labuschagne and Albertyn, 2007).

4.1.3 PCR amplification of *CYP52A13* and *CYP52A17* from *Candida tropicalis* ATCC20336

The polymerase chain reaction (PCR) was performed under standard reaction conditions using an automated 2400 PCR system (Perkin Elmer). Specific primers (Table 4.2) were used to amplify *CYP52A13* and *CYP52A17* genes from *Candida tropicalis* ATCC20336 genomic DNA. The PCR mixture (50 µl) contained 1x polymerase buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 10 mM of each dNTP, 100 pmol of each primer, 100 ng of DNA template and 2.5 U *Taq* DNA polymerase (Roche). The following cycle profile was used: an initial DNA denaturation at 94 °C for 2 min, followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, primer extension at 72 °C for 1 minute and finally primer extension at 72 °C for 7 min. This was followed by cooling at 4 °C.

The PCR products were separated by electrophoresis on a 0.8% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide. The appropriate size bands were sliced from the gel and purified using the GFX^{Tm} PCR DNA and Gel Purification kit according to the instruction manual (Amersham Bioscience). The purified DNA samples were ligated into a pGem-T Easy vector (Promega) according to the standard protocol provided by the supplier. The ligation mixture was transformed into XL10 gold *E. coli* competent cells. The positive clones were grown in LB medium supplemented with 100 µg ml^{-1} ampicilin. Plasmid was isolated from each clone by the alkaline lysis miniprep method (Sambrook *et al*., 1989). The presence of the appropriate sized insert was determined by cleavage of each isolated plasmid with *Eco*RI, which cleaves on either side of the ligation site of the pGem-T Easy vector.

Clones with the correct size insert were double digested with *Bam*HI and *Avr*II. The released insert was ligated into JMP62 vector that was double digested with *Bam*H1 (Roche) and *Avr*II (Biolabs). The ligation mixture was incubated at room temperature for 16 h before transformation into XL10 gold *E. coli* competent cells.

Table 4.2: Primers used in the study

Name	Sequence (5'-3')
CYP52A17 FP	GGG **GGA TCC** ATG ATT GAA CAA CTC CTA G
CYP52A17 RP	CC**C CTA GG**C TAG TCA AAC TTG ACA ATA GC
CYP52A13 FP:	GG **GGA TCC** ATG ACT GTA CAC GAT ATT ATC G
CYP52A13 RP	CC**C CTA GG** C TAG TCA AAC TTG ACA ATA GC

The specific primers for *CYP52A13* and *CYP52A17* genes were designed using available sequences from NCBI. The forward primers have the *Bahm*HI (bold and underlined) restriction site and the reverse primer has the *Avr*II (bold and underlined) restriction site

4.1.4 Transformation of XL10 gold *E. coli* competent cells with JMP62 vector, JMP62-*CYP52A13* and JMP62-*CYP52A17*

Escherichia coli XL10 gold (TetrD(*mcrA*)*183* D(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F´ *proAB lacI*q*Z*D*M15* Tn*10* (Tetr) Amy Camr) competent cells were used for transformation. The 1.5 ml eppendorf tubes containing 50 µl competent cells (OD reading between 0.6 and 0.9) were thawed on ice. When thawed, 10 µl of plasmid was aliquoted to the tube and gently mixed with a pipette. The mixture was incubated on ice for 30 min while the LB plates were placed in the laminar air flow chamber. The mixture was heat shocked in a water bath at 42 °C for 35 s, incubated on ice for 2 min and then 800 µl SOC (5 ml LB with 50 µl of 2M Mg^{2+} solution (20.33 g $MgCl_2.H_2O$, 24.65 g $MgSO_4$.7H2O and 100 ml dH_2O – filter sterilized) and 100 µl of 1 M glucose) was added. The mixture was incubated at 37 °C for 1 h while shaking at 250 rpm. After 1 h incubation, the mixture was centrifuged at 12 000 x *g* for 30 s. The supernatant was removed leaving approximately 100 µl and mixed properly with a pipette. The cells were plated out on LB plates containing kanamycin (25 µg/ml) and incubated at 37 °C for approximately 20 h.

4.1.5 Preparation of competent cells of *Yarrowia lipolytica*

Competent cells of *Yarrowia lipolytica* CTY021 and CTY022 were prepared as described by Barth and Gaillardin (1996). Fresh cells were inoculated in YPD broth (1% yeast extract, 1% peptone and 1% glucose) containing 50 mM citrate buffer pH 4 and incubated at 25 °C for 8 h. The concentration of the cells was determined by counting in a hemacytometer. The cells were inoculated into YPD broth (25 ml) in 250 ml flasks at a concentration of 1 x 10^6 cell/ml and incubated at 25 °C on a shaker at 250 rpm. The cells were grown to a concentration of 1 x 10^8 cells/ml. The cells were centrifuged at 5000 x *g* for 10 min and washed twice with 10 ml TE buffer (50 mM Tris, 5 mM EDTA, pH 8). Cells were resuspended to a final concentration of 5 x 10^7 cells/ml in 0.1 M lithium acetate buffer, pH 6 and

incubated at 28 ºC for 1 h on a rotary shaker. The cells were harvested by centrifugation and resuspended in 0.1 M lithium acetate buffer at a concentration of 5 x 10^8 cell/ml.

4.1.6 Transformation of *Yarrowia lipolytica* with JMP62 carrying *CYP52A13* or *CYP52A17*

JMP62 vector containing *CYP52A13* or *CYP52A17* genes were digested with *Not*I (Roche) restriction enzyme which cut within the zeta elements of the vector. The fragments carrying the genes were transformed into *Yarrowia lipolytica* CTY021 and CTY022 by the following procedure. Competent cells (100 µl), carrier DNA (25 µg) and digested vector (2 µg) were added into a 2 ml eppendorf tube and mixed gently with a pipette. The mixture was incubated in a 28 °C water bath for 15 min (without shaking), 700 µl of 40% PEG (polyethylene glycol) 4000 (in 0.1 M lithium acetate buffer, pH 6) was added and the mixture incubated in a 28 °C water bath with gentle shaking for 1 h. The cells were heat shocked by incubation at 39 °C for 10 min, before 1 ml 0.1 M lithium acetate buffer (pH 6) was added and the suspension mixed gently by inversion of the tubes. The cells were harvested by centrifugation at 2500 x *g* for 5 min and resuspended in 200 µl 0.1 M lithium acetate buffer (pH 6). For selection using the *URA*3 marker, the cells were plated on YNBcasa plates [0.17% YNB without amino acids and $(NH_4)_2SO_4$, 0.4% NH_4Cl, 1% glucose, 0.2% casamino acids, 50 mM phosphate buffer and 2% agar].

4.1.6 Southern Hybridization

Total DNA (5 µg) isolated from the strains which had been transformed with JMP62 containing the *CYP52A13* or *CYP52A17* genes, was digested with *Eco*R1 restriction enzyme for 1 hr at 37 °C. The digested DNA was electrophoresed on 1% agarose gel at 100 V for 2 h. The DNA was depurinated by washing with 0.25 M HCl for 15 min. Denaturation was performed by washing the gel twice for

30 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH). The gel was neutralized in 0.5 M Tris buffer (pH 7.4) containing 1.5 M NaCl for 30 min. The DNA was blotted onto a 0.22 micron nylon membrane by capillary transfer. The membrane was washed in 10 x SSC (1.5 M NaCl, 0.15 M Na-citrate, pH 7) for 15 min and allowed to dry. The DNA was cross-linked to the membrane by exposure to UV light (254 nm) using a GS Gene linker (Bio-rad).

Approximately 1.5 kb PCR product from the *CYP52A13* or *CYP52A17* genes was digoxigenin labelled using the Dig DNA labeling kit according to the suppliers protocol (Roche) and hybridization was performed at 65 °C for 24 h. Post hybridization was done under the following conditions: 2 x 5 min in 0.1% SSC, 0.1% SDS at room temperature, followed by washing of the membrane 2 x 15 min in 0.1% SSC, 0.1% SDS at room temperature. Detection was performed as described by the supplier's protocol (Roche).

4.2 Part B Biotransformation experiments

4.2.1 Biotransformations in shake flasks

Y. lipolytica transformants were grown in YPD_2 broth (1% w/v yeast extract, 1% w/v peptone, 2% w/v glucose) in shake flasks at 30 °C. Substrate (3% v/v alkanes) was added 24 h after main-culture inoculation. Glucose (0.5% w/v) was added 24 h after substrate addition and every 24 h thereafter as co-substrate. The pH was adjusted to 8 every 24 h. Phenol red was used as an indicator. Samples were taken at regular intervals for GC analysis.

Y. lipolytica CTY026, CTY021:*CYP52A13*-clone 3 and CTY021:*CYP52A17*-clone 4 were grown in YPD_2 broth in shake flasks at 30 °C. Inducers (0.1% v/v) were added 24 h after main-culture inoculation. 4-Hexylbenzoic acid (0.1% v/v) was added as substrate 24 h later. The pH was adjusted to 8 every 24 h. Phenol red

was used as an indicator. Samples were taken at regular intervals for TLC and GC analysis.

4.2.1 Biotransformations in Sixfors multireator

Y. lipolytica CTY026 and CTY021:CYP52A13-clone 3 were grown in YPD_2 broth media in shake flasks at 30 °C for 24 h. The cultures (4.6 ml) were then transferred to 230 ml YPD_4 broth in 300 ml Sixfors multi-reactor vessels (Infors, AG Rittergasse 27, CH-4103 Bottmingen, Switzerland). Three vessels were inoculated with *Y. lipolytica* CTY026 and three vessels with CTY021:CYP52A13-clone 3. The cultures were grown at 25 °C, at a maximum stirrer speed of 1100 r min^{-1} and supplied with a flow rate of 0.6 slpm (standard liters per minute) of air. The fermentation process was divided into a growth and conversion phase. Cultures were grown for 24 h during the growth phase with pH values starting at 6 and not adjusted during the growth phase. The pH was allowed to decrease and then increase. After the pH just started to increase the substrate (3% v/v hexadecane) was added 30 h (CTY026) and 35 h (CTY021:*CYP52A13*-clone 3) after inoculation. The pH was raised to 8.0 and maintained between pH 8 and 9 during the conversion phase by the addition of 1 M NaOH. Glucose (0.5% w/v) was added 24 h after substrate addition and every 24 h thereafter as co-substrate. With each addition the amount of glucose added was reduced by 10%.

