BIOTRANSFORMATION OF ALKYLBENZENES AND ALKYLCYCLOHEXANES BY GENETICALLY ENGINEERED *YARROWIA LIPOLYTICA* STRAINS

BY

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“The greatest personal limitation is to be found not in the things you want to do and can't, but in the things you've never considered doing.”

Richard Bandler

I dedicate this thesis to my mother. I would not have done all this without your unending support. I am truly blessed to have you. I love you very much.

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List of abbreviations

CPR	Cytochrome P450 reductase
CYP	Cytochrome P450 (gene)
DDT	1,4-Dithio-DL-theitol-solution
EDTA	Ethylenediaminetetraacetic acid disodium salt
FAD	Flavin adenine dinucleotide
FALDH	Fatty alcohol dehydrogenase
FAO	Fatty alcohol oxidase
FMN	Flavin mononucleotide
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HCl	Hydrochloric acid
KCN	Potassium cyanide
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	Sodium hydroxide
PMSF	Phenylmethanesulfonylflouride
P450	Cytochrome P450 monooxygenase
TMSH	Trimethylsulfonium hydroxide

Chapter 1

Introduction to the study

Yarrowia lipolytica is a non-pathogenic ascomycetous yeast. It is one of the most studied 'non-conventional' yeast species, in terms of its genetics, molecular biology and biotechnological applications. The interest in studying this yeast arose from its ability to utilise ***n***-paraffins as sole carbon source (Barth and Gaillardin, 1997). ***Y. lipolytica*** has been classified as Generally Regarded As Safe (GRAS) by the American Food and Drug Administration (FDA) (Fickers ***et al.***, 2005). This yeast has been investigated for the biotransformation of hydrophobic compounds into value added products. These hydrophobic compounds include alkanes, fatty acids and their derivatives, monoterpenes, monoterpenoids, sterols and steroids (Fickers ***et al.***, 2005). Examples of products are dicarboxylic acids and γ-lactones (Juretzek ***et al.***, 2001).

The ability of ***Y. lipolytica*** to utilise hydrocarbons such as alkanes for the production of single-cell protein and large-scale production of metabolites such as citric acid and 2-ketoglutaric acid, has resulted in a good understanding of its large-scale cultivation on these substrates in bioreactors (Madzak ***et al.***, 2004; Fickers ***et al.***, 2005). ***Y. lipolytica*** has also been studied for foreign gene expression and protein secretion, as it is capable of secreting large proteins in high amounts. In the past years, several genes have been cloned and expressed in ***Y. lipolytica*** and the gene products characterised (Barth and Gaillardin, 1997; Juretzek ***et al.***, 2001). Several promoters for the heterologous expression have also been studied (Juretzek ***et al.***, 2000). The complete genome sequence of ***Y. lipolytica*** strain E150 was also determined by the Génolevures Consortium in France (Fickers ***et al.***, 2005).

Y. lipolytica strain E150 has been used in our research group for the heterologous expression of four cytochrome P450 (***CYP***) genes. These genes were ***CYP53B1***, a benzoate ***para***-hydroxylase from ***Rhodotorula minuta***, ***CYP52F1*** and ***CYP52F2***, alkane hydroxylases from ***Y. lipolytica*** and

CYP557A1, a putative alkane or fatty acid hydroxylase from ***Rhodotorula retinophila***. These genes were cloned under control of either the isocitrate lyase (***ICL***) promoter or the acyl-CoA oxidase (***POX2***) promoter (Fickers ***et*** al., 2005; Shiningavamwe ***et al***., 2006). The ***POX2*** promoter is induced by alkanes, fatty acids and fatty acid derivatives while the ***ICL*** promoter is strongly induced by ethanol and acetate in addition to alkanes, fatty acids and fatty acid derivatives (Juretzek ***et al***., 2000; Madzak ***et al***., 2004).

Expression of cytochrome P450 in a foreign host often requires the coexpression of the cytochrome P450 reductase (CPR) (Dong and Porter, 1996). The P450 and the CPR must be in a correct ratio for the transfer of electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to the P450 (Backes and Kelley, 2003). All the strains mentioned below except one, therefore also had additional copies of the ***Y. lipolytica CPR*** cloned under the ***ICL*** promoter.

The strains overexpressing ***CYP*** genes under the ***POX2*** promoter (***pPOX2***) were constructed by Dr. A.N. Shiningavamwe and Dr. M.E. Setati. The strains with ***CYP*** genes cloned under the ***ICL*** promoter (***pICL***) were constructed by Mr. C.W. Theron. All strains were constructed in the laboratory of Prof. J. Albertyn in the department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa. The routes followed for the construction of the different strains are explained in Fig. 1.1.

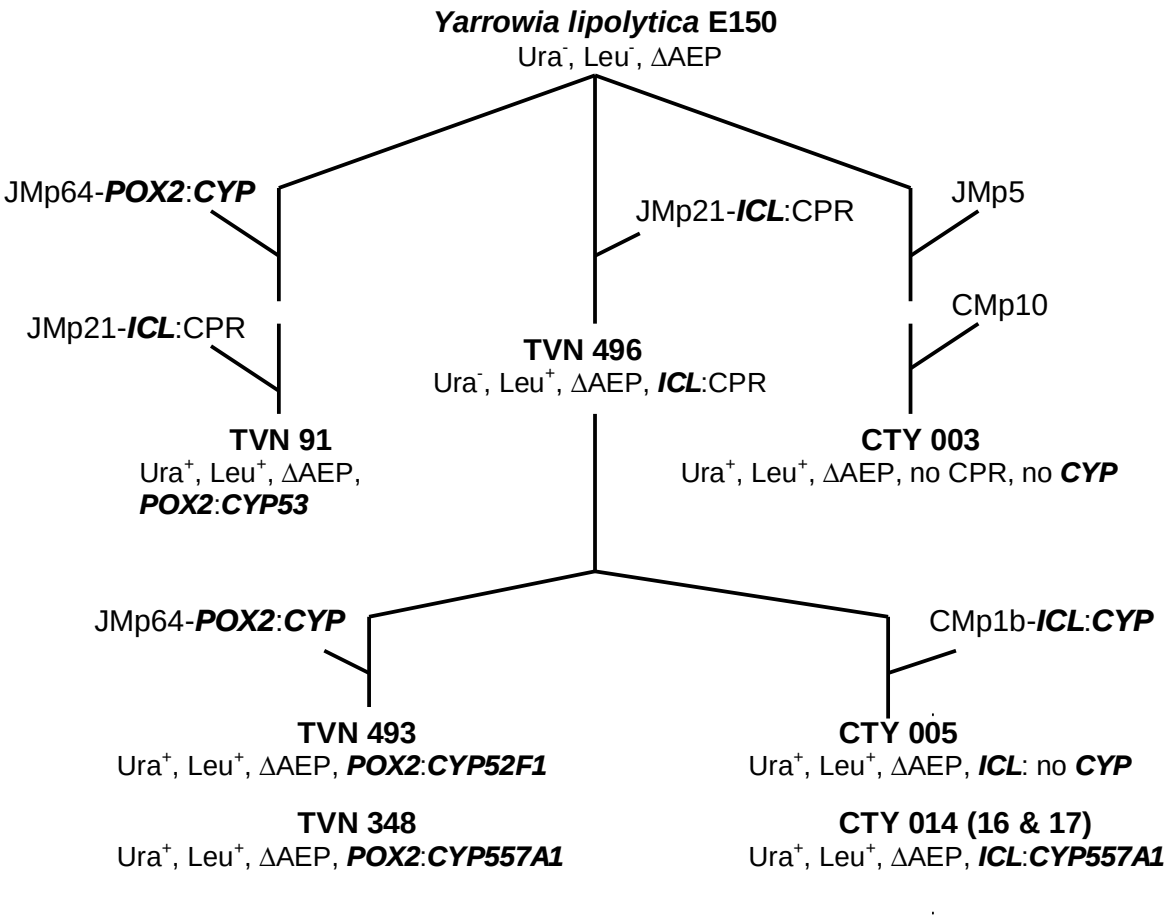


Figure 1.1: Construction of ***Yarrowia lipolytica*** strains with different cytochrome P450s and additional cytochrome P450 reductase cloned under either the ***POX2*** or ***ICL*** promoters (Theron, 2007). Construction of control strains is also shown.

The JMp64 vector (Nthangeni ***et al***., 2004) was used for the cloning of multiple copies of ***CYP53B1*** under ***pPOX2*** into ***Y***. ***lipolytica*** E150 strain. This strain contains zeta sequences for homologous recombination. The vector also contains a defective ***ura3d4*** marker to ensure multiple integrations. An additional copy of the ***YlCPR*** gene was cloned under ***pICL*** using the JMp21 vector with selective ***LEU2*** marker for single insertion (Nthangeni ***et al***., 2004). The resulting strain was TVN 91 (Shiningavamwe ***et al***., 2006).

Y. ***lipolytica*** strains, TVN 348 and TVN 493, were constructed from TVN 496 which was derived from E150 by first cloning an additional copy of the ***YlCPR*** gene under the control of ***pICL***, using the JMp21 vector. The JMp64 vector was used for cloning ***CYP557A1*** under ***pPOX2***. This resulted in the strain TVN 348 (Shininganvamwe, 2004). The same vector was used for the cloning of ***CYP52F1*** under ***pPOX2*** to construct the strain TVN 493 (Setati, unpublished results).

In whole cell biotransformation experiments benzoate ***para***-hydroxylase activity was detected with TVN 91 containing the cloned ***CYP53B1.*** Activity was however disappointingly low (Shiningavamwe, ***et al***., 2006). This could be due to the induction of the wild-type P450s by the fatty acids used to induce ***pPOX2***. ***Y. lipolytica*** has twelve genes (***Alk1*** to ***Alk12***) encoding for putative alkane and fatty acid hydroxylases belonging to the CYP52 family of cytochrome P450 monooxygenases (Iida ***et al***., 2000; Fickers ***et al***., 2005). Experiments to detect the effect of the cloned putative fatty acid hydroxylase encoding gene ***CYP557A1*** and the proven alkane hydroxylase encoding gene ***CYP52F1*** were not successful when using alkylbenzenes as substrates, because no significantly increased hydroxylase activity was detected after induction with fatty acids. It was however observed that the use of ethanol as an inducer of the ***YlCPR*** cloned under ***pICL***, delayed the induction of the wild-type P450s (Van Rooyen, 2005; Obiero, 2006; Shiningavamwe, ***et al***., 2006).

Vectors were subsequently constructed to allow the expression of cloned cytochrome P450s under the ***ICL*** promoter (Theron, 2007). These vectors are CMp1b, CMp10 and JMp5. ***Y***. ***lipolytica*** strains E150 and TVN 496 were used

as the parental strains in the construction of control strains and strains with additional cytochrome P450s cloned under ***pICL***. The vectors JMp5 and CMp10 were used for generating the control strain CTY 003 by cloning the empty vectors into the strain E150. CTY 003 therefore has no additional ***CPR*** or ***CYP*** genes cloned. CMp1b was used for the cloning of ***CYP557A1*** under ***pICL*** into TVN 496 generating the strains CTY 014(16) and CTY 014(17). The empty CMp1b vector was used for constructing the control strain CTY 005. This strain had no additional ***CYP*** genes cloned, but had an additional ***YlCPR*** cloned under ***pICL*** (Theron, 2007).

The main aim of this project was to use whole cell biotransformations to compare hydroxylase activities of the different strains of ***Y***. ***lipolytica*** in which cytochrome P450s were cloned under ***pPOX2*** and ***pICL***. Alkylbenzenes and alkylcyclohexanes were used as substrates, because during alkane degradation by ***Y***. ***lipolytica*** no products accumulate that can be used to measure the hydroxylation activity. It has previously been shown that phenylacetic acid and benzoic acid accumulate from alkylbenzenes (Van Rooyen, 2005; Obiero, 2006; Shiningavamwe, ***et al***., 2006). A literature review was also prepared to summarise information available on the hydroxylation of the alkyl side chains of alkylbenzenes and alkylcyclohexanes.

Chapter 2

Side chain hydroxylation of alkylbenzenes and alkylcyclohexanes by alkane degrading microorganisms

2.1 Introduction

Hydrocarbons are organic compounds that consist of carbon and hydrogen. They originate from biogenic and geological processes and are the main constituents of crude oil, fossil fuels and creosotes, the waste products of coal gasification. They are also found as secondary metabolites in plants and microbes (Holliger and Zehnder, 1996; Cheng ***et al***., 2002; Prenafeta-Boldú ***et al***., 2006).

Hydrocarbons comprise simple compounds such as alkanes and monoaromatic hydrocarbons, as well as complex compounds such as polycyclic aromatic hydrocarbons (PAHs). The monoaromatics are commonly known as BTEX (benzene, toluene, ethylbenzene and xylene), and the PAHs include naphthalene, anthracene and phenanthrene (Holliger and Zehnder, 1996; Prenafeta-Boldú ***et al***., 2006).

Alkylbenzenes are alkylated aromatic hydrocarbons (Fig. 2.1a). They are major components of fossil fuels such as petroleum and coal (Prenafeta-Boldú ***et al***., 2006). These compounds are considered major environmental pollutants. They are of major concern because of their potential carcinogenicity (Holliger and Zehnder, 1996).

On the other hand, alkylcyclohexanes are alkylated aliphatic cyclic compounds (Fig. 2.1b). The main source of alkylcyclohexanes is also petroleum. They are however, also found as secondary metabolites in plants and microbes. These compounds are used in herbicides and insecticides, and are of importance in the aroma and fragrance industry (Cheng ***et al***., 2002). Monoterpenes are C10 alkylcyclohexanes that are important flavour and

fragrance compounds (van Rensburg ***et al***., 1997; Haudenschild ***et al***., 2000; Duetz ***et al***., 2003).

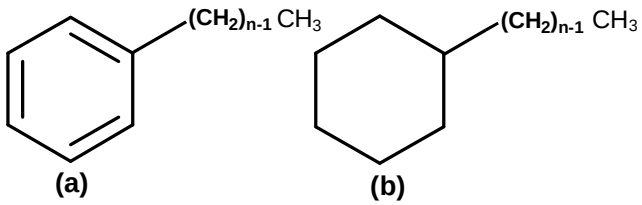


Figure 2.1: General structures of (a) alkylbenzene and (b) alkylcyclohexane with n denoting the number of carbon atoms on the alkyl side chain.

A wide biodiversity of microorganisms are adapted to metabolise alkylbenzenes and alkylcyclohexanes by diverse degradation pathways. Traditionally, the biodegradation of aromatic hydrocarbons by fungi have been considered a cometabolism, but recently a number of fungi isolated from hydrocarbon polluted areas have been shown to utilise these compounds as the sole carbon and energy source (Prenafeta-Boldú ***et al***., 2006).

Aromatic hydrocarbons have non-activated carbon-hydrogen bonds and hydroxylation is the most useful reaction in the activation of these bonds. Oxygenases carry out the introduction of oxygen in various organic molecules (van Beilen ***et al***., 2003a). Microbial hydroxylations have been used for years in the industrial production of fine chemicals such as pharmaceutical products (i.e. hydroxylation of steroids) and for bioremediation processes. Hydroxylation is one of the most widespread enzymatic activities. Hydroxylases occur in all forms of life from bacteria to humans (Holland and Weber, 2000).

Hydroxylation involves the conversion of a carbon-hydrogen bond to a carbon-hydroxyl bond. Various enzymes catalyse hydroxylation reactions. In nature, these enzymes include dioxygenases, lipooxygenases and monooxygenases (Li ***et al***., 2002). The cytochrome P450 monooxygenases (P450s) are of particular interest since they are able to introduce atomic

oxygen into allylic positions, double bonds and even into non-activated carbon-hydrogen bonds under mild conditions (Urlacher ***et al***., 2004; Liu ***et al***., 2006).

The aim of this review was to investigate different alkane degrading microorganisms capable of hydroxylating alkylbenzenes and alkylcyclohexanes at the side chains. Also to differentiate between the specific cytochrome P450 enzymes involved in these hydroxylations.

2.2 Cytochrome P450 monooxygenases

Cytochrome P450 monooxygenases (P450s) derive their name from the absorption band at 450 nm of the reduced carbon-monoxide-bound form (Werck-Reichhart and Feyereisen, 2000). The unusual spectral feature of cytochrome P450s is due to a cysteine thiolate group forming the fifth ligand of the heme iron (Hannemann ***et al***., 2007). They are a group of heme proteins that are widespread in nature (Kitazume ***et al***., 2002b). P450 encoding genes are found in the genomes of virtually all organisms and the number of characterised P450s in plants has exploded in recent years (Werck-Reichhart and Feyereisen, 2000).

Most eukaryotic cytochrome P450 monoxygenases receive electrons from an NADPH cytochrome P450 reductase (CPR), a flavoprotein which contains the flavin cofactors FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide). The P450 and CPR are N-terminally fixed to the endoplasmic reticulum. The CPR transfers the hydride ion of the NADPH to the FAD, which transfers the electron to FMN. FMN in turn reduces the heme centre of the P450 in order for the P450 to activate the molecular oxygen. One atom of the oxygen is incorporated into the substrate, while the other is reduced to water (Maurer ***et al***., 2005; Van Bogaert ***et al***., 2007). The overall reaction is as follows:

$$\mathbf{R\text{-}H + O_2 + 2H^+ + 2e^- \rightarrow R\text{-}OH + H_2O}$$

Where, RH is the hydrocarbon, ROH is the hydroxylated product and the reducing equivalents are from NADPH (De Mot and Parret, 2002).

Cytochrome P450 monooxygenases can catalyse the initial terminal hydroxylation of alkanes (Luykx ***et al***., 2003). In ***n***-alkane-assimilating yeast the P450s that catalyse the terminal hydroxylation of ***n***-alkanes and fatty acids, belong to the CYP52 family (Ohkuma ***et al***., 1998; Iida ***et al***., 2000). In the ***n***-alkane-assimilating yeast, ***Candida maltosa*** eight P450 ***ALK*** genes: ***Alk1- Alk8***, have been identified (Ohkuma ***et al***., 1998; Kogure ***et al***., 2005). In ***Candida tropicalis*** ATCC 20336, ten ***CYP52*** genes have been isolated and characterised (Craft ***et al***., 2003). ***Candida albicans*** contains 10 putative cytochrome P450 (CYP) genes. One of the P450 enzymes is a putative alkane/fatty acid hydroxylase belonging to the CYP52 family, and it is encoded by CYP52A21 (Kim ***et al***., 2007). Genome sequencing has revealed twelve cytochrome P450 genes (***YlALK1*** to ***YlALK12***) in ***Yarrowia lipolytica*** strain E150. Eight of these genes (***ALK1*** to ***ALK8***) belong to CYP52 family (Fickers ***et al***., 2005). ***YlALK1*** catalyses oxidation of short chain ***n***-alkanes such as ***n***-decane and ***YlALK2*** catalyses longer chain ***n***-alkanes (Iida ***et al***., 2000).

In bacteria, the CYP153 family has been shown to mediate the terminal hydroxylation of ***n***-alkanes. This P450 has been isolated from ***n***-alkane degrading bacteria such as ***Acinetobacter*** sp. EB104 (CYP153A1), ***Alcanivorax borkumensis*** SK2 (P450balk), ***Mycobacterium*** sp. strain HXN-1500 (CYP153A6) and ***Sphingomonas*** sp. HXN-200 (which has five ***CYP153*** genes) (Kubota ***et al***., 2005; van Beilen ***et al***., 2005, 2006; Funhoff ***et al***., 2006; van Beilen and Funhoff 2007).

The use of P450 enzymes in industrial processes is currently restricted to whole-cell biotransformation as the system is more stable and because of the simultaneous cofactor regeneration in the cellular metabolism. However, there might be further degradation of products and toxicity of the intermediates and products to the cell. The recovery of products might also be complicated,

especially from complex fermentation broth. Therefore, the use of isolated oxygenases can be advantageous (Maurer ***et al***., 2005).

2.2.1 Classes of cytochrome P450s

Cytochrome P450s can be classified into different classes according to their redox partners. Hannemann and colleagues compiled a classification of classical as well as recently discovered cytochrome P450 redox systems according to their compositions (Table 2.1) (Hannemann ***et al***., 2007). Only four of these classes are discussed here. Class I enzymes are three component systems consisting of a P450, an iron sulphur protein (ferredoxin) and a FAD-containing reductase (Fig. 2.2). They are found commonly in bacteria as soluble enzymes. The electron transfer from NADPH to the P450 protein is ***via*** the reductase and the ferredoxin (Kitazume ***et al***., 2002b; Roberts ***et al***., 2002). In eukaryotes, class I enzymes are also found associated with the inner membrane of mitochondria (Werck-Reichhart and Feyereisen, 2000).

Class II enzymes are microsomal two component systems, comprising of a reductase containing both FAD and FMN and a P450. They are common in eukaryotes (Roberts ***et al***., 2002). Electron transfer from NADPH to the P450 is mediated by the FAD and FMN-containing reductase (De Mot and Parret, 2002).

The class VIII enzymes are made up of the same components as the class II systems, but all the components are linked together. These enzymes are self-sufficient hence; do not require an additional protein for electron transfer from NADPH (Hannemann ***et al***., 2007; Roberts ***et al***., 2002). Class VII P450s are also self-sufficient. The system consists of an FMN-containing reductase with a ferredoxin-like centre fused to the P450 (Roberts ***et al***., 2002).

Table 2.1: Classes of P450 systems classified depending on the topology of the protein components involved in the electron transfer to the P450 enzyme (adapted from Hannemann ***et al***., 2007).

Class/source	Electron transport chain	Localization/remarks
Class I Bacterial Mitochondrial	NAD(P)H ► [FdR] ► [Fdx] [a] ► [P450] NAD(P)H ► [FdR] ► [Fdx] ► [P450]	Cytosolic, soluble P450: inner mitochondrial membrane, FdR: membrane associated, Fdx: mitochondrial matrix, soluble
Class II Bacterial Microsomal A Microsomal B Microsomal C	NADH ► [CPR] ► [P450] NAD(P)H ► [CPR] ► [P450] NAD(P)H ► [CPR] ► [cytb5] ► [P450] NADH ► [cytb5Red] ► [cytb5] ► [P450]	Cytosolic, soluble; ***Streptomyces carbophilus*** Membrane anchored, ER Membrane anchored, ER Membrane anchored, ER
Class III Bacterial	NAD(P)H ► [FdR] ► [Fldx] ► [P450]	Cytosolic, soluble; ***Citrobacter braakii***
Class IV Bacterial	Pyruvate, CoA ► [OFOR] ► [Fdx] ► [P450]	Cytosolic, soluble; ***Sulfolobus tokadaii***
Class V Bacterial	NADH ► [FdR] ► [Fdx-P450]	Cytosolic, soluble; ***Methylococcus capsulatus***
Class VI Bacterial	NAD(P)H ► [FdR] ► [Fldx-P450]	Cytosolic, soluble; ***Rhodococcus rhodochrous*** strain 11Y
Class VII Bacterial	NADH ► [PFOR-P450]	Cytosolic, soluble; ***Rhodococcus*** sp. strain NCIMB 9784, ***Burkholderia*** sp., ***Ralstonia metallidurans***
Class VIII Bacterial, fungi	NAD(P)H ► [CPR-P450]	Cytosolic, soluble; ***Bacillus megaterium***, ***Fusarium oxysporum***
Class IX Only NADH dependent, fungi	NADH ► [P450]	Cytosolic, soluble; ***Fusarium oxysporum***
Class X Independent in plants/mammals	[P450]	Membrane bound, ER

Abbreviated protein components contain the following redox centres: Fdx (iron-sulfur-cluster); FdR, Ferredoxin reductase (FAD); CPR, cytochrome P450 reductase (FAD, FMN); Fldx, Flavodoxin (FMN); OFOR, 2-oxoacid: ferredoxin oxidoreductase (thiamine pyrophosphate, [4Fe-4S] cluster); PFOR, phthatate-family oxygenase reductase (FMN, [2Fe-2S] cluster).

[a] Fdx containing iron-sulfur-cluster of [2Fe-2S], [3Fe-4S], [4Fe-4S], [3Fe-4S], /[4Fe-4S] type.

