EVALUATION OF *YARROWIA LIPOLYTICA* AS A HOST FOR CYTOCHROME P450 MONOOXYGENASE EXPRESSION

By

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B.Sc. (U.O.N); M.Sc. (U.B)

Submitted in fulfilment of the requirements for the degree

PHILOSOPHIAE DOCTOR

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June, 2006

Promoter: Prof. M.S. Smit

"It's not that I'm so smart, it's just that I stay with problems longer."

Albert Einstein **(1879-1955)**

This thesis is dedicated to my late mother, Mrs. Beldine Obiero and my dad, Mr. Gabriel Obiero whose unwavering support and encouragement has been my pillar of strength.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude toward the following people:

Prof. M.S. Smit for her invaluable guidance, patience and constructive criticism during the course of the study. Thanks for the patience and determination throughout this project.

Mr. P.J. Botes for his technical assistance with the chemical analyses.

Dr. E. setati for her valuable and motivating discussions.

All my colleagues in Biotransformation Research Group.

Fellow students and **staff** in the Department of Microbial, Biochemical and Food Biotechnology, University of Free State

My family and **friends** for being there for me throughout the study and for their much needed words of encouragement and support.

National Research Foundation (NRF) for the financial support of this project.

The Heavenly Father without whom this work would not have been successful.

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AA	Acetanilide
BA	Benzoic acid
CA	***trans***-Cinnamic acid
c/a	Casamino acids
ClBA	Chlorobenzoic acid
CYP450	Cytochrome P450 monooxygenase
CPR	Cytochrome P450 reductase
Dwt	Dry weight
GC	Gas chromatography
GC-MS	Gas chromatography-Mass spectrometry
HB	Hexzylbenzene
HCl	Hydrochloric acid
MS	Mass spectrometry
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
OD	Optical density
PAA	Phenylacetic acid
***p*-AAP**	***para***-acetaminophene
PAH	Poly aromatic hydrocarbons
pHBA	***para***-hydroxybenzoic acid
PN	Phenylnonane
TLC	Thin layer chromatography
TMSH	Trimethylsulfonium hydroxide
YE	Yeast extract

CHAPTER ONE

1 Introduction

1.1 Biocatalytic hydroxylations

Biocatalytic hydroxylation of unactivated carbons is of special interest in organic synthesis. It involves the use of biological systems to introduce a hydroxyl group to a nonfunctionalized carbon centre to form a carbon-hydroxyl bond (Flitsch ***et al***., 1999). It is one of the most widespread enzymatic activities occurring in all forms of life including both prokaryotes and eukaryotes (Wong, 1998; Li ***et al***., 2002). This reaction plays a key role in the oxidative metabolism of many organic compounds and leads to the formation of both beneficial pharmaceutical products as well as degradation of harmful environmental pollutants (Holland ***et al***., 2000).

Biocatalytic hydroxylations have been used for many years in the industrial production of fine chemicals and for bioremediation processes. In spite of their ubiquity, hydroxylation reactions are still some of the least understood of all enzymatic reactions, because for the most part the hydroxylase enzymes are membrane bound multiprotein complexes, making them difficult to isolate and often unstable in the isolated form. Recent advances in enzyme technology and genetic manipulation, has had a significant impact on the study of hydroxylating enzymes (Holland ***et al***., 2000).

Biohydroxylation reactions are catalyzed by various types of enzymes (Ayala and Torres, 2004). In nature, the different types of hydroxylating enzymes include dioxygenases, lipooxygenases as well as monooxygenases (CYP450 monooxygenases, methane monooxygenases and integral membrane di-iron alkane hydroxylases) (**Table** 1.1). Dioxygenases catalyze the regiospecific and steriospecific insertion of two oxygen atoms from molecular oxygen into a substrate (Figure 1.1:d). They can be co-factor dependent or independent while lipoxygenases are non-heme iron dioxygenases that catalyze specific hydroxylation of polyunsaturated fatty acids, yielding chiral 1,3-***cis***-***trans***-diene-5-hydroxyperoxides (**Figure** 1.1e) (Li ***et al***., 2002). Examples of these hydroxylation reactions, their substrates and the hydroxylation products are illustrated in **Figure** 1.1.

The focus of this study will be on monooxygenase reactions. These enzymes use molecular oxygen to insert one oxygen atom into a substrate with the other oxygen atom reduced to water. The reaction usually requires a metal center, and electron transfer from a reduced cofactor (van Beilen and Funhoff, 2005). Biological hydroxylations are mainly catalyzed by P450s. P450s are heme-thiolate proteins which comprise a very large enzyme family. They contribute to vital processes in the cell such as carbon assimilation in microorganisms, biosynthesis of hormones and detoxification of xenobiotics in animals and plants (Werck-Reichhart and Feyeisen, 2000). Many P450 enzymes catalyze monooxygenation of various hydrophobic compounds such as fatty acids (Matsunaga ***et al***., 2002). P450s use electrons from NAD(P)H to catalyze activation of molecular oxygen leading to the regiospecific and stereospecific oxidative attack of substrates (Capdevila ***et al***., 1984; Sutter ***et al***., 1990; Vogel ***et al***., 1992).

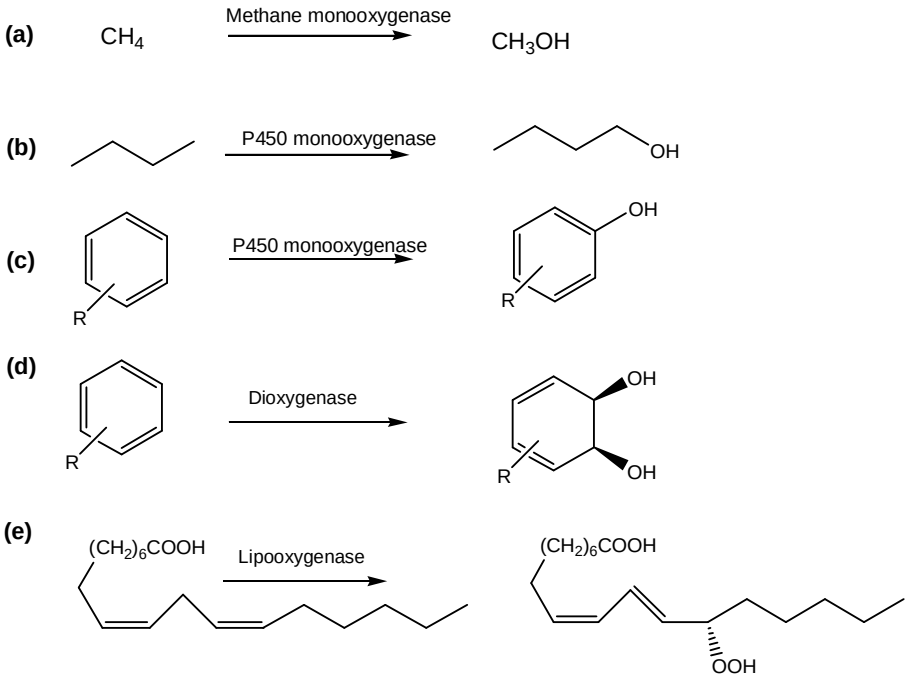


Figure 1.1: Scheme showing different oxygenase catalyzed hydroxylations. (**a**) Via methane monooxyganase, (**b**) &(**c**) via P450 monooxygenases, (**d**) via co-factor dependent dioxygenase and (e) via cofactor-independent dioxygenase (Adapted from Li ***et al***., 2002; van Beilen ***et al***., 2005).

Table 1.1. Table showing enzymes that hydroxylate different hydrocarbons, their host organisms and substrate range (Table adapted from van Beilen ***et al***., 2003; van Beilen ***et al***., 2005).

Enzyme	Composition and cofactors	Examples of host organism	Substrate range	Ref.
Cytochrome P450 (CYP52) monooxygenase	Microsomal oxygenase: P450 heme reductase:FAD,FMN,NADPH	***Candida maltosa***, ***Candida tropicalis***, ***Yarrowia lipolytica***	C_{10}-C_{16}	Craft ***et al***., 2003
Integral membrane alkane hydroxylase	Membrane hydroxylase: dinuclear iron Rubredoxin: iron Rubredoxin reductase: FAD,NADH	***Acitobacter, Burkhlderia, Rhodococcus***	C_5-C_{16}	Smit ***et al***., 2002
Soluble methane monooxygenase	$\alpha_2\beta_2\gamma_2$ structure Hydroxylase: dinuclear iron Rubredoxin: iron Rubredoxin reductase:FAD, NADH regulatory subunit	***Methylisinus trichosporium OB3b, Methylococcus capsulatus***	C_1-C_{10}	Baik ***et al***., 2003
Particulate methane monooxygenase	Putative $\alpha_2\beta_2\gamma_2$ structure	All known methanotrophs	C_1-C_5	Lieberman ***et al*** ., 2004
Butane monooxygenase	$\alpha_2\beta_2\gamma_2$ structure Hydroxylase: dinuclear iron Reductase [2Fe-2S], FAD, NADH regulatory subunit	***Pseudomonas butanovora***	C_2-C_8	Sluis ***et al***., 2002
Peroxidases	No co-factor: heme-iron centre	***Pseudomonas fluorescens***	Naphthalene	Bühler ***et al***., 2004; Li ***et al***., 2002; van de Velde ***et al***., 2001

1.2 Advantages of biocatalytic hydroxylation

Biocatalytic hydroxylation has several advantages over chemical synthesis. The use of microbial hydroxylations has a long history, but the exploitation of this process for the production of pharmaceutical products is a more recent development (Holland ***et al***., 2000). Examples of pharmaceutical products produced through microbial hydroxylation reactions include pravastatin and cortisone (Guengerich, 2002). The enzymes involved allow for the highly chemo-, regio-, and enantiospecific functionalization of readily available petrochemicals as well as xenobiotics and larger molecules, such as steroids, under mild conditions (Shilov and Shulpin, 1997; Carelli ***et al***., 1999; Hartmann and Enst, 2000; Bühler ***et al***., 2004). Their chemical counterparts on the other hand do not exist or do not have the required regiospecificity and stereopecificity (Li ***et al***., 2002). Chemical functionalizations usually require the use of expensive and hazardous reactants and catalysts and often yield product mixtures, which complicate product isolation (Shilov and Shulpin, 1997; Carelli ***et al***., 1999; Hartmann and Enst, 2000; Bühler ***et al***., 2004). The oxygenases use oxygen as a cheap, environmentally friendly oxidant compared to toxic chemical oxidants. They can be used to prepare chiral building blocks and intermediates, which are important in organic synthesis (Li ***et al***., 2002).