4.2.2 Biomass determination using the centrifugation method

Microcentrifuge tubes (1.5 ml) were dried at 119 °C for 24 h and weighed to 4 decimal places. Aliquots (1 ml) of the fermentation broth samples were transferred into the pre-weighed microcentrifuge tubes and centrifuged at 10 000 x *g* for 10 min Supernatants were discarded and the biomass remaining in the microcentrifuge tubes were washed with a mixture (1 ml) of cyclohexane (2 ml), 5 M NaOH (400 µl) and distilled water (4 ml) to remove residual dodecane and dioic acid. The cells were then centrifuged at 10 000 x *g* for 10 min Supernatants

were discarded and pellets dried at 119 °C for 24 h. Dried microcentrifuge tubes with biomass were weighed to 4 decimal places (Gumede, personal communication).

4.2.2 Extraction and analysis

Samples (500 µl) were taken at regular intervals, 1 M NaOH (500 µl) was added, samples were vorted (10 min) and aliquots (500 µl) transferred to new tubes. These aliquots were neutralised with 1M HCl (500 µl) and vortexed (10 min). Samples were acidified to pH 3 with 1M HCl (200 µl). The diluted samples were extracted with ethyl acetate (600 µl) containing 0.1% w/v myristic acid as internal standard and centrifuged (10 000 x *g* for 10 min). Aliquots of the extracts (50 µl) were methylated with trimethylsulfonium hydroxide (50 µl) and analyzed with gas chromatography.

GC (Gas Chromatography) analysis of methylated samples was carried out on a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a CP-Wax CB column (Chrompack) measuring 30 m x 0.53 mm x 1 µm. GC conditions were as follows: initial oven temperature was 120 °C held for 5 min, increasing at 10 °C min^{-1} to a final temperature of 250 °C held for 12 min. Flow through the column was at 6 ml/min hydrogen with a split ratio of 1:50. The temperature of the FID detector was 280 °C.

For TLC (Thin Layer Chromatography) analysis samples (10 µl) were spotted onto Alugram Sil G/UV_{245} TLC plates (Machery-Nagel) containing a fluorescent indicator. Plates were developed using a mobile phase consisting of di-n-butyl-ether (Merck), formic acid (Merck) and water in a 90:7:3 ratio. Plates were visualized under short wavelength UV-light or stained using a bromocresol green stain consisting of 0.1 g bromocresolgreen (Fluka), ethanol (500 ml) and 0.1 M NaOH (5 ml). Before staining with bromocresolgreen, plates were heated in an

oven at 110 °C for 10 to 15 min. After staining plates were again heated at 110 °C until spots developed.

4.3 Results and Discussion

4.3.1 Cloning of *CYP52A13* and *CYP52A17* genes

The *CYP52A17* and *CYP52A13* genes were amplified from genomic DNA of *C. tropicalis* ATCC20336, using specific primers (Table 4.2). The PCR products were sub-cloned into the pGEM-T Easy vector, re-cloned into the JMP62 (Figure 4.1) vector using the *Bam*HI and *Avr*II restriction sites and transformed into *E. coli* XL10 gold competent cells. Positive transformants were identified by selecting for kanamycin resistance and confirmed by colony PCR. To confirm the correct size of the inserts, plasmids were isolated from positive clones and subjected to double digestion with *Bam*HI and *Avr*II restriction enzymes. Restriction analysis (Figure 4.2) gave expected profiles for both the vector and the inserts. The expected insert size for *CYP52A17* was 1560 bp and for *CYP52A13* 1575 bp, so that bands were expected at approximately 1.5 kb. Clone 35 from the transformation with JMP62:*CYP52A17* (Figure 4.2 (A)) and clone 9 from the transformation with JMP62*:CYP52A13* (Figure 4.2 (B)) were chosen for further transformation into *Yarrowia lipolytica* strains (Table 4.1) using the lithium chloride method of Barth and Gillardin, (1996). Before transformation, the bacterial cassette was removed by *Not*I digestion.

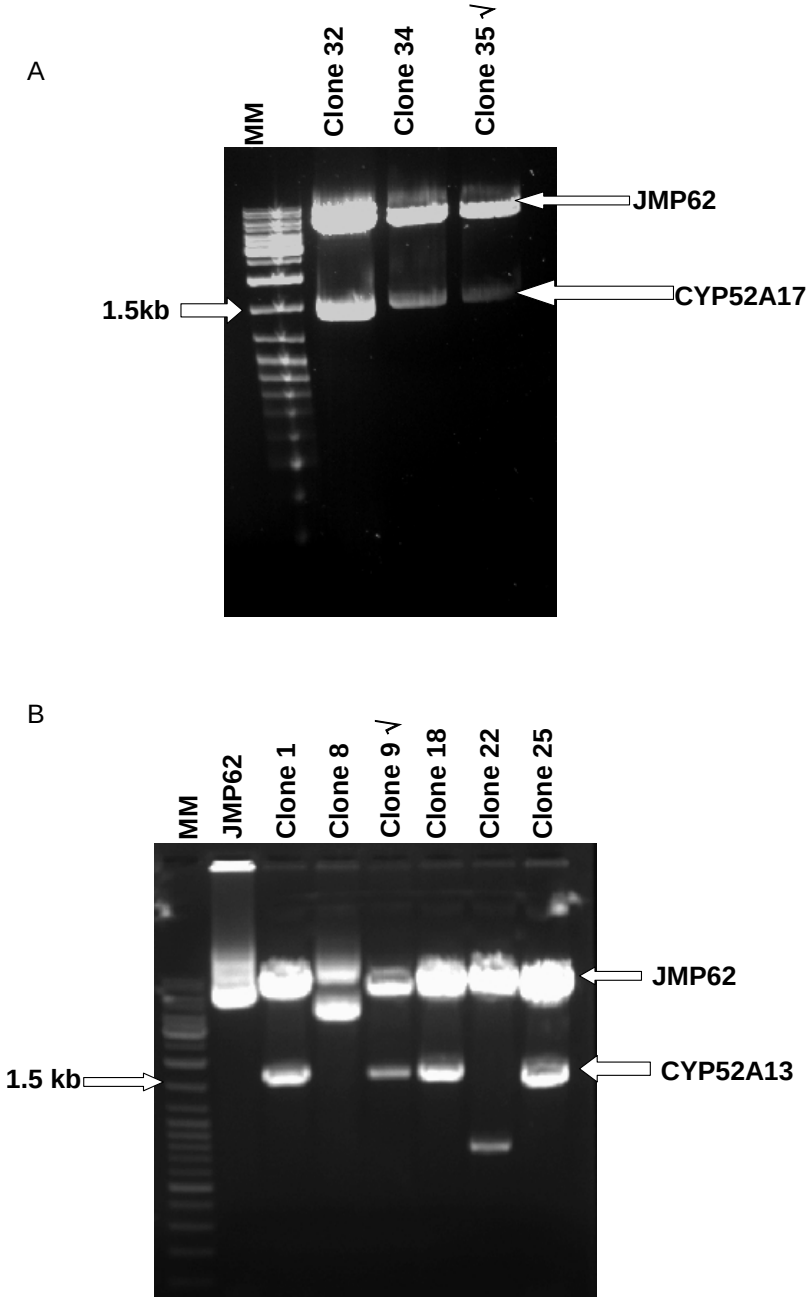


Figure 4.2: Digestion of plasmid DNA from *E. coli* strains transformed with JMP62:*CYP52A17* (A) and JMP62:*CYP52A13* (B) with *Bam*HI and *Avr*II.

4.3.2 Screening for strains containing cloned *CYP52A13* and *CYP52A17* genes

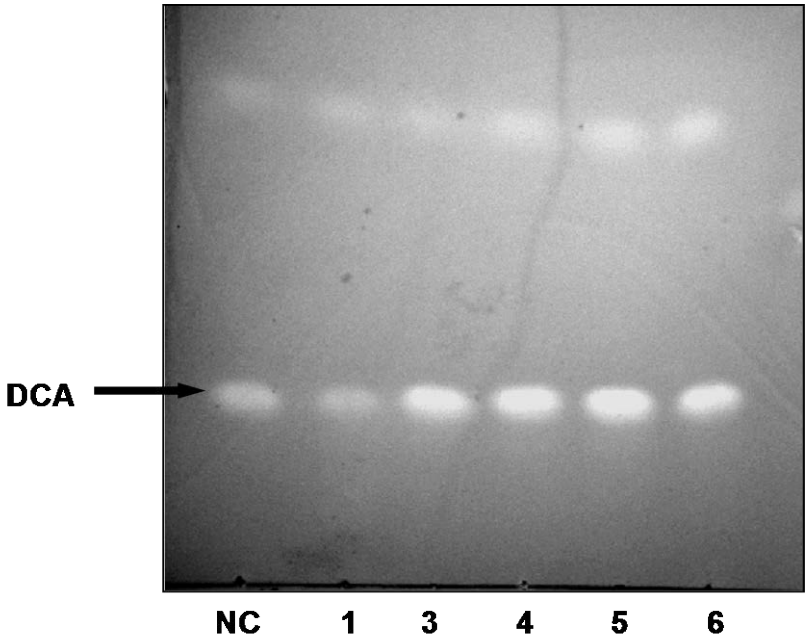
The JMP62:*CYP52A17* and JMP62*:CYP52A13* vectors were integrated into *Yarrowia lipolytica* CTY021 and CTY022 strains by non homologous integration. The CTY022 strain has an extra *Y. lipoltyca* CPR which is cloned under the *pPOX2* promoter while CT021 has two extra *Y. lipoltyca* CPR which are cloned under *pPOX2* and *pICL* promoters (Table 4.1). The *pPOX2* promoter is induced by fatty acids and alkanes and repressed by glucose. The *ICL* promoter is induced by fatty acids, alkanes, ethanol and sodium acetate. The parental strain *Y. lipolytica* W29 (French isolate), as well as CTY021 and CTY022, do not have the 714 bp long terminal repeats (LTRs) termed ‘’zeta’’ elements. However, zeta elements in the vector were shown to enhance non homologous integration into *Y. lipolytica* stains devoid of Ylt1 retrotransposon (Madzak *et al*., 2004). The transformants were grown on YNBcasa agar plates and screened using *ura3d1* defective marker. The clones that grew fast on YNBcasa agar plates were further screened for DCA production.

4-Hexylbenzoic acid was initially used to screen for transformants expressing *CYP52A13* and *CYP52A17*. However, biotransformation of 4-hexylbenzoic acid after induction with oleic acid was not significantly increased in the transformants when compared to the control strain (results not shown). In the next round of screening dodecane (3% (v/v)) was used as substrate. To confirm the presence of the cloned *CYP52* genes, genomic DNA was isolated from the clones which produced the most and least diacid. The resulting genomic DNA was subjected to *Eco*RI digestion and southern blot analysis was performed using DIG labelled PCR products of the respective genes as probes.