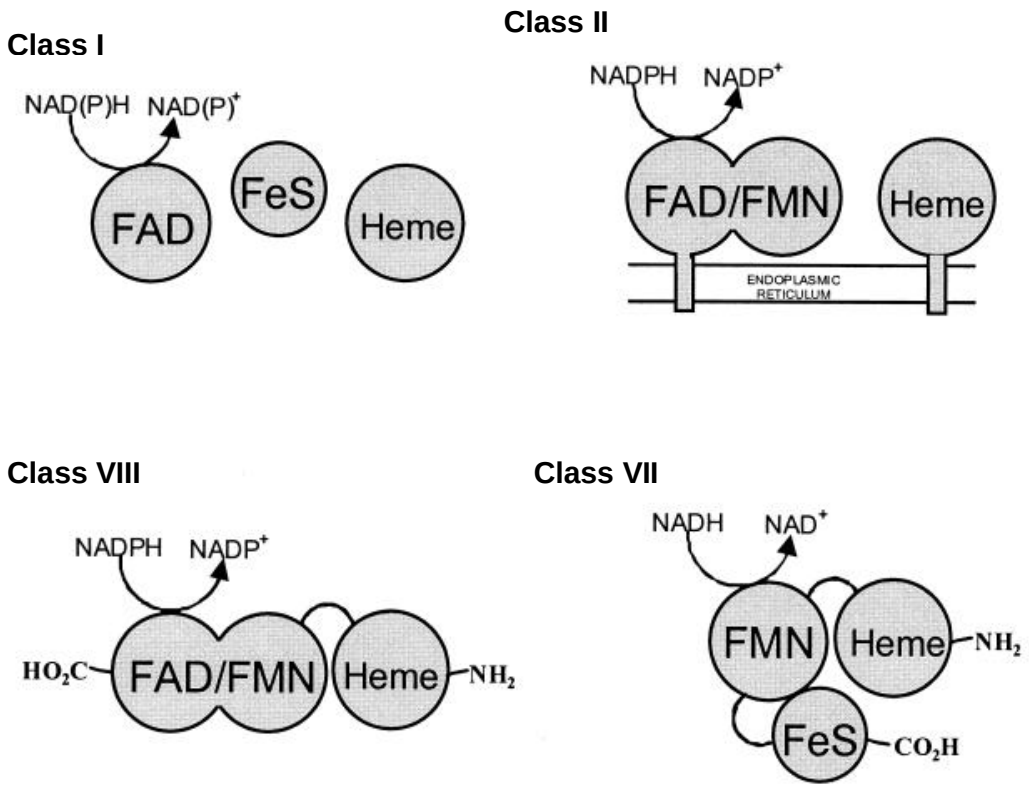


Figure 2.2: Schematic representation of the different classes of cytochrome P450 systems. Adapted from Roberts ***et al***., 2002.

2.3 Side chain hydroxylation of alkylbenzenes

Side chain hydroxylation of alkylbenzenes can be at the benzylic (Fig. 2.3a), subterminal (Fig. 2.3b) or terminal position (Fig. 2.3c) (Adam ***et al***., 2001; Luykx ***et al***., 2003; Uzura ***et al***., 2001).

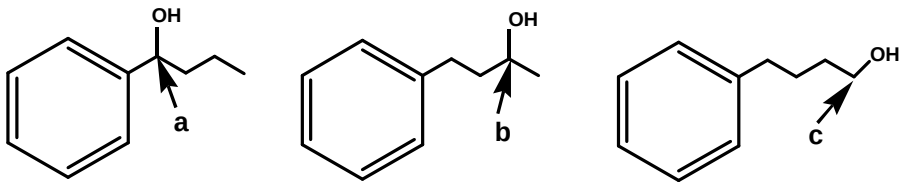


Figure 2.3: Side chain hydroxylation of alkylbenzenes at the benzylic position (a), the subterminal position (b) and the terminal position (c).

2.3.1 Benzylic hydroxylations

2.3.1.1 Toluene hydroxylation

Toluene and other related hydrocarbons such as xylene, fluorene and benzene are abundant environmental pollutants, hence the interest in their degradation. ***Cladosporium shaerospermum*** was the first fungus to be isolated growing on toluene as a carbon and energy source (Luykx ***et al***., 2003). Luykx and co-workers suggested that the initial hydroxylation of toluene by ***C***. ***shaerospermum*** is catalysed by toluene monooxygenase (TOMO). They postulated that the hydroxylation is at the methyl group due to the preference of TOMO for alkylated benzenes. However, the products were not identified.

They presumed TOMO to be a cytochrome P450 as its activity was inhibited by carbon monoxide and the reduced carbon monoxide difference spectrum showed a maximum peak of 451 nm. Furthermore, it required NADPH and molecular oxygen to oxidise toluene. TOMO activity was induced by the addition of toluene to a culture of ***C***. ***shaerospermum*** pre-grown on glucose and was highest at pH 7.5 and 35ºC. The highest activity was measured in the presence of NADPH, O_2, FAD and FMN. The specific activity of TOMO was higher in the microsomes than in the cytosolic fractions. Therefore, TOMO was identified as a membrane bound cytochrome P450 associated with its membrane bound CPR (Luykx ***et al***., 2003).

Holland ***et al***., 1988, showed that toluene is converted into benzyl alcohol by the fungi ***Mortierella isabellina*** NRRL 1757, and ***Helminthosporium*** sp. strain NRRL 4671. The ***Helminthosporium*** sp. seemed to be capable of further biotransformation of the benzyl alcohol. This was evidenced by low recoveries obtained in control experiments using the alcohol as a substrate. They suggested that these hydroxylations are performed by a cytochrome P450-dependend hydroxylase enzyme (Holland ***et al***., 1988).

Another microorganism that can hydroxylate toluene is ***Pseudomonas oleovorans*** Gpo1 (also known as ***Pseudomonas putida*** Gpo1). Toluene was oxidised ***in vivo*** by resting cells and only when present in concentration of 1% v/v. ***P. oleovorans*** has an alkane hydroxylase system named AlkB (van Beilen ***et al***., 1994, 2001). AlkB is an integral membrane-bound diiron protein. It is a three-component system in which the AlkB is the catalytic component, AlkT, the NADPH-dependent rubredoxin reductase and AlkG, the rubredoxin. The rubredoxin contains an iron atom with four cysteines as ligands. The electron transfer from NADPH is mediated by the rubredoxin reductase via FAD to the rubredoxin, which in turn transfer the electrons to the hydroxylase (van Beilen ***et al***., 1994, 2001, 2003b).

Recently a new member of the NADPH-dependent class VII cytochrome P450 monooxygenase was identified in ***Rhodococcus ruber*** strain DSM 44319. The enzyme was successfully expressed and characterised in ***Escherichia coli***. It was shown to hydroxylate toluene to benzyl alcohol (Fig. 2.4). The enzyme is a self-sufficient P450 monooxygenase. In the presence of NADPH, the enzyme showed activity towards polycyclic aromatic hydrocarbons and alkyl aromatics (Table 2.2). Alkyl aromatics like toluene, ***m***-xylene and ethylbenzene were hydroxylated exclusively at the side chains (Liu ***et al***., 2006).

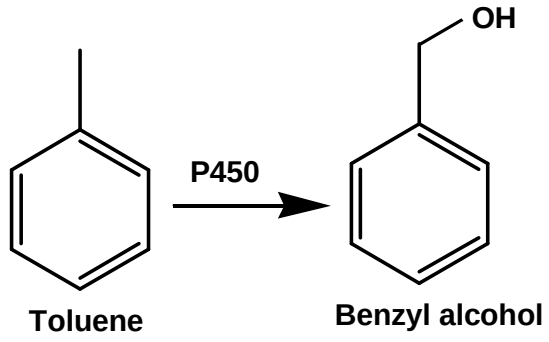


Figure 2.4: Hydroxylation of toluene by an NADPH-dependent class VII cytochrome P450 monooxygenase (Liu ***et al***., 2006).

Table 2.2: Substrates, products and product formation rates of the P450 monooxygenase from ***R***. ***ruber*** DSM 44319 taken from Liu ***et al***., 2006

Substrate	Product	Product formation rate (nmol x nmol^{-1} P450 x min^{-1})
7-Ethoxycoumarin	7-Hydroxycoumarin	0.917±0.05
Acenaphthene	1-Acenaphthenol	0.079±0.01
Flourene	9-Flourenol	0.04±0.005
Naphthalene	1-Naphthol	0.106±0.01
Indene	1-Indenol	nd
Toluene	Benzyl alcohol	0.301±0.01
Ethyl benzene	1-Phenylethyl alcohol	nd
	2-Phenylethyl alcohol	nd

nd Not determined

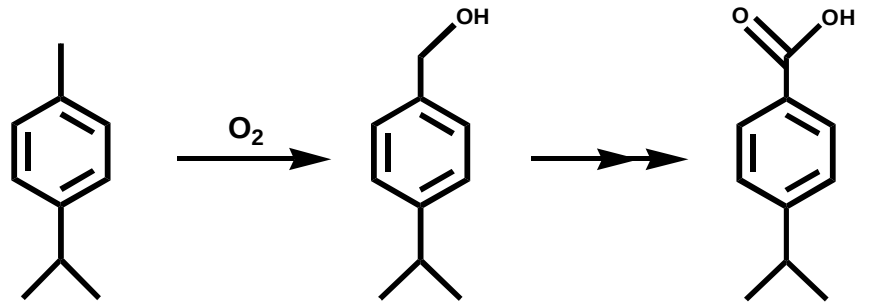
2.3.1.2 *p*-Cymene hydroxylation

Nishio and colleagues did a study on ***p***-cymene, and they showed that the initial hydroxylation is catalysed by cymene monooxygenase (CMO) from ***P***. ***putida*** F1 producing isopropylbenzyl alcohol (Fig. 2.5). CMO is a two-component enzyme made up of a hydroxylase (CymA1) and a reductase (CymA2), which catalyses the insertion of one atom of molecular oxygen into the methyl group. Subsequent oxidation leads to the formation of isopropylbenzoic acid (Nishio ***et al***., 2001).

Recently Funhoff and co-workers demonstrated the hydroxylation of ***p***-cymene at the methyl group to ***p***-cumic alcohol (***p***-isopropylbenzyl alcohol). The hydroxylase was shown to be a member of the CYP153 family, namely CYP153A6 from ***Mycobacterium*** sp. HXN-1500. The turnover of this hydroxylation was 38.9 min^{-1} (Funhoff ***et al***., 2006). In the hydroxylation of ***p***-cymene, the methyl group is preferred over the bulky isopropyl group. This could be that the substrate binding pockets of the involved enzymes are shaped such that linear alkanes are preferred, and the isopropyl group introduces steric hindrance (Nishio ***et al***., 2001; Funhoff ***et al***., 2006).

CYP153 enzymes are class I P450 proteins that require the two component electron delivering protein system (ferredoxin and ferredoxin reductase protein). Eight ***CYP153*** genes have been functionally expressed in ***P***. ***putida*** and ***Pseudomonas fluorescens***. This has allowed the host to use aliphatic alkanes ranging from pentane to dodecane as carbon and energy source. These enzymes are the first soluble enzymes that specifically display hydroxylating activity towards the terminal position of alkanes (Funhoff ***et al***., 2006).

Studies done in our laboratory also demonstrated the conversion of ***p***-cymene to isopropylbenzoic acid using ***Y***. ***lipolytica*** strains (unpublished results). In this case alkane hydroxylating cytochrome P450s belonging to the CYP52 family are probably also responsible for the hydroxylation of ***p***-cymene.



***p*-Cymene** ***p*-Isopropylbenzyl alcohol** ***p*-Isopropylbenzoic acid**

Figure 2.5: Hydroxylation of ***p***-cymene. The initial hydroxylation to isopropylbenzyl alcohol is by a cymene monooxygenase in the case of ***P. putida*** F1, and cytochrome P450 monooxygenases such as CYP153s in bacteria and CYP52s in ***Y. lipolytica.***

2.3.1.3 Ethylbenzene and propylbenzene hydroxylation

In the case of ethylbenzene and propylbenzene, hydroxylation can occur at positions other than the benzylic position. However, resting cells of ***Fusarium moniliforme*** strain MS31 oxidise the side chains of ethylbenzene and propylbenzene at the benzylic position, producing 1-phenylethanol and 1-phenylpropanol respectively (Fig. 2.6). The maximum bioconversion of

propylbenzene by ***F***. ***moniliforme*** was at 25 - 30ºC and pH 7. The biotransformations were done with resting cells (0.1 g) resuspended in 2 mL of 25 mM potassium phosphate buffer. 200 µmol of ethylbenzene (21 µL) and propylbenzene (24 µL) yielded 13.4 µmol and 16.2 µmol of corresponding alcohols respectively (Uzura ***et al***., 2001a, b).

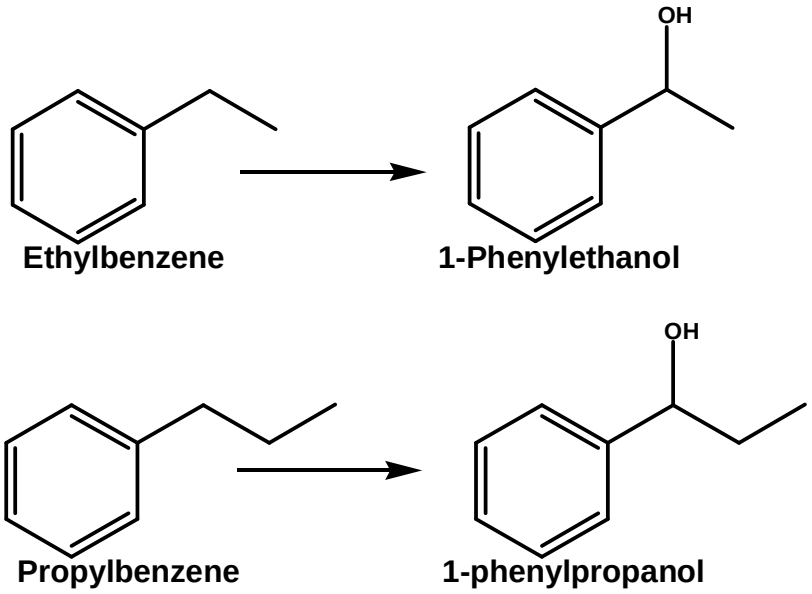


Figure 2.6: The scheme showing hydroxylation of ethylbenzene and propylbenzene to their corresponding benzylic alcohols (Adam ***et al***., 2001; Uzura ***et al***., 2001c).

Other examples of fungi that are able to transform ethylbenzene to 1-phenylethanol are ***M***. ***isabellina*** NRRL 1757 and ***Cunninghamella echinulata*** var. ***elegans*** ATCC 2629 (Holland ***et al***., 1988). ***Bacillus megaterium*** hydroxylated propylbenzene at the benzylic position producing 1-phenylpropanol (Adam ***et al***., 2001).

The enzyme involved in the hydroxylations by ***F***. ***moniliforme*** might be a cytochrome P450 monooxygenase. The biotransformations were done with cell free extracts (0.2 g) resuspended in 2 mL of 25 mM potassium phosphate buffer. The hydroxylation occurred with 5% (v/v) oxygen and increased with concentrations of oxygen up to 50% (v/v). The reaction also required reducing equivalents and the addition of NADPH gave higher activity than when NADH was added. Addition of FAD and FMN increased the activity further, and the

hydroxylation was inhibited by carbon monoxide. These characteristics match those of a microsomal cytochrome P450 monooxygenase system containing a NADPH-cytochrome P450 reductase (Uzura ***et al***., 2001c).

One interesting example is the hydroxylation of ethylbenzene by NADPH-dependent class VII P450 from ***Rhodococcus ruber.*** The hydroxylation was not regioselective, but occurred at both the benzylic and terminal positions to yield a mixture of 1-phenylethyl alcohol and 2-phenylethyl alcohol (Fig. 2.7). The ability of this self-sufficient enzyme to perform such hydroxylations makes it a potential candidate for biodegradation of pollutants and an attractive biocatalyst for synthesis (Liu ***et al***., 2006).

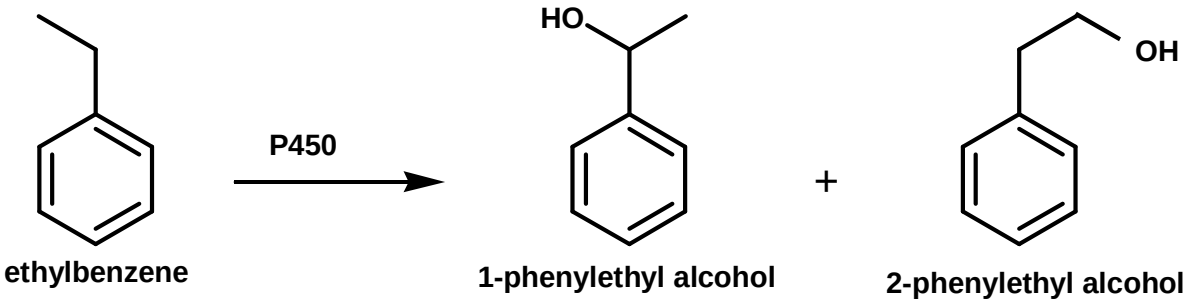


Figure 2.7: Hydroxylation positions of ethylbenzene to 1-phenylethyl alcohol and 2-phenylethyl alcohol, by an NADPH-dependent class VII P450 identified from ***Rhodococcus ruber*** (Liu ***et al***., 2006).

2.3.2 Benzylic and subterminal hydroxylations

B. ***megaterium*** has been shown to hydroxylate pentylbenzene at both the benzylic and subterminal positions (Fig. 2.8) (Adam ***et al***., 2001).

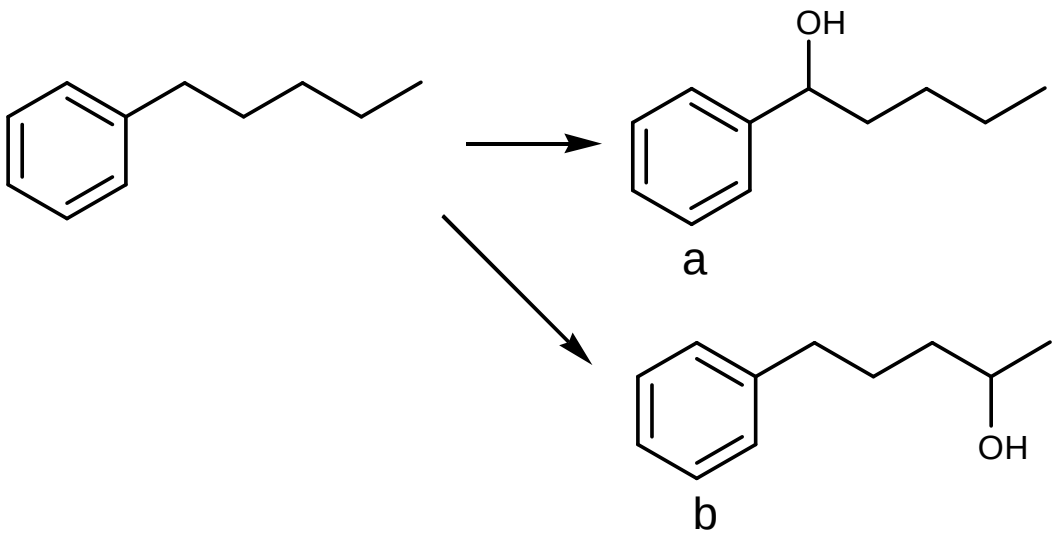


Figure 2.8: Hydroxylation positions of pentylbenzene by ***Bacillus megaterium***. (a) Is the benzylic position and (b) subterminal position (Adam ***et al***., 2001).

F. ***moniliforme*** also gives benzylic and subterminal hydroxylation of butylbenzene but the main product was 3.1 mM 1-phenylbutan-2-ol (Fig. 2.9a) while the products of benzylic hydroxylation was 0.8 mM 1-phenylbutanol (Fig. 2.9b) and subterminal hydroxylation produced 1.5 mM 4-phenylbutan-2-ol (Fig. 2.9c). The conversion ratios of these products were 3.1%, 0.8% and 1.5% respectively (Uzura ***et al***., 2001c).

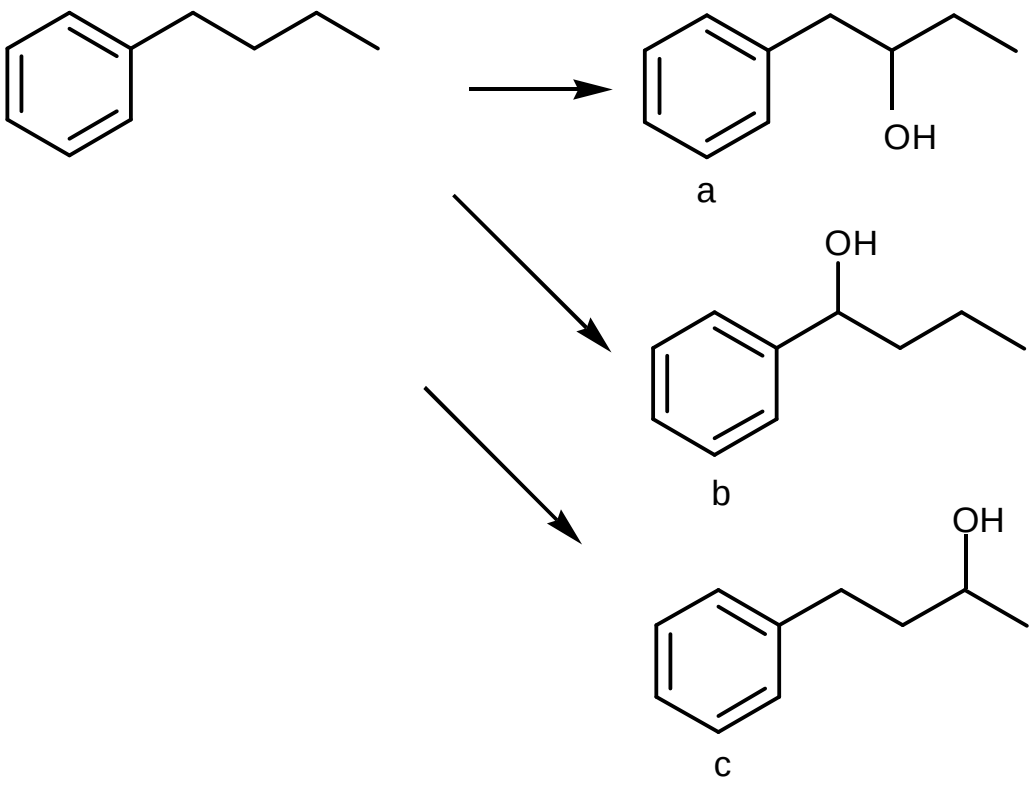


Figure 2.9: Hydroxylation positions of butylbenzene by ***Fusarium moniliforme*** (Uzura ***et al***., 2001c).

Subterminal hydroxylations of alkylbenzenes by ***B***. ***megaterium*** and ***F***. ***moniliforme*** are of interest, because fatty acid hydroxylases that give subterminal hydroxylation of long chain fatty acids, namely P450 BM3 (CYP102A1) and CYP505 have been cloned from ***B***. ***megaterium*** and ***F***. ***oxysporum*** respectively (Appel ***et al***., 2001; Kitazume ***et al***., 2000, 2002). It would be interesting to investigate whether these enzymes give subterminal hydroxylation of alkylbenzenes.

These enzymes are totally unrelated but are similar in that they are self sufficient, meaning the heme and the reductase domains are fused. Moreover, their specific activity is 100 - 1000 fold higher than their counterparts (Lentz, ***et al***., 2004). However, they have significant differences. BM-3 is soluble and CYP505 was purified from membrane fractions (Appel ***et al***., 2001; Kitazume ***et al***., 2000, 2002a).

2.3.3 Terminal hydroxylation of alkylbenzenes

Alkylbenzenes are hydroxylated at the end of the alkyl chain by alkane utilising bacteria and yeasts to form the corresponding phenylalkanol, which is further oxidised and degraded by β-oxidation to either benzoic acid or phenylacetic acid (Fig. 2.10). The final product depends on the length of the alkyl chain. If the side chain is even numbered, phenylacetic acid is formed, and if it is odd numbered it is degraded to benzoic acid (Dutta and Harayama, 2001; Beam and Perry, 1974).