1.3 Issues involving the application of hydroxylating enzymes

The number of production processes involving both biocatalytic hydroxylations and metabolic engineering has been steadily increasing over the recent years. The development of these oxygenase based bioprocesses to industrial scale, however, faces various draw backs that are not experienced by biocatalytic processes involving easy to use enzymes such as hydrolases, isomerases, or lyases (van Beilen ***et al***., 2003; Bühler and Schmid, 2004). Most oxygenases are quite unstable, consisting of multiple components, of which some are membrane bound. Due to the nature of their reactions, they also require expensive cofactors such as NAD(P)H (Duetz ***et al***., 2001, Bühler and Schmid, 2004). These factors constrict the use of isolated enzymes in practical applications, because they would require sophiscated cofactor regeneration systems (Faber, 2000; Bühler and Schmid, 2004). Thus, during the past two

decades, efforts towards industrial applications of oxygenases have mainly focused on whole-cell biocatalysis (Bühler and Schmid, 2004).

Whole-cell oxygenase-based bioprocesses are also quite complex because various physiological affects must also be taken into account. These reactions are often carried out in two-liquid phase bioconversion media since the substrates and the products are hydrophobic and water-insoluble, which makes them difficult to work with (van Beilen ***et al***., 2003). Product degradation, cofactor recycling and toxicity of substrates and products have to be considered (Bühler and Schmid, 2004). Bioprocess engineering of the hydroxylating enzymes also faces further challenges such as downstream processing and the high oxygen requirements of the whole-cell biocatalysts leading to the danger of explosion hazard (van Beilen ***et al***., 2003).

For the industrial application of hydroxylating enzymes, critical intrinsic enzyme properties including low enzymatic rates, uncoupling, and multiple oxidation of the substrate has to be considered. Since the specific catalytic rate $\boldsymbol{k}_{cat}$, of most cofactor dependent oxygenases are relatively low compared to hydrolytic enzymes, optimization of the productivity is always a major challenge (Bühler and Schmid, 2004). NAD(P)H-dependent oxygenases have low turn over frequencies (i.e. $\boldsymbol{k}_{cat}$) in the range of 0.2-75 s^{-1}, while for other enzymes these frequencies are in the order of 600,000 s^{-1} (Ayala and Torres, 2004). Cofactor independent oxygenases acting on single electron donors such as catechol dioxygenase and lactate 2-monooxygenase have been reported to have significantly higher $\boldsymbol{k}_{cat}$ values as compared to cofactor dependent oxygenases (Bühler and Schmid, 2004). It has been hypothesized that the electron transfer step is relatively slow, as NAD(P)H-independent oxygenases show 10-fold to 1000-fold higher frequencies (Ayala and Torres, 2004).

All the mentioned issues (**Figure** 1.2; **Table** 1.2) make the discovery and development of a suitable oxygenase biocatalyst and the transfer of oxygenase-based processes from laboratory-scale to industrial-scale demanding and time consuming (van Beilen ***et al***., 2003). The two main approaches being explored to overcome these limitations include discovery of new enzymes and the improvement of known enzymes (Ayala and Torres, 2004).

Very few economic analyses have been performed regarding the application of monooxygenases in production of hydroxylated products. The estimation of the minimum costs for large-scale biohydroxylations suggests that combining adequate reactor operations and high catalytic activity at low cell densities might reduce the cost five-fold. The cost might be further reduced if the catalytic activity is doubled without loss of stability (Ayala and Torres, 2004).

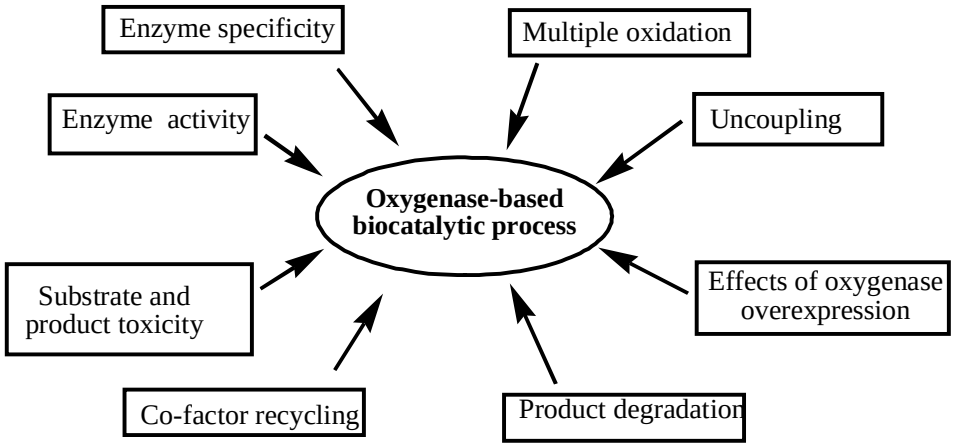


Figure 1.2: Properties influencing development of biocatalytic hydroxylation processes, which includes both enzymatic and physiological factors (Taken from Bühler and Schmid, 2004)

Several oxygenases catalyze multiple oxidations of hydrocarbon substrates, which is a problem if a specific alcohol is the desired product. This multiple oxidation activity may be due to a low specificity resulting in hydroxylation of multiple sites or in overoxidation of an alcohol product to the corresponding aldehyde, ketone or acid (Bühler and Schmid, 2004). In the case where degradation of the desired product takes place, it is necessary to block through-conversion of the intermediates by addition of enzyme inhibitors. However due to economics and an increase in process complexity, the use of enzyme inhibitors is mainly restricted to small scale applications and characterization of metabolic pathways (Arp, 1999; Bühler and Schmid, 2004). Mutagenesis of the wild type strains or overexpression of the desired gene into a host that is not able to degrade the product is also a means of accumulating the desired products (Bühler and Schmid, 2004).

Table 1.2. Issues and challenges in the application of hydroxylating enzymes (Adapted and modified from van Beilen ***et al***., 2003; Bühler and Schmid, 2004).

Issue/challange	Explanation/cause/comment	Solution	References
Low K_{cat}	Balance between optimal activity,selectivity and overoxidation (speculative)	Fusions between oxygenase and electron transfer component; directed evolution	van Beilen ***et al***., 2003;Duetz, WA., 2001
Uncoupling	Bad fitting of substrate, product binding to active site	Protein engineering and directed evolution. Product removal by ***in situ*** product recovery	van Beilen ***et al***., 2003 ;Jones ***et al***, 2001; Meyer, A. ***et al***., 2002.
Multiple oxidation	Product might also be substrate. Overoxidation	Addition of enzyme inhibitors. Deletion of pathways by mutagenesis. Heterologus expression in suitable host	van Beilen ***et al***., 2003;Buhler and Schmid, 2004
Overexpression	Multiple components. Differences in molecular environment from wild type, co-factor incorporation and genetic stability	Addition of co-factor precussors, protein engineering and multicistronic expression vectors	Guengerich, ***et a***l., 1997; Bühler and Schmid, 2004.
Substrate uptake and product toxicity	Hydrophobic substrates disrupt cell membranes. They are also water insoluble. General toxicity of more polar compounds. Uptake systems for larger substrates have not been characterized yet	Substrates and products dissolved in apolar liquids. New hosts with altered uptake profiles. Co-expression of uptake systems/porins. Regulating substrate addition and ***in situ*** product removal	Held ***et al***.,1999 ; Bühler and Schmid, 2004
Cofactor recycling	Capacity of cell metabolism may become limiting at higher specific oxygenase activities.Results due to reduced NAD(P)H regeneration rate	Co-expression of suitable dehydrogenase. Reduce activity of terminal electron chain. Electrochemical methods	Bühler and Schmid, 2004
Product degradation	Degradation of desired product .No dead end product	Heterologous expression in suitable host. Need for suitable substrate.	Bühler and Schmid, 2004
Enzyme activity	Specific catalytic rate low, K_{cat}. Specificity of enzyme	High-level expression in host. Protein engineering and mutagenesis	Bühler and Schmid, 2004

1.4 Heterologous Expression of CYP450s

Heterologous expression is a solution for some of the problems involving the application of hydroxylating enzymes, but in most cases, heterologous overexpressions of oxygenases in a recombinant host results in lower activities as compared to the wild type strain. This could be due to the differences in the molecular environment of the recombinant host and the wild type strain, which might complicate the stable and functional expression of oxygenases in recombinants. Some of the critical factors for the overproduction of the active hydroxylating enzymes include protein folding, protein stability, genetic stability, the ratio of multiple components, cofactor incorporation, the interactions of membrane-associated components of the host membrane, the requirements for additional proteins, and the formation of reactive oxygen species (Bühler and Schmid, 2004).

1.5 *Y. lipolytica* as a host for heterologous expression of CYP450 monooxygenases

A variety of expression hosts, which include bacteria, yeasts and mammalian cells, have been used to successfully overexpress catalytically active foreign proteins with variable success (Gonzalez ***et al***., 1995). Microorganisms such as ***Escherichia coli, Saccharomyces diastaticus, Saccharomyces cerevisiae, Schizosaccharomyces pombe*** and ***Y. lipolytica*** have all been tested for heterologous expression of CYP450s (Murakami ***et al***., 1990; Liu ***et al***., 1998). Amongst these, ***Y. lipolytica*** has been reported to be one of the most attractive host organisms for protein expression (Müller ***et al***., 1998; Nicaud ***et al***., 2002). The main factors influencing the choice of expression host include expense, ease of use and yield required (Gonzalez ***et al***., 1995).

Y. lipolytica is a dimorphic, non-pathogenic yeast with significant capacities for high molecular weight protein secretion (Juretzek ***et al.***, 2001). It is capable of forming yeast cells, pseudohyphae and septate hyphae. Accumulation of large amounts of data on its behaviour in large fermentors coupled with its genetic stability (Barth and Gaillardin, 1996) has led to its development as one of the most extensively studied “non-conventional” yeasts, which is currently used as a model for various studies. It is known for its efficient degradation of hydrophobic

substrates such as alkanes, fatty acids, fats and oils (Juretzek ***et al.***, 2001), and has been classified as Generally Regarded As Safe (GRAS) by the American Food and Drug Administration for citric acid production (Fickers ***et al***., 2005). Due to its specific properties, such as the very efficient utilization of hydrocarbon substrates and the high secretion capacity of metabolites and proteins, this yeast has been used in different applications mainly focusing on bioconversion processes for the chemical and food industries (Fickers ***et al***., 2005) for citric acid production. Further more, the alkane utilizing yeasts such as ***Y. lipolytica*** or ***Candida maltosa*** could be useful for efficient biotransformation of hydrophobic substrates into value added products, such as dicarboxylic acids and other hydroxylated products (Barth and Gaillardin, 1996; Maursberger ***et al.***, 1996; van Rensburg ***et al***., 1997; Juretzek, 1999; Juretzek ***et al***., 2001).