From the CTY022 clones transformed with JMP62:*CYP52A17* (Figure 4.3 (A) and (B)), four clones showed significantly improved DCA production after 96 h when compared with the control strain CTY029 (17.7 mM) namely clone 3 (26.2

mM), clone 4 (23.5 mM), clone 5 (27.6 mM) and clone 6 (24.9 mM). However, according to the southern blot analysis (Figure 4.7 (A)); none of these clones contained the cloned *CYP52A17* gene. None of the CTY022 clones transformed with JMP62:*CYP52A13* showed improved DCA production (Figure 4.4 (A) and (B)). However, southern blot analysis showed that two transformants (clone 6 and clone 7) contained the cloned *CYP52A13* gene, while the least performing strain (clone 1) did not have the *CYP52A13* gene (Figure 4.7 (B)).

A



B

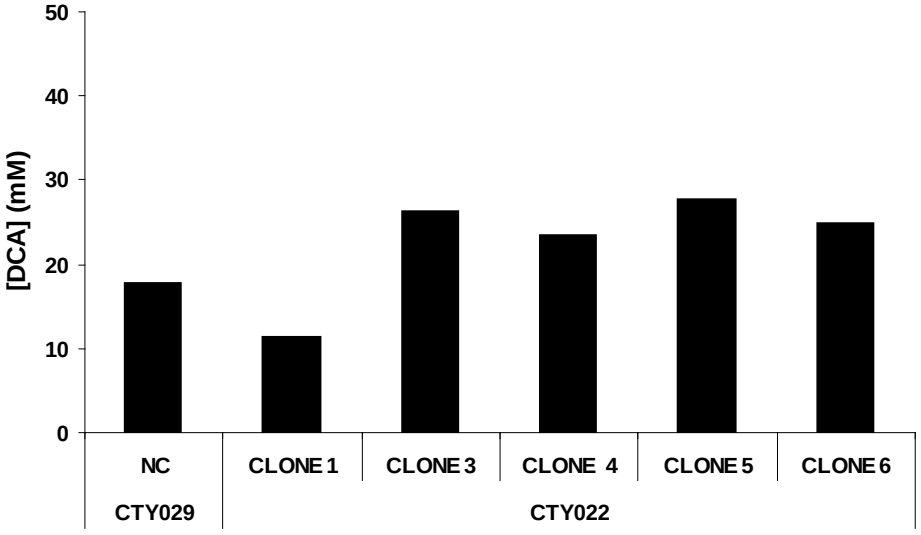
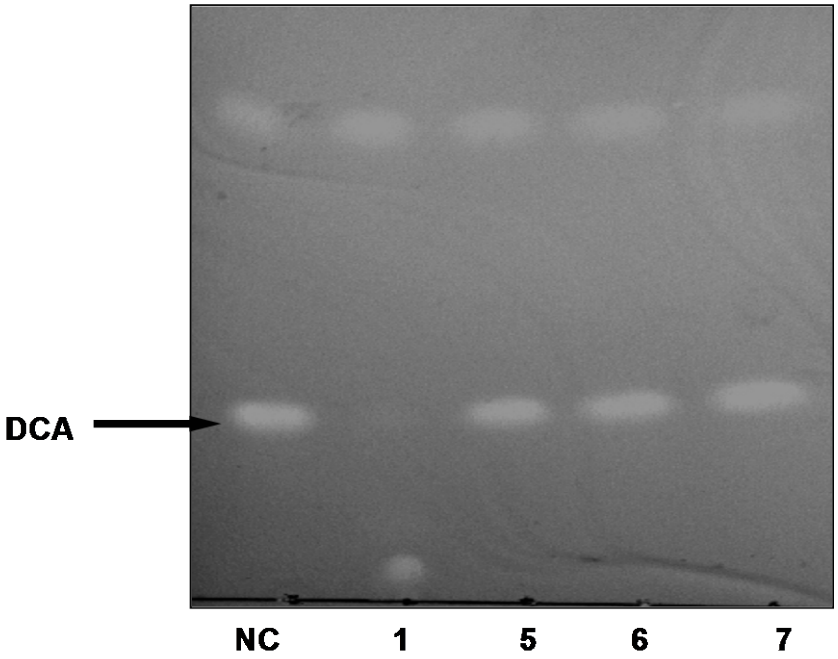


Figure 4.3: DCA production after 96 hours (shown (A) TLC and (B) GC results) from clones of *Y. lipolytica* CTY022 transformed with *JMP62-CYP52A17*. CTY029 was used as a control strain.

A



B

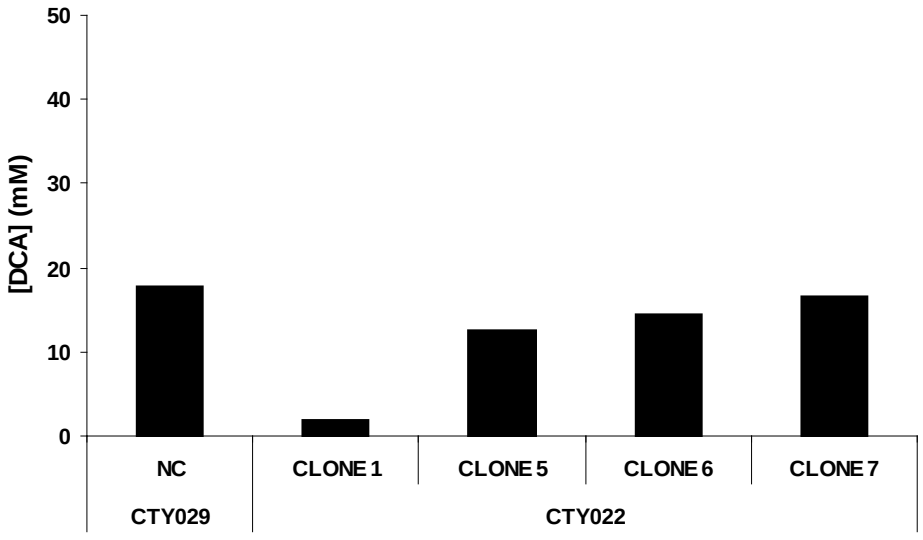
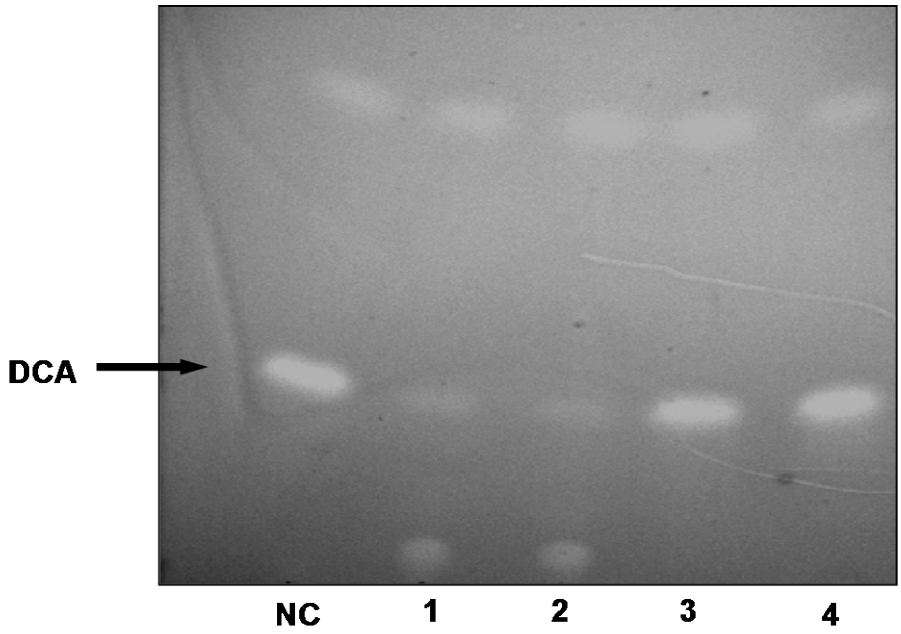


Figure 4.4: DCA production after 96 hours (shown (A) TLC and (B) GC results) from clones of *Y. lipolytica* CTY022 transformed with *JMP62-CYP52A13*. CTY029 was used as a control strain.

None of the CTY021 clones transformed with JMP62:*CYP52A17* showed improved DCA production compared to the control strain CTY026 (Figure 4.5 (A)

and (B)), although southern blot analysis showed that the best performing strain (clone 5) contained up to three copies of the *CYP52A17* gene (Figure 4.7 (A)). One of the CTY021 clones transformed with JMP62:*CYP52A13* (clone 3) showed a 62 % improvement in DCA production (49.2 mM) (Figure 4.6 (A) and (B)). Southern blot analysis (Figure 4.7 (B)) confirmed that this clone had at least two copies of the cloned *CYP52A13* gene.