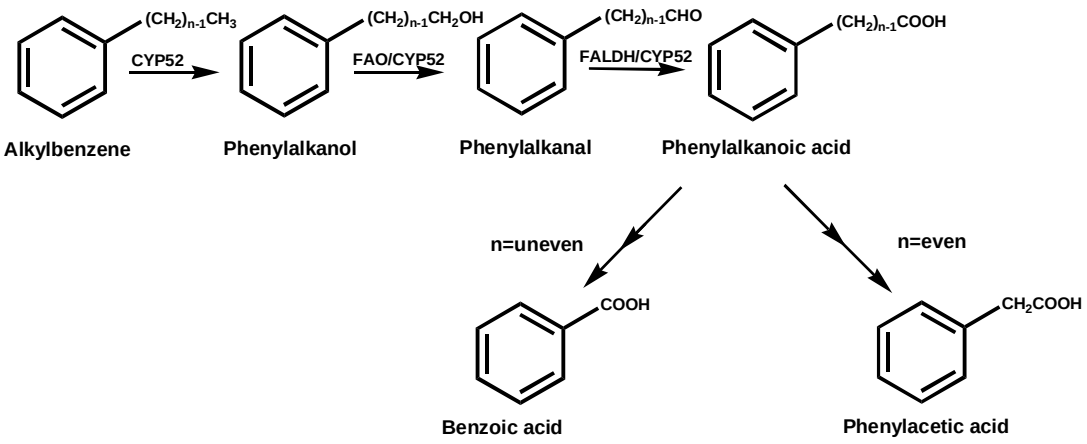


Figure 2.10: General degradation reaction scheme of alkylbenzenes with n the number of carbon atoms on the alkyl side chain. Alkylbenzenes are oxidised to either benzoic acid or phenylacetic acid, depending on the number of carbon atoms in the side chain. If n is uneven, benzoic acid is formed and if n is even, phenylacetic acid is formed (adapted from van Rooyen, 2005).

The terminal oxidation of alkylbenzenes in yeasts such as ***Yarrowia lipolytica*** and ***Candida maltosa*** is catalysed by a CYP52 enzyme, although it has been shown to have an inhibitory effect on growth in the latter (Mauersberger, 1996). The alkylbenzene is hydroxylated to phenylalkanol. The subsequent oxidations by fatty alcohol oxidase (FAO) and fatty alcohol dehydrogenase (FALDH) result in phenylalkanal and phenylalkanoic acid respectively. With alkanes, these further oxidations have been shown to be catalysed by CYP52s as well (Craft ***et al***., 2003).

Work done in our research laboratory demonstrated that ***Y. lipolytica*** strains degraded hexylbenzene and decylbenzene to phenylacetic acid (Van Rooyen, 2004). The effect of additional cloned alkane hydroxylases, ***CYP52F1*** and ***CYP52F2*** could not be conclusively demonstrated. In another study strains overexpressing ***CYP52F1***, ***CYP557A1*** and ***CYP53B1*** were tested for the biotransformation of phenylnonane. All these strains transformed phenylnonane to benzoic acid (Obiero, 2006).

The cytochrome P450 CYP153 family catalyse the terminal hydroxylation of ***n***-alkanes. Kubota ***et al***., 2005, isolated CYP153 genes from ***Alkanivorax borkumensis*** SK2 (designated P450balk) and expressed these genes as chimeras with the reductase component of the self-sufficient class VII P450 from ***Rhodococcus*** sp. NCIMB 9784 in ***Escherichia coli***. These chimeric self-sufficient P450s converted butylbenzene to 4-phenyl-1-butanol (Fig. 2.11) (Kubota ***et al***., 2005).

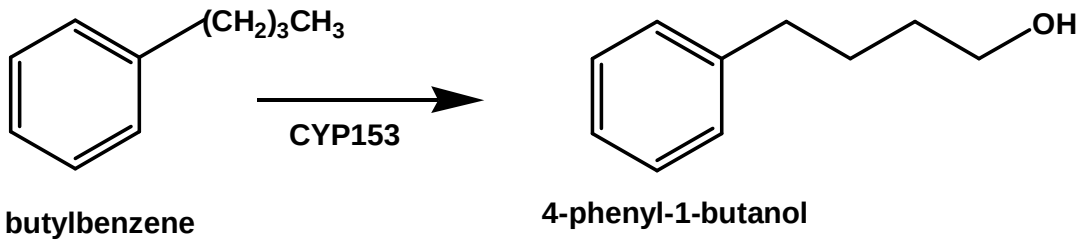


Figure 2.11: Terminal hydroxylation of butylbenzene to 4-phenyl-1-butanol by CYP153 P450 monooxygenase from the bacterium ***Alcanivorax borkumensis*** (Kubota ***et al***., 2005).

The alkane hydroxylase system (AlkB) of ***Pseudomonas oleovorans*** GPo1 has also been shown to hydroxylate alkylbenzenes. Examples of substrates accepted are ethylbenzene, propylbenzene and butylbenzene, which were hydroxylated to 2-phenyl-1-ethanol, 3-phenyl-1-propanol and 4-phenyl-1-butanol respectively (Fig. 2.12) (van Beilen ***et al***., 1994).

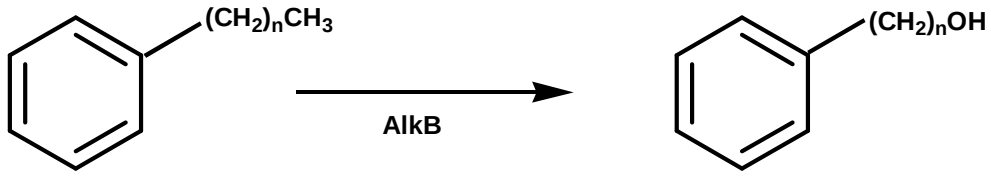


Figure 2.12: Hydroxylation of alkylbenzenes by the alkane hydroxylase system of ***P. oleovorans*** Gpo1. n=1 is ethylbenzene, and the product of hydroxylation is 2-phenyl-1-ethanol; n=2 is propylbenzene and the product is 3-phenyl-1-propanol; n=3 is butylbenzene and the product is 4-phenyl-1-butanol (van Beilen ***et al***., 1994).

2.4 Hydroxylation of alkylcyclohexanes

2.4.1 Limonene hydroxylation

D-Limonene is the main constituent of orange and lemon peel oil. Biotransformation of limonene is of interest because it is the starting compound for industrially relevant fine chemicals and flavour compounds, such as carveol, carvone and perillyl alcohol. It can be hydroxylated at several positions by a number of microorganisms carrying different hydroxylases (Duetz ***et al***., 2001).

A number of alkane degrading organisms have been found to hydroxylate limonene at the 7 position (Fig. 2.13) (van Beilen ***et al***., 2005). A strain of ***Bacillus stearothermophilus*** degrades limonene via perillyl alcohol and perillyl aldehyde (Cheng ***et al***., 1995). The production of perillyl alcohol is interesting because of its anticarcinogenic properties. Since it is present in low levels in few plant oils, an alternative synthetic source is desirable (van Beilen ***et al***., 2005; Funhoff ***et al***., 2006).

Mycobacterium sp. Strain HXN-1500 hydroxylates limonene to perillyl alcohol. The hydroxylation activity towards limonene was 31.2 min^{-1} (Funhoff ***et*** al., 2006). The oxygenase responsible for this was identified as a cytochrome P450 belonging to the CYP153 family. This enzyme was purified and expressed in ***P***. ***putida.*** The fragment cloned, encoded a P450, a ferredoxin

and a ferredoxin reductase (van Beilen ***et al***., 2005). The hydroxylation activity in the cell extracts towards limonene was 31.2 min^{-1} (Funhoff ***et*** al., 2006).

Studies in our research group were conducted using ***Y***. ***lipolytica*** and it hydroxylated limonene to perillic acid. Inhibitor studies using ***Y***. ***lipolytica*** had indicated the possibility of cytochrome P450 monooxygenase involvement in the initial hydroxylation of limonene (Moleleki, 1998).

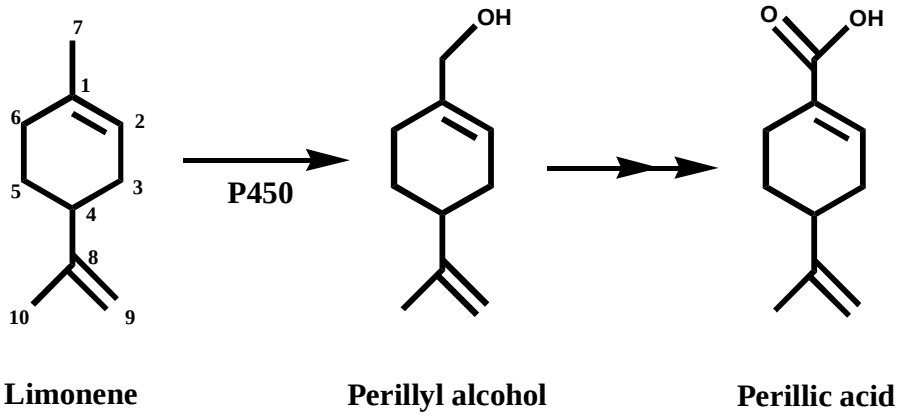


Figure 2.13: Hydroxylation of limonene at the 7 position producing perillic acid. The initial hydroxylation is by cytochrome P450 monooxygenase, resulting in perillyl alcohol.

2.4.2 Hydroxylation of *n*-alkylcyclohexanes

n-Alkylcyclohexanes are components of crude oil. These hydrocarbons are degraded by a number of bacteria and fungi. The oxidation is generally at the terminal methyl group of the ***n***-alkyl side chain. The compounds are initially hydroxylated to a carboxylic acid, which is finally transformed to carboxylic or acetic acid derivatives through β-oxidation (Beam and Perry, 1974; Dutta and Harayama, 2001).

Beam and Perry, 1974, conducted studies on the oxidation and assimilation of ***n***-alkyl substituted cycloalkanes by ***Mycobacterium convolutum*** strain R-22. This strain utilised heptadecylcyclohexane and dodecylcyclohexane as the sole carbon and energy source (Fig. 2.14). However, they did not identify the enzyme responsible in the initial hydroxylation. The relative amount of

cyclohexylacetic acid and cyclohexylpropanoic acid produced during dodecyl- and heptadecylcyclohexane degradation by the ***M***. ***convolutum*** strain R-22 was quantified. Dodecyl- and heptadecylcyclohexane (0.2%) were added in 2 L Erlenmeyer flasks containing 500 mL medium. The 12-carbon ***n***-alkyl-substituted cycloparaffin yielded 7.4 mg of cyclohexylacetic acid, and 21 µg of cyclohexylacetic acid was obtained from the 17-carbon ***n***-alkyl-substituted heptadecylcyclohexane. Equal yield of total cell was obtained on these substrates (Beam and Perry, 1974).

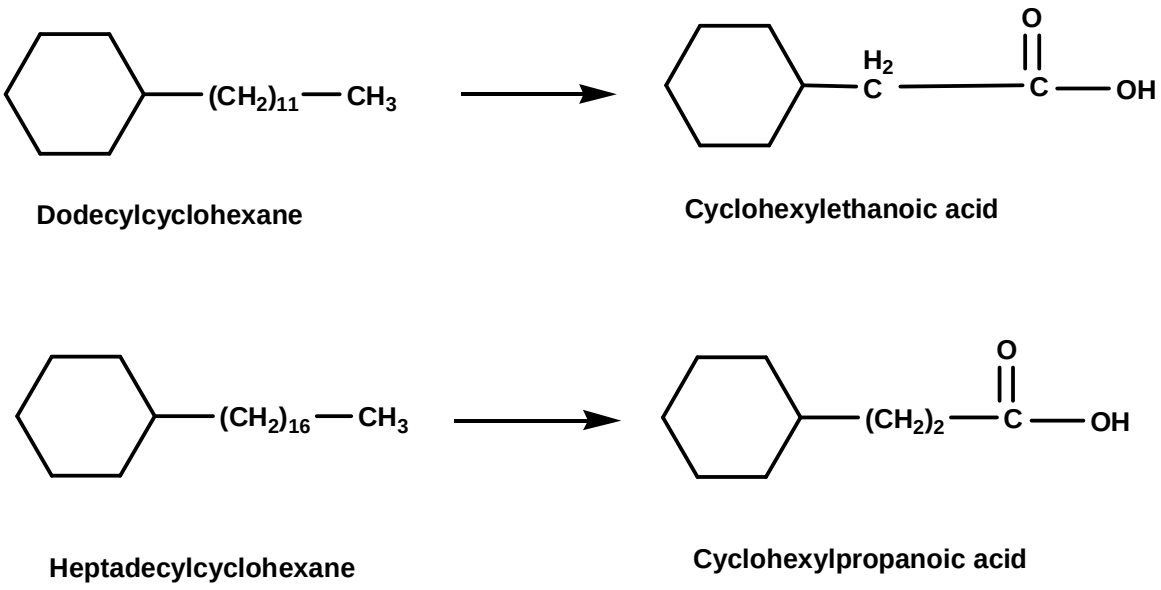


Figure 2.14: Oxidation of dodecylcyclohexane and heptadecylcyclohexane by ***Mycobacterium convolutum*** strain R-22 (Beam and Perry, 1974).

A study on ***Acinetobacter*** sp. ODDK71, which is a long chain ***n***-alkane degrading bacterium, showed the ability to degrade ***n***-alkylcyclohexanes (alkyl side chain length ≥12) when hexadecane was used as a co-substrate. Dodecylcyclohexane was degraded to cyclohexanecarboxylic acid and cyclohexylacetic acid as final products. These products showed that the substrate was oxidised at the alkyl side chain. There was also a ring oxidation that gave 4-dodecylcyclohexanone as a product. Tetradecylcyclohexane was also tested and the degradation also followed both the ring oxidation and alkyl side chain oxidation pathways. Dodecylcyclohexane and tetradecylcyclohexane were not degraded when hexadecane was not used as

a co-substrate (Fig. 2.15A). Both ***n***-alkylcyclohexanes were degraded by co-metabolism with hexadecane (Fig. 2.15B). In the co-metabolism experiments, hexadecane (0.05% w/v) was used as a growth substrate and dodecylcyclohexane (0.05% w/v) or tetradecylcyclohexane (0.05% w/v) was used as a co-substrate. The residual hexadecane was 4.7% and dodecyclohexane 62% after 72 h cultivation. Tetradecyclohexane after 72 h of cultivation was 43.7% and hexadecane, 5.9% (Koma ***et al***., 2003).

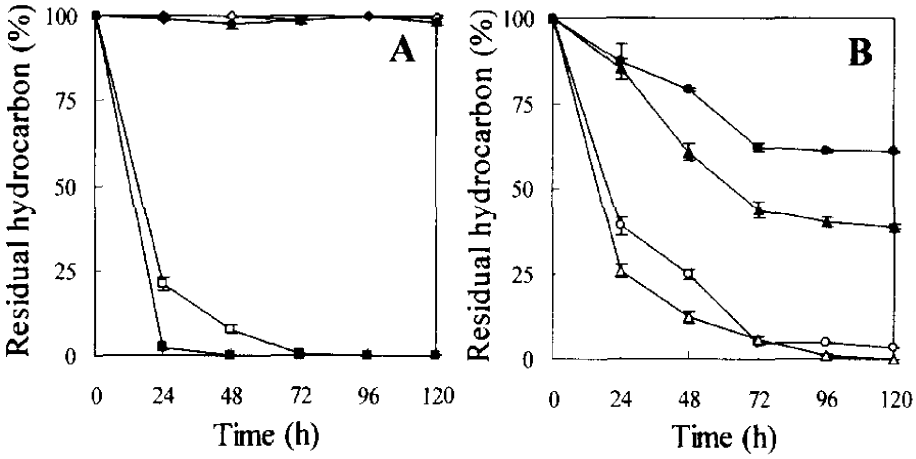


Figure 2.15: Time course experiments of dodecylcyclohexane and tetradecylcyclohexane degradation with and without hexadecane by strain ODDK71. (A) The residual hydrocarbons when each hydrocarbon was used as a sole carbon and energy source. Open squares, closed squares, open diamonds, and closed diamonds indicate the residual hexadecane (0.1% w/v addition), hexadecane (0.05% w/v addition), dodecylcyclohexane (0.05% w/v addition), and tetradecylcyclohexane (0.05% w/v addition), respectively. (B) Open circles and triangles indicate the residual hexadecane during dodecylcyclohexane and tetradecylcyclohexane cometabolism, respectively. Closed circles and triangles indicate the residual dodecylcyclohexane and tetradecylcyclohexane, respectively. Adapted from figure 1 (Koma ***et al***., 2003).

In another study, ***Rhodococcus*** sp. NDKK48 was shown to completely degrade dodecylcyclohexane without co-oxidation via an alkyl side chain oxidation and ring oxidation pathways. It was also able to degrade substrates with short alkyl side chains (e.g. methylcyclohexane) but this required a co-

substrate. The degradation involved a Baeyer-Villiger oxidation (Koma ***et al***., 2005).

Another example is the degradation of ***n***-octadecylcyclohexane and ***n***-nonadecylcyclohexane by ***Alcanivorax*** sp. Strain MBIC 4326. Dutta and Harayama did studies with ***n***-octadecylcyclohexane and ***n***-nonadecylcyclohexane which were transformed by more than one pathway (Fig. 2.16). One pathway involves terminal oxidation of the methyl group of the alkyl side chain to a carboxylic acid. Subsequent β-oxidation yielded cylcyclohexaneacetic acid from ***n***-octadecylcyclohexane and cyclohexanecarboxylic acid from ***n***-nonadecylcyclohexane.

The formation of cylcyclohexaneacetic acid from ***n***-nonadecylcyclohexane as well as the formation of cyclohexanecarboxylic acid from ***n***-octadecylcyclohexane cannot be explained by simple β-oxidation. The degradation of ***n***-nonadecylcyclohexane is interesting because of the final transformation of cyclohexanecarboxylic acid to benzoic acid as this is the first report thus far. Further genetic and biochemical studies are required to clarify enzymes involved (Dutta and Harayama, 2001).

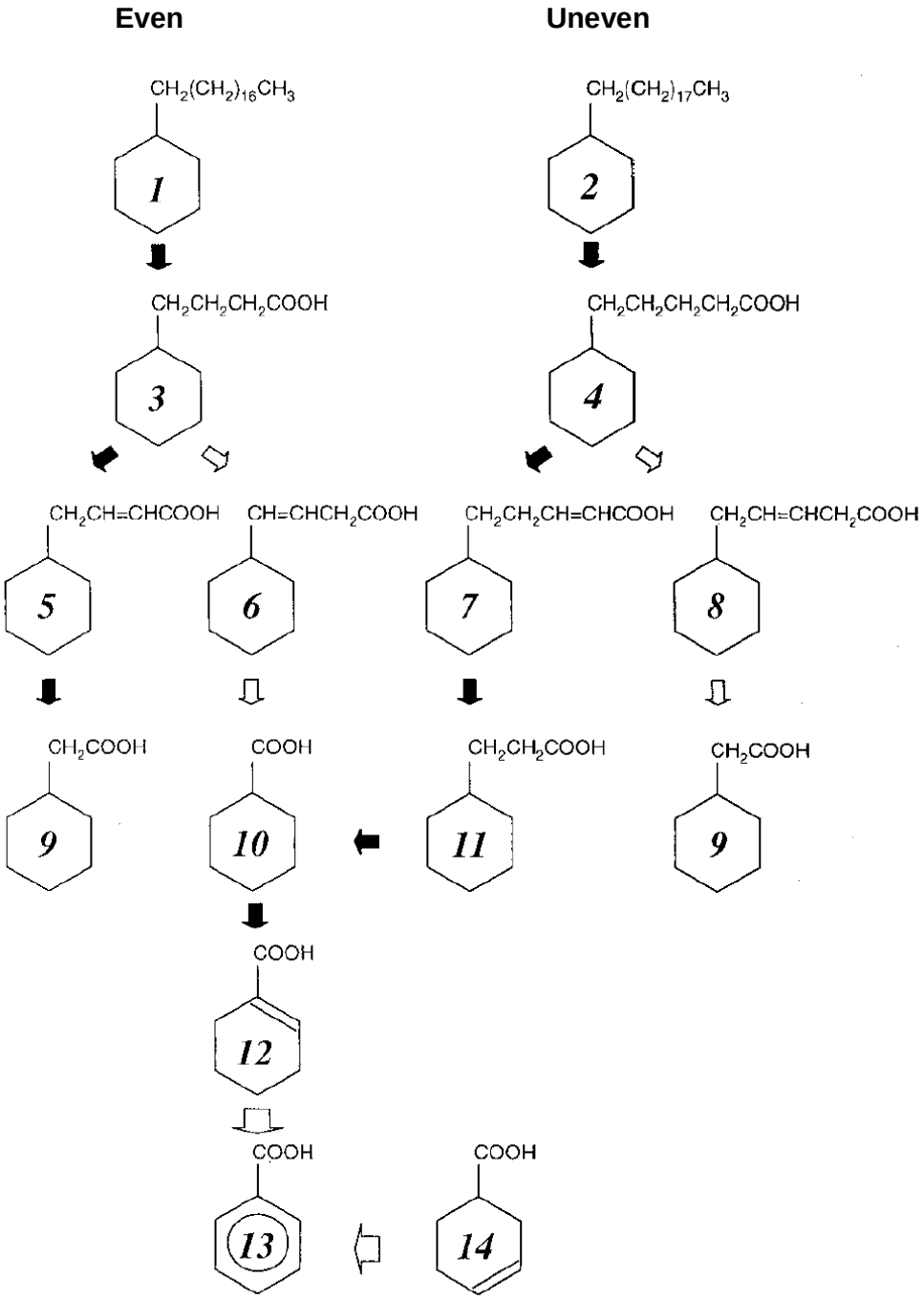


Figure 2.16: Adapted from figure 1 (Dutta and Harayama, 2001). Proposed pathways for the degradation of ***n***-alkylcyclohexanes by ***Alcanivorax*** sp. Strain MBIC 4326. Solid arrows indicate β-oxidation routes shown to be major metabolic routes, while open arrows indicate minor routes. Larger open arrows indicate novel metabolic routes. (1) Represents ***n***-octadecylcyclohexane, (2) ***n***-nonadecylcyclohexane (9) cyclohexaneacetic acid, (10) cyclohexanecarboxylic acid and (13) benzoic acid.

In the last two examples of alkylcyclohexanes hydroxylations, the enzymes involved were not stated. The alkylcyclohexanes in both cases are hydroxylated at the terminal end. ***Mycobacterium*** and ***Alcanivorax*** species are known to have AlkB-related alkane hydroxylases as well as CYP153 enzymes (van Beilen ***et al***., 2006). Both systems are capable of terminal hydroxylations, hence the previous hydroxylations could be either by AlkB or CYP153.

The alkane hydroxylase system (AlkB) of ***Pseudomonas oleovorans*** GPo1 has also been shown to oxidise alkylcyclohexanes (Fig. 2.17). However, this system unlike the P450s does not hydroxylate these compounds on the side chain, but on the alicyclic ring (van Beilen ***et al***., 1994).

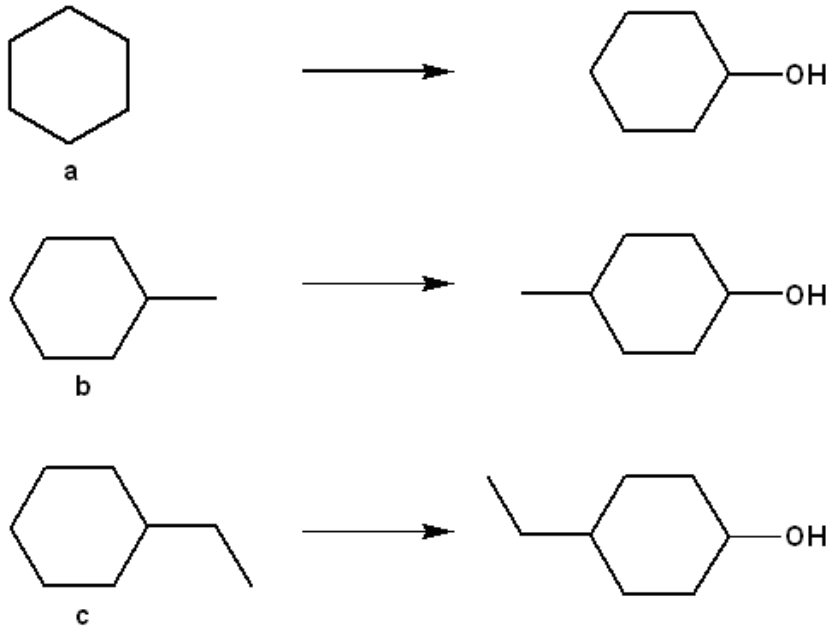


Figure 2.17: Hydroxylation of alkylcyclohexanes by the alkane hydroxylase system (alkB) of ***P***. ***oleovorans*** Gpo1. Substrate and products names are: (a) cyclohexane, cyclohexanol; (b) methylcyclohexane, ***trans***-4-methylcyclohexanol; (c) ethylcyclohexane, ***trans***-4-ethylcyclohexanol (van Beilen ***et al***., 1994).