Alkane-utilising yeasts, such as ***Y. lipolytica*** exhibit a high catalytic activity of their cytochrome P450 monooxygenases (P450s), catalyzing the hydroxylation of different hydrocarbon substrates. This is supported by an efficient sub cellular organization, which facilitates substrate and product transport, a proliferation of the endoplasmic reticulum as well as an efficient electron transfer system (Mauersberger ***et al***., 1987; Mauersberger ***et al***., 1996; Fickers ***et al***., 2005).

A recent evaluation of several yeasts has revealed that ***Y. lipolytica*** is a highly attractive alternative for secretion and expression cloning (Müller ***et al***., 1998; Juretzek ***et al***., 2001). The recent development of genetic tools for ***Y. lipolytica*** has enabled it to be used to overexpress foreign proteins***.*** These include several strong and regulated promoters, which are now available for heterologous gene expression (Juretzet ***et al***., 2001). A literature search on successful overexpression of CYP450s in ***Y. lipolytica*** yielded three cases. A further four CYP450s were cloned into ***Y. lipolytica*** in our laboratory (**Table** 1.3). For the expressed P450 to be fully functional, a cell must have adequate heme biosynthetic capabilities, ample intracellular membranes and other enzymatic components such as the flavoprotein NADPH- P450 oxidoreductase (Schenkman ***et al***., 1993; Gonzalez ***et al***., 1995). As an alkane degrading yeast ***Y. lipolytica*** already meets these requirements and one would expect it to be a good host for

the overexpression of CYP450s yielding useful recombinant whole cell biocatalyst for hydroxylation reactions.

Table 1.3. ***CYP450s*** overexpressed in ***Y. lipolytica.***

Additional CYP genes inserted			
CYP gene cloned	**Source**	**Hydroxylase activity**	**Reference (Cloned by)**
CYP17A	Bovine	17α steroid hydroxylase	Juretzek ***et al***., 2000
CYP74	Green bell pepper	Fatty acid hydroperoxide lyase	Bourel ***et al***., 2004
CYP1A1	Human	Hydroxylation of polycyclic aromatic hydrocarbons	Nthangeni ***et al***., 2004
CYP53B1	***Rhodotorula minuta***	Benzoate-***para*** hydroxylase	Shiningavamwe, 2003
CYP52F1	***Y. lipolytica***	Alkane hydroxylase	Setati, 2004
CYP52F2	***Y. lipolytica***	Alkane hydroxylase	Setati, 2004
CYP 557A1	***Rhodotorula retinophila***	Putative fatty acid hydroxylase	Shiningavamwe, 2004

1.6 Aim of the study

The principle aim of this study was to evaluate ***Y. lipolytica*** as a recombinant whole-cell biocatalyst for hydroxylation reactions by using available ***Y. lipolytica*** strains overexpressing the ***CYP1A1; CYP53B1; CYP52F1 and CYP557A1*** genes. Hydroxylase activity of the genetically engineered strains was to be compared with activity in wild type yeasts expressing the relevant CYP450s.

The specific aims of this study were to:

(1) Compile a literature review focused on CYP450 catalyzed hydroxylation reactions by both wild type and genetically engineered organisms

(2) Evaluate the suitability of different substrates for the evaluation of different CYP450s. Factors such as toxicity, further degradation, analysis and cost were to be considered

(3) Determine problems and limitations as well as factors influencing ***Y. lipolytica*** as a host for CYP450 expression

(4) Quantify microbial CYP450 hydroxylation reactions in bioreactor experiments to determine, maximum reaction rates and yields under controlled conditions

CHAPTER TWO

2.1 Hydroxylation reactions in nature

Enzymatic hydroxylation involves the introduction of a hydroxyl group(s) into various substrates and is catalyzed by different types of enzymes. Amongst these are the heme-containing cytochrome P450 monooxygenases and the non-heme enzymes such as methane monooxygenase, alkane hydroxylase and fatty acid desaturases (Ayala and Torres, 2004) as well as the flavin dependent monooxygenases which catalyze hydroxylation of various aromatic compounds. Hydroxylating enzymes are involved in a plethora of metabolic processes, both anabolic and catabolic, and collectively interact with an enormous variety of substrates (Mot and Parret, 2002). While oxygenated compounds such as sugars need not to be hydroxylated, the catabolism of compounds such as alkanes, lipids and aromatic compounds is often initiated by hydroxylation reactions (Hayaishi, 1969).

Since enzymatic hydroxylation is a key part of oxidative metabolism of many organic compounds, it continues to be an area of intensive study. It has also been used for many years in the industrial production of fine chemicals and for bioremediation processes (Holland and Weber, 2000). In this review examples of hydroxylation reactions in nature will focus on eukaryotes, specifically fungi and plants. Similar and even more diverse activities are found in prokaryotes.

2.1.1 Hydroxylating enzymes in fungal membrane integrity

Sterols are important constituents of biological membranes and the reaction sequence for their biosynthesis is well established in mammals and plants. In most fungi the end product of sterol synthesis is 24-methylcholesta-5,7,22-trien3*β*-ol (ergosterol). The synthesis of ergosterol has been worked out by extensive studies using the yeast ***Saccharomyces cerevisiae*** and species from other fungal taxa (Méjanelle ***et al***., 2001). Reaction sequences for ergosterol biosynthesis (**Figure** 2.1) downstream from lanosterol may vary according to fungal taxa. The removal of the 14α-methyl group (**Figure** 2.1,

compound 1 to 2) is an essential step of sterol biosynthesis in all eukaryotes. It is catalyzed by CYP450s belonging to the CYP51a subfamily of CYP450s (Hurtado ***et al***., 1999). Ergosterol is the predominant lipid molecule in yeast cells that regulate membrane fluidity, permeability and the activity of most membrane bound enzymes. The functional activities of ergosterol thus play an important role in cellular growth. Natural lipids of many fungi including ***S. cerevisiae*** are mostly composed of sterols and squalene, the precursor of sterols (Mejanelle ***et al***., 2001).

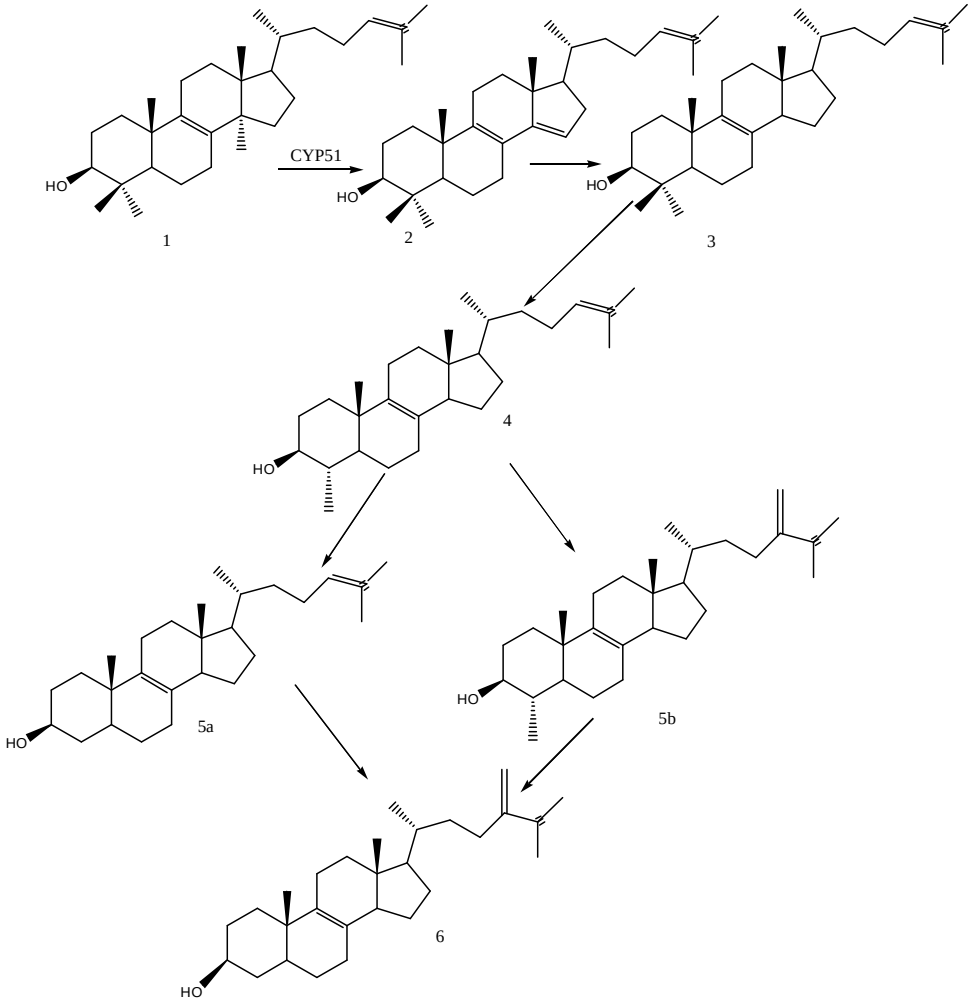


Figure 2.1: Predominant reaction sequences for ergosterol (6) biosynthesis downstream from lanosterol (1). The CYP51 is involved in the demethylation, step (1) to (2) (Adapted from Hurtado ***et al***., 1999)

Azole compounds such as flucanazole, miconazole and clotrimazole, which are all commonly used antifungals, target and inhibit the ergosterol biosynthesis pathway in yeasts by inhibiting 14α-demethylation (Hurtado ***et al***., 1999; Parveen ***et al***., 2004). Studies on the order of potency of antifungal drugs, suggests that CYP450s such as CYP51 sterol demethylase and CYP121, are the actual target enzymes for these azole drugs (Mclean ***et al***., 2002). The physiological role of CYP121 remains unclear, but one of its closest relatives is P450 eryF (CYP107A1) from ***Saccharopolyspora erythraea***. This enzyme is involved in erythromycin biosynthesis, suggesting that CYP121 perhaps plays a role in polyketide synthesis (Munro ***et al***., 2003).