A



B

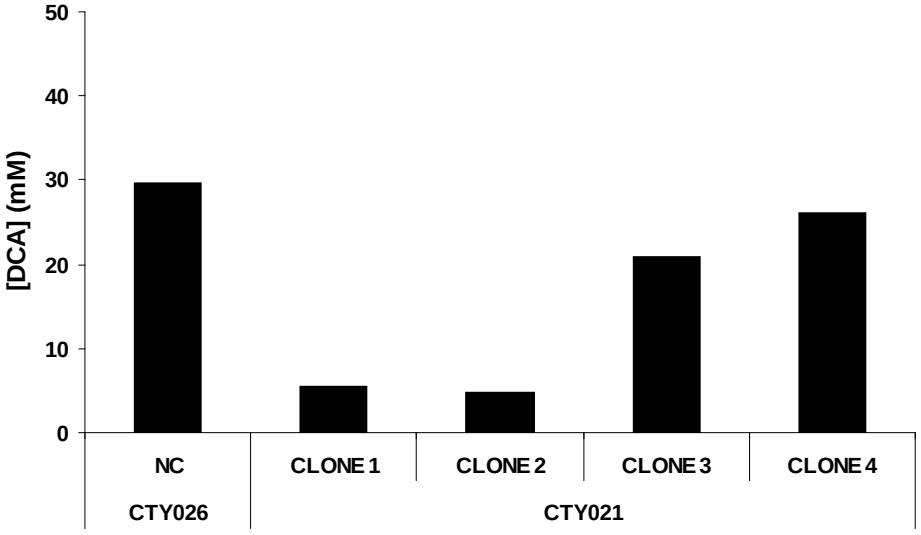
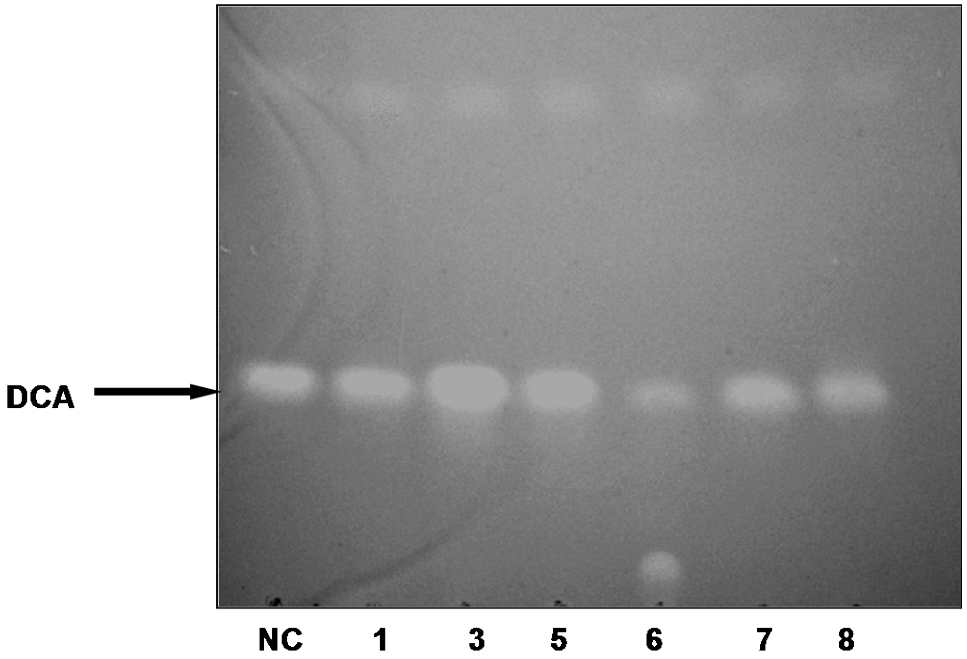


Figure 4.5: DCA production after 96 hours (shown (A) TLC and (B) GC results) from clones of *Y. lipolytica* CTY021 transformed with *JMP62-CYP52A17*. CTY026 was used as a control strain.

A



B

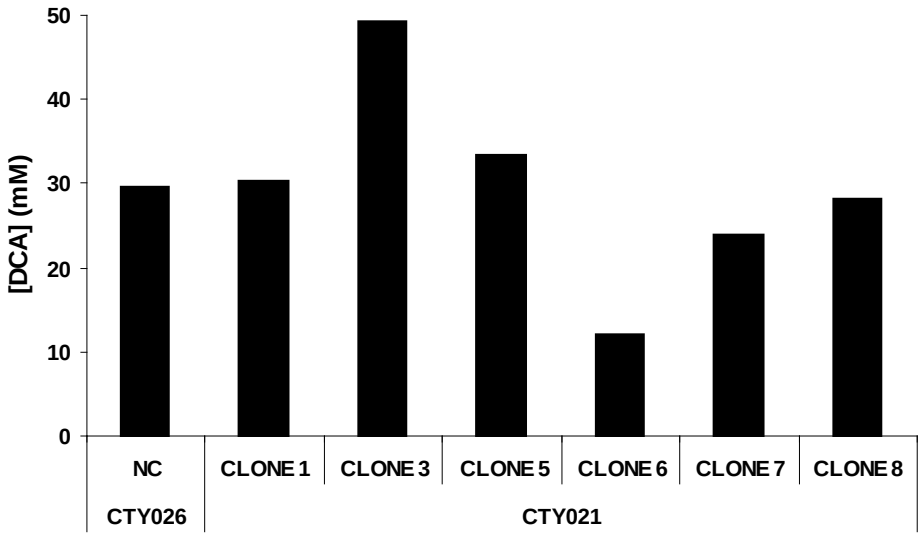
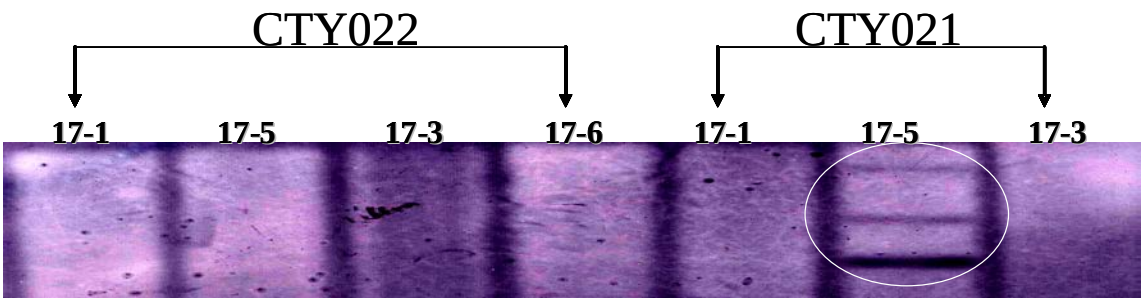


Figure 4.6: DCA production after 96 hours (shown (A) TLC and (B) GC results) from clones of *Y. lipolytica* CTY021 transformed with *JMP62-CYP52A13*. CTY026 was used as a control strain.

A



B

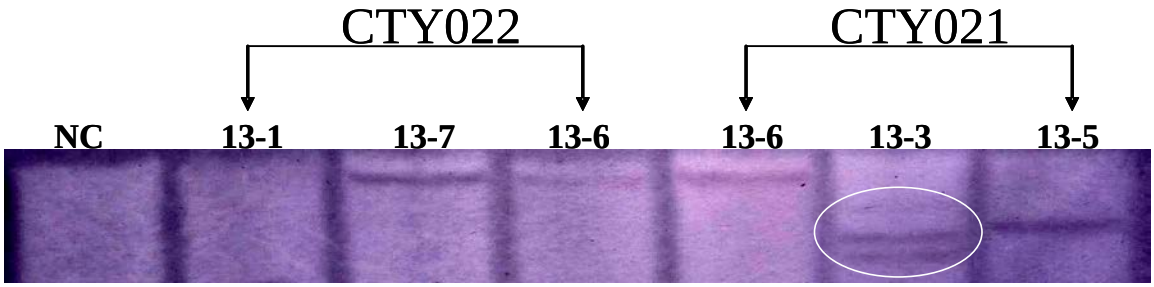


Figure 4.7: Southern blot analysis of clones of Y*. lipolytica* CTY022 and CTY021 transformed with (A) JMP62:*CYP52A17* and (B) *JMP62:CYP52A13.*

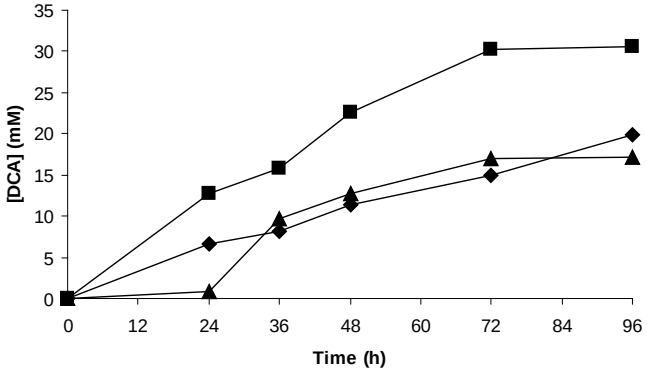
4.3.3 Dioic acid production from different chain length *n*-alkanes

Y*. lipolytica* (β-oxidation blocked) mutants have been reported to produce significant amounts of α,ω-dicarboxylic acids (DCA) (Smit *et al*., 2005). However, the DCA concentrations reported by Smit *et al*., 2005 were low compared with concentrations reported for *C. tropicalis* (Picataggio *et al*., 1992). The best performing clones containing the cloned genes, CTY021-JMP62:*CYP52A13* (clone-3), CTY021-JMP62:*CYP52A17* (clone-4) and the control strain CTY026, were evaluated for the production of α,ω-dicarboxylic acids from *n*-dodecane (C_{12}) (3% v/v), *n*-tetradecane (C_{14}) (3% v/v) and *n*-hexadecane (C_{16}) (3% v/v).

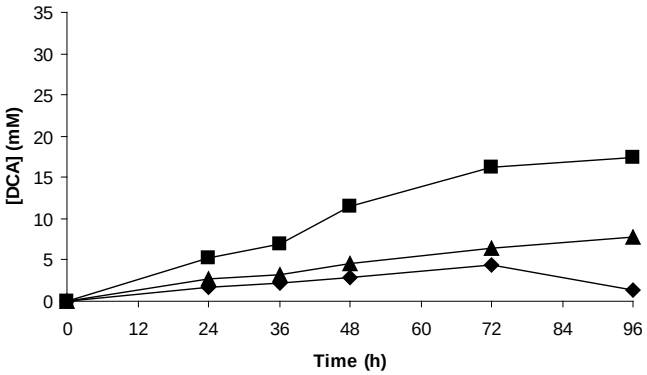
Although DCA production (30.5 mM) from *n*-dodecane was this time lower than in the previous experiment, the results obtained with this experiment confirmed

that the strain with CYP52A13 cloned (CTY021-JMP62:*CYP52A13,* clone-3) gave improved DCA production (Figure 4.8 (A)). In the case of *n*-dodecane DCA production (30.5 mM) was improved by 60%, in the case of *n*-tetradecane (17.4 mM) (Figure 4.8 (B), it was improved by 92% and in the case of *n*-hexadecane (9.3 mM) (Figure 4.8 (C), it was improved by 84%. The highest DCA accumulation was obtained from *n*-dodecane as a substrate, although the highest improvement was seen with *n*-tetradecane (Figure 4.9). We did not obtain improved DCA accumulation in the CTY021 clone transformed with JMP62:*CYP52A17* (Figure 4.8 (A, B and C). This could be due to (1) *n*-alkanes are not substrates for *CYP52A17* gene or (2) the *CYP52A17* gene has CUG codons at positions 61 and 490 which in most yeasts (including *Yarrowia lipolytica*) code for leucine, but *Candida* sp., have unusual codon usage where CUG codes for serine instead of leucine (Eschenfeldt *et al*., 2003). Since CUG codon usage was not modified, we do not know the effect of the changed amino acid on the function of the protein.