2.5 Conclusion

Alkylbenzenes and alkylcyclohexanes are of major concern as they are environmental pollutants and potential carcinogens. Some of these compounds namely the monoterpenes are important in aroma, fragrance and pharmaceutical industries. Many alkylated compounds are natural compounds hence; many microorganisms have the ability to utilise them as carbon sources.

The degradation of alkylbenzenes and alkylcyclohexanes are useful in bioremediation and in the production of desired intermediates for industrial purposes. As these compounds are inert, they need to be activated first for enzymatic reactions. Chemical reactions can be used to activate them, but they are often unspecific and result in undesired by-products. Chemical reactions also require high temperatures and these make chemical reactions unattractive to industrial purposes.

A number of microorganisms hydroxylate alkylbenzenes and alkylcyclohexanes at different positions, namely the benzylic, in-chain, subterminal and terminal positions. Hydroxylation reactions activate the inert bonds of the alkylated hydrocarbons. The enzymes that can catalyse these reactions have been shown to be cytochrome P450 monooxygenases or diiron monooxygenases. Terminal hydroxylations have in most cases been shown to be catalysed by alkane hydroxylases belonging to the CYP153 or CYP52 families of P450s or to the diiron alkane hydroxylases referred to as AlkB. Even the benzylic methyl group of toluene, ***p***-cymene and limonene have been shown to be hydroxylated by alkane hydroxylases. Enzymes responsible for hydroxylation at the benzylic, in-chain and subterminal positions of the alkyl chains have not been as well characterised. Given that alkane hydroxylases give terminal hydroxylation of alkylbenzenes, and that some organisms such ***Y. lipolytica*** accumulate benzoic acid and phenylacetic acid from alkylbenzenes, the use of these substrates as model compounds for studying alkane hydroxylases should be further investigated.

Chapter 3

3.1 Part A: Materials and Methods

3.1.1 Microorganisms

Yeast strains were constructed in the laboratory of Prof J. Albertyn in the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State in Bloemfontein, South Africa, and are maintained in the MIRCEN Yeast Culture Collection of the University of the Free State. The distinguishing properties of the different strains are summarised in Table 3.1. Working cultures were maintained on YP_2D_2 agar plates containing 10 g yeast extract (Biolab), 20 g peptone (Biolab), 20 g glucose (Saarchem) and 20 g agar (Biolab) per litre distilled water. Frozen stock cultures were prepared by growing the cultures in test tubes containing 5 mL YP_2D_2 media (as described above) for 24 h. Aliquots (750 µL) of these cultures were transferred to 1.5 mL microcentrifuge tubes, 250 µL of glycerol (Saarchem) was added and the suspensions were mixed. The stock cultures were stored at -70°C.

Table 3.1: Yeast strains used in this study

		Additional CYP genes		
Strain	**Strain number**	**Gene cloned**	**Gene source**	**Hydroxylase activity**
Yarrowia lipolytica	TVN 91[1]	CYP53B1 (multiple copies)	***Rhodotorula minuta***	Benzoate ***para*** hydroxylase
Yarrowia lipolytica	TVN 348[1]	CYP557A1 (multiple copies)	***Yarrowia lipolytica***	Putative fatty acid hydroxylase
Yarrowia lipolytica	TVN 493[2]	CYP52F2 (multiple copies)	***Yarrowia lipolytica***	Alkane hydroxylase
Yarrowia lipolytica	CTY 003[3]	None	n.a	n.a.
Yarrowia lipolytica	CTY 005 [3]	None	n.a.	n.a.

Yarrowia lipolytica	*CTY 014(16)[3]	CYP557A1 (multiple copies)	***Rhodotorula retinophila***	Putative fatty acid hydroxylase
Yarrowia lipolytica	*CTY 014(17)[3]	CYP557A1 (multiple copies)	***Rhodotorula retinophila***	Putative fatty acid hydroxylase

1. Strains were constructed by Dr. A.N. Shiningavamwe.
2. Strains were constructed by Dr. M.E. Setati.
3. Strains were constructed by Mr. C.W. Theron.

* Different transformants from the same transformation experiment.

3.1.2 Growth media

YP_2D_2 broth contained (per litre of distilled water) 10 g yeast extract, 20 g peptone and 20 g glucose for the seed cultures and main cultures. All chemicals were from Merck.

3.1.3 Growth conditions

Cultures grown for 48 h on YP_2D_2 agar plates were used to inoculate Erlenmeyer flasks (250 mL) containing YP_2D_2 broth (25 mL). The seed cultures were incubated on a rotary shaker at 180 rpm at 25ºC for 24 h. Seed cultures (2.5 mL) were then used to inoculate YP_2D_2 broth (25 mL) containing 50 mM phosphate buffer (pH 8) in Erlenmeyer flasks (500 mL). The main cultures were incubated at the same conditions as above.

3.1.4 Turbidity measurements

Samples (500 µL) were transferred to microcentrifuge tubes (1.5 mL) and vortexed for 5 min. Cells were appropriately diluted (to obtain maximum OD of 0.5) in physiological salt solution (0.9% w/v NaCl) before optical densities were measured in microtitre plates at 620 nm using a Labsystems iEMS microtitre plate reader MF (Thermo Bio Analysis Company, Helsinki Finland).

3.1.5 Dry weights measurement

Samples (4 mL) were collected in test tubes. 2 mL cyclohexane (to dissolve the hydrophobic substrates from the cells) and 400 µL NaOH (5 M) (to dissolve organic acid substrates or products) were added, before vortexing for 5 min. The solution was vacuum filtered through pre weighed glass fibre filters (GF50 47MM BX200, Schleicher and Schuell). The cells were 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). The filters were oven dried at 110ºC and cooled in a desiccator before weighing (Shiningavamwe ***et al***., 2006).

3.1.6 Extraction and analysis of biotransformation products

Samples (500 µL) were taken at intervals and acidified to pH < 3, by adding 5 M HCl (70 µL). Ethyl acetate (600 µL) containing myristic acid (0.1% w/v) was added as an internal standard. The samples were vortexed for 5 min, and then centrifuged at 10000 rpm for 10 min. The organic top layer was transferred into new microcentrifuge tubes. Aliquots of the extracts (50 µL) were transferred to GC vials, and methylated with the same volume of trimethylsulfonium hydroxide (TMSH) (Smit ***et al***., 2005).

Gas Chromatography (GC) analysis was carried out on the methylated samples using a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionisation detector (FID) and a supelco wax 10 CB column measuring 30 m x 0.53 mm. The initial temperature of the oven was 120°C held for 5 min and then increasing at 10°C min^{-1} to a final temperature of 250°C where it was kept for 12 min. The inlet temperature was 200°C and the flow of hydrogen (carrier gas) through the column was 6 mL min^{-1}. The sample volume was 1 µL. Standard curves were used to determine the concentrations of substrates and products. The retention times and conversion factors used for calculations of substrates and products concentration are summarised in Table 3.2.

Table 3.2: Summary of substrate and product retention times and the conversion factors (m) used for calculating concentrations with the formula: concentration compound = m x (area compound peak)/(area myristic acid peak). Conversion factors apply for a myristic acid internal standard concentration of 10 g L^{-1}.

Substrate/Product	Retention time (min)	Conversion factor (g L^{-1})
Myristic acid	11.38	1
Hexylbenzene	3.74	1.07
Phenylacetic acid	7.74	0.88
Nonylbenzene	8.63	0.98
p-Hydroxybenzoic acid	11.71	0.85
Butylbenzene	1.43	0.90
Benzoic acid	4.68	1.01
Pentylbenzene	1.95	3.15
Heptylbenzene	4.79	3.51
Cyclohexylacetic acid	4.17	0.97
Perillic acid	13.67	1.10

For confirmation of products formed, Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the methylated samples was carried out on a Finnigan Trace GC ultra chromatograph, with a 60 m x 0.32 mm HP5MS column, with helium (He) as a carrier gas at 1 mL min^{-1}. The column temperature at the start was 80°C for 1 min and then increased by 8°C min^{-1} until a final temperature of 280°C. The inlet temperature was at 200^{o}C. The sample volume was also 1 µL.

For glucose determination, samples (1 mL) were taken at intervals up to 48 h growth. Samples were centrifuged for 5 min at 10 000 rpm. The supernatant was filtered using 0.45 µm nylon filter (Uniflo). The concentration was determined by HPLC (High Performance Liquid Chromatography) by injecting filtered samples (20 µL) on a Waters Breeze HPLC system equipped with a differential refractive index detector. The column used was Waters SUGARPACK1 (300 m x 7.8 mm) at 84°C. The mobile phase was deionised water. The concentrations were then calculated by using a standard curve.

3.1.7 Cell harvesting

Cultures (50 mL) were transferred into pre weighed sterile falcon tubes (50 mL). The cells were centrifuged using a Beckman model J2-21 centrifuge, at 4500 rpm for 5 min. The supernatants were discarded. The pellets were resuspended in 50 mL phosphate buffer (50 mM, pH 8) and centrifuged as described above. This was repeated three times. After the last wash, the cells were resuspended in 50 mL phosphate buffer (50 mM, pH 8) and transferred into Erlenmeyer flasks for biotransformations.

3.1.8 Biotransformations under bioreactor conditions

Batch cultivations were carried out using 300 mL bench top bioreactors, Sixfors, (Infors AG Rittergasse 27 CH-4103 Bottmingen, Switzerland) with a working volume of 200 mL. The cultivation parameters were: temperature was maintained at 25ºC, dissolved oxygen at 30 to 40% of saturation, agitation range 500 to 1000 rpm and cultivation time 96 h. The aeration and agitation rates were continuously adjusted to maintain the dissolved oxygen concentration at 30 to 40% of saturation. This was done to ensure that oxygen supply did not limit hydroxylase activity. The dissolved oxygen in the culture was monitored with a polarographic pO_2 electrode (Mettler, Toledo, Halstead, UK). Aeration was maintained at the desired level by manual adjustment of the stirrer speed within the preset ranges as indicated above. The pH was controlled and maintained at pH 8.2 by using hydrochloric acid (1 M) and sodium hydroxide (1 M).

3.1.9 Cytochrome P450 reductase (CPR) and cytochrome P450 activity (CYP) assays

3.1.9.1 Isolation of microsomes

Cultures (20 mL) were transferred to centrifuge tubes and centrifuged at 861 x ***g*** for 5 min at 4ºC. The supernatant was discarded and the pellet washed twice in 20 mL ice cold wash buffer (50 mM Tris-HCl buffer, pH 7.4 kept at

4ºC). The pellet collected after the last wash was resuspended in 3 mL lysis buffer (50 mM Tris-HCl buffer, 40% v/v glycerol, 10 mM EDTA, 5 mM PMSF, 10 mM DTT, 1 µM FAD, 1 µM FMN). Acid washed glass beads (400-500 microns) (ca. 4 g) were added to the cells (1:1 ratio (w/v)). Cells were vortexed for 30 s on high speed and then placed on ice for 1 min. The cycle was repeated 30 times.

After lysis, 27 mL ice cold dilution buffer (50 mM Tris-HCl, 0.5 M sorbitol and 5 mM PMSF) was added. The PMSF was added to the buffer just before use. The suspension was vortexed for 1 min and then centrifuged for 5 min at 5000 x ***g*** and 4ºC. The supernatant was transferred to clean centrifuge tubes (autoclaved) and centrifuged for 15 min at 12000 x ***g*** and 4ºC. The supernatant was transferred to clean centrifuge tubes (autoclaved) and 1 M calcium chloride (600 µL) was added. The microsomes precipitated and the suspension was again centrifuged for 20 min at 20000 x g and 4ºC. The pellet was resuspended in 3 mL ice cold microsomal resuspension buffer (50 mM Tris-HCl, 0.5 M sorbitol and 30% v/v glycerol). This suspension was used for CPR and cytochrome P450 activity assays. The protein concentration was also determined (Kappeli ***et al***., 1982; Scheller ***et al***., 1996; He and Chen, 2005).

3.1.9.2 CPR Assays

Reaction mixtures contained 10 µL protein suspension, 50 µL phosphate buffer (1M, pH 7.2), 3.3 µL KCN (1 M), 50 µL cytochrome c (50 µM) and 10 µL NADPH (1.1 mM) made up to 1 mL with sterile distilled water (Beaufay ***et al***., 1974). NADPH was added last. Cytochrome c was omitted from the blank and NADPH was omitted from the control. The solutions were incubated for 10 min at room temperature in the dark while absorbance was measured at 550 nm using a Beckman Coulter DU 800 spectrophotometer. Volumetric rates were determined by recording changes in absorbance for 5 min and calculating the slopes of the absorbance against time graphs. Blank rates were subtracted from the tests. A change in cytochrome c concentration was

calculated by using the extinction coefficient (21 mM^{-1} cm^{-1}). Specific activities were calculated by dividing the volumetric rate by protein concentration.

3.1.9.3 CYP Assays

Reaction mixtures contained 10 µL protein suspension, 50 µL phosphate buffer (1M, pH 7.2), substrate dissolved in 50 µL ethanol (0.5 mM) and 10 µL NADPH (0.1 mM) made up to 1 mL with sterile distilled water (Eiben ***et al***., 2007). NADPH was added last. NADPH was omitted from the blank and ethanol (50 µL) without substrate was used for the control. The solutions were incubated for 6 min at room temperature while absorbance was measured at 340 nm every 1 min using quartz cuvets. A Beckman Coulter DU 800 spectrophotometer was used to determine the absorbance. The volumetric hydroxylase activity was determined by subtracting the rate of change in absorbance per min of the control from the rate of change in absorbance per min of test. This was then divided by the extinction coefficient (6.2 mM^{-1} cm^{-1}). The volumetric hydroxylase activity was divided by protein concentration (mg L^{-1}) to determine the specific activity.

3.1.9.4 Protein estimation

Bradford reagent (Bradford, 1976) was used to determine protein concentrations. The assays for protein determinations were prepared according to Table 3.3. The absorbance was measured at 595 nm. These were used to construct standard curve, which was used to calculate protein concentrations.

Table 3.3: Experimental assay for protein concentration determination

Solution number	*Protein solution (µL)	Protein added to assay mixture (µg)	Sterile water (µL)	Bradford reagent (mL)
1	10	5	90	1
2	20	10	80	1
3	30	15	70	1
4	40	20	60	1
5	50	25	50	1
Blank	-	-	100	1
Test	5	-	95	1
Test	5	-	95	1

*Solution 1 – 5 contained albumin

*The test contained 5 µL protein sample

3.1.10 Chemicals

A stock solution of 10% (v/v) oleic acid (Saarchem) was prepared by dissolving 10 mL oleic acid, 0.1 mL NaOH (5 M) (Merck) and 0.5% v/v (0.5 mL) of tween 80 (polyoxyethylene-sorbitan monooleate) (Sigma) in 90 mL distilled water. The suspension was stirred for 24 h, autoclaved for 15 min at 121°C and stirred for another 24 h. A stock solution of 10% (w/v) sodium acetate was prepared by dissolving 10 g of anhydrous sodium acetate (Merck) in 100 mL distilled water. The solution was autoclaved.

Phosphate buffer (0.5 M, pH 8) was prepared by dissolving 17.4 g di-potassium hydrogen phosphate (K_2HPO_4) (Merck) in distilled water (100 mL) and 13.6 g potassium dihydrogen phosphate (KH_2PO_4) (Merck) in distilled water (100 mL). KH_2PO_4 solution (5.3 mL) was added to K_2HPO_4 solution (94.7 mL). This buffer was autoclaved.

Phenol red pH indicator was prepared by dissolving phenol red (50 mg) in distilled water (20 mL), 1N NaOH (200 µL, pH 10.6) was added to the solution

and it was autoclaved. The phenol-red solution (40 µL) was added to cultures (5 mL).

Bradford reagent was prepared by dissolving Brilliant Blue **G-**250 (10 mg) in 95% ethanol (5 mL). Ortho phosphoric acid (10 mL, 85% (w/v)) was added to the solution and it was diluted to a final volume of 100 mL with sterile distilled water. This was filtered through Whatman no. 1 filter paper and stored at 4ºC.

Albumin (50 mg) was dissolved in sterile distilled water (90 mL) and the volume was made up to 100 mL. The solution was stored at 4ºC.

Butylbenzene (Fluka), butylcyclohexane (Sigma-Aldrich), dodecane (Fluka), ethanol (Merck), ethyl acetate (Merck), heptylbenzene (Sigma-Aldrich), hexylbenzene (Fluka), (+)-limonene (Fluka), nonylbenzene (Sigma-Aldrich) and pentylbenzene (Fluka) were used directly.

3.2 Part B: Biotransformation procedures

3.2.1 Biotransformation of hexylbenzene by resting cells of *Yarrowia lipolytica* strain TVN 348

Y. ***lipolitica*** (TVN 348) was used for the biotransformation of hexylbenzene. Seed cultures were inoculated and incubated for 24 h on a shaker at 25°C. Optical densities were determined and main cultures were inoculated using 2.5 mL seed cultures. Main cultures were incubated in 25 mL YP_2D_2 broth in 500 mL Erlenmeyer flasks under the same conditions. After 24 h growth the following inducers were added to different flasks: (i) ethanol (1% v/v); (ii) ethanol (1% v/v) and hexylbenzene (60 mM); (iii) hexylbenzene (60 mM); (iv) oleic acid (2% w/v). A fifth and sixth flask received no inducer. The cultures were incubated for further 24 h. Samples for OD measurements and glucose assimilation were taken regularly. Samples for biomass determination were taken after 48 h of growth before the rest of the cultures were harvested and resuspended in 25 mL potassium phosphate buffer (50 mM, pH 8). Hexylbenzene (60 mM) was added to five of the flasks and in the sixth flask

that had not received an inducer before harvesting, ethanol (1% v/v) was added together with hexylbenzene (60 mM). Samples were taken at regular intervals until 72 h after substrate addition for GC analysis. Biomass was determined at the end of the experiment.

3.2.2 Biotransformation of hexylbenzene and nonylbenzene using oleic acid induced resting cells

Y. lipolitica strains TVN 91, TVN 348, TVN 493 and CTY 005 were inoculated and seed cultures and main cultures were grown as before. The same procedures as above were carried out to prepare resting cells except that only oleic acid (2% w/v) was used as an inducer. Hexylbenzene and nonylbenzene (60 mM) were added as substrates to resuspended resting cells. Samples for GC analysis were taken at regular intervals for 72 h. Biomass determination was carried out at the end of the experiment.

3.2.3 Biotransformation of butylbenzene and hexylbenzene using ethanol as an inducer

Y. lipolitica strains CTY 005, CTY 003, CTY 014(16) and CTY 014(17) were used for the biotransformation of butylbenzene and hexylbenzene. Seed cultures and main cultures were inoculated as before. Ethanol (2% v/v) was added as an inducer after 24 h growth. OD's and biomass were determined as before. In this experiment, cells were not harvested. Substrates were added after 24 h of induction (30 mM butylbenzene and 60 mM hexylbenzene). The pH of the cultures was monitored with phenol red as an indicator and adjusted to pH 8 every 12 h. Samples were taken at regular intervals for 72 h for GC analysis.

3.2.4 Biotransformation of hexylbenzene using sodium acetate as an inducer

Y. lipolitica strains CTY 005 and CTY 014(17) were used for the biotransformation of hexylbenzene. Seed cultures and main cultures were

grown as before. In this experiment, cells were not harvested. Two sets of cultures were prepared. Sodium acetate (2% w/v) was added as an inducer after 24 h growth. After 24 h of induction hexylbenzene (60 mM) was added to all the flasks. To one set of flasks, sodium acetate (1% w/v) was again added with substrate addition and every 12 h thereafter until the end of the biotransformation. OD's and biomass were determined as before. The pH of the cultures was monitored with phenol red and adjusted to pH 8 every 12 h. Samples were taken at regular intervals for 96 h. In a repeat experiment, hexylbenzene was added at a lower concentration of 30 mM.

3.2.5 Biotransformation of pentylbenzene and heptylbenzene

Y. lipolitica strains CTY 005 and CTY 014(17) were used again for the biotransformation of pentylbenzene (30 mM) and heptylbenzene (60 mM) using the same procedure as described above. Sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h thereafter.

3.2.6 Biotransformation of hexylbenzene and nonylbenzene under bioreactor conditions

Y. lipolitica strains CTY 005 and CTY 014(17) were used in this experiment. The seed culture was prepared as described before and used to inoculate the bioreactors to a starting OD of 0.2. The cultivation medium was YP_2D_2 (buffered at pH 8 using potassium phosphate buffer (0.5 mM)). The working volume of the bioreactors was 200 mL. OD's and biomass were determined as previously described. The temperature was maintained at 25ºC and aeration between 30 to 40% of saturation. The pH was maintained at 8.2. Sodium acetate (2% w/v) was added after 24 h of inoculation and substrates (30 mM) were added 24 h later (after 48 h growth). Sodium acetate (1% w/v) was added again at substrate addition and every 12 h thereafter. Samples for GC analysis were taken at regular intervals until 96 h of growth.

3.2.7 Biotransformation of alkylcyclohexanes

Y. lipolitica strains CTY 005 and CTY 014(17) were used for the biotransformation of butylcyclohexane and limonene. Seed cultures and main cultures were inoculated and grown as before. Two sets of cultures were prepared for each substrate. To one set of cultures dodecane (2% v/v) was added as an inducer after 24 h growth, while to the other set glucose (2% w/v) was added to supply an equivalent amount of energy. Cultures were incubated for a further 24 h and then the substrates butylcyclohexane (30 mM) and (+)-limonene (30 mM) were added. Regular sampling for GC analysis was done until 48 h after substrate addition in the butylcyclohexane experiment and until 72 h in the limonene experiment.

Chapter 4

Results

4.1 Experiments with strains having *CYP* genes cloned under *pPOX2*

Y. ***lipolytica*** strains with multiple copies of different ***CYP*** genes cloned under ***pPOX2*** had previously been tested for the biotransformation of alkylbenzenes (van Rooyen, 2005). The biotransformation reactions were carried out with early stationary phase cultures with inducers added after 24 h and alkylbenzene substrates 24 h later. Cells were not harvested. It was difficult to determine whether the substrates selectively induced the cloned ***CYP*** genes or whether the activities observed were due to the endogenous ***CYP*** genes. At the beginning of this study, experiments were conducted using harvested cells in order to observe the difference when cells were harvested after induction and if induction can take place after cells were harvested and resuspended in a buffer.

4.1.1 Biotransformation of hexylbenzene by differently induced resting cells of *Yarrowia lipolytica* strain TVN 348

In this experiment harvested resting cells of ***Y***. ***lipolytica*** strain TVN 348 were tested for the degradation of hexylbenzene. Multiple copies of ***CYP557A1***, a putative alkane or fatty acid hydroxylase from ***Rhodotorula retinophila*** was previously cloned into TVN 348 under ***pPOX2*** which is induced by alkanes and fatty acids (Juretzek ***et al***., 2000; Madzak ***et al***., 2004). This strain also has an additional CPR (cytochrome P450 reductase) cloned under ***pICL*** which is induced by ethanol and acetate as well as alkanes and fatty acids (Juretzek ***et al***., 2000).