2.1.2 Catabolism of hydrophobic substrates

In nature, the catabolism of hydrophobic substrates such as alkanes, fatty acids and triglycerides, is a quite complex process. In eukaryotes, this process involves several metabolic pathways, which take place in different subcellular compartments (Mauersberger ***et al***., 1996; Fickers ***et al.,*** 2005). Of the various hydrocarbons, ***n***-alkanes and branched alkanes of intermediate lengths (C10-C20) are the preferred substrates for microorganisms and tend to be mostly degradable. Longer chain alkanes (>20) are hydrophobic solids and are much more difficult to degrade, while cycloalkane degradation is much slower than the corresponding ***n***- and branched alkanes (Aichberger ***et al***., 2005).
An important characteristic of alkane assimilation by yeast is the metabolic flow of carbon from alkane substrates to synthesis of cellular components via fatty acids, which is quite different from the case of conventional substrates like glucose (Fickers ***et al***., 2005). In yeasts, the alkane assimilation mainly occurs via the monoterminal, diterminal or subterminal oxidation pathways (**Figure** 2.2). Alkane assimilation by microorganisms is divided into several steps (Fukui ***et al***., 1981; Fickers ***et al***., 2005). The initial step after being incorporated into the cell is usually the hydroxylation of the alkane by monooxygenases to the corresponding alcohol. This is followed by further oxidations leading to the corresponding dicarboxylic acids, which are subsequently degraded to acetyl-CoA and propionyl-CoA (in the case of odd-chain alkanes) ***via*** the *β*–oxidation pathway (Fickers ***et al***., 2005).

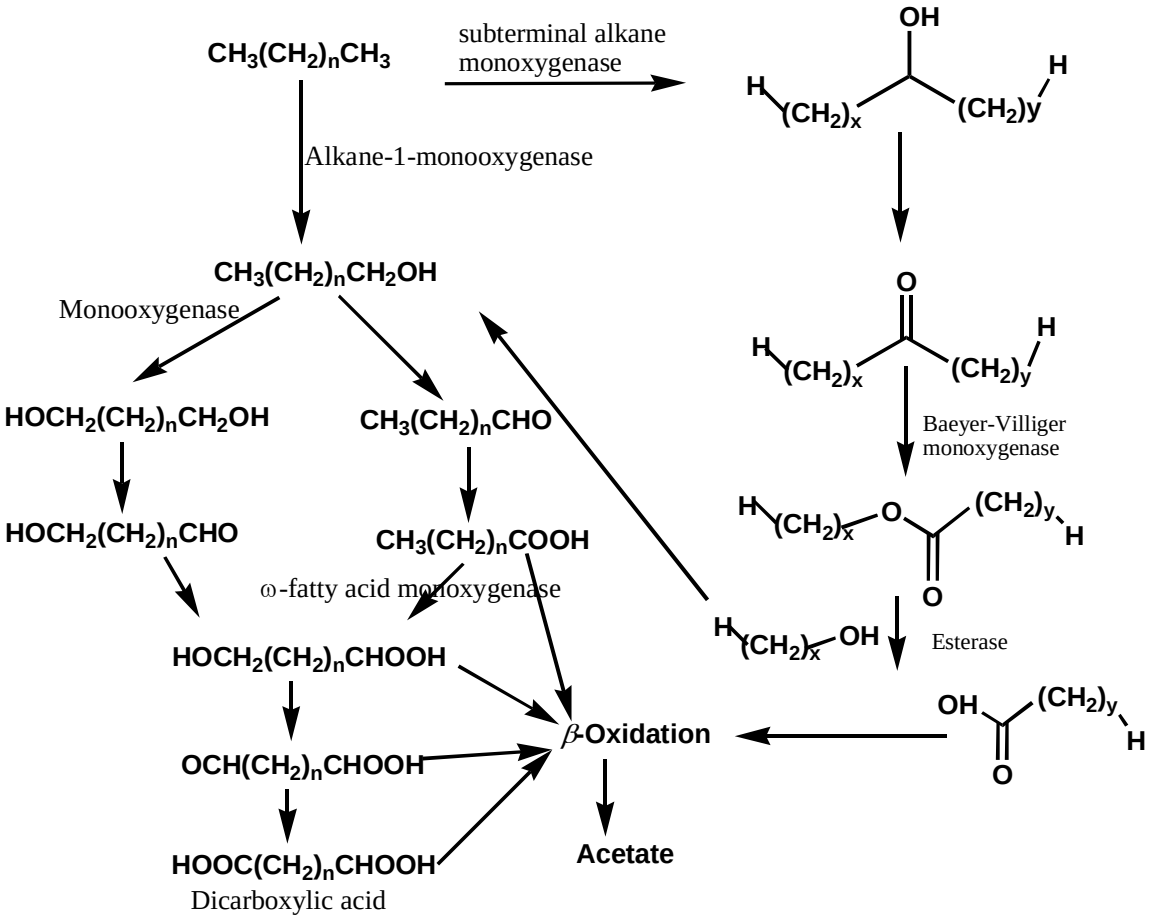


Figure 2.2: Monoterminal, diterminal and subterminal alkane degradation pathways in fungi. The different types of hydroxylating enzymes are highlighted. (Adapted from Casey ***et al***., 1990; van Beilen ***et al***., 2003)

Aromatic compounds, which are some of the heaviest burdens at polluted sites, pose a real challenge for microbial degradation due to their toxicity at high concentrations (Smith, 1980). In spite of their toxic properties, a number of microorganisms can under aerobic conditions utilise these compounds as sole sources of carbon and energy (Paller ***et al***., 1995).

Yeasts such as ***Debaryomyces***, ***Trichosporon*** and ***Candida*** mainly catabolize phenols under strictly aerobic conditions ***via*** its oxidation products (**Figure** 2.2) (Hölzel ***et al***., 1995). In the catabolism of aromatic compounds, some crucial steps such as ring hydroxylation and ring cleavage are common for all the degradation pathways (Bertini ***et***

al., 1996). The ascomycetous yeast***, Debaryomyces hansenii*** has been reported to metabolize benzenoid compounds such as phenol, dihydroxybenzenes (catechol, resorcinol) and dihydroxybenzoic acids (Yadav and Loper, 1999).

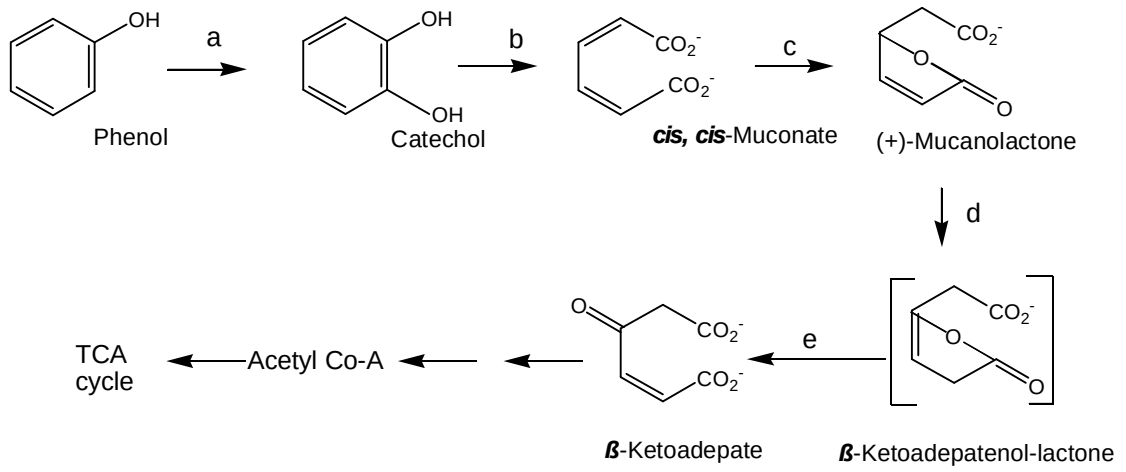


Figure 2.3: Proposed pathway for the degradation of phenol by the yeast ***Trichosporon cutaneum*** (a) Phenol hydroxylase; (b) catechol 1,2-oxygenase; (c) ***cis cis***-muconate lactonase; (d-e) delactonising activities, proposed in analogy to bacterial systems - (+)-mucanolactone isomerase, and enol-lactone hydrolase (Gaal ***et al***., 1979; Yadav and Loper, 1999)

Durham ***et al***. (1984) reported that the basidiomycetous yeast, ***Rhodotorula graminis*** could utilise benzoic acid via the *β*-ketoadipate pathway. The most crucial step in the degradation of benzoic acid is the initial hydroxylation by a benzoate-4-hydroxylase (a CYP450) at the ***para*** position to form 4-hydroxybenzoate. This is further oxidised by 4-hydroxy-benzoate-3-hydroxylase (a flavin dependent monooxygenase) to form 3,4-dihydroxybenzoate, which undergoes further catabolism to give TCA products as illustrated (**Figure** 2.4).

Eppink ***et al***. (1997) however suggested a completely different pathway for the metabolism of 4-hydroxybenzoate by ***Candida parapsilosis*** CBS604 (**Figure** 2.5). This yeast metabolised 4-hydroxybenzoate by oxidative decarboxylation to give 1,4-dihydroxybenzene. This reaction and subsequent conversion of 1,4-dihydroxybenzene to 1,2,4-trihydroxybenzene were catalysed by two distinct flavin dependent monooxgenases.

The catabolism of 2,4-dihydroxybenzoate and 3,4-dihydroxybenzoate in ***C. parapsilosis*** also proceeded through 1,2,4-trihydroxybenzene. It was reported that ***C. parapsilosis*** could also grow on 2,4-dihydroxybenzoate and 3,4-dihydroxybenzoate as sole carbon sources. (Eppink ***et al***., 1997).

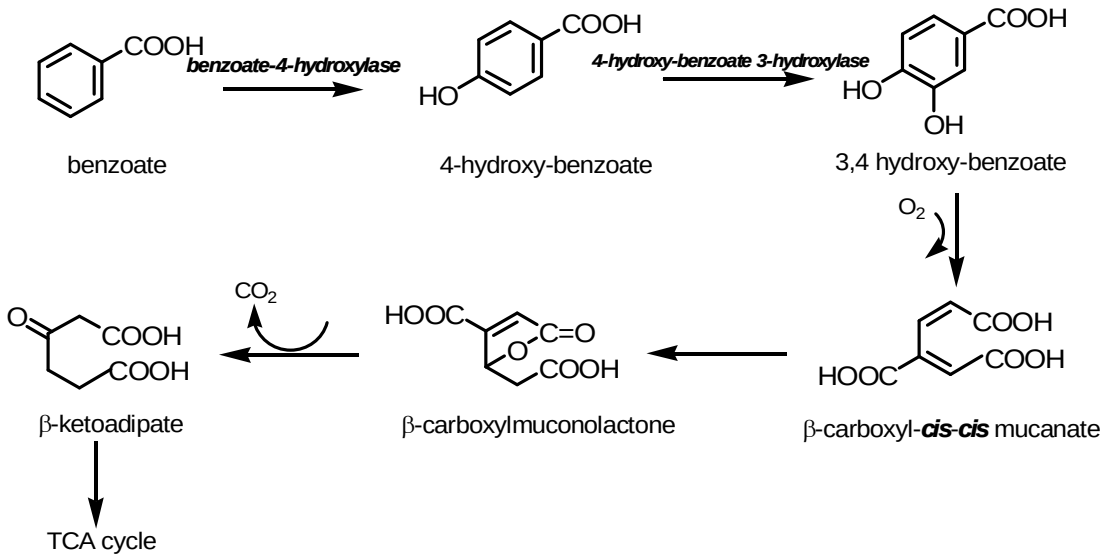


Figure 2.4: Catabolism of benzoic acid by ***R. graminis***. Adapted from (Durham ***et al***., 1984).