A



B



C

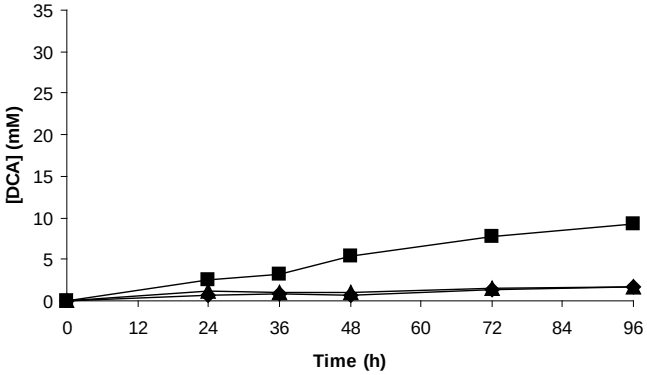


Figure 4.8: Biotransformation of C_{12} (A), C_{14} (B) and C_{16} (C) by CTY026 (◆), CTY021-CYP52A13 (clone 3) (■), and CTY021-CYP52A17 (clone 4) (▲). DCA conversions were done using C12 standard.

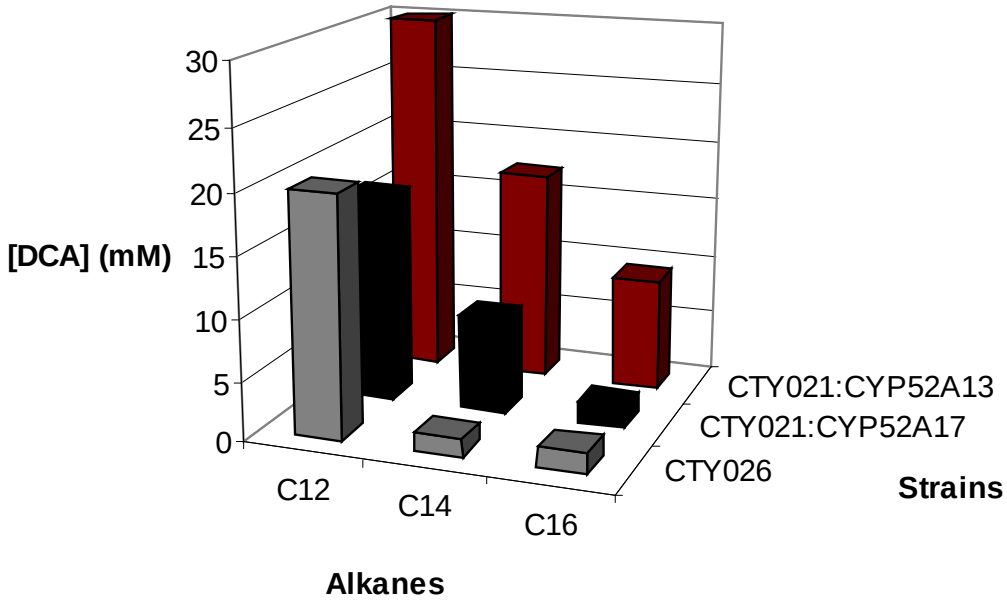


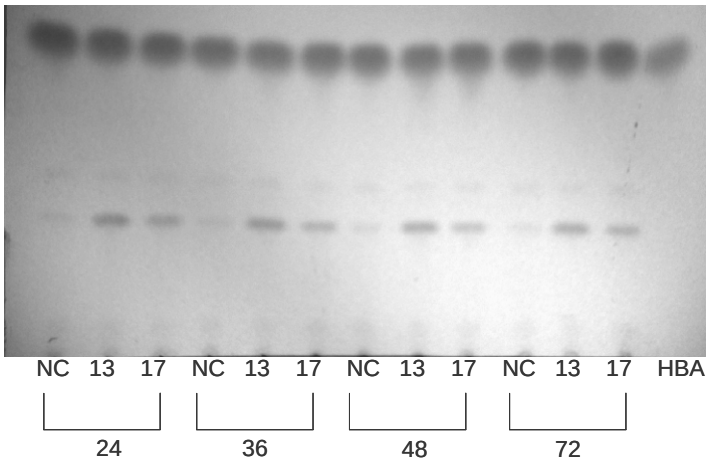
Figure 4.9: Alkanes preference of CYP52A17 and CYP52A13 P450 enzymes after 96 h of biotransformation. *n*-Dodecane (C_{12}) 3% (v/v), *n*-tetradecane (C_{14}) 3% (v/v) and *n*-hexadecane (C_{16}) 3% (v/v) were added as substrates after 24 h growth. DCA conversions were done using C_{12} standard.

4.3.4 Biotransformation of 4-hexylbenzoic acid after induction with hexadecane (C_{16}), palmetic acid (C_{16} FA) and oleic acid ($C_{18:1}$)

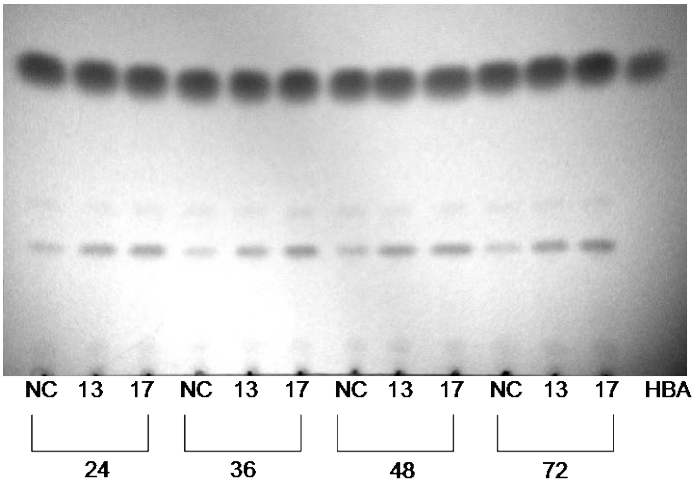
4-Hexylbenzoic acid biotransformation showed that this substrate might be used as marker substrate for expression of *Candida tropicalis* ATCC20336 P450 genes in *Yarrowia lipolytica* strains (Chapter 3). Initial efforts to use 4-hexylbenzoic acid as a substrate to screen for transformants containing the cloned genes did not show a significant difference in transformants. This experiment was done to confirm these initial results. Hexadecane, palmetic acid and oleic acid are strong inducers of the *pPOX2* promoter under which the *CYP52A13* and *CYP52A17* genes were cloned (Madzak *et al*., 2004). Although biotransformation of 4-hexylbenzoic acid yielded low product formation (similar to what was previously observed with the *Y. lipolytica* W29 strain when oleic acid

was added as inducer), we were able to show slightly improved product formation by the CTY021 strains with *CYP52A17* and *CYP52A13* cloned (Figure 4.10 (A, B, C)). These results were disappointing, because we had expected with the strains with CYP52A13 and CYP52A17 cloned, significant improvement in conversions, similar to what had been observed with *C. tropicalis* ATCC20336. From these results it appears as if the wild-type CYP52s of *Y. lipolytica*, induced by oleic acid, palmetic acid and *n*-hexadecane, are still more strongly expressed than the cloned ones and that these *Y. lipolytica* CYP52s do not have high activity towards hexylbenzoic acid.

A



B



C

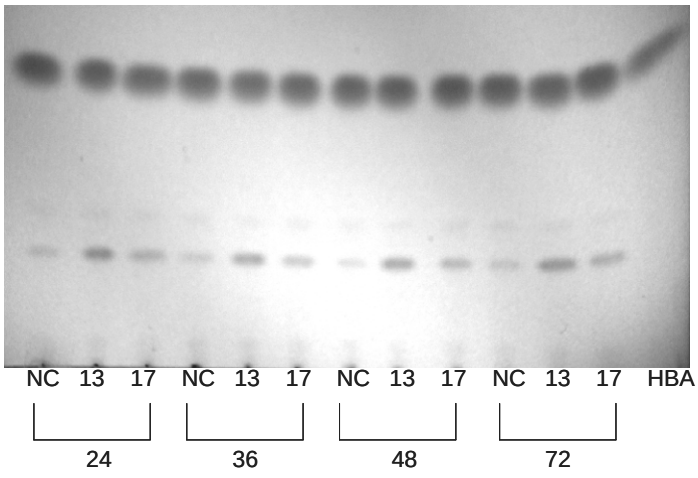


Figure 4.10: Biotransformation of 4-hexylbenzoic acid after induction with *n*-hexadecane (A), palmitic acid (B) and oleic acids (C). Strains used: NC - CTY026, 13 - CTY021-CYP52A13 (clone 3), and 17 - CTY021-CYP52A17 (clone 4).

4.3.4 DCA production from hexadecane in bioreactors using CTY026 and CTY021:*CYP52A13* (clone3)

CTY021:*CYP52A13* (clone3) was the most promising clone in the biotransformation of *n*-alkanes. In the biotransformation of *n*-hexadecane, we observed improved DCA production only with CTY021:*CYP52A13* (9.3 mM), while the control strain CTY026 had very low activity towards hexadecane (1.6 mM DCA). We therefore decided it would be best to use *n*-hexadecane as substrate in a set of bioreactor experiments using the Sixfors multireactor. To examine the repeatability of our results the bioreactor study was done in triplicate. The growth phase and the conversion phase will be discussed separately.

To initiate the growth phase, CTY026 and CTY021:CYP52A13 were inoculated (10% v/v) from overnight YPD_2 shake flask cultures into the reactors containing 230 ml YPD_4 broth. In all the reactors temperature was kept constant at 25 °C and the pH was not controlled until the substrate was added. The stirrer speed was initially set at 300 rpm. In response to a decrease in oxygen concentration (measured as pO_2) the stirrer speed was gradually ramped up until the maximum speed was reached at 1100 rpm (to maintain pO_2 above 30%). Active growth and glucose utilization decreased the dissolved oxygen concentration and by increasing stirrer speed aeration was increased. Once the glucose was depleted the oxygen concentration increased again. Also once the stirrer speed reached the maximum speed, growth became oxygen limited. The optical densities of the strains in the six reactors were also measured at regular intervals. Dry weight determinations, of samples taken just before substrate addition, were done in triplicates.

The conversion phase was initiated by adding hexadecane (3% v/v) as substrate. In DCA production experiments the substrate is usually added when the pH starts to increase (Mobley and Shank 2000); probably indicating that the glucose

is depleted, and that the organism is using the peptone in the broth. Thus *n*-hexadecane (3% v/v) was added after 30 h growth to the three reactors with CTY026 and 5 hours later to the three reactors with CTY021:*CYP52A13*. Just before substrate addition the pH of the broth was increased to pH 8 using NaOH (1 M). The stirrer speed and temperature were kept constant at 1100 rpm and 25 °C respectively. To supply energy and carbon for cell maintenance and energy for DCA production glucose was added 24 h after substrate addition and then every 24 h. The first time 0.5% w/v glucose was added and every time thereafter the additional glucose added was reduced by 10%. Thus during the conversion phase the oxygen and pH profiles of the reactors inform us of the behavior of the organisms in response to additional glucose with both pO_2 and pH dropping in response to glucose addition. The pO_2 levels higher than 100% are due to the hydrophobic substrate increasing the solubility of oxygen in the medium (Clarke *et al*., 2006). The significant differences in the pO_2 levels in the different reactors can be ascribed to differences in airflow and mixing between the reactors (Gumede, personal communication).