After 24 h growth in YP_2D_2 broth, different compounds (oleic acid, ethanol, hexylbenzene, ethanol and hexylbenzene) were added as inducers and/or substrates (Fig. 4.1). In two flasks nothing was added. After a further 24 h of

incubation, cells were harvested and resuspended in buffer. Turbidity measurements as well as glucose assimilation were determined at regular intervals during the first 48 h of growth until just before cells were harvested. Induction with oleic acid (2% w/v) yielded the highest biomass (12.4 g L^{-1}), while the addition of hexylbenzene resulted in the lowest biomass (6.7 g L^{-1}), possibly indicating that hexylbenzene affected the viability of the cells. Biomass was almost the same when nothing was added (11.2 g L^{-1}) and when ethanol was added (11.4 g L^{-1}), while biomass obtained when ethanol was added with hexylbenzene (9.7 g L^{-1}) seemed to indicate that ethanol counteracted the negative effect of the hexylbenzene. OD measurements at 48 h did not reflect the biomass measurements (Fig. 4.1a). The flasks with hexylbenzene (60 mM) added and with oleic acid (2% w/v) added gave the highest ODs (between 11 and 12) and the other three flasks reached an OD of 9. Oleic acid and hexylbenzene are both insoluble in water and it is possible that this affected the OD measurements. The amount of glucose left at 48 h was 8.2 g L^{-1} in the flask that had hexylbenzene added, while 4.8 g L^{-1} glucose was left in the flask with oleic acid added (Fig. 4.1b). In the flasks with nothing added to the glucose was consumed after 48 h and with only ethanol added 1.5 g L^{-1} was left. The glucose results support the conclusion from the biomass results, that hexylbenzene added after 24 h growth was probably toxic to the cells.

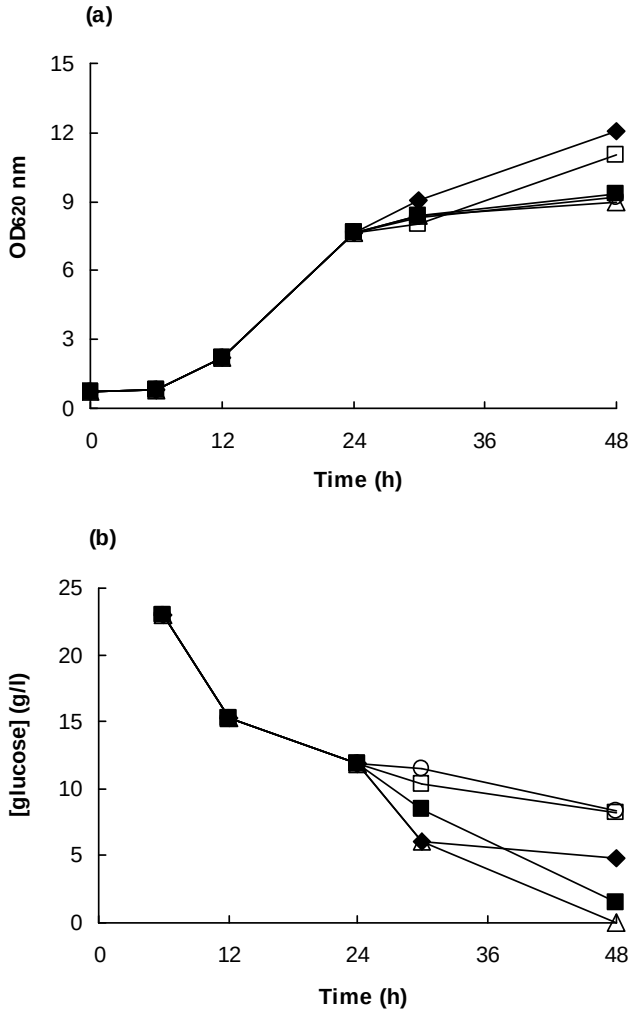


Figure 4.1: Graph A shows the growth at 620 nm and graph B shows the assimilation of glucose by ***Y. lipolytica*** TVN 348. After 24h growth different compounds were added as inducers in five different flasks: No inducer was added (△); ethanol (1% v/v) (■); ethanol (1% v/v) and hexylbenzene (60 mM) (○); hexylbenzene (60 mM) (□) and oleic acid (2% w/v) (◆). Dry weights when cells were harvested: no inducer added 11.2 g L^{-1}, after ethanol induction 11.4 g L^{-1}, induction with ethanol and hexylbenzene 9.7 g L^{-1}, induction with hexylbenzene 6.7 g L^{-1} and with oleic acid 12.4 g L^{-1}.

After the harvested cells had been resuspended, hexylbenzene (60 mM) was added as substrate to six flasks. In the one case where nothing had been added before harvesting, ethanol (1% v/v) and hexylbezene (60 mM) were added to the resuspended cells.

When samples taken at regular intervals were subjected to GC analysis, less than 30 mM of hexylbenzene was recovered from all the samples (Fig. 4.2).

This can be ascribed to poor sampling of the hydrophobic substrate. At the end of the experiment, hexylbenzene almost completely disappeared from two flasks; the one in which the cells had been induced with oleic acid and the one where ethanol was the inducer. Hexylbenzene (only one sampling point) almost disappeared from the flask with the cells that had not received any inducer, but to which ethanol and hexylbenzene were added after resuspension. In the two cases where cells were either induced with hexylbenzene or with hexylbenzene and ethanol, there was still about 30 mM hexylbenzene left after 72 h of the biotransformation reactions, again supporting the idea that hexylbenzene added before harvesting had affected the viability of the cells (Fig. 4.2a).

The samples from the three flasks with cells induced with hexylbenzene, uninduced cells with only hexylbenzene added and uninduced cells with ethanol and hexylbenzene added, contained insignificant amounts of phenylacetic acid. Only in the case where cells had been induced with oleic acid, almost all the hexylbenzene was converted to phenylacetic acid (58 mM). Cells induced with ethanol produced a maximum of only 27 mM of phenylacetic acid after 48 h and the product (15 mM) was less at 72 h (Fig. 4.2b).

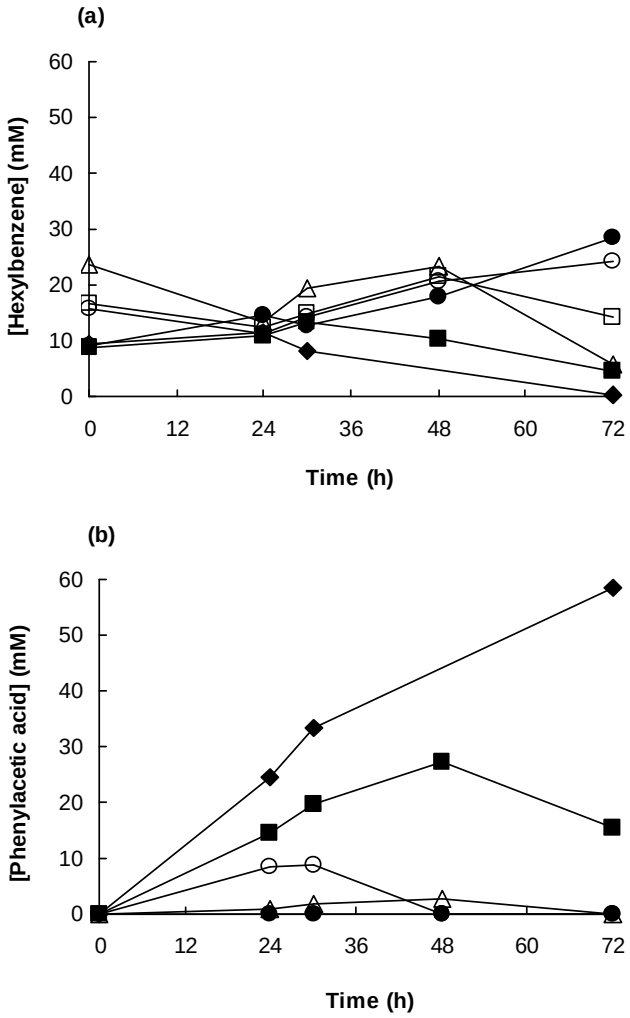


Figure 4.2: The biotransformation of hexylbenzene (60 mM) by resting cells of ***Y. lipolytica*** TVN 348. After 24h growth different compounds were added as inducers: ethanol (1% v/v) (■); ethanol (1% v/v) and hexylbenzene (60 mM) (○); hexylbenzene (60 mM) (●); oleic acid (2% w/v) (◆); No inducer was added to two cultures. After the cells had been resuspended, ethanol (1% v/v) and hexylbenzene (60 mM) (△) were added to cells from one of the latter cultures, while only hexylbenzene (60 mM) was added to cells from the other culture (□). Hexylbenzene (60 mM) was also added to cells from all the induced cultures. Graph A shows remaining hexylbenzene and graph B shows formation of phenylacetic acid. Dry weights after cell harvesting (i.e. before substrate addition) were after ethanol induction 6.58 g L^{-1}, after induction with ethanol and hexylbenzene 6.38 g L^{-1}, after induction with oleic acid 8.28 g L^{-1}, and when no inducer was added it was (△) 8.48 g $L^{-1}$and (□) 6.5 g L^{-1}.

4.1.2 Biotransformation of hexylbenzene and nonylbenzene using oleic acid as an inducer

In the next experiment four strains of ***Y. lipolytica*** TVN 91, TVN 348, TVN 493 and CTY 005 were tested for the biotransformation of hexylbenzene (60 mM) and nonylbenzene (60 mM) by resting cells that had been induced with oleic acid. All the strains had an extra CPR cloned under ***pICL*** and all except CTY 005 had extra cytochrome P450s cloned under ***pPOX2***. TVN 91 had multiple copies of ***CYP53B1***, a benzoate ***para*** hydroxylase from ***Rhodotorula minuta*** cloned. Multiple copies of ***CYP557A1***, a putative alkane or fatty acid hydroxylase from ***Rhodotorula retinophila*** had been cloned into TVN 348 and TVN 493 had multiple copies of ***CYP52F2***, an alkane hydroxylase from ***Y. lipolytica*** cloned.

Oleic acid (2% w/v) was added as an inducer before cells were harvested and resuspended and substrates added. In this experiment, we expected to observe differences in the alkylbenzene biotransformation activities due to the different cloned P450 genes. In the flasks where hexylbenzene was added as a substrate, almost 53 mM of hexylbenzene was recovered with TVN 348, with the other strains about 40 mM was recovered (Fig. 4.3a). The flasks with CTY 005, TVN 348 and TVN 493 had no hexylbenzene in the 48 h and 72 h samples, but hexylbenzene only disappeared at 72 h in the flask with TVN 91. All the strains except TVN 91 showed maximum phenylacetic acid production (ca. 45 mM) after 48 h. TVN 91 only showed the maximum phenylacetic acid production (42 mM) after 72 h (Fig. 4.3b).

Nonylbenzene was also tested using resting cells of the same strains treated in the same way. At 72 h nonylbenzene disappeared from all the flasks except the one with TVN 91 (Fig. 4.4a). The maximum concentration of benzoic acid accumulated (ca. 27 mM) was almost the same in all the flasks except the one containing TVN 91 (Fig. 4.4b). TVN 91 also converted some of the benzoic acid to small amounts of ***para***-hydroxybenzoic acid. This indicated that benzoate ***para***-hydroxylase activity was induced by oleic acid and retained after the cells were harvested (Obiero, 2006). When benzoic acid

and ***para***-hydroxybenzoic acid produced by TVN 91 were added together, the sum of products (19 mM) was still less than the benzoic acid produced by other strains (ca. 27 mM).

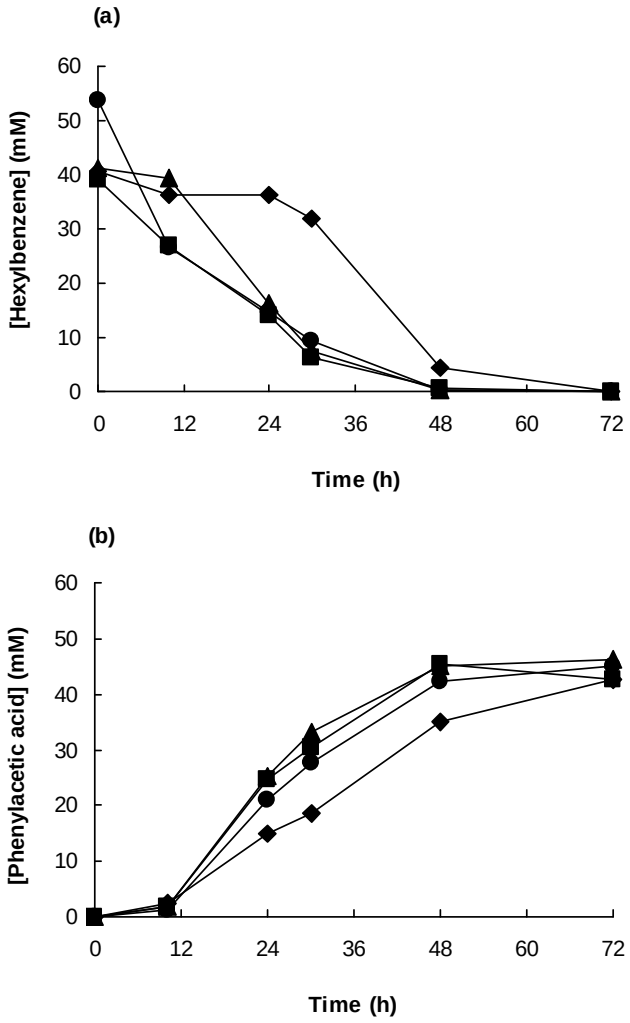


Figure 4.3: The biotransformation of hexylbenzene (60 mM) by resting cells of ***Y. lipolytica*** strains. Oleic acid (2% w/v) was added as an inducer after 24 h of growth and cells were harvested 24 h later. Graph A shows the degradation of hexylbenzene and B the formation of phenylacetic acid by TVN 91 (◆), TVN 348 (●), TVN 493 (▲) and CTY005 (■). Dry weights of resuspended cells: TVN 91 (9.3 g L^{-1}), TVN 348 (15.9 g L^{-1}), TVN 493 (13.8 g L^{-1}) and CTY005 (16 g L^{-1}).

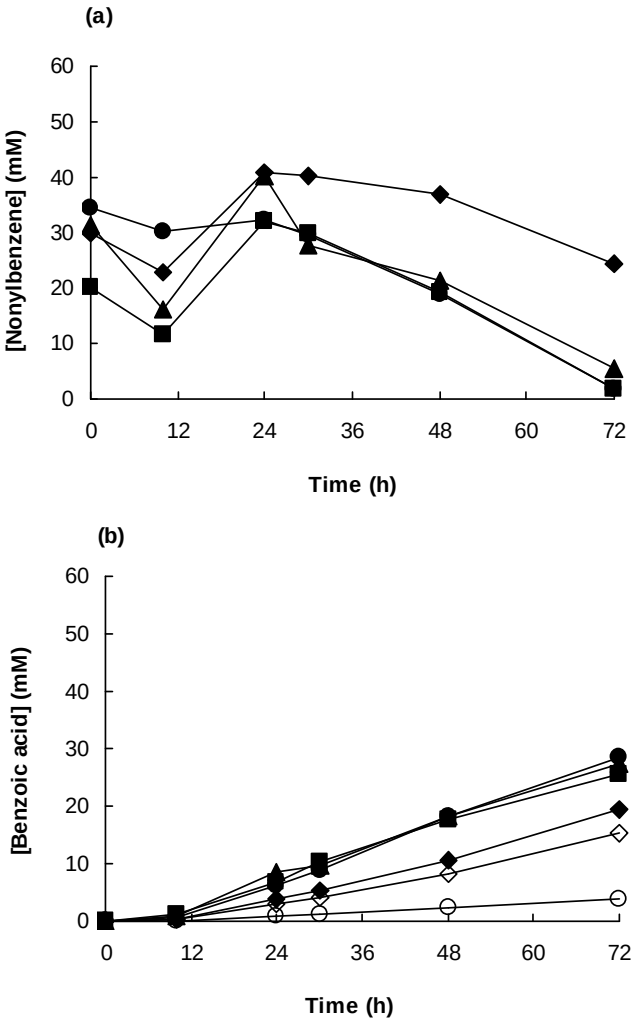


Figure 4.4: The biotransformation of nonylbenzene (60 mM) by ***Y. lipolytica*** strains. Oleic acid (2% w/v) was added as an inducer after 24 h of growth. Graph A shows the degradation of nonylbenzene and B the formation of benzoic acid by TVN 348 (●), TVN 493 (▲), CTY005 (■) as well as ***p***-hydroxybenzoic acid production by TVN 91 (○), benzoic acid production by TVN 91 (◇) and sum of products produced by TVN 91 (◆).

TVN 91 was the only strain that showed significantly lower activity with both hexylbenzene and nonylbenzene. However, the flasks with TVN 91 contained less biomass and when the specific activities were calculated, all the strains had almost the same activities except TVN 493, which had slightly higher activities with both hexylbenzene and nonylbenzene (Table 4.1).

Table 4.1: Specific activities calculated for the biotransformation of hexylbenzene and nonylbenzene as substrates

Strain	Biomass of resuspended cells ($g\ L^{-1}$)	Specific activity ($\mu mol\ min^{-1}\ g\ dry\ weight^{-1}$)	
		Hexylbenzene	Nonylbenzene
TVN 91	9.3	1.45	0.48
TVN 348	15.9	1.47	0.45
TVN 493	13.8	1.93	0.61
CTY 005	16	1.56	0.52

4.2. Experiments with strains having *CYP* genes cloned under *pICL*

4.2.1 Cytochrome P450 reductase (CPR) and Cytochrome P450 (CYP) Assays

Y. lipolytica strains CTY 005, CTY 014(16) and CTY 014(17) were used to conduct enzyme assays with microsomal fractions (Table 4.2) obtained from cells harvested after induction with ethanol. CTY 005 was used as a control strain because it had no additional ***CYP*** genes cloned. The two strains, CTY 014(16) and CTY 014(17) had ***CYP557A1,*** a putative fatty acid hydroxylase cloned under the control of ***pICL***. All the strains had additional ***CPR*** genes cloned under ***pICL***.

CPR activity was determined by measuring the rate of cytochrome c reduction. This is measured by an increase in absorbance at 550 nm. The same CPR activity was expected for all three strains, since all three strains had been derived from the same strain TVN 496 with an additional CPR gene cloned under ***pICL***. However, CTY 014(17) had a specific activity (0.09 µmol min^{-1} mg $protein^{-1}$), which was higher than that of the other two strains.

Table 4.2: Specific activities obtained from the CPR and CYP assays

Strain	Microsomal protein (mg protein ml culture^{-1})	Specific enzyme activity (µmol min^{-1} mg protein^{-1})			
		CPR	CYP		
			Dodecane	Hexylbenzene	Nonylbenzene
CTY 005	0.19	0.07	0.017	0.008	0.018
CTY 014(16)	0.20	0.06	0.001	0.005	0.018
CTY 014(17)	0.15	0.09	0.015	0.068	0.046

Hydroxylase activities were determined with the same microsomal fractions by following the consumption of NADPH in the presence of three substrates namely dodecane, hexylbenzene and nonylbenzene. NADPH concentration is determined by measuring absorbance at 340 nm. With hexylbenzene and nonylbenzene as substrates CTY 014(17) showed CYP activity that was higher than the activity observed with CTY 005 and CTY 014(16). With dodecane as substrate CTY 014(17) and CTY 005 had similar activities, while CTY 014(16) had very low activity. It should be noted that when southern hybridisation experiments were performed with CTY 014(16) and CTY 014(17) to detect the presence of the cloned ***CYP557A1*** gene, CTY 014(16) did not give the expected result but CTY 014(17) showed the presence of the cloned ***CYP557A1*** gene (K. Syed, personal communication).

It was expected that strains with CYP557A1 cloned should show increased activity with both dodecane and the alkylbenzene, since the CYP557A1 gene had been cloned from decane grown cells of ***Rhodotorula retinophila*** (Shiningavamwe, 2004). The activities obtained with the alkylbenzenes were thus as expected, but not the results obtained with dodecane. The results obtained with this experiment should be treated with caution, since it is based on single determination. However, similar results were obtained in a second experiment.

4.2.2 Biotransformation of butylbenzene and hexylbenzene when using ethanol as an inducer

The results obtained with the microsomal fractions were promising, but literature searches revealed that it is not common practice to use NADPH consumption to measure P450 hydroxylase activity with crude microsomal fractions. It was therefore decided to rather continue with whole cell biotransformation experiments.

Biotransformation of butylbenzene and hexylbenzene was subsequently tested using ***Y. lipolytica*** strains CTY 003, CTY 014(16), CTY 014(17) and CTY 005. CTY 005 and CTY 003 were used as controls. CTY 003 had neither any ***CYP*** nor ***CPR*** gene cloned. After 24 h of growth in YP_2D_2 broth ethanol (2% v/v) was added as inducer and 24 h later (48 h growth) the substrates, butylbenzene (30 mM) and hexylbenzene (60 mM) were added. Thus, in this experiment cells were not harvested, because in the preceding experiments with harvested cells hexylbenzene had disappeared from the flasks without concomitant formation of phenylacetic acid when ethanol was added as inducer. It was also difficult to obtain reproducible results with the harvested cells because activity was easily lost during harvesting (data not shown).

Figure 4.5a shows the disappearance of butylbenzene from all the flasks 48 h after substrate addition. There is a difference observed between the activities of the strains in the formation of phenylacetic acid (Fig. 4.5b). CTY 014(16) and CTY 014(17), which are the strains with ***CYP557A1*** genes cloned had more product formed compared to the control strains, CTY 003 and CTY 005. The highest concentration of phenylacetic acid accumulated (4.7 mM) was obtained in the biotransformation with CTY 014(17) after 72 h. The flask with CTY 014(16) accumulated only 2.9 mM and the other two strains only accumulated around 1 mM of phenylacetic acid. Table 4.3 shows the volumetric and specific activities of these strains. CTY 014(17) had the highest volumetric and specific activities (0.077 mM h^{-1} and 0.12 µmol min^{-1} g dry $weight^{-1}$ respectively). CTY 014(16) showed almost the same activities as CTY 014(17). This did not fit in with the results obtained with the CYP activity

assays and with southern hybridisation analysis. The two control strains had lower activities when compared with the strains with extra ***CYP557A1*** genes cloned. The amount of product accumulated did not account for the amount of substrate added (30 mM). It is possible that butylbenzene might be evaporating, as it is volatile.

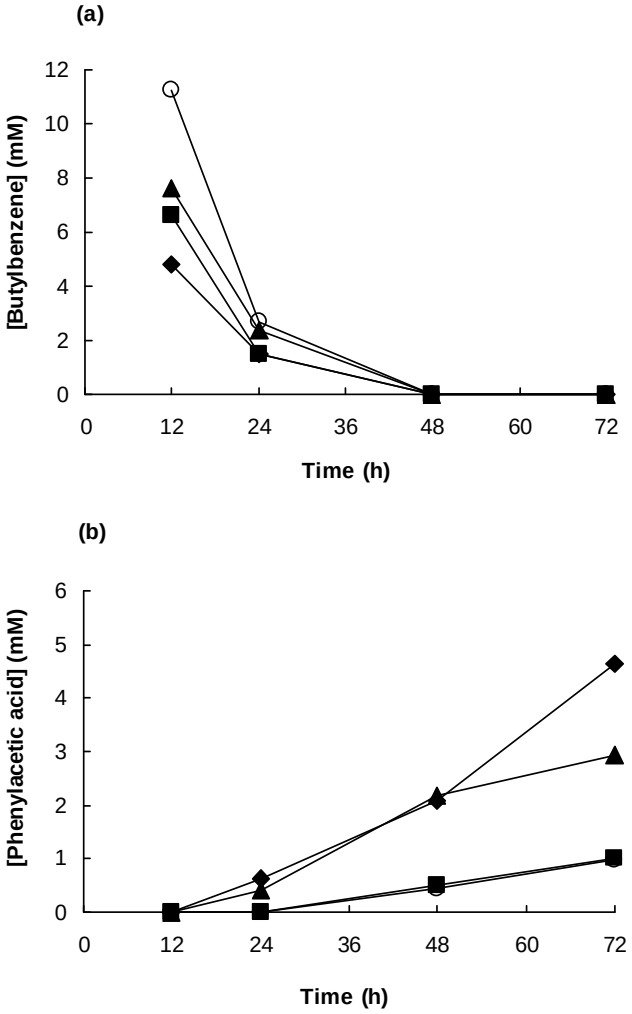


Figure 4.5: The biotransformation of butylbenzene (30 mM) by ***Y. lipolytica*** strains. Ethanol (2% v/v) was added as an inducer after 24 h of growth. Graph A shows the degradation of butylbenzene and B the formation of phenylacetic acid, CTY 003 (○), CTY 014(16) (▲), CTY 014(17) (◆) and CTY 005 (■). Dry weights of cells before substrate addition: CTY 003 (8.95 g L^{-1}), CTY 005 (11.75 g L^{-1}), CTY 014(16) (8.63 g L^{-1}) and CTY 014(17) (11 g L^{-1}).