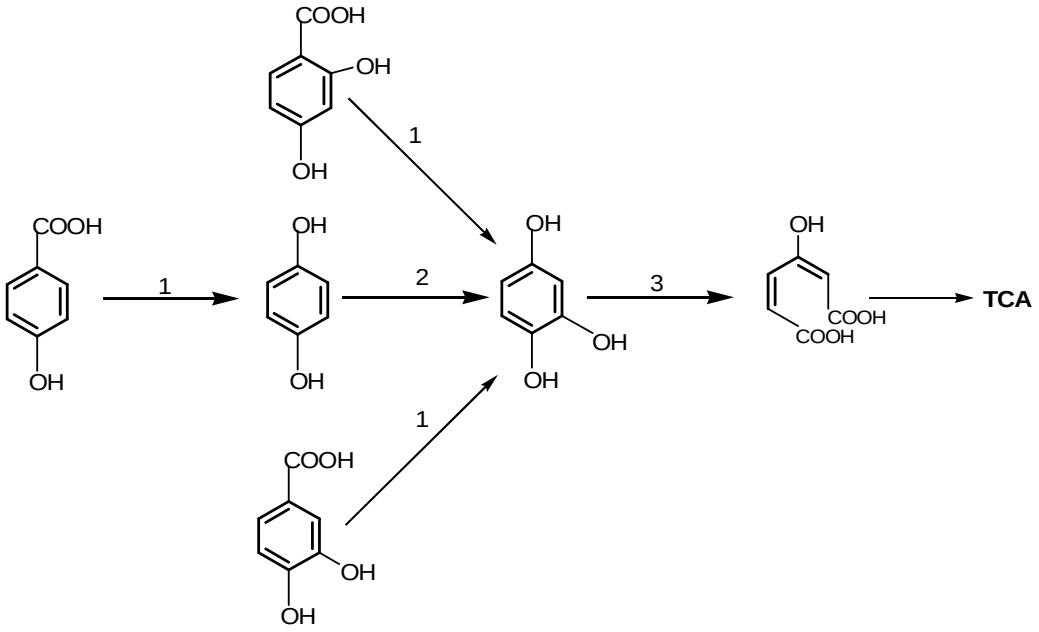


Figure 2.5: Reaction sequence for the catabolism of 4-hydroxybenzoate, 2,4-dihydroxybenzoate, and 3,4-dihydroxybenzoate in ***C. parapsilosis*** CBS604. Enzymes: 1,4-dihydroxybenzoate (dicarboxylating); 2, phenol monoxygenase; 3, trihydroxy 1,2-dioxygenase (Adapted from Eppink ***et al***.,1997)

Chlorobenzoates, which are another class of persistent environmental pollutants, may be introduced to the soil directly through application as herbicides or insecticides. Many bacteria have been isolated which posses the ability to degrade these chlorinated aromatic compounds. ***Pseudomonas aeruginosa*** strain (3mT) isolated by Ajithkumar ***et al***., (1998) degraded high concentrations of 3-chlorobenzoate (up to 8 g/l) and 4-chlorobenzoate (up to 12 g/l) respectively. However, the information about aromatic and chloroaromatic degradation is very limited for yeasts (Ajithkumar ***et al***., 1998). Walker (1973) reported the oxidation of halogenophenols by a strain of ***Rhodotorula glutinis.*** Chlorinated aromatic compounds are degraded through oxidation by yeasts and other eukaryotic cells (Neilson, 1990; Sun ***et al***., 2000). The aerobic dehalogenation of chlorinated aromatic compounds in yeasts may involve oxidative dehalogenation reactions and is carried out by a cytochrome monooxygenase system (Sun ***et al***., 2000). Yeasts such as ***Rhodotorula rubra*** are much more regulated in the uptake and metabolism of chlorinated aromatic compounds than bacteria. In ***R. rubra***, it is the hydroxylase system that is involved in the degradation of aromatic pollutants. The aerobic dehalogenation of chlorinated aromatic compounds in microorganisms may involve either oxidative or hydrolytic dehalogenation reactions. ***R. rubra*** is able to completely degrade 2-chrorobenzoate to TCA products (**Figure** 2.6) while 3-chlorobenzoate is hydroxylated to 3-chloro 4-hydroxybenzoate as the dead end product. This yeast is however not at all able to degrade or transform 4-chlorobenzoate (Sun ***et al***., 2000).

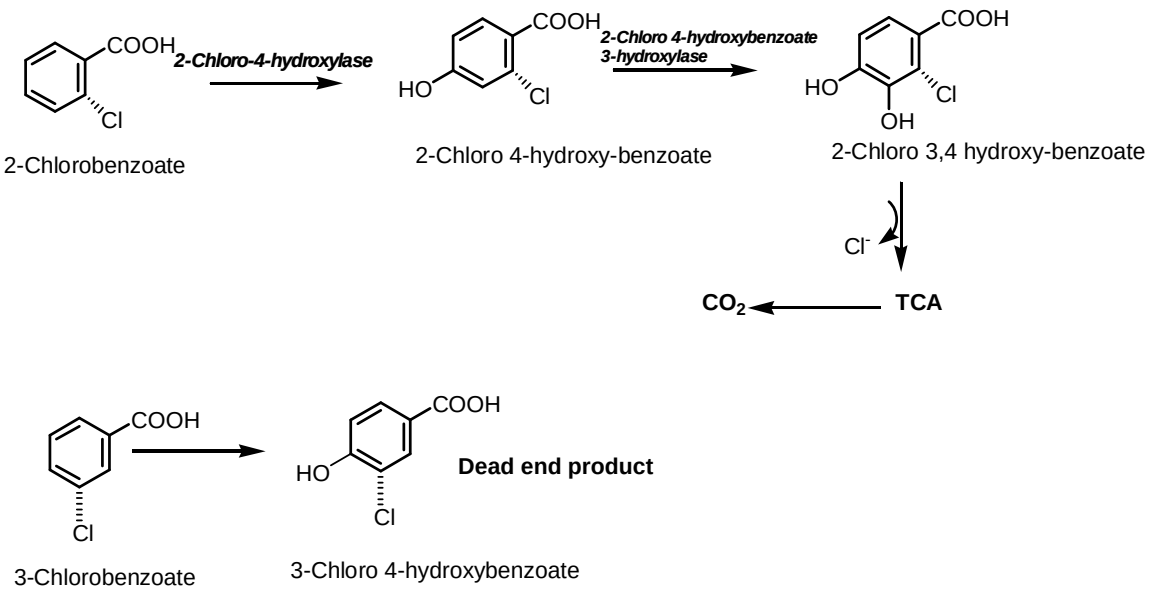


Figure 2.6: Proposed catabolism of chlorinated benzoic acids by ***R. rubra*** Y-1529. 2-Chlorobenzoate is completely degraded, but hydroxylation of 3-chlorobenzoate yields a dead end product (Adapted from Sun ***et al***., 2000).

2.1.3 Hydroxylating enzymes in the synthesis of secondary metabolites

Evolution of various hydroxylating enzymes is related to the complex and versatile chemistry developed by higher plants, animals and microorganisms to synthesize a large variety of secondary metabolites. An interesting example is the P450 monooxygenases which catalyze most of the oxidation steps in plant secondary metabolism (Kahn and Durst 2000; Morant ***et al***., 2003). CYP450s form the largest class of plant enzymes and several hundreds of CYP450 encoding genes are probably present in the genomes of most plant species (Werck-Reichhart ***et al***., 2000).

A large proportion of the most complex regiospecific and stereospecific reactions in the biosynthesis of bioactive compounds are catalyzed by hydroxylating enzymes (Morant ***et al***., 2003). Several genes encoding CYP450s that catalyze branch-point reactions in flavonoid biosynthesis have recently been characterised. Hydroxylation reactions in the biosynthesis of shikimate coumaroyl esters from phenylalanine in ***Arabidopsis*** is catalyzed by P450s belonging to the CYP73A and CYP98A subfamilies (**Figure** 2.6) (Schoch ***et al***., 2001; Morant ***et al***., 2003).

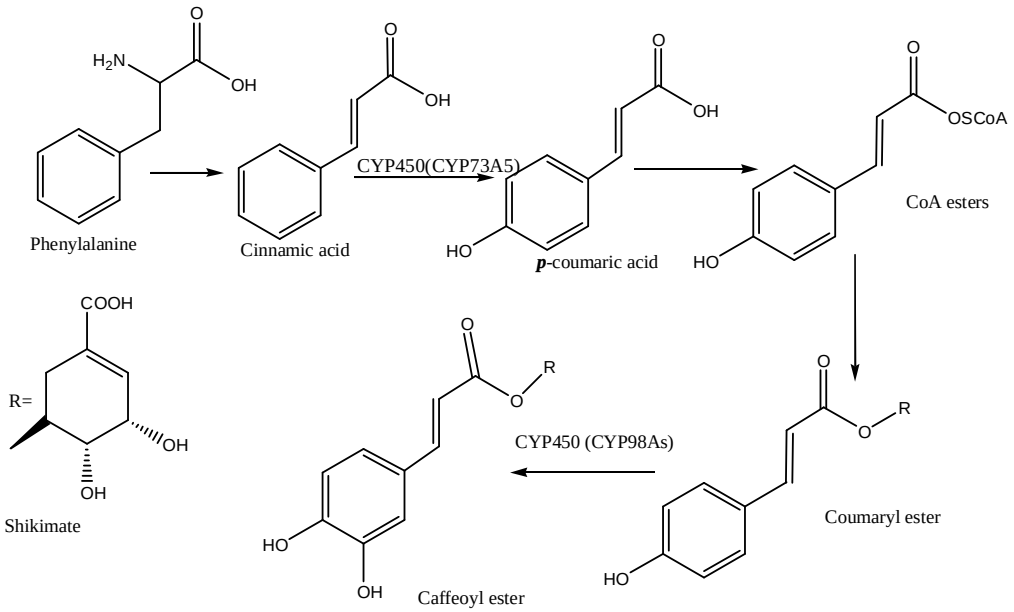


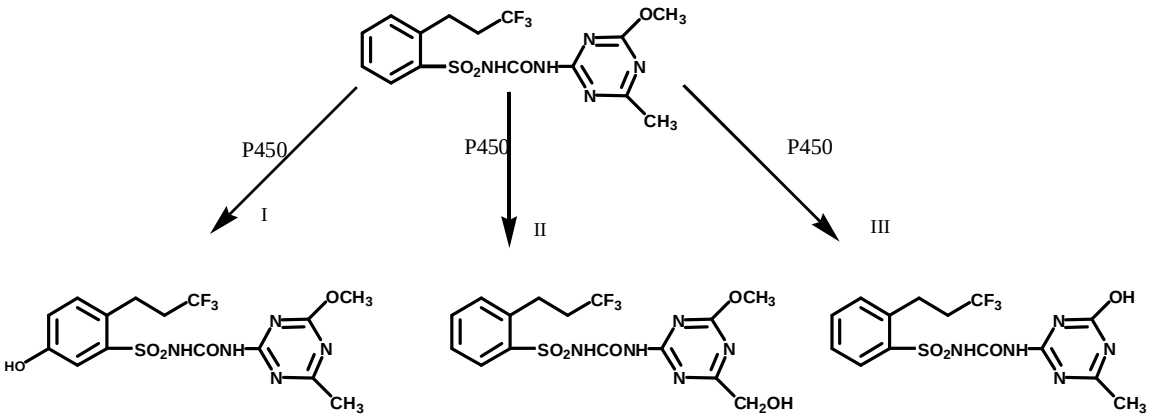
Figure 2.7: Schematic representation of the synthesis of secondary metabolites from phenylalanine showing the hydroxylating role of CYP450 in the metabolic pathways (Adapted from Morant ***et al***., 2003).