In order to understand the improvements in DCA production from CTY021:CYP52A13 – clone3 in our previous results, we firstly examined the discrepancies in each set of triplicates and then analyzed the differences between the control strain (CTY026) and the strain with *CYP52A13* gene cloned (CTY021:CYP52A13 – clone3).

In the reactors with the control strain (CTY026) (Figure 4.11), the pO_2 concentration was almost the same for the first 8.55 h until the maximum stirrer speed (1100 rpm) was reached. Thereafter the pO_2 in reactor 3 decreased more slowly than the pO_2 in reactors 1 and 2. A minimum pO_2 of 23.5% to 25.5% was maintained for 12 h. This is considered the phase of active growth. After 25.15 h the pO2 concentration and pH started to increase. Hexadecane was added 5 h later, that was after 30 h of growth to start the conversion phase. After substrate addition the pO_2 leveled off at different concentrations - reactor 1: 119.4%,

reactor 2: 87.7%, reactor 3: 131.4%. The OD measurements just before the substrate was added (after 30 h growth) were different (reactor 1: 13.4, reactor 2: 14.3 and reactor 3: 16). The highest OD measurement was obtained with control strain in reactor 3. However when the biomass determinations (Figure 4.13) were done, the culture in reactor 2 (21 g l^{-1}) gave the highest dry weight per litter compared to reactor 3 (18.4 g l^{-1}) and reactor 1 (17.2 g l^{-1}). After 48 h of growth the culture in reactor 3 (OD 12.2) was the one which grew better compared to reactor 2 (OD 11.4) and reactor 1 (OD 9.4).

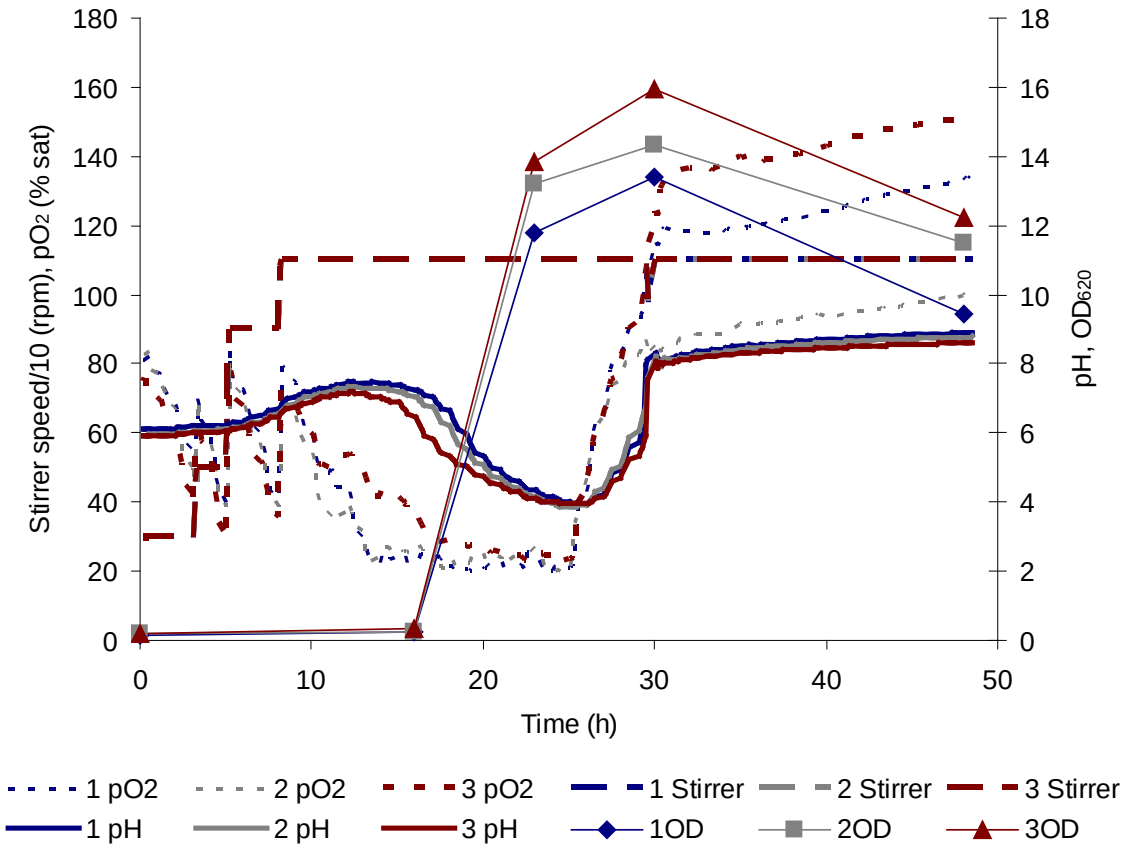


Figure 4.11: Growth, pH, pO_2 and agitation profiles for the growth phase of CTY026, the control strain, in three different reactors. The pH, pO_2 and agitation in each reactor were monitored every two minutes while the OD readings were taken at different time intervals.

In the reactors with test strain (CTY021:CYP52A13-clone 3) (Figure 4.12), similar profiles were observed. The pO_2 concentration was also almost the same but for

only 8.05 h until the maximum stirrer speed (1100 rpm) was reached. Thereafter the pO_2 concentration also decreased but decreased differently in all the reactors. The minimum pO_2 levels reached were different for each reactor (reactor 1: 26.5%, reactor 2: 17.7% and reactor 3: 16.7%) but the duration at minimum pO2 for reactor 1 and reactor 3 was short compared to reactor 2. Overall the duration at minimum pO_2 was short compared to the control strain. The substrate was also added when the pH started to increase. With this strain it only happened after 35 h of growth. The pO_2 after substrate addition was again different for the different reactors, reactor 1 and 3 was 122.8% and reactor 2 was 104.5%. The growth in all the reactors was the same until the end of the growth phase. The organism in reactor 1 had an OD of 11 before substrate addition (after 35 h) and 7.9 after 48 h, reactor 2 12.2 and 10.8 and finally reactor 3 12.2 and 9. The control strain grew better than the test strain. Biomass just before substrate addition (i.e. after 30 h growth for CTY026 and after 35 h growth for CTY021:CYP52A13) was within experimental error the same (Figure 4.13), although the behavior of CTY021:CYP52A13 during the growth phase was different to that of the control strain (Figure 4.11 and 4.12).

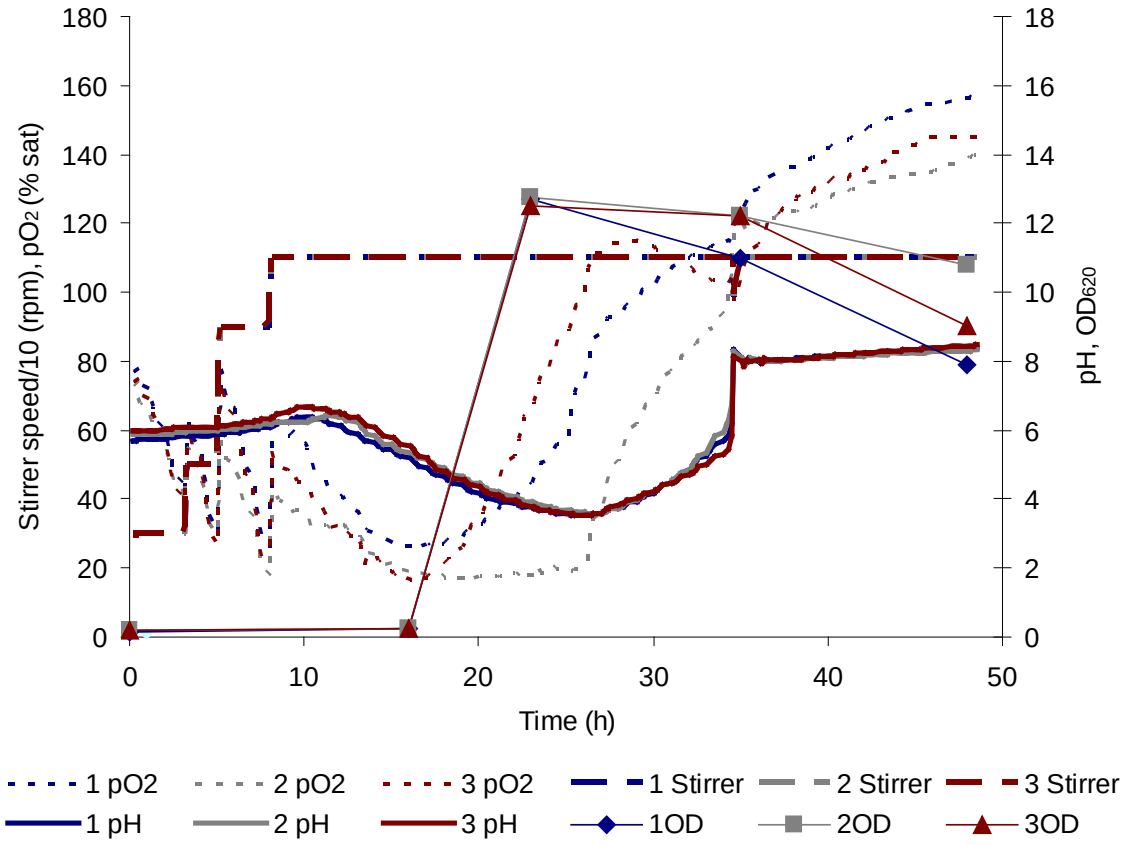


Figure 4.12: Growth, pH, pO_2 and agitation profiles for the growth phase of CTY021:CYP52A13-clone3, the control strain, in three different reactors. The pH, pO_2 and agitation in each reactor were monitored every two minutes while the OD readings were taken at different time intervals