Table 4.3: Volumetric and specific activities obtained from the biotransformation of butylbenzene and hexylbenzene as substrates

Strain	*Biomass ($g\ L^{-1}$)	Volumetric activity ($mM\ h^{-1}$)		Specific activity ($\mu mol\ min^{-1}\ g\ dry\ weight^{-1}$)	
		BB	HB	BB	HB
CTY 003	8.95	0.017	0.49	0.032	0.91
CTY 005	11.75	0.018	1.02	0.025	1.45
CTY 014(16)	8.63	0.052	0.52	0.10	1.00
CTY 014(17)	11.00	0.077	0.49	0.12	0.74

***** Biomass at 48 h of growth

BB-Butylbenzene

HB- Hexylbenzene

In the biotransformation of hexylbenzene, less than 25 mM was initially recovered from all the flasks and it disappeared almost completely in all the samples at 72 h (Fig. 4.6a). CTY 005 converted almost all the hexylbenzene to phenylacetic acid (60 mM) but the other three strains only accumulated 30 mM of phenylacetic acid although all the substrate was consumed at 72 h (Fig. 4.6a and b). In this experiment phenylbutanoic acid was for the first time observed as an intermediate in the degradation of hexylbenzene. CTY 005, CTY 014(16) and CTY 014(17) accumulated between 1.4 mM and 2 mM at 24 and 48 h but the concentration dropped at 72 h. CTY 003 accumulated a maximum of only 0.5 mM phenylbutanoic acid at 72 h.

CTY 005 had the highest volumetric and specific activities for product formation from hexylbenzene (Table 4.3). CTY 014(16) and CTY 014(17) had approximately the same volumetric activities, but CTY 014(17) had lower specific activity. When considering the specific activities of product formation CTY 003 and CTY 014(17) had almost the same activity, but hexylbenzene disappeared more quickly from the flask with CTY 014(17) and phenylbutonoic acid was produced and consumed more quickly by CTY 014(17). Comparing the results from the biotransformation of butylbenzene and hexylbenzene, the strains have higher activity towards hexylbenzene.

Repeat experiments were done with ethanol as an inducer, but the results obtained were variable and biotransformation of butylbenzene was not observed again. The addition of ethanol resulted in a drop in pH and it was difficult to monitor and control the pH in shake flasks. Before substrate addition the pH was between 6.7 and 7.5. This could be the reason why the results were variable.

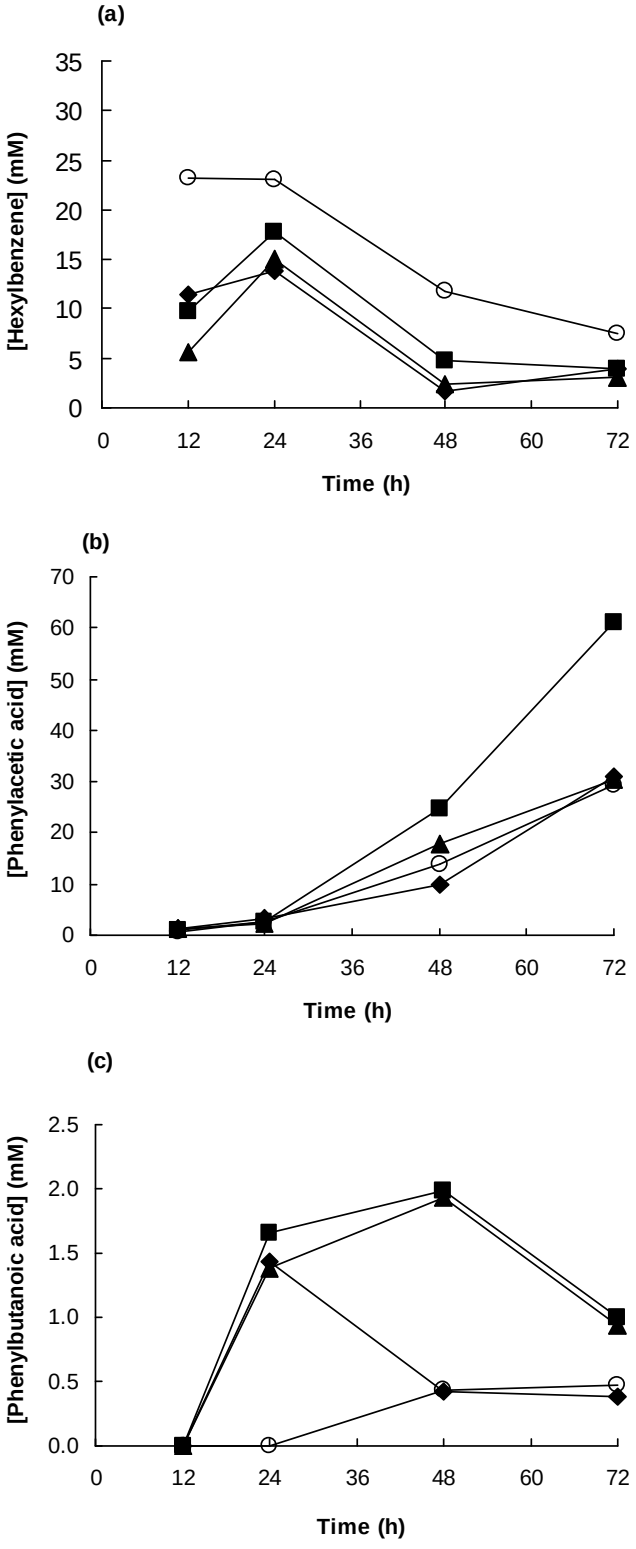


Figure 4.6: The biotransformation of hexylbenzene (60 mM) by ***Y. lipolytica*** strains. Ethanol (2% v/v) was added as an inducer after 24 h of growth. CTY 003 (○), CTY 014(16) (▲), CTY 014(17) (◆) and CTY 005 (■). Graph A shows the degradation of hexylbenzene and B the formation of phenylacetic acid and (c) formation of phenylbutanoic acid.

4.2.3 Biotransformation of pentylbenzene, hexylbenzene and heptylbenzene by *Y. lipolytica* strain CTY 005 when using ethanol as an inducer

The biotransformation of pentylbenzene, hexylbenzene and heptylbenzene was tested using only the control strain CTY 005 (Fig. 4.7). Pentylbenzene and heptylbenzene were chosen because ***Y. lipolytica*** cannot degrade benzoic acid, which is the product of the biotransformation of these substrates (Obiero, 2006). It was hoped that improved product recovery would be achieved. Ethanol (1%) was added as an inducer after 24 h of growth. Only two sets of samples were analysed (72 h and 96 h). With pentylbenzene 23 mM of substrate was extracted from the 96 h samples while for hexylbenzene and heptylbenzene the amount of substrate left at 96 h was between 14 and 16 mM. Pentylbenzene gave the lowest conversion with a maximum of 5 mM benzoic acid detected at 72 h. The maximum concentration of benzoic acid obtained from heptylbenzene was 22 mM while hexylbenzene was converted to three times more phenylacetic acid (62 mM). The results obtained showed that both pentylbenzene and heptylbenzene did not evaporate. The lower activity towards pentylbenzene might be because it is toxic to the cells or because pentylbenzene does not induce the endogenous P450 encoding genes. However, more product was formed than from butylbenzene. Hexylbenzene appears to be the preferred substrate for ***Y. lipolytica*** since activity towards heptylbenzene was also lower. With the oleic acid induced resting cells, activity towards nonylbenzene had also been lower than the activity towards hexylbenzene.

There must have been an error with substrate addition or with analysis in this experiment since the sum of product and substrate recovered from the hexylbenzene transformation was more than 60 mM. Results obtained in this experiment should therefore be interpreted with caution.

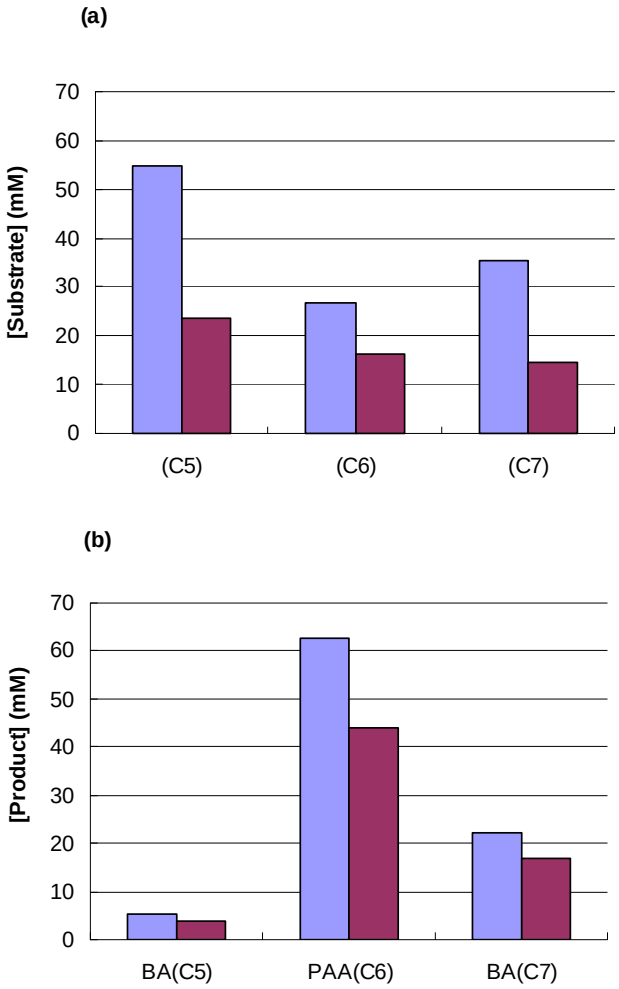


Figure 4.7: The biotransformation of pentylbenzene (C5, 60 mM), hexylbenzene (C6, 60 mM) and heptylbenzene (C7, 60 mM) by ***Y. lipolytica*** strains CTY 005. Ethanol (1% v/v) was added as an inducer after 24 h growth. Graph A shows the utilization of substrates and graph B the formation of products at 72 (■) and 96 h (■).Biomass at 48 h of growth was 10 g L^{-1}

4.2.4 Biotransformation of hexylbenzene when using sodium acetate as an inducer

Sodium acetate is also an inducer of ***pICL*** (Juretzek ***et al***., 2000). ***Y. lipolytica*** strains CTY 005 and CTY 014(17) were tested for the biotransformation of hexylbenzene after induction with sodium acetate. Two sets of flasks were prepared. To both sets sodium acetate (2% w/v) was added after 24 h of growth and 24 h after induction hexylbenzene (60 mM) was added as a substrate. To one set of flasks, sodium acetate (2% w/v) was also added at

substrate addition and then every 12 h thereafter. In the case where sodium acetate was added once, the concentration of hexylbenzene extracted from the samples remained at ca. 30 mM throughout the biotransformation (Fig. 4.8) and the amount of phenylacetic acid produced was not significant (around 2 mM). In the set of flasks where sodium acetate was also added at substrate addition and every 12 h thereafter hexylbenzene (60 mM) was completely consumed after 96 h. At substrate addition (48 h growth) the biomass of CTY 005 was 9 – 10 g L^{-1} while the biomass of CTY 014(17) was 10 – 11 g L^{-1}.

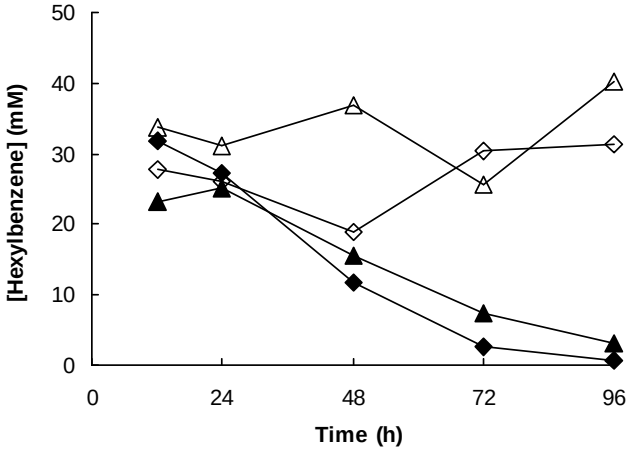


Figure 4.8: The biotransformation of hexylbenzene (60 mM) by ***Y. lipolytica*** strains. CTY 005 (△) and CTY 014(17) (◇), when sodium acetate (2% w/v) was added once as an inducer after 24 hours of growth. CTY 005 (▲) and CTY 014(17) (◆) when sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h thereafter.

Hexylbenzene was again converted to phenylacetic acid and phenylbutanoic acid (Fig. 4.9). There was a difference between the strains as CTY 014(17) produced more acids than CTY 005. Also in this case the amount of acid products recovered was considerably less than the amount of substrate added. The highest concentrations of acid products (sum of phenylacetic acid and phenylbutanoic acid) recovered were 19 mM from CTY 014(17) and only 9 mM from CTY 005. Four repeat experiments gave variable results and similar results were obtained in only one of these experiments. In three

experiments, phenylacetic acid concentrations were less than 5 mM for both strains and in the best experiment of the four about 8 mM of phenylacetic acid was accumulated by CTY 005 and 6 mM by CTY 014(17).

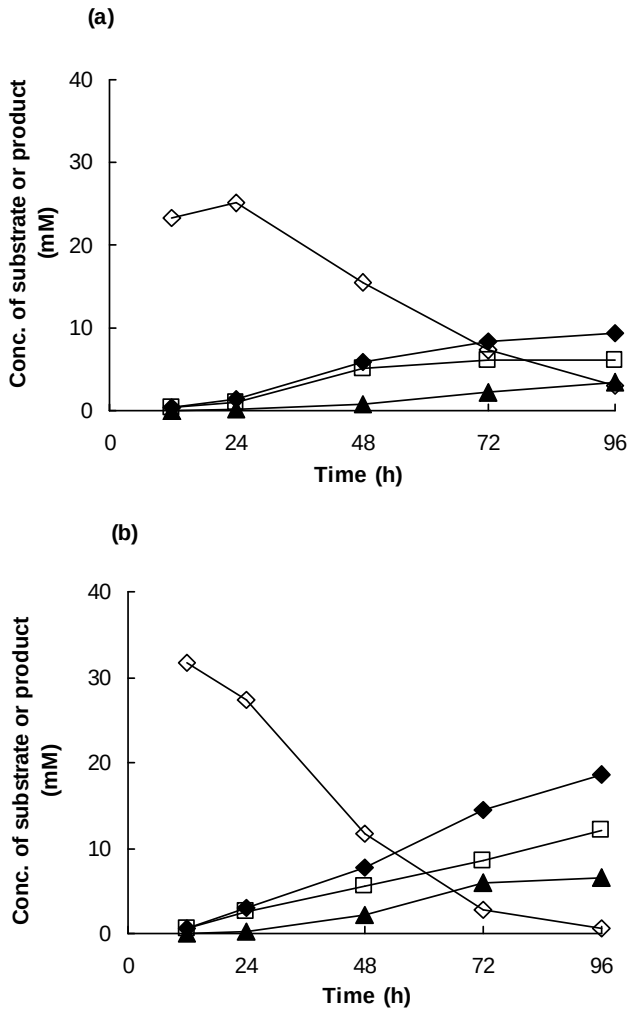


Figure 4.9: The biotransformation of hexylbenzene (◇), sum of acid products (phenylacetic acid and phenylbutanoic acid) (◆), formation of phenylbutanoic acid (▲) and phenylacetic acid (□) by ***Y. lipolytica*** strains. (A) CTY 005 and (B) CTY 014(17). Sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h thereafter.

In the experiment that gave similar results, hexylbenzene (30 mM) was consumed after 96 hours of biotransformation (Fig. 4.10a). In this experiment, GC-MS was used to confirm the formation of phenylhexanoic acid and phenylbutanoic acid as products (Fig. 4.12 and 4.13). This was the only

experiment where phenylhexanoic acid was also detected as a product. Phenylhexanoic acid was formed only transiently. It was present in only one sample, the 48 h sample, of CTY 014(17). The phenylhexanoic acid concentration in this sample was 4 mM (Fig. 4.10b). This acid never reached a concentration of more than 1 mM in the control strain (Fig. 4.10a).

CTY 014(17) appeared to have the fastest product formation since it accumulated more than 1 mM of phenylhexanoic acid and the total acid products reached 9 mM after 48 h, while CTY 005 reached only 7 mM at 72 h. No substrate was extracted from the 96 h samples for both strains. However, the product formed did not account for the substrate that was consumed and the total acid products concentrations also decreased after 72 h. Less than 5 mM of the substrate was recovered from all the samples.

The amount of the product recovered was expected to be the same as the amount of substrate added, that is a 100% conversion of a substrate into a product is expected. However, in the last sets of experiments there was a low concentration of products recovered even though all the substrate was apparently consumed. The possible explanations for this might be poor sampling because the substrate is not clinging to the cells, evaporation or degradation of the phenylacetic acid or incorporation of phenylalkanoic acids into lipids or other secondary metabolites. The experiment where sodium acetate was added once as an inducer showed that evaporation could not have been the problem since 30 mM of hexylbenzene was extracted throughout the experiment.

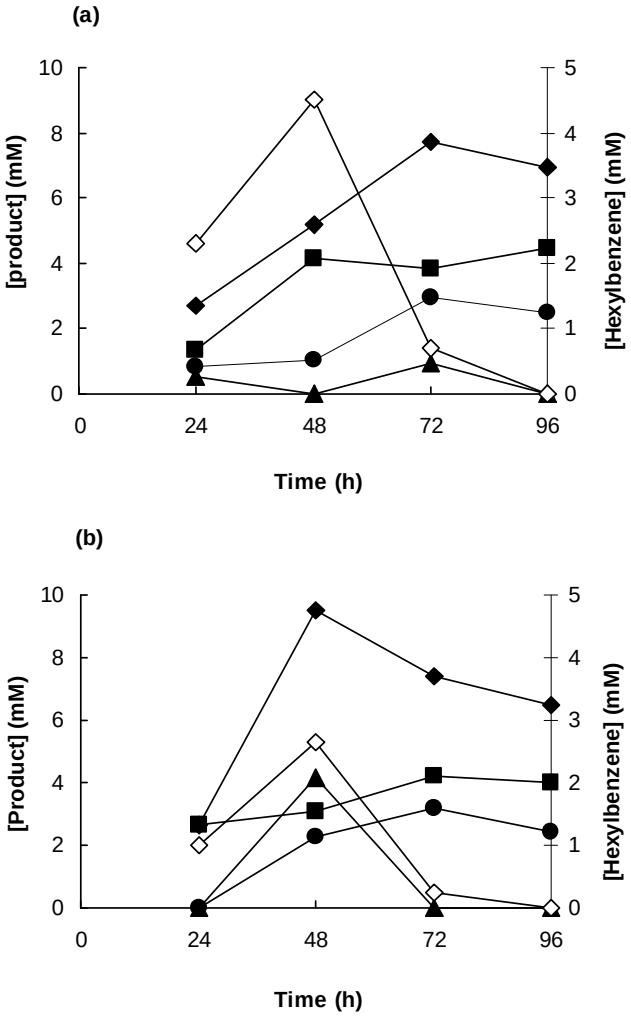


Figure 4.10: The biotransformation of hexylbenzene (30 mM) by ***Y. lipolytica*** strains CTY 005 (A) and CTY 014(17) (B). Sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h thereafter. Degradation of hexylbenzene (◇), sum of acid products (◆), formation of phenylbutanoic acid (●), phenylhexanoic acid (▲) and phenylacetic acid (■). CTY 005 had a biomass of 9.11 g L^{-1} at substrate addition (48 h of growth) and CTY 014(17) 8.39 g L^{-1}.

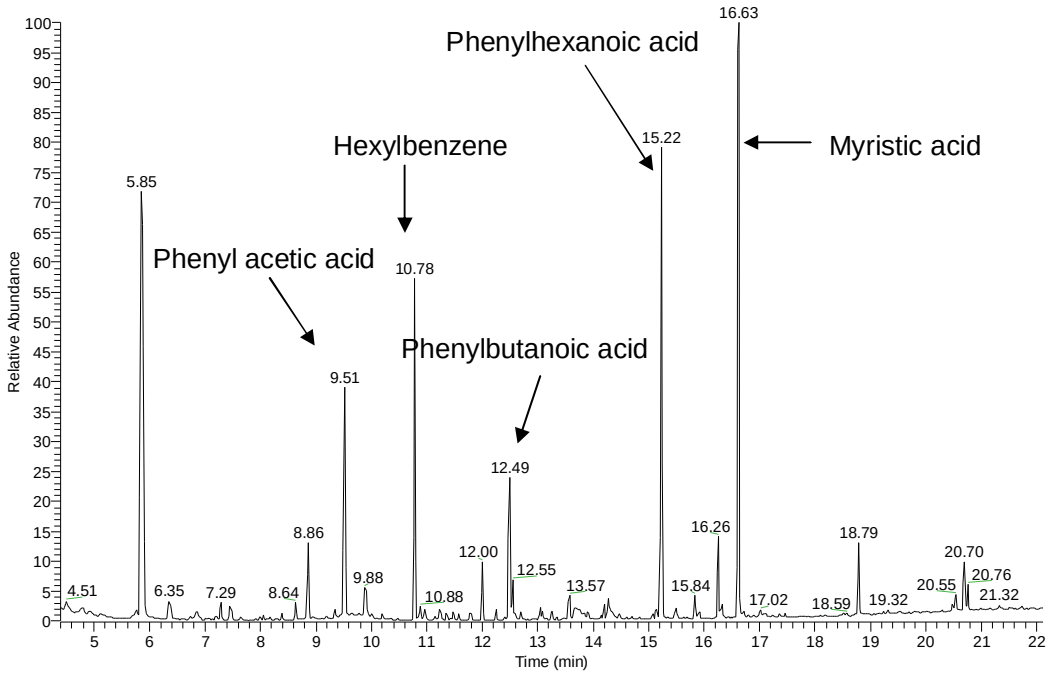


Figure 4.11: GC chromatogram of methylated extract from hexylbenzene biotransformation by ***Y. lipolytica*** strain CTY 014(17) (48 h sample).

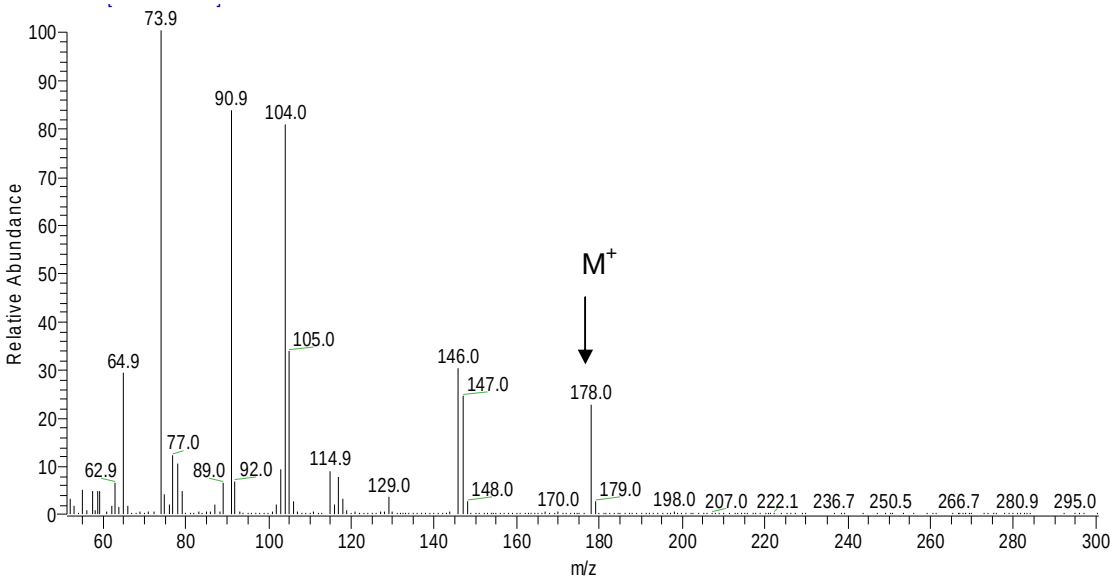


Figure 4.12: Mass spectrum of methyl ester of phenylbutanoic acid from the biotransformation of hexylbenzene by ***Y. lipolytica*** strain CTY 014(17) (48 h sample).