2.1.4 Hydroxylating enzymes in herbicide and insecticide detoxification reactions

An indication that CYP450s might be involved in herbicide metabolism came from the analysis of herbicide residues formed ***in vivo.*** Among the major metabolites of most classes of herbicides are aryl- or alkyl-hydroxylated, and ***N***-, ***S***- or ***O***-dealkylated products and their glucose conjugates. Most herbicides e.g. prosulfuron and dichlofop, can be converted by CYP450s into several metabolites. In wheat, prosulfuron is metabolised via phenyl-ring hydroxylation, alkyl hydroxylation or ***O***-demethylation while the metabolism of diclofop involves a hydroxylation with simultaneous migration of the halogen atom to an adjacent position (**Figure** 2.8). Both these processes are CYP450 mediated (Werck-Reichhart ***et al***., 2000). In most cases herbicide resistance appears to result from an increase in metabolism due to formation of polar products, which are as a result of CYP450 involvement. Tremendous amounts of work mostly performed on major crop plants have confirmed the important role of CYP450s in the oxidation of most classes of herbicides (Barret ***et al***., 1995; Werck-Reichhart ***et al***., 2000).

Among the best-characterized insect CYP450 systems used for detoxification of plant allelochemicals are the set of CYP6B isoenzymes from ***Lepidoptera*** species that have the ability to metabolise toxic furanocoumarins. In addition to detoxification of allelochemicals, another crop pest, ***Helicoverpa zea*** (corn earworm) has the ability to detoxify a large variety of insecticides including carbamates and pyrethroids. Even though, the detoxification of pyrethroids has been shown to be CYP450-mediated (Li ***et al***., 2000b; Sasabe ***et al***., 2005), the exact relationship between CYP450s mediating allelochemical detoxification and insecticide detoxifications is only beginning to emerge (Sasabe ***et al***., 2005).

(a) Prosulfuron



(b) Diclofop

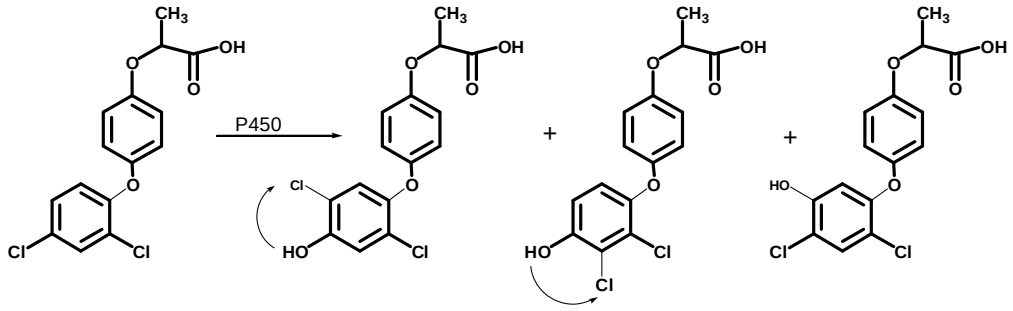


Figure 2.8: Examples of P450-catalyzed oxygenations of herbicides. (a) In wheat, prosulfuron is metabolised via phenyl-ring hydroxylation, alkyl hydroxylation and O-demethylation of the triazine. (b) Metabolism of diclofop by P450s results in hydroxylation with simultaneous halogen atom migrations 'NIH shift' (Adapted from Werck-Reichhart ***et al***., 2000).

2.2 Hydroxylating enzymes

This section of this literature review will focus on hydroxylating enzymes acting on unactivated carbons. Enzymes capable of hydroxylating unactivated carbons can often also hydroxylate activated carbons, but some enzymes such as the flavin dependent monooxygenases can only hydroxylate activated carbons which include aromatic carbons, olefinic carbons, benzylic and allylic carbons.

Several enzyme systems capable of hydroxylating unactivated carbons under aerobic conditions have evolved in both eukaryotes and prokaryotes. These enzymes, which are ubiquitous in nature, include; integral membrane diiron alkane hydroxylases (e.g. AlkB), soluble diiron methane monooxygenases (sMMO), membrane bound copper containing (and possibly iron-containing) methane monooxygenases (pMMO) and cytochrome P450 monooxygenases (van Beilen ***et al***., 2005). These enzymes catalyze some of the most difficult reactions to perform selectively by conventional chemical routes (Ayala and Torres, 2004).

2.2.1 Diiron monooxygenases

These are monooxygenases characterised by the presence of a diiron cluster in their hydroxylase component. The function of the metallic centre is to activate the dioxygen during the oxidation of substrates (Ayala and Torres, 2004). The diiron monooxygenases include enzymes such as: Toluene monooxygenase, phenol hydroxylase, methane monooxygenase and alkane hydroxylase. For each of these enzymes, the oxygenase subunit contains a carboxylate-bridged diiron centre at the active site. Due to immense interest in the nature of the diiron centre at the active site of these enzymes and its function in catalysis, members of this family have been subjected to extensive research in recent years (Leahy ***et al***., 2003). The reaction mechanism for hydroxylation of alkanes by diiron monooxygenases is a complicated process. It has been reported that the extreme oxidation state of the iron dimer complex makes a large number of intermediate states possible, and also opens up a large number of possible reaction pathways (Siegbahn, 2001).

From available structural information, it appears that these enzyme systems all contain three to four components: a dimeric hydroxylase protein composed of two or three subunits in a $(\alpha\beta\gamma)_2$ or $\alpha\beta$ quaternary structure, an NADH oxidoreductase with an N-terminal chloroplast-type ferrodoxin domain and a C-terminal reductase domain with FAD- and NAD(P)-ribose binding regions, a small effector or coupling protein with no prosthetic groups, and in some cases a ferrodoxin protein (Leahy ***et al***., 2003).

The study of diiron monooxygenases has been motivated by their importance in expanding the substrate range to include a diverse array of hydrocarbons, amongst them hazardous compounds such as benzene and trichloroethylene (Badr ***et al***., 1992; Leahy ***et al***., 2003).

2.2.1.1 Methane monooxygenases

Diiron monooxygenases include the thoroughly characterized but relatively rare, soluble methane monooxygenase (sMMO) (Kopp and Lippard, 2002; Baik ***et al***., 2003; van Beilen ***et al***., 2005). These enzymes are unique among the diiron monoxygenases in their capacity to oxidize the highly stable methane molecule to methanol (Leahy ***et al***., 2003). Besides the activation of methane, sMMO is able to oxidize saturated and unsaturated alkanes and halogenated, aromatic and heterocyclic compounds (Baik ***et al*** 2003; van Beilen ***et al***., 2005). However, the microorganisms producing methane monooxygenases can only catalyse the oxidation of medium chain alkanes but are unable to grow on any of the alkanes as the only carbon source (Ayala and Torres, 2004).

Two forms of methane monooxygenases have been described: the particulate (pMMO) and the soluble (sMMO) methane monooxygenase, though there appears to be no evolutionary relationship between these two. While pMMO is a membrane bound protein produced by all known methanotrophs, sMMO is expressed only in a subset of these microorganisms and has wider substrate specificity. It has however been shown that, pMMO, which is an iron-copper containing protein, is produced only under copper sufficiency while sMMO which is an iron-containing enzyme is produced only under copper

limitation (Ayala and Torres, 2004). sMMO has been extensively characterised as a dimeric hydroxylase protein and is comprised of three components which include: an oxygenase, a reductase and a coupling protein (Leahy ***et al***., 2003; Ayala and Torres, 2004). Green ***et al.,*** (1989) reported the relative hydroxylation rates of sMMO from ***Methylococcus capsulatus*** for short chain alkanes (**Table** 2.1). On the other hand, pMMO has been poorly characterized due to its instability and strict purification conditions (Ayala and Torres, 2004).

Table 2.1. Relative rates for hydroxylation of alkanes catalyzed by sMMO as reported by Green ***et al***., 1989. Adapted from (Ayala and Torres 2004).

Substrate	Major product	Relative rate ($K^{substrate}/K^{ethane}$)
Pentane	2-pentanol	1
Hexane	hexanol	0.72
Heptane	heptanol	0.54
Neopentane	Neopentanol	0.36
2-Methylpropane	2-Methyl-2-propanol	1.5
2,3-Dimethylpentane	3,2-Dimethyl-3-propanol	0.91

The ability of sMMO to hydroxylate unactivated C-H bonds has led to many comparisons with cytochrome P450. In the sMMO reaction mechanism (**Figure** 2.9), O_2 is assumed to add to the Fe_2 (II, II) reduced form of MMO to give two peroxo species (**1** and **2**). After breaking the O-O bond, the bis-μ-oxo Fe (IV) dimer, **3** can undergo a rearrangement to the key Fe^{III}-O-Fe^{V}=O intermediate, **4**. This is followed by proton abstraction (**4-5**) which is rapidly followed by reaction of the transient methyl radical with the metal centre to form a weak Fe-CH_3 bond (**6**). Finally, rearrangement and the loss of methanol leads to a Fe^{III}-OH-Fe^{III} dimer that requires reduction to return to the Fe_2 (II, II) starting species (Siegbahn and Crabtree, 1997).