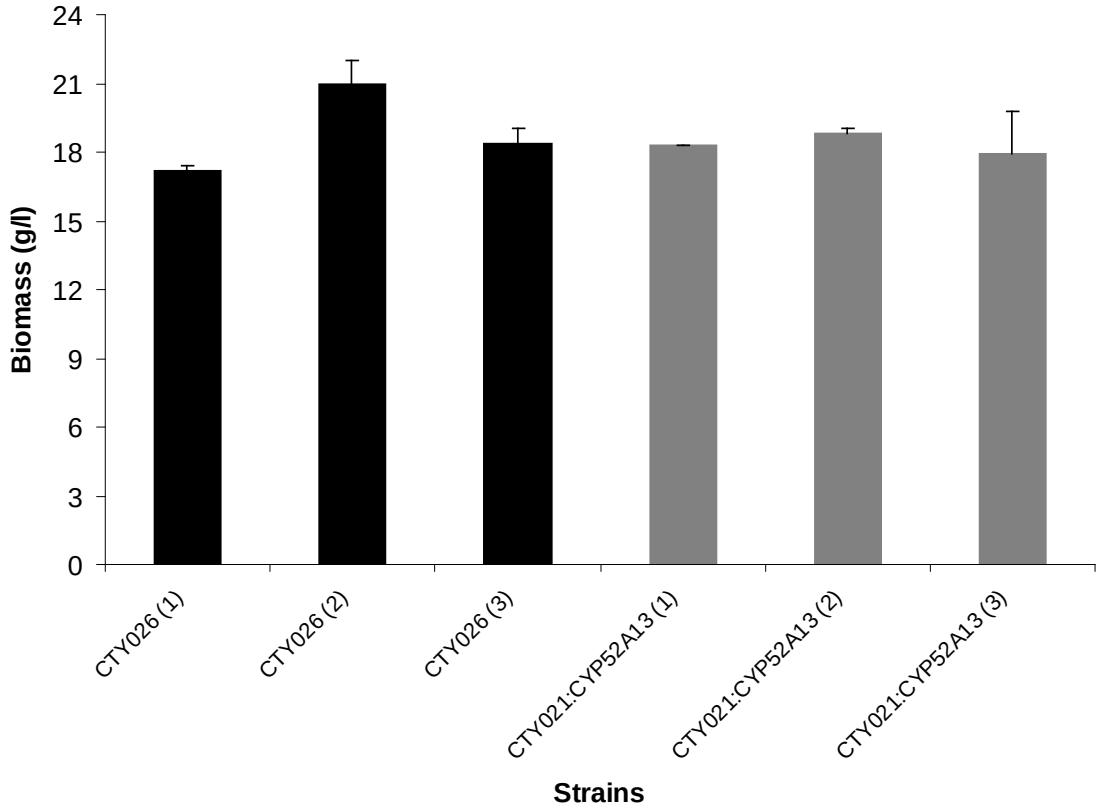


Figure 4.13: Biomass determination of CTY026 (after 30 h of growth) and CTY021:CYP52A13-clone3 after 35 h of growth. The biomass determinations were done in triplicates.

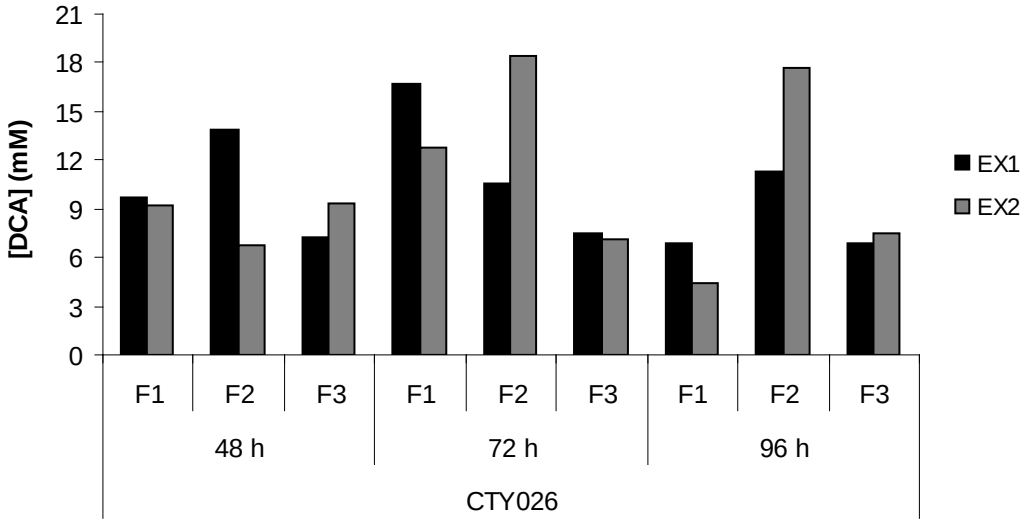
We could not draw any firm conclusions from the biotransformation of hexadecane in the bioreactors, because extractions of duplicate samples taken from the same reactor at the same time gave large differences in DCA concentrations (Figure 4.14 (A) and (B)). DCA extractions from CTY026 showed that the highest DCA that can be accumulated by this strain was probably 18 mM and this was observed 72 h after substrate addition in reactor 2. The highest DCA concentration extracted from the reactors with the CTY021:CYP52A13 strain was 21 mM and this was extracted 48 h after substrate addition in reactor 3. From these extraction results, it seemed that the CTY021:CYP52A13 strain probably produced more DCA than the control strain. Unfortunately duplicate extractions of the same sample in almost all the cases gave different DCA

concentrations. This difference between extracts of duplicate samples taken from the same reactor at the same time might be due to the insolubility of the DCA product. The longer the carbon chain length of the DCA (for example C_{16} DCA) the more insoluble the product is in water as well as in organic solvents and the more likely it is that the product will crystallize.

When averages were calculated for the duplicate extracts product formation in two of the reactors containing the control strain CTY026 and all three reactors containing the test strain CTY021:*CYP52A13* gave similar DCA production profiles (Figure 4.15 and 4.16). During the first 40 h after substrate addition DCA production with both strains was comparable less than obtained with strain CTY021:CYP52A13 in shake flasks (results not shown). In the samples taken 48 h after substrate addition there was in all the reactors a significant increase in DCA content to an average of 10 mM in the reactors with CTY026 and an average of 13 mM in the reactors with CTY021:CYP52A13. In the reactors with CTY021:CYP52A13 DCA production then apparently leveled off reaching an average maximum of only 15 mM. However, it continued to increase in two of the reactors with CTY026 to also reach an average maximum of ca. 15 mM. The average maximum DCA produced by CTY021:*CYP52A13* (clone3) strain in the reactors (Figure 4.16) was comparable to the DCA accumulated in one of the shake flasks experiment (results not shown). In contrast, with the control strain (CTY026) DCA accumulation was significantly increased from 1.2 (Figure 4.9 C) in shake flasks to 15 mM in two of the reactors (Figure 4.15). Although one might conclude from the DCA extracted 48 h after substrate addition was more in the reactors containing CTY021:CYP52A13 it was from bioreactor studies, with the problems of extractions and differences between reactors, difficult to conclude that cloning of *CYP52A13* gene improved DCA production. It is important to note that in the bioreactor experiments substrate addition was in the case of the test strain (CTY021:CYP52A13) five hours later than in the case of the control strain (CTY026) because substrate was only added when pH started to increase and this happened 5 h later with the test strain. It is possible that the rates at which

test and control strains reached end of active growth were also in the shake flasks different and that the substrate was perhaps added a little too late to the control strain, explaining why in shake flasks DCA production by the control strain was less.

A



B

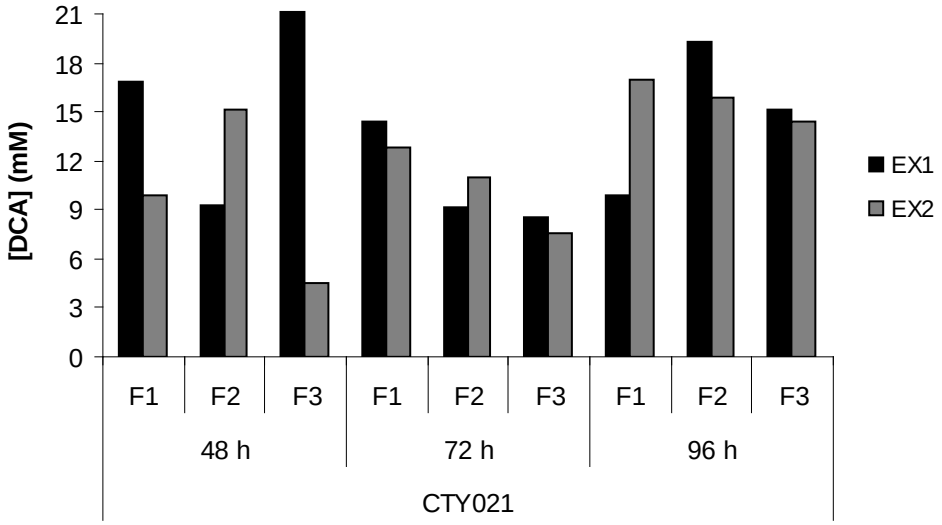


Figure 4.14: Extractions of C_{16} DCA. Extractions of the same stored samples were done on two separate occasions. Samples were stored at -20 °C.

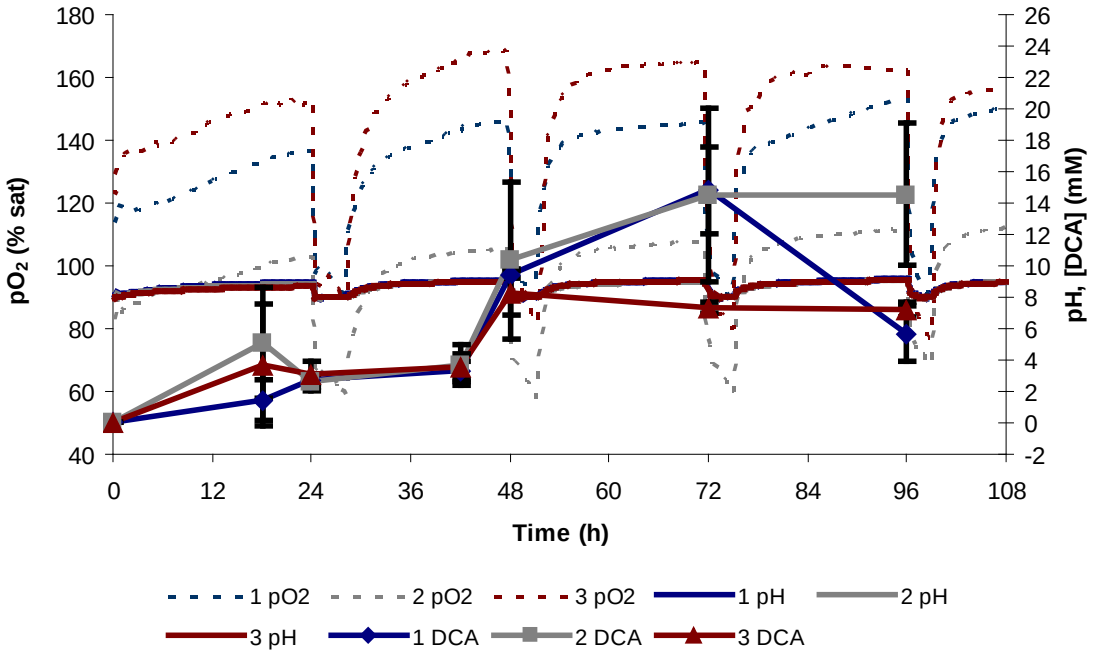


Figure 4.15: Product formation, pO_2 and pH profiles for the biotransformation of hexadecane to C_{16} DCA by control strain CTY026. The results are for three different reactors. Extractions of the same sample were done on two separate occasions.