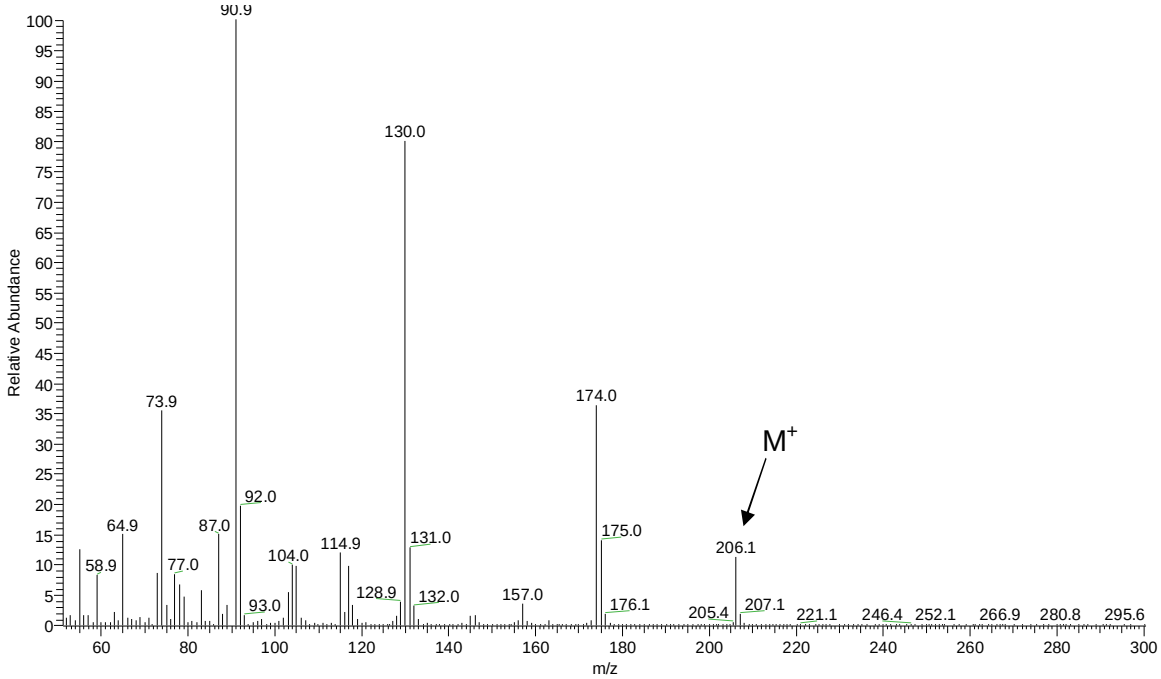


Figure 4.13: Mass spectrum of methyl ester of phenylhexanoic acid from the biotransformation of hexylbenzene by ***Y. lipolytica*** strain CTY 014(17) (48 h sample).

4.2.5 Biotransformation of pentylbenzene and heptylbenzene when using sodium acetate as an inducer

The biotransformation of pentylbenzene and heptylbenzene was tested in a subsequent experiment using strains CTY 005 and CTY 014(17) with frequent addition of sodium acetate as described above.

With both pentylbenzene and heptylbenzene, very little substrate was extracted from the samples. Less than 3 mM pentylbenzene and less than 5 mM heptylbenzene was recovered from all samples. All the pentylbenzene disappeared at 96 h and heptylbenzene remained at about 4 mM (Fig. 4.14 and Fig. 4.15). Benzoic acid recovery from both pentylbenzene (Fig. 4.14b) and heptylbenzene (Fig. 4.15b), was also very low. Only about 6 mM was accumulated from 30 mM of pentylbenzene and 8 mM from 60 mM heptylbenzene. There was no difference between the activities of the strains as both the control (CTY 005) and the test strain (CTY 014(17)) produced almost the same amounts of benzoic acid. The biomass of both the strains at substrate addition was also the same (CTY 005 10.8 g L^{-1} and CTY 014(17) 10.1 g L^{-1}). The poor recovery of substrate from the samples might have been due to poor sampling. A possible explanation for this might have been that the cells did not become hydrophobic in the presence of these substrates as with hexylbenzene and that the hydrophobic substrates did not cling to the cells, but floated on top, despite vigorous shaking before sampling.

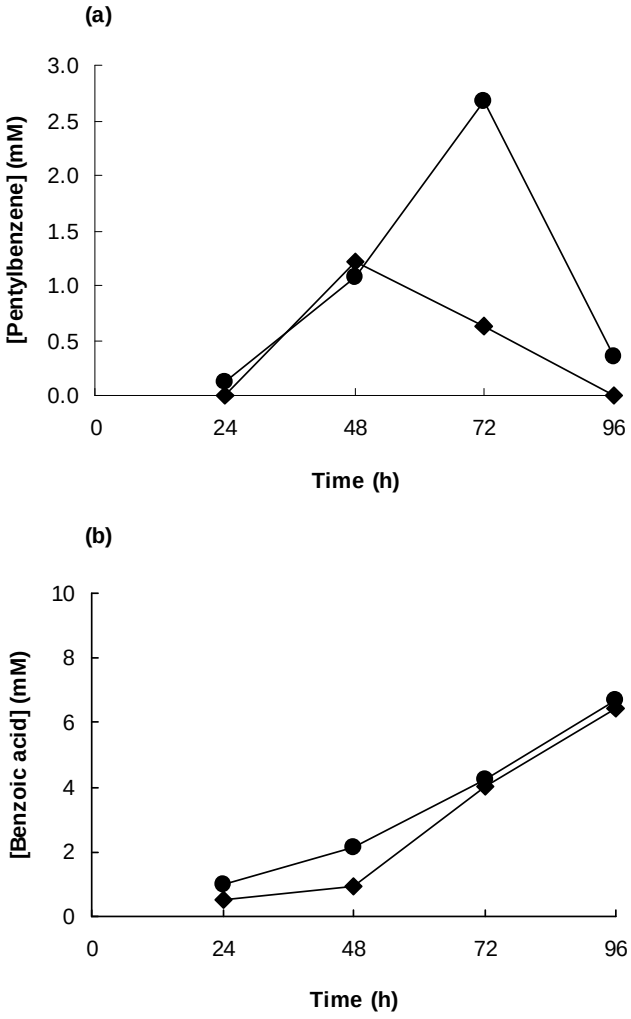


Figure 4.14: The biotransformation of pentylbenzene (30 mM) by ***Y. lipolytica*** strains CTY 005 (●) and CTY 014(17) (◆). Sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h there after. Biomass at 48 h of growth: CTY 005 10.8 g $L^{-1}$and CTY 014(17). 10.1 g L^{-1}.

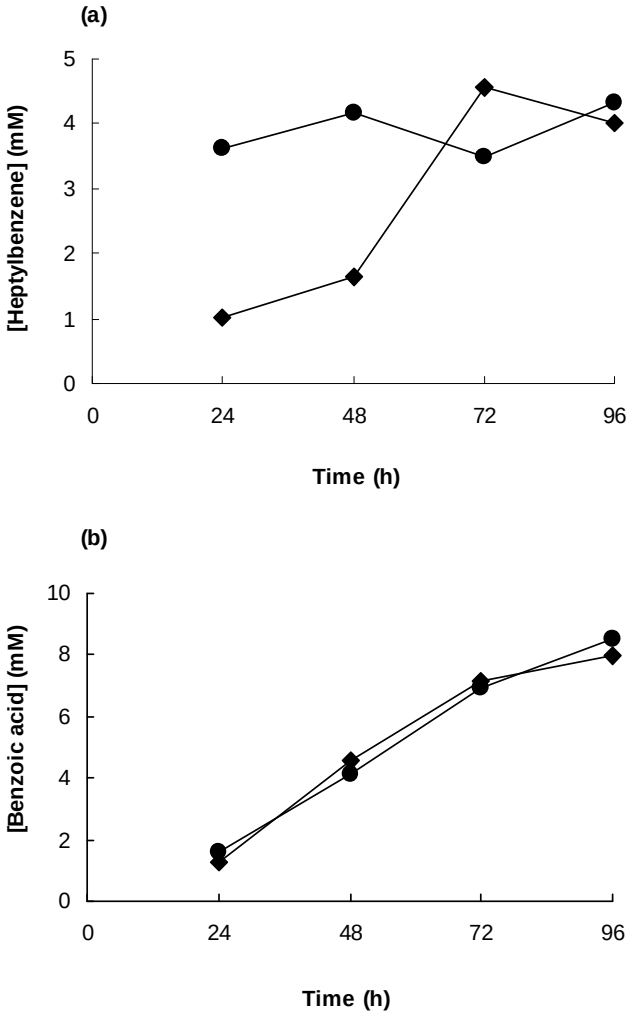


Figure 4.15: The biotransformation of heptylbenzene (60 mM) by ***Y. lipolytica*** strains CTY 005 (●) and CTY 014(17) (◆). Sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h there after. Biomass at 48 h of growth: CTY 005 10.8 g $L^{-1}$and CTY 014(17). 10.1 g L^{-1}.

4.2.6 Biotransformation of hexylbenzene and nonylbenzene under bioreactor conditions

After evaluating the biotransformation of alkylbenzenes in the experiments conducted in shake flasks and not being able to detect the expected difference in activities between strains with and without additional cytochrome P450s cloned, the next experiment was done using the Sixfors multireactor

system. The Sixfors multireactor system consists of six 300 mL reactor flasks connected together and they can be operated at the same time. The Sixfor system is connected to IRIS software, which can communicate directly with the computer running this software. The software makes it possible to control and/or monitor temperature, aeration, stirring and pH during the run.

Since it is difficult to control and maintain parameters like pH and oxygen transfer in a shake flask, this experiment was conducted in bioreactors where these conditions can be controlled as desired. The objective was to see if the differences between the strains would be observed. Improved sampling can also be expected from a properly stirred bioreactor. The reactors are also equipped with water cooled condensers which should minimize loss of volatile substrates through evaporation. Sodium acetate was added frequently as in the previous experiments and CTY 005 and CTY 014(17) were tested for the biotransformation of hexylbenzene and nonylbenzene.

4.2.6.1 Biotransformation of hexylbenzene when using sodium acetate as an inducer

The bioreactor parameters and results obtained with hexylbenzene as substrate are summarised in figure 4.16. In both graphs the changes in the stirrer speed was due to attempts to maintain the aeration at 70% of saturation. The drop in the relative oxygen concentration (pO_2) at 24 h of growth occured when sodium acetate (2% w/v) was added as an inducer. The change at 48 h of growth occurred when hexylbenzene (30 mM) and sodium acetate (1% w/v) were added. Further changes corresponded to the 12 hourly additions of sodium acetate (1% w/v). With both strains, the substrate was consumed after 96 h but CTY 005 accumulated only 5.1 mM phenylacetic acid (Fig. 4.16A) and CTY 014(17) only 5.7 mM (Fig. 4.16B). Consumption of hexylbenzene and formation of phenylacetic acid is also compared in Fig. 4.17. The results show that the behaviour of the two strains and their biotransformation activities were similar.

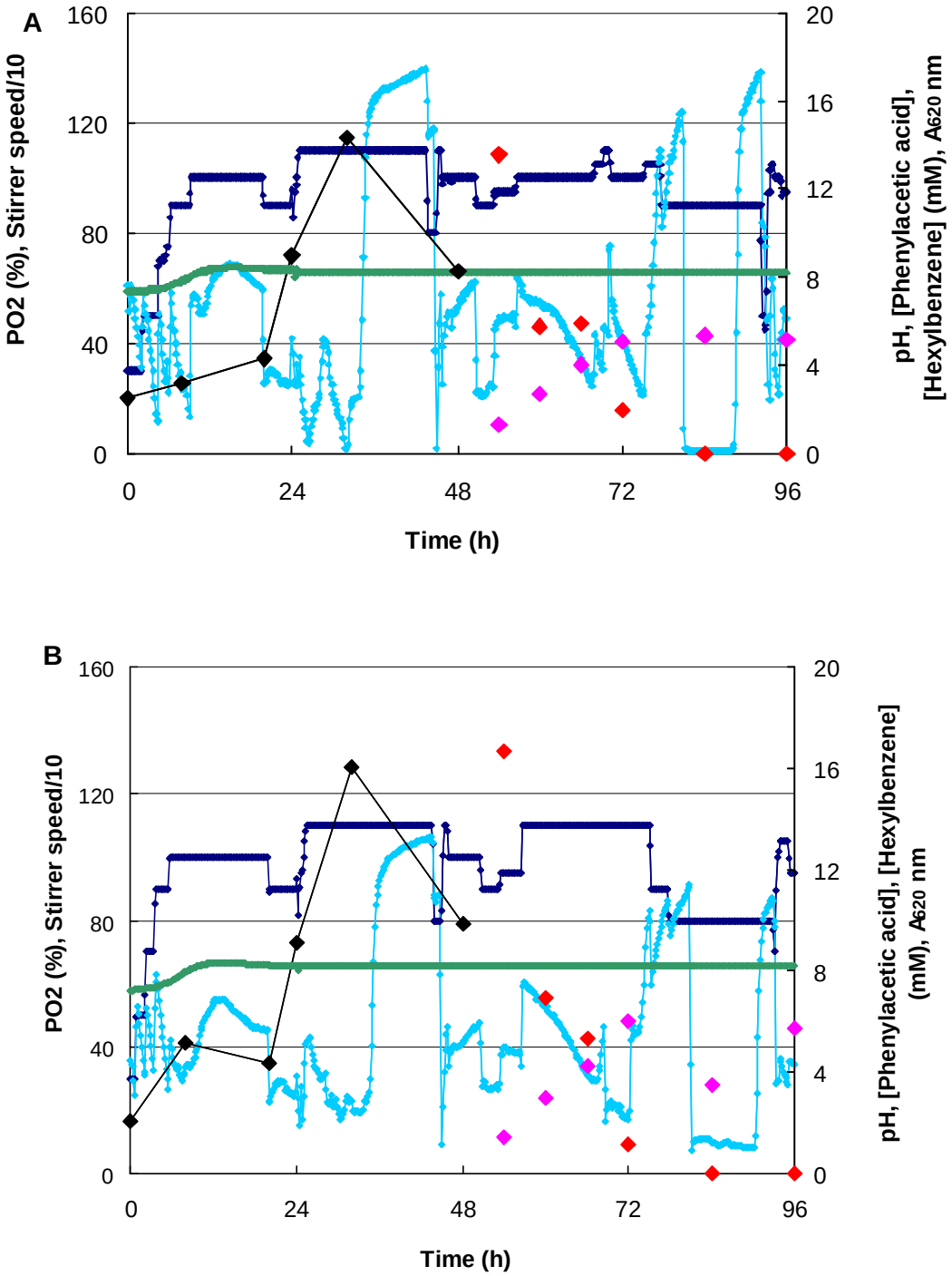


Figure 4.16: The biotransformation of hexylbenzene (60 mM) by ***Y. lipolytica*** strains CTY 005 (A) and CTY 014(17) (B). Sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h thereafter. (◆) Stirrer speed/10, (◆) relative oxygen concentration (pO_2) as percentage of saturation of broth without cells, (◆) pH, (◆) nonylbenzene concentration (mM) and (◆) benzoic acid concentration (mM), (◆) growth (OD at 620 nm). Time of inoculation is 0 h. Biomass at time of substrate addition (48 h) was for CTY 005 8.6 g L^{-1} and CTY 014(17) 10.8 g L^{-1}.

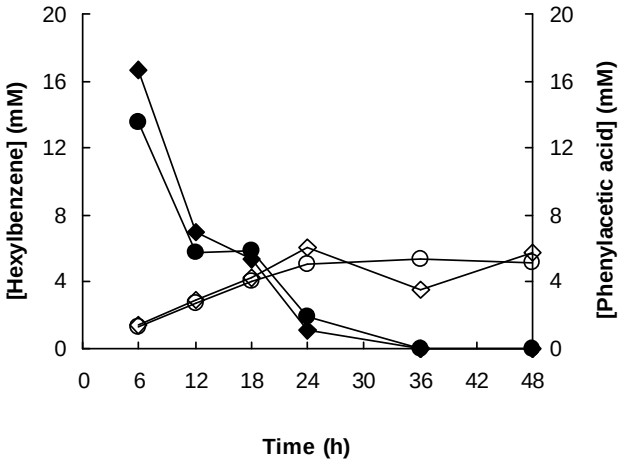


Figure 4.17: The biotransformation of hexylbenzene (30 mM) and formation of phenylacetic acid by ***Y. lipolytica*** strains CTY 005 and CTY 014(17). Hexylbenzene degradation by CTY 014(17) (◆) and by CTY 005 (●). Formation of phenylacetic acid by CTY 014(17) (◇) and CTY 005 (○).

4.2.6.2. Biotransformation of nonylbenzene when using sodium acetate as an inducer

In the same bioreactor run, nonylbenzene (30 mM) was tested using the same strains. Nonylbenzene yields benzoic acid as product and in previous experiments (results not shown) higher activities were observed with nonylbenzene than with pentylbenzene and heptylbenzene. Figure 4.19 shows that the two strains started to behave differently after 24 h. In the biotransformation conducted with CTY 005, the pO_2 did not respond to the addition of nonylbenzene and sodium acetate (Fig. 4.19A) as happened with CTY 014(17) (Fig. 4.19B) or in the biotransformations with hexylbenzene. Very little benzoic acid was produced by CTY 005 and between 30 mM and 25 mM nonylbenzene was extracted from all the samples. It appeared as if respiration ceased after the first addition of sodium acetate. These results at least confirmed that the substrate was not evaporating from the bioreactor. In the biotransformation with CTY 014(17) all the nonylbenzene was consumed within 48 h of addition (Fig. 4.18). However, a maximum of only 12.8 mM of benzoic acid was extracted after 96 h.

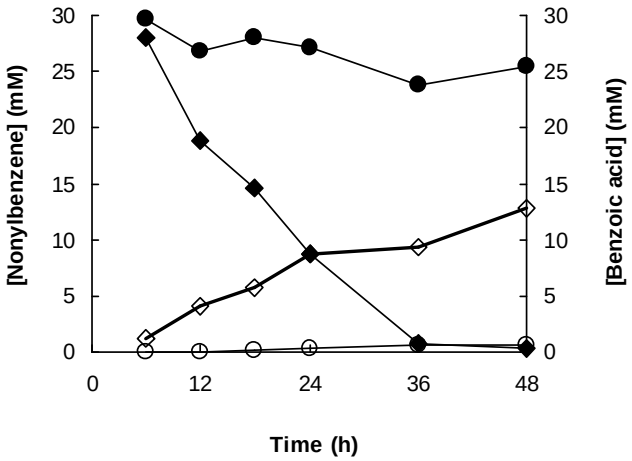


Figure 4.18: The biotransformation of nonylbenzene (30 mM) and formation of benzoic acid by ***Y. lipolytica*** strains CTY 005 and CTY 014(17). Nonylbenzene degradation by CTY 014(17) (◆) and by CTY 005 (●). Formation of benzoic acid by CTY 014(17) (◇) and CTY 005 (○).

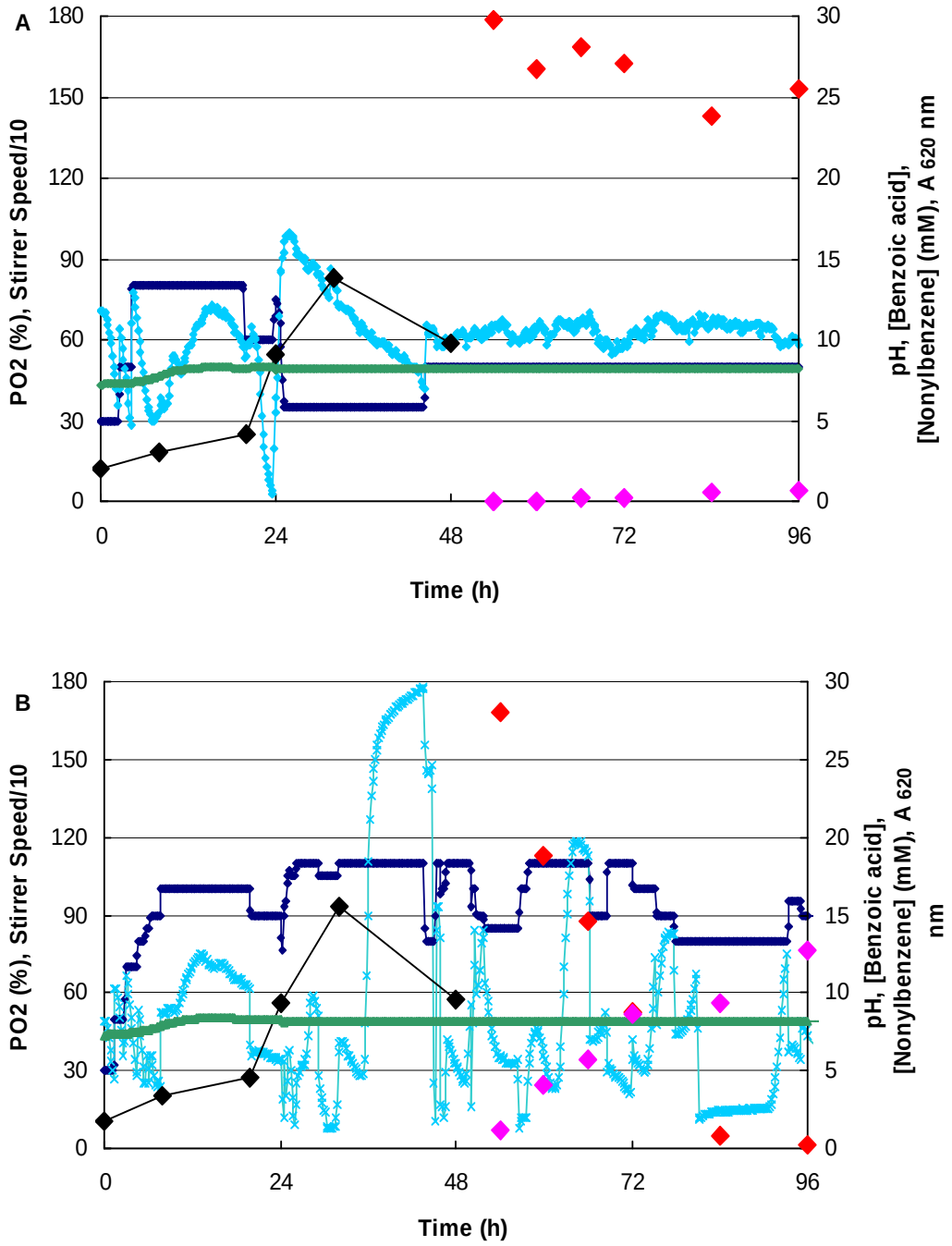


Figure 4.19: The biotransformation of nonylbenzene (30 mM) by ***Y. lipolytica*** strains CTY 005 (A) and CTY 014(17) (B). Sodium acetate (2% w/v) was added as an inducer after 24 h growth and sodium acetate (1% w/v) was added again at substrate addition (48 h growth) and every 12 h thereafter. (◆) Stirrer speed/10, (◆) relative oxygen concentration (pO_2) as percentage of saturation of broth without cells, (◆) pH, (◆) nonylbenzene concentration (mM) and (◆) benzoic acid concentration (mM), (◆) growth (OD at 620 nm). Time of inoculation is 0 h. Biomass at time of substrate addition (48 h) was for CTY 005 8.6 g L^{-1} and CTY 014(17) 10.8 g L^{-1}.

The results obtained with CTY 005 and nonylbenzene indicated that it was possible to recover all the substrate when the cells were not metabolically active. These results discounted poor sampling and evaporation as possible explanations for the lack of recovery of added hexylbenzene and nonylbenzene, either as unreacted substrate or acid products, from the other bioreactors. The other possibilities might be that the acid products are being incorporated into cellular lipids or lipoproteins or the complete degradation of these products. Degradation seems unlikely since these results were obtained when sodium acetate was added continuously, supplying the cells with more than enough carbon and energy. The continuous addition of sodium acetate however might stimulate lipid or citric acid production. This might either lead to the incorporation of products such as phenylhexanoic acid into the lipids or the complete degradation of the substrate followed by production of lipids or citric acid from the resulting acetyl CoA.