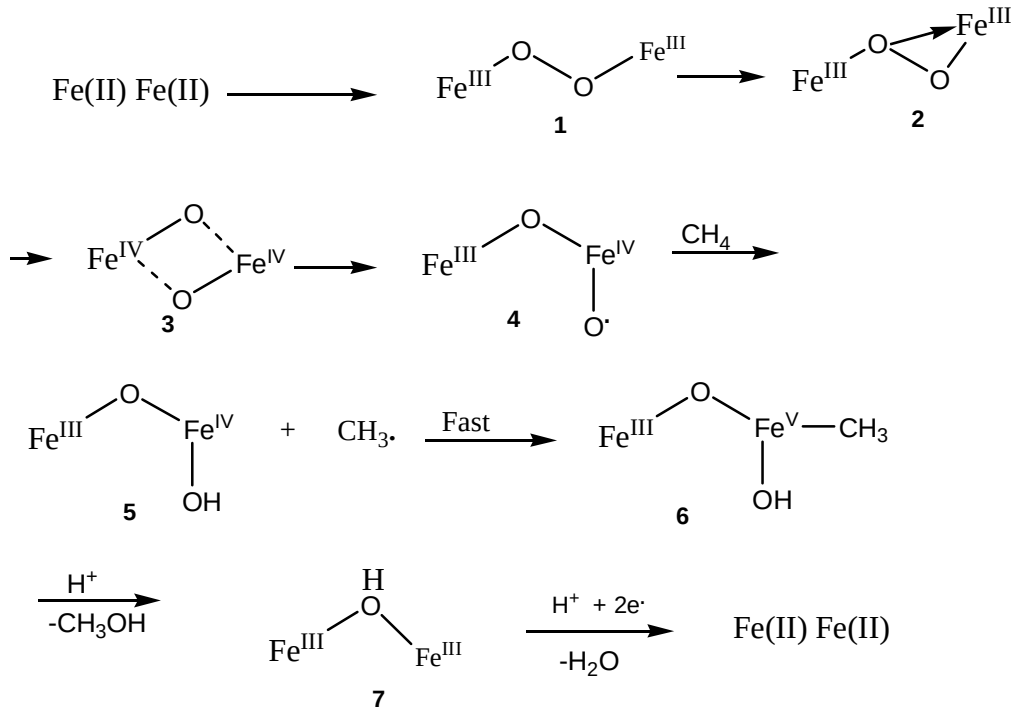


Figure 2.9: Reaction mechanism of methane monooxygenases as an example of diiron monooxygenases (Adapted from Siegbahn and Crabtree, 1997).

2.2.1.2 Alkane hydroxylases

The diiron alkane monooxygenase system (Alk) is present in a wide variety of microorganisms, which include ***Burkhalderia cepacia***, ***Pseudomonas***, ***Acinetobacter*** and ***Rhodococcus*** strains. It is a three-component monooxygenase system, comprising a hydroxylase, a rubredoxin and a rubredoxin reductase. While the hydroxylase system is membrane bound, both the rubredoxin and the rubredoxin reductase are soluble, cytoplasmic proteins. This enzyme complex which has the ability to oxidize medium to long chain linear alkanes using reducing equivalents from NADH or NADPH belongs to a group of diiron enzymes with a conserved set of eight histidine residues, which are necessary for activity. Members of this group include xylene monooxygenases, fatty acid desaturases, fatty acid monooxygenases, steroid oxygenases and decarbonylases. Most microorganisms producing alk enzymes can also use medium chain alkanes as energy and carbon source (Ayala and Torres, 2004). The reaction mechanism of alkane hydroxylase system is the same as that of sMMO.

2.2.2 Cytochrome P450 monooxygenases

Another class of enzymes capable of oxidizing unactivated carbons are the cytochrome P450 monooxygenases. These enzymes unlike sMMO and Alk described above are heme proteins. CYP450s are found throughout nature. The hydroxylase complex is composed of a CYP450 monooxygenase and a NADPH: CYP oxidoreductase (Ayala and Torres, 2004). Prokaryotic CYP450s are soluble proteins while eukaryotic CYP450s are anchored in membranes of the endoplasmic reticulum (ER) or inner mitochondrial membranes by hydrophobic N-termini (Nelson and Strobel, 1988; Scheller ***et al***., 1994; Menzel ***et al***., 1996).

CYP450s comprise a highly divergent gene superfamily that maintains secondary and tertiary structural conservations but very little primary sequence conservations (Schuler and Werck-Reichhart, 2003). The highly conserved regions in CYP450s include the helix I (HI) and the heme binding region (HR2) domains. The heme-binding domain has a consensus of Phe-X-X-Gly-X-Arg-X-Cys-X-Gly. Here, the conserved cysteine acts as a fifth ligand to the heme iron. Meanwhile, the helix I region has a consensus sequence of Ala/Gly-Gly-X-Asp/Glu-Thr-Thr/Ser and corresponds to the proton transfer groove on the distal side of the heme and is implicated in substrate recognition (Gotoh, 1992).

The most promising and thoroughly researched member of the CYP450 enzyme family is the non-specific bacterial CYP450 BM-3 (CYP102) from ***Bacillus megaterium*** (Van Beilen and Funhoff, 2005). This is a soluble single polypeptide chain which unlike, MMO, Alk or other CYP450s has the hydroxylase and reductase domain as components of the same polypeptide (Ayala and Torres, 2004). The wild-type BM-3 is a fatty acid hydroxylase which catalyses subterminal hydroxylation of fatty acids. A mutant of BM3, BM-3139-3, engineered by Glieder and co-workers (2002) is the fastest known enzyme for alkane hydroxylation (**Table** 2.2). This mutant BM3 is over 17 times faster than the sMMO or Alk enzymatic systems.

Table 2.2. Alkane hydroxylation rates of different enzymatic systems as reported by Glieder ***et al***., 2002. Adapted from Ayala and Torres, 2004.

Enzymatic system	Substrate	Maximum rate (min^{-1})
P450 BM-3 139-3	Hexane	3800
P450 MB3	Hexane	182
Alk	Octane	210
sMMO	Methane	222

2.2.2.1 Reaction mechanism of CYP450s

The essential step in the oxidation of a substrate by a CYP450 is the addition of one atom of molecular oxygen, which is activated by a reduced heme, to the substrate. The second oxygen atom is reduced to water, by accepting electrons from NAD(P)H via a flavoprotein or ferrodoxin. The activation of oxygen which takes place at the iron-protoporphyrin IX (heme) is common in all P450s. The commonly accepted mechanism for hydroxylation by CYP450 is illustrated in **Figure** 2.10. The heme is inactive in its ferric form when water forms the sixth ligand (1). The catalytic mechanism involves six steps. The first step involves the binding of the substrate to the P450 active site with displacement of water (2). The ferric enzyme is then reduced to the ferrous state by a one-electron transfer (3). Molecular oxygen then binds, resulting in a ferrous-dioxy species (4). The transfer of a second electron and a proton to the active site generates an iron-hydroxoperoxo intermediate (5), (Fe^{III}-OOH species). The cleavage of this O-O bond releases water and an activated iron-oxo ferryl species (6) (Fe^{V}=O) is formed. This very reactive iron-oxo ferryl species (6) oxidises the substrate and the product is subsequently released (Urlacher ***et al***., 2004). The properties of this iron-oxo ferryl species (6) -the last active Fe-intermediate in the catalytic cycle - are still poorly understood although crystal structures recently confirmed its existence (Schlichting ***et al***., 2000;Urlacher ***et al***., 2004). In support of the similarity between cytochrome P450 and the heme peroxidase chemistry, the high valent intermediate can also be generated through the reaction of the Fe^{III} enzyme with peroxides through a peroxide shunt (Costas ***et al***., 2000).

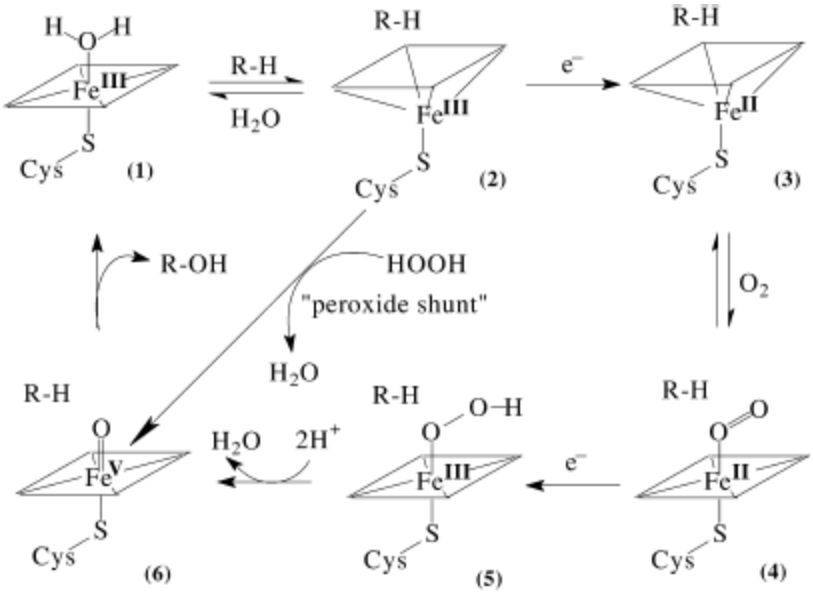


Figure 2.10: A schematic illustration of the catalytic cycle for Cytochrome P450 enzymes (Taken from Urlacher ***et al***., 2004).

2.2.2.2 Distribution of cytochrome P450 monooxygenases

The origin of the CYP450 superfamily probably lies in prokaryotes, before the advent of eukaryotes and before the accumulation of molecular oxygen in the atmosphere. Although ***Escherichia coli*** has no CYP450 encoding gene, ***Mycobacterium tuberculosis*** has 20, baker's yeast has 3, and the fruit fly, ***Drosophila melanogaster*** has 83 CYP450 genes and seven pseudogenes. Higher plants have more CYP450 encoding genes, with about 286 genes in ***Arabidopsis thaliana***, while humans have up to 55 genes. Only one CYP450 gene family, ***CYP51***, coding for the 14-α demethylases which are important in the biosynthesis of sterols is conserved across phyla, from plants to fungi and animals (Werck-Reichhart and Feyereisen, 2000).

2.2.2.3 Classification of cytochrome P450 monooxygenases

Members of the CYP450 monooxygenase superfamily are subdivided and classified following recommendations of a nomenclature committee (Nelson ***et al***., 1996; Werck-Reichhart and Feyereisen, 2000) on the basis of their amino acid identity, phylogenic

criteria and gene organization. The diversity of the cytochrome P450 superfamily arose by an extensive process of gene duplications and probable, but less well documented, cases of gene amplifications, conversions, genome duplications, gene loss and lateral transfers (Werck-Reichhart and Feyereisen, 2000). These enzymes vary in their subcellular locations, organismal ranges and the identities of their electron transfer partners (Rupasinghe ***et al***., 2003).

Not all CYP450s catalyze hydroxylation reactions. One type of CYP450 initially identified in the fungus ***Fusarium oxysporium*** catalyses the reduction of nitric oxide (NO) to nitrous oxide (N_2O) using NAD(P)H as the direct electron donor (Mansuy, 1998). This enzyme (CYP55A) called P450nor or nitric oxide reductase is a soluble CYP450 that receives its electrons directly from reduced pyridine nucleotides without intervention of an electron carrier (Peterson and Graham, 1998; Werck-Reichhart and Feyereisen, 2000). According to Werck-Reichhart and Feyereisen (2000) these CYP450s are classified as class IV CYP450s. Class III enzymes according to Werck-Reichhart and Feyereisen (2000) are self-sufficient CYP450s that do not require molecular oxygen or any external electron source. They catalyze the rearrangement or dehydration of alkylhydroperoxides or alkylperoxides initially generated by dioxygenases. None of these CYP450s has been crystallized to date (Werck-Reichhart and Feyereisen, 2000). Examples in this class include the mammalian enzyme, thromboxane synthase, CYP5A1 and the plant enzyme, allene oxide synthase, CYP74A1 (Peterson and Graham, 1998). The latter two classes (III and IV respectively) might be considered as the remains of the most ancestral forms of CYP450 involved in detoxification of harmful activated oxygen species (Werck-Reichhart and Feyereisen, 2000).

The hydroxylating CYP450s, which this review focuses on, are also divided into four classes (**Figure** 2.11) depending on the mode of delivery of the electrons to the P450 catalytic site. Classes I and II in this classification are the same as the class I and II CYP450s described by Werck-Reichhart and Feyereisen (2000), but classes III and IV are different. The classification of the interactions with redox partners is unrelated to CYP450 evolutionary history (Werck-Reichhart and Feyereisen, 2000). If the assumption is made

that here is a ‘structural core’ common in all P450s, then the charge distribution and topography of the ‘redox-partner binding region’ of each class of CYP450 must vary to accommodate the various types of redox partners (Peterson and Graham, 1998). The reactions catalyzed usually require an external source of reducing equivalents, NAD(P)H, and auxiliary proteins to transfer electrons to the P450 (Miles ***et al***., 2000).

Class I and IV CYP450 monooxygenases

Class I CYP450s have two electron transfer proteins: an FAD-containing NAD(P)H-dependant ferredoxin reductase and ferredoxin which is an iron sulfur protein [2Fe-2S] (Werck-Reichhart and Feyereisen, 2000). Most bacterial and all of the eukaryotic mitochondrial P450 systems are three component protein systems belonging to class I (Miles ***et al***., 2000). Examples in this class of enzymes include the well-characterised camphor hydroxylase, P450cam or CYP101 (Schlitchting ***et al***., 2000; Werck-Reichhart and Feyereisen, 2000; Mot ***et al***, 2002). Eukaryotic class I enzymes are found associated with the inner membrane of mitochondria of vertebrates, insects and nematodes (Rupasinghe ***et al***., 2003) and catalyze several steps in the biosynthesis of steroidal hormones and vitamin D_3 (Werck-Reichhart and Feyereisen, 2000). The class I enzymes channel electrons from NAD(P)H to FAD (in the ferredoxin reductase) to [2Fe-2S] (in ferredoxin) to heme to O_2 (De Mot and Parret, 2002).

Self sufficient CYP450s with CYP450 domains that are related to class I CYP450s have been discovered in ***Rhodococcus*** spp***.*** and other actinomycetes (De Mot and Parrot, 2002). These CYP450s labelled class IV CYP450s by Roberts ***et al***. (2003) are soluble, one component enzymes that contain an NADPH-dependent FMN-containing reductase and ferredoxin fused to the heme domain. The reductase domains of these enzymes are more similar to the reductase subunits of the “phtalate family” of mono- and dioxygenases than to the reductases of the class I CYP450s. In this class of CYP450s electrons are thus proposed to flow from NADH to FMN to [2Fe-2S] to heme to O_2 (De Mot and Parret, 2002).

Class II and III CYP450 monooxygenases

Class II proteins require only one electron transfer partner, a reductase which contains both FAD and FMN (Werck-Reichhart and Feyereisen, 2000). This class may be composed of a membrane bound, two-component system where the redox partner is an NADH-dependent diflavin (FAD and FMN containing) reductase and the P450 protein (**Figure** 2.11, class II) or a single component system in which the catalytic P450 and the diflavin reductases are all fused in a single polypeptide (**Figure** 2.11, class III) (Miles ***et al***., 2000). Examples of class III enzymes include CYP102, which is the self-sufficient water-soluble P450 monooxygenase from ***Bacillus megaterium*** (P450-BM-3) in which the catalytic P450 domain and the diflavin reductase are naturally fused in a single polypeptide (Williams ***et al.,*** 2000; Werck-Reichhart and Feyereisen, 2000; Appel ***et al***., 2001; Munro ***et al***., 2002). This enzyme has been functionally expressed in ***Escherichia coli*** and its known function include the subterminal hydroxylation of saturated and epoxidation of unsaturated medium- and long chain fatty acids (Li and Poulos, 1997; Appel ***et al***., 2001). Its specific activity in this reaction is about 1000-fold higher than eukaryotic P450 monooxygenases of similar specificity e.g. CYP52A3 and CYP52A4 from ***Candida maltosa*** (Scheller ***et al***., 1994; Appel ***et al***., 2001). Underlying the high activity of P450 BM3 is the efficiency of electron transfer from NAD(P)H cofactor through the reductase to the P450 heme (Munro ***et al***., 2002). Other examples of class III enzymes include the eukaryotic fatty acid hydroxylase, CYP505A1 from the fungus ***Fusarium oxysporum*** (De Mot and Parret, 2002).

Class II enzymes are extremely diverse in their functions and are very common in eukaryotes, where they are associated with the endoplasmic reticulum (Miles ***et al***., 2000; Werck-Reichhart and Feyereisen, 2000). In fungi, they are involved in the synthesis of membrane sterols and mycotoxins, detoxification of phytoalexins, and the metabolism of lipid carbon sources (Feyereisen, 1999; Werck-Reichhart and Feyereisen, 2000). In animals, they are involved in different physiological functions, which include many aspects of the biosynthesis and catabolism of all types of hormones, retinoic acid and oxylipins (Feyereinsen, 1999) while in plants, they are involved in biosynthesis or catabolism of all

types of hormones, in the oxygenation of fatty acids for the synthesis of cutins, and in different pathways of secondary metabolism.

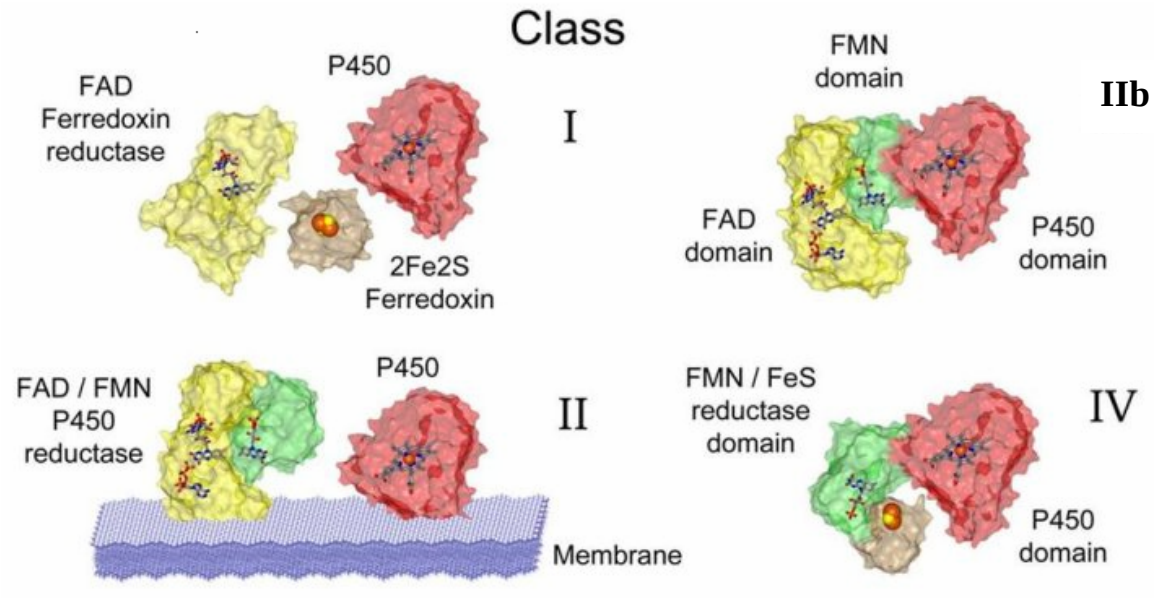


Figure 2.11: Different classes of P450 super family. Adapted from http://www.chem.ed.ac.uk/chapman/p450.html on 6-11-2005.

2.2.2.4 Recombinant CYP450s

Heterologous expression systems have proven to be very important in CYP450 research, permitting investigation of many CYP450s identified only by recombinant (cloning) technologies (Waterman ***et al***., 1995). Catalytically active CYP450 enzymes have been successfully overexpressed in mammalian, bacterial and yeast cells (Gonzalez ***et al***., 1995). This overproduction of CYP450 proteins has both biotechnological and therapeutic significance (Sawada and Kamataki, 1998). In order to produce recombinant CYP450s, a variety of expression vectors have been used resulting in both transient and stable expression systems (Gonzalez ***et al***., 1995). Recent studies on CYP450 monooxygenases have produced excellent results due to new approaches based on gene technology, and prompt identification of new molecular forms by cDNA cloning and analysis of gene regulation mechanisms leading to enzyme induction (Sawada and Kamataki, 1998).

The main catalytic functions of individual CYP450s can be analyzed by the method of heterologous expression of its cDNA (Sawada and Kamataki, 1998). Some of the important factors (Table 3), which have immense impact on recombinant CYP450s include, host physiology, cofactor regeneration, substrate/product toxicity and transport, and strain stability (Van Beilen ***et al***., 2003). However, heterologous expression in foreign hosts usually lead to a lower k_{cat} so that more enzyme needs to be produced in the recombinant strain to obtain the same specific whole-cell activity as in the wild type (Duetz ***et al***., 2001). As the demand for expressing more complex proteins develops, and more transformation systems become available for new organisms, the range of possible new