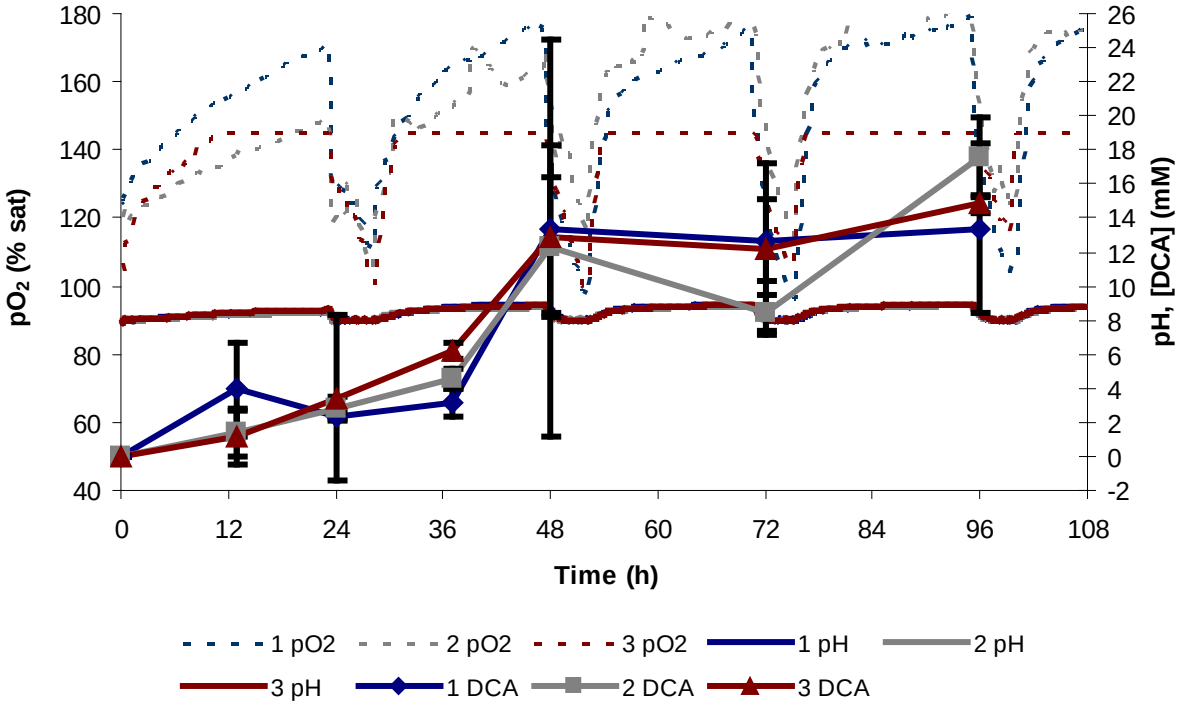


Figure 4.16: Product formation, pO_2 and pH profiles for the biotransformation of hexadecane to C_{16} DCA by CTY021 expressing two copies of *CYP52A13* gene. The results are for three different reactors. Extractions of the same sample were done on two separate occasions.

4.4 Conclusion and future prospects

The *CYP52A13* and *CYP52A17* genes from *Candida tropicalis* ATCC20336 were cloned into two *Y. lipolytica* strains with β-oxidation disrupted. One of the transformants with *CYP52A13* cloned consistently showed in three shake flask experiments significantly improved hydroxylase activity towards *n*-alkanes. The strains in which CYP52A17 was cloned did not show improved hydroxylase activity towards *n*-alkanes, although one clone had according to Southern blot analysis three copies of the gene. CYP52A13 apparently prefers *n*-tetradecane as substrate (90% improvement in activity), although higher DCA production was observed with *n*-dodecane (30.5 mM C_{12} DCA produced).

Hexadecane was used as substrate in a set of bioreactor experiments conducted in triplicate using a Sixfors multireactor. Hexadecane was selected as substrate, because in shake flask experiments the difference between the control and test strains was the largest with hexadecane as substrate. Unfortunately the low solubility of the C_{16}DCA resulted in a large variation in C_{16}DCA concentrations so that no firm conclusions could be drawn from this set of experiments, although it appeared as if the strain with CYP52A13 cloned produced DCA slightly faster. The DCA concentrations obtained in this study were still very low (maximum 21 mM) compared to the DCA concentrations reported in the literature.

However, these results combined with the results of Eschenfeldt *et al*., 2003, might indicate that CYP52A17 is likely a fatty acid hydroxylase while CYP52A13 might also hydroxylate *n*-alkanes. In future work we will use CO-difference spectra to compare expression levels of P450s in strains with *CYP52A17* and *CYP52A13* cloned with the control strain CTY026. We will also further characterize CYP52A17 and CYP52A13 by expression of the genes in *Saccharomyces cerevisiae.* Microsomal fractions and an $NADP^+$-cofactor regeneration systems will be used to test activities towards a broader range of fatty acids and alkanes. We will also investigate DCA production thoroughly

using bioreactors and different chain length *n*-alkanes as substrates, once DCA extraction has been optimized.

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Summary

Yarrowia lypolytica, *Candida tropicalis*, *Candida maltosa* and *Candida cloacae* are extensively studied *n*-alkane degrading yeasts and are widely used in various industrial processes (Madzek *et al*., 2004, Mobley, 1999). Cytochrome P450 monooxygenases belonging to the CYP52 family are responsible for the terminal hydroxylation of *n*-alkanes and fatty acids. *Candida* species have been successfully used in the synthesis of long-chain α,ω-dicarboxylic acids (DCAs) which are difficult to produce using chemical processes (Wache *et al*., 2006 Mobley, 1999). These processes, which yield more than 100 g l^{-1} DCA at volumetric rates up to 1.9 g $l^{-1}h^{-1}$, are regarded as the most successful P450 dependent biotransformation processes developed thus far (Julsing *et al*., 2008). Because *C*. *tropicalis* and *C*. *maltosa* are related to *Candida albicans* (Eschenfeldt *et al*., 2003), they are regarded as potential pathogens. However, *Y*. *lipolytica* has GRAS status for a number of processes and the entire genome sequence is known (Fickers *et al*., 2005). Although *Y*. *lipolytica* has been shown to produce DCA, none of its mutant strains have produced DCA concentrations close to the DCA produced by *Candida* sp. (Kogure *et al*., 2007, Smit *et al*., 2005).

In order to understand the differences in terminal hydroxylase activity that contribute to differences in DCA production, we compared the wild-type strains *Y*. *lipolytica* W29 and *C*. *tropicalis* ATTC20336 by using alkylbenzenes, 4-hexylbenzoic acid and 4-nonyloxybenzoic acid as substrates. We also cloned and expressed *CYP52A13* and *CYP52A17* from *Candida tropicalis* ATTC20336 into *Y*. *lipolytica* CTY021 and CTY022, two β-oxidation disrupted strains. Dodecane, tetradecane, hexadecane and 4-hexylbenzoic acid were used as substrates. One experiment was done in bioreactors using a control strain and a strain expressing CYP52A13.

The experiments with the wild-type strains showed that *Yarrowia lipolytica* W29 and *Candida tropicalis* ATTC20336 responded differently to hydrocarbons. These differences are probably due to differences in the alkane and fatty acid hydroxylases of the two yeasts. In the biotransformation of alkylbenzenes by *Y. lipolytica* W29, nonylbenzene was the only substrate significantly converted to benzoic acid. The highest product formation (20.6 mM benzoic acid) was observed when the cultures were not induced with oleic acid or *n*-alkanes (C_{12}, C_{14}). In the case of *C. tropicalis* ATTC20336, both nonylbenzene and hexylbenzene were accepted as substrates and *n*-dodecane and oleic acid, reported inducers of *CYP52* genes (Craft *et al*., 2003), significantly enhanced biotransformation of these substrates. Biotransformation of 4-hexylbenzoic acid by *Y. lipolytica* also occurred in the absence of an inducer, while in *C. tropicalis* it was only converted to the corresponding DCA after induction with oleic acid. This meant 4-hexylbenzoic acid could possibly be used as a marker substrate for monitoring the expression of *C. tropicalis CYP52* genes in *Y. lipolytica* W29. Biotransformation of 4-nonyloxybenzoic acid by both these strains occurred when cultures were induced with oleic acid.

The *CYP52A17* and *CYP52A13* were cloned under the *pPOX2* promoter into *Yarrowia lipolytica* strains with β-oxidation disrupted. A transformant with at least three copies of the *CYP52A17* gene cloned had relatively low activity towards alkanes when compared to the control strain. However, the CTY021:CYP52A13 transformant with at least two copies of the *CYP52A13* gene showed improved activity towards *n*-alkanes. The biggest improvement (92 %) was observed with *n*-tetradecane. TLC analyses showed that after induction with oleic acid, palmitic acid and *n*-hexadecane slightly more product was formed from 4-hexylbenzoic acid by the strain with CYP52A13 cloned than by the control strain. However, this improvement was much less than anticipated and 4-hexylbenzoic acid was in the end not used to screen for strains expressing the cloned *CYP52* genes.

A bioreactor study was conducted in triplicate in a Sixfors multireactor to compare C_{16}DCA production form hexadecane by the control strain and the strain with *CYP52A13* cloned. The control strain (CTY026) and test strain CTY021:CYP52A13 grew differently. Although there was an indication that the strain with *CYP52A13* cloned produced DCA faster, results were too varaible to reach a firm conclusion that cloning of the CYP52A13 gene significantly improved DCA production by *Y. lipolytica*.

Keywords: *Yarrowia lipolytica*, *Candida tropicalis*, alkylbenzene, *n*-alkanes, fatty acids, DCA, 4-hexylbenzoic acid, 4-nonyloxybenoic acid, CYP52A13, CYP52A17.