4.2.7 Biotransformation of alkylcyclohexanes

The degradation of alkylcyclohexanes by several bacteria have been reported in the past and their degradation pathways have been investigated (Beam and Perry, 1974; Dutta and Harayama, 2001; Koma ***et al***., 2003; 2005). It was shown that cyclohexanecarboxylic acid is formed from odd numbered alkylcyclohexanes, and cyclohexylacetic acid is formed from even numbered alkylcyclohexanes (Beam and Perry, 1974). Thus biotransformation of butylcyclohexane yielded cyclohexylacetic acid in studies conducted by Beam and Perry (1974), Dutta and Harayama (2001) and Koma and colleagues (2003; 2005). No literature information was found about the biotransformation of alkycyclohexanes such as butylcyclohexane by either yeasts or fungi. However, it has been reported that biotransformation of limonene by alkane degrading yeasts yielded perillic acid (van Rensburg ***et al***., 1997).

Biotransformation of limonene and butylcyclohexane were tested in the next set of experiments. It was hoped that the degradation/disappearance of acid products would not occur as was the case in the biotransformation of alkylbenzenes by ***Y***. ***lipolytica*** strains. The strains used in the previous

experiments were used. In a bioreactor experiment with butylcyclohexane and limonene as substrates sodium acetate was added as an inducer (2% w/v at 24 h of growth and 1% w/v at substrate (30 mM) addition and every 12 h thereafter). In another set, dodecane (2% v/v) was added at 24 h of growth as an inducer and butylcyclohexane (30 mM) as substrate 24 h later. Product formation in these experiments was very low and the results are therefore not reported in detail. In the sodium acetate induced cultures where butylcyclohexane was added as substrate no product was detected. The highest concentration of cyclohexylacetic acid formed in the dodecane induced cultures was 1.9 mM and the two strains gave similar results. In the biotransformation of limonene, a maximum of only 0. 9 mM of perillic acid was detected in the 24 h samples. Both the substrates (butylcyclohexane and limonene) could not be detected in the GC analysis because of their volatility. These compounds are more volatile than alkylbenzenes and the GC conditions were not optimized to detect them.

The next experiment was conducted in shake flasks, using the same strains. In one set of flasks dodecane (2% v/v) was added after 24 h of growth as an inducer and in another set, glucose (2% w/v) was added to supply an equivalent amount of carbon and energy. In the case where dodecane was added, CTY 005 accumulated 4.3 mM (Fig. 4.20a) and CTY 014(17) 6.1 mM of cyclohexylacetic acid (Fig. 4.21a) from butylcyclohexane. When glucose was added as an additional carbon source, CTY 005 only accumulated 1.9 mM (Fig. 4.20b) and CTY 014(17) 2.1 mM of cyclohexylacetic acid (Fig. 4.21b). These maximum concentrations were obtained at 48 h after substrate addition. However at 72 h the product concentrations again decreased. This was unexpected and is very difficult to explain. A second product was formed from butylcyclohexane. GC-MS analysis had shown that this product is a hydroxylated cyclohexylacetic acid, but there was no match for this product in the mass spectra database (R.K. Gudiminchi, personal communication). This product is most likely a product of β-oxidation that could not be completed (Fig. 4.22). The highest concentration of this product was accumulated by CTY 014(17) in the dodecane induced cells after 72 h of substrate addition (2

mM). When specific activities were calculated for the dodecane induced cultures based on the total acid products formed during the first 24 h, the activities of 0.39 and 0.36 μmol min^{-1} g dry $weight^{-1}$ for CTY 005 and CTY 014(17), respectively, were practically the same, although higher product concentrations were accumulated by CTY 014(17).

Comparison of the results obtained with dodecane and glucose, shows that butylcyclohexane does not very efficiently induce the hydroxylase responsible for its biotransformation and that butylcyclohexane is also not a very good substrate for the hydroxylases induced by dodecane.

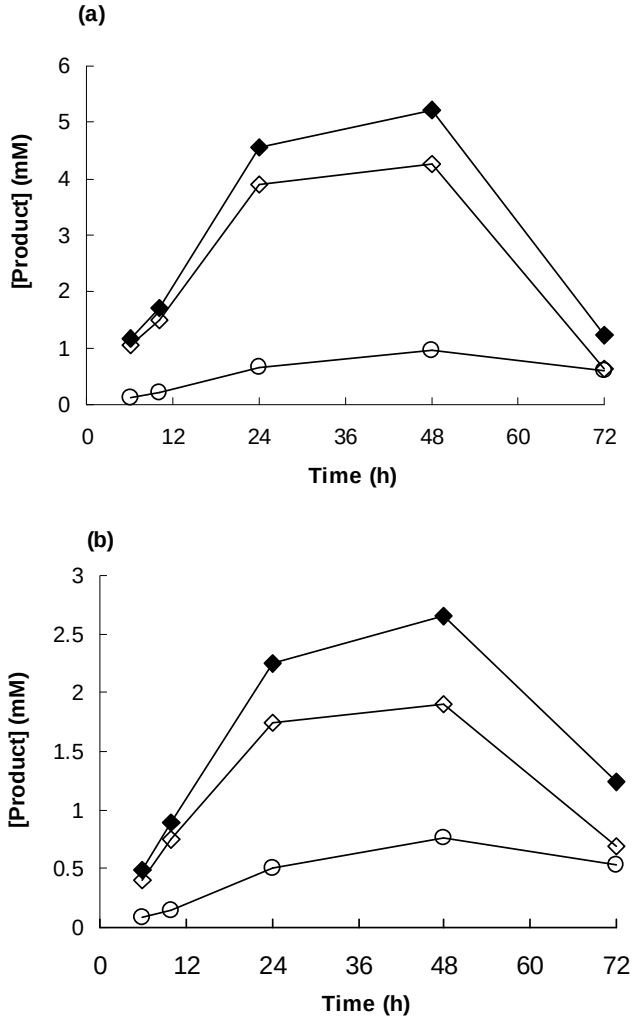


Figure 4.20: The biotransformation of butylcyclohexane by CTY 005. Sum of products (◆), Formation of cyclohexylacetic acid (◇), and formation of hydroxylated cyclohexylacetic acid (○). (A) Dodecane (2% v/v) added as an inducer at 24 h of growth and (B) glucose (2% w/v) added as an additional energy source at 24 h of growth. The biomass at 48 h when dodecane was added as an inducer was 8.3 g L^{-1} and when glucose was added as an additional energy source it was 10.6 g L^{-1}.

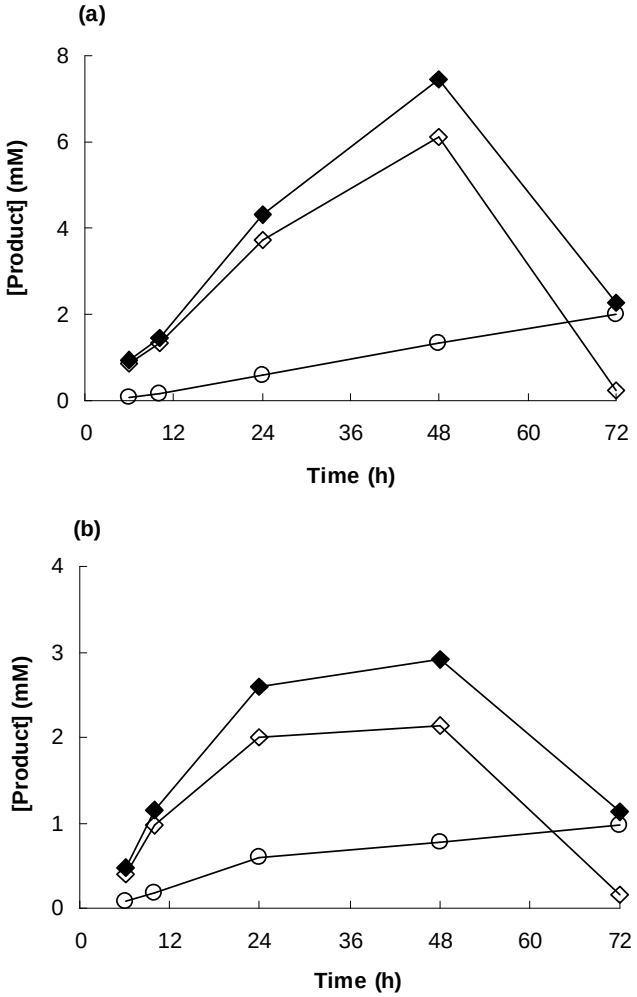


Figure 4.21: The biotransformation of butylcyclohexane by CTY 014(17). Sum of products (◆), Formation of cyclohexylacetic (◇), and formation of product 2 (○). (A) Dodecane (2% v/v) added as an inducer at 24 h of growth and (B) glucose (2% w/v) added as an additional energy source at 24 h of growth. The biomass at 48 h when dodecane was added as an inducer was 8.8 g L^{-1} and when glucose was added as an additional energy source it was 12.1 g L^{-1}.

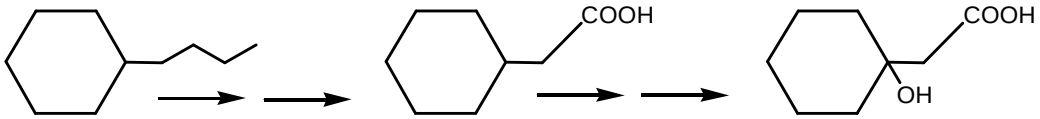


Figure 4.22: Proposed pathway for butylcyclohexane hydroxylation.

Biotransformation of limonene (30 mM) was also tested under similar conditions. There was again a difference observed between when dodecane was added as inducer and when glucose was added to supply approximately an equivalent amount of carbon and energy. Dodecane addition again resulted in much higher activity than glucose (Fig. 4.23). However there was no significant difference between the activities of the two strains. The amounts of perillic acid formed in this experiment was similar to results previously reported when a wild type strain of ***Y. lipolytica*** (CBS 599T) was used for the biotransformation of limonene to perillic acid. In these experiments up to 6 mM of perillic acid was produced (van Rensburg ***et al***., 1997).

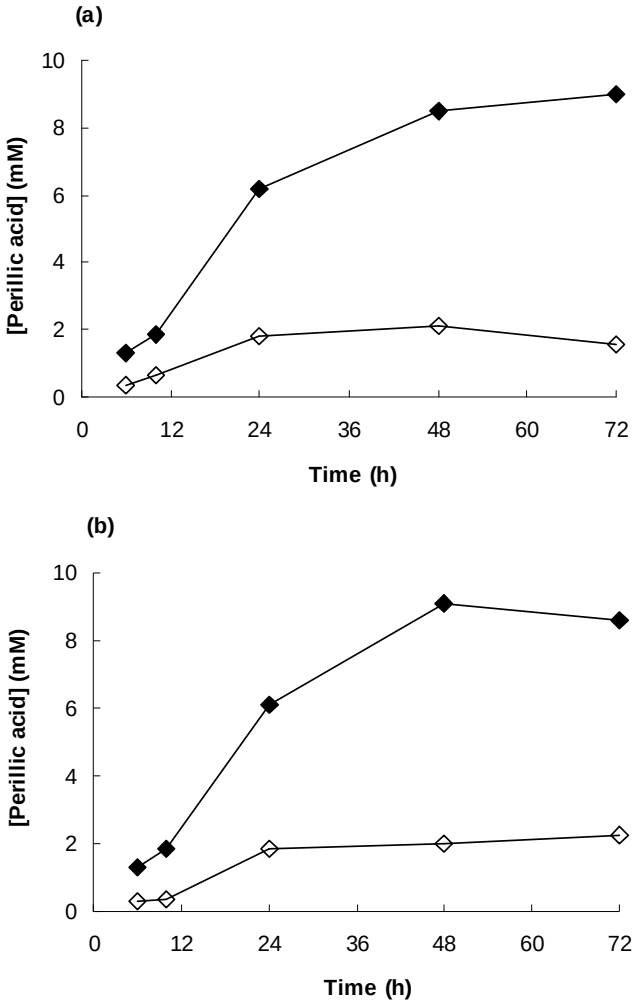


Figure 4.23: The biotransformation of limonene by CTY 005 and CTY 014(17). Formation of perillic acid in dodecane (2% v/v) induced cells (◆), formation of perillic acid when glucose (2% w/v) was added (◇). Biotransformation by CTY 005 (A) and CTY 014(17) (B). The biomass at 48 h of CTY 005 when dodecane was added as an inducer was 9.1 g L^{-1} and 8.1 g L^{-1} for CTY 014(17). When glucose was added as an additional energy source the biomass for CTY 005 was 13.0 g L^{-1} and for CTY 014(17) it was 11.2 g L^{-1}.

Chapter 5

Discussions

The aim of this study was to investigate the biotransformation of alkylbenzenes and alkylcyclohexanes to detect the effect that alkane hydroxylases cloned into ***Y. lipolytica*** have on hydroxylase activity. Cytochrome P450 genes had been cloned into ***Y. lipolytica*** under control of two different promoters; ***pPOX2*** and ***pICL***. ***pPOX2*** is induced by alkanes and fatty acids while ***pICL*** is strongly induced by ethanol and acetate as well as alkanes and fatty acids (Juretzek ***et al***., 2000; Madzak ***et al***., 2004).

It is difficult to study alkane degradation in alkane degrading yeasts because the substrates are not soluble in water causing problems with sampling from shake flasks and because the alkane is completely degraded. Hexylbenzenes and alkylcyclohexanes are attractive alternatives because they are degraded to cyclic acid products that are not easily degraded. In previous studies it was shown that ***Y. lipolytica*** transforms hexylbenzene to phenylacetic acid (Van Rooyen, 2005) and nonylbenzene to benzoic acid (Obiero, 2006). These products were easily quantified with GC analysis and approximately 100% conversion to products was often obtained.

Previous studies using strains with alkane hydroxylating P450s cloned under ***pPOX2*** did not conclusively show the difference between the wild type and cloned P450s activities when alkylbenzenes were added directly to the cultures after induction with alkanes or fatty acids (van Rooyen, 2005). In the current study cells were harvested after induction with oleic acid. When specific activities were calculated for biotransformations using these resting cells, the strain with multiple copies of ***CYP52F1***, an alkane hydroxylase proven to hydroxylate both short and long chain alkanes (Iida ***et al***., 2000), showed an approximately 20% increase in activity with both hexylbenzene and nonylbenzene as substrates when compared to the control strain and a strain with ***CYP557A1***, a putative alkane and/or fatty acid hydroxylase cloned. Unfortunately these results could not be repeated because in some

experiments the harvested cells had very little activity. The reason for this could not be determined. It might have been because activity was lost during harvesting or because oleic acid was toxic to ***Y***. ***lipolytica*** when it was not added correctly. For instance, the concentration of oleic acid in the stock solution might have been too high or the pH too low.

Subsequently experiments were conducted with strains with the ***CYP557A1*** gene, encoding a putative alkane and/or fatty acid hydroxylase, cloned under ***pICL.*** Ethanol and sodium acetate were tested as inducers with butylbenzene, pentylbenzene, hexylbenzene, heptylbenzene, and nonylbenzene as substrates. When ethanol was added once after 24 h growth, the strain with the cloned ***CYP557A1*** gene showed in two experiments higher activity. The first experiment was when CYP activity was determined with microsomal fractions using hexylbenzene and nonylbenzene as substrates and the second was the biotransformation of butylbenzene by cultures to which substrate was added directly 24 h after induction. The CYP activity was determined by following the consumption of NADPH. This is not a standard procedure for measuring CYP activity with microsomal fractions (Ansede and Thakker, 2004). The experiment with butylbenzene could not be repeated. In subsequent experiments no product was detected. When ethanol was added once and hexylbenzene used as substrate the control strain had higher activity than the test strains. However the test strains consumed the substrate at the same rates as the control strain and all the substrate was consumed but without an equal amount of product formed. Thus the results were inconclusive.

When ethanol was added once as an inducer and butylbenzene, pentylbenzene, hexylbenzene and heptylbenzene were used as substrates, the fastest product formation was observed with hexylbenzene. Also when cells were induced with oleic acid and hexylbenzene and nonylbenzene were used as substrates, product formation was higher with hexylbenzene. The enzyme assays with microsomal fractions of ethanol induced cells also gave higher activity with hexylbenzene than with nonylbenzene. All these results

point to hexylbenzene being the preferred substrate for the hydroxylases involved.

When sodium acetate was added once as an inducer, no transformation of hexylbenzene was observed. With multiple additions of sodium acetate the strain with cloned ***CYP557A1*** showed higher activity in two shake flask experiments when hexylbenzene was used as a substrate. However, in two other shake flask experiments and in one bioreactor experiment, there was no significant difference between the strains. In shake flask experiments with pentylbenzene, hexylbenzene and nonylbenzene as substrates there was also no difference between the strains.

In all the experiments where there were multiple additions of sodium acetate, substrates were consumed without concomitant formation of products. It is difficult to interpret these results because it was shown that the disappearance of the substrates cannot be attributed to evaporation. Possible explanations for the consumption of substrates without the formation of detectable products could be that the acid products are being incorporated into cellular lipids or lipoproteins or the complete degradation of the products. The continuous addition of sodium acetate might stimulate lipid or citric acid production leading to either the incorporation of products such as phenylhexanoic acid into the lipids or the complete degradation of the substrate followed by the synthesis of lipids or citric acid from the resulting acetyl CoA.

It is of interest to note that phenylhexanoic acid and phenylbutanoic acid as intermediates in the β-oxidation of hexylbenzene were for the first time observed in this study when ethanol (phenylbutanoic acid) and sodium acetate (phenylhexanoic acid and phenylbutanoic acid) were added as inducers. This probably points to the inhibition of β-oxidation when ethanol is present and might indicate that these intermediates are incorporated into lipids or lipoproteins when these citric acid cycle intermediates are used as inducers.

Phenylacetic acid might be degraded by ***Y. lipolytica*** since there is a putative phenylacetate 2-hydroxylase gene present in its genome (M.S. Smit, personal communication). However, there is no benzoate ***para***-hydroxylase gene present in the ***Y. lipolytica*** genome and previous research where benzoic acid was used as substrate had shown that benzoic acid is not degraded by ***Y. lipolytica*** (Obiero, 2006, Shiningavamwe ***et al***., 2006).

In the biotransformation experiments with butylcyclohexane and limonene the expected products, cyclohexylacetic acid and perillic acid were obtained. The activities were relatively low compared to the activities obtained with hexylbenzene and nonylbenzene. Because alkylcyclohexanes are more volatile than alkylbenzenes, problems were encountered with GC analysis and it is also very likely that these substrates evaporated in the experiments conducted in shake flasks. It is even possible that these substrates evaporated from the bioreactors, since tap water was used to cool the condensers. The alkylcyclohexanes do not hold much promise for future work because of these problems and because they also did not reveal differences between the control and test strains.

This M.Sc. study was largely exploratory. A number of problems that must be solved as well as experiments that should be done in future have been identified. It is important to note that bioreactor experiments demonstrated that neither sodium acetate nor ethanol (R.K. Gudiminchi, personal communication) inhibited the induction of the endogenous P450s that hydroxylate alkylbenzenes. Previous results in shake flasks which indicated that ethanol delayed induction of hydroxylases (van Rooyen, 2005) should probably be ascribed to insufficient pH control. It is however clear that multiple addition of sodium acetate and ethanol R.K. Gudiminchi, personal communication), cannot be used to distinguish between the strains, since it activates the complete consumption of substrates without formation of detectable products.

Single additions of ethanol and sodium acetate should still be tested with bioreactor experiments. However there were indications that even with a

single addition of ethanol some strains consumed all the hexylbenzene, while converting only 50% to phenylacetic acid. At this stage it thus appears as if the ***ICL*** promoter is not useful to distinguish between cloned and endogenous P450s in whole cell experiments. More experiments should be done with microsomal fractions to proof expression of the cloned ***CYP557A1*** and to show that it has activity towards alkanes and alkylbenzenes.

The strains with the cloned P450s under ***pPOX2*** should also be tested in the bioreactors with direct addition of substrates and with harvested cells. Oleic acid induction should give more reproducible results in the bioreactors, because it will be possible to monitor and control the pH more accurately. The problem of consumption of substrates without formation of detectable products might also be solved by using strains with β-oxidation blocked through deletion of ***POX*** genes (Smit ***et al.,*** 2005).

Chapter 6

Conclusions

- Biotransformation of alkylbenzenes by ***Y. lipolytica*** strains having ***CYP*** genes encoding alkane and/or fatty acid hydroxylases cloned under the ***pPOX2*** or ***pICL*** promoters was investigated. Control and test strains accumulated phenylacetic acid from butylbenzene and hexylbenzene, while benzoic acid was accumulated from pentylbenzene, heptylbenzene and nonylbenzene.

- The highest activities were observed with hexylbenzene as substrate, indicating that it is probably the preferred substrate for the alkane hydroxylases involved in the biotransformation reactions.

- Neither with the addition of oleic acid as inducer of the ***pPOX2*** nor with the use of either ethanol or sodium acetate as inducers of the ***pICL*** was it possible to clearly demonstrate that the cloned cytochrome P450s had an effect on hydroxylase activity.

- The lack of reproducibility of results was a serious problem. In some cases activity was apparently lost during harvesting and the difficulty to control pH in shake flasks probably also contributed to the lack of repeatability of results.

- When ethanol and sodium acetate were used as inducers the alkylbenzenes were consumed without the equivalent formation of detectable products, further complicating the interpretation of results. This problem was worse with multiple additions of sodium acetate.

- This study demonstrated that ethanol and sodium acetate do not inhibit the induction of the endogenous alkane hydroxylases. Previous results

where this appeared to be the case should probably be ascribed to insufficient pH control in shake flasks.

- Biotransformation of alkylcyclohexanes by ***Y. lipolytica*** yielded cyclohexylacetic acid and a hydroxylated derivative of cyclohexylacetic acid from butylcyclohexane while perillic acid was accumulated from limonene. Activity towards these substrates was very low and the activities of the test strain with ***CYP557A1*** cloned under pICL and the control strain were similar. The volatility of these substrates caused problems with the GC analyses and probably also with evaporation from flasks.

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Summary

Y. ***lipolytica*** has the ability to utilise hydrophobic hydrocarbons as carbon sources. It is also an attractive host for heterologous expression of cytochrome P450 (***CYP***) genes. ***Y. lipolytica*** strains with ***CYP*** genes cloned under control of two different promoters, ***pPOX2*** and ***pICL*** were used in this study. The purpose of this project was to detect the effect of cloned alkane hydroxylases in ***Y***. ***lipolytica***. Alkylbenzenes and alkylcyclohexanes were used to compare the hydroxylase activities of the genetically engineered strains with control strains.

Butylbenzene and hexylbenzene were transformed to phenylacetic acid while pentylbenzene, heptylbenene and nonylbenzene yielded benzoic acid as product. Butylcyclohexane and limonene were transformed to cyclohexylacetic acid and perillic acid, respectively, as major products. The activity towards hexylbenzene was highest. Phenylhexanoic acid and phenylbutanoic acid were also for the first time observed as intermediates in the biotransformation of hexylbenzene.

Y. ***lipolytica*** strains expressing alkane hydroxylases under ***pPOX2*** were induced with oleic acid and harvested. A strain with multiple copies of a proven alkane hydroxylase, cloned, had in one experiment higher activity than the other strains, towards both hexylbenzene and nonylbenzene. However, these results could not be confirmed, because in subsequent experiments the resting cells had very low activities.

Ethanol and sodium acetate were used as inducers in the experiments conducted with ***Y***. ***lipolytica*** strains with CYP557A1, a putative alkane and/or fatty acid hydroxylase, cloned under ***pICL***. The substrates were added directly to the cells. With single addition of ethanol, the strain with cloned ***CYP557A1*** had in one whole cell experiment with butylbenzene as substrate higher activity than the control strains.

With multiple additions of sodium acetate the strain with cloned ***CYP557A1*** showed higher activity in two shake flask experiments when hexylbenzene was used as a substrate. However, when ethanol and sodium acetate were used as inducers the alkylbenzenes were often consumed without the equivalent formation of detectable products, complicating the interpretation of results. This also happened in bioreactor experiments.

Biotransformation of alkylcyclohexanes also did not demonstrate the effect of the cloned gene, since the activities of the test strain with ***CYP557A1*** cloned under ***pICL*** and the control strain were similar. Alkylcyclohexanes are not promising substrates to distinguish between hydroxylase activity of the cloned genes as they are very volatile and activity towards them is relatively low.

Keywords: alkylbenzenes, alkylcyclohexanes, benzoic acid, CPR, CYP, cytochrome P450 monooxygenases, hydroxylase activity, ***ICL*** promoter, phenylacetic acid, ***POX2*** promoter, ***Yarrowia lipolytica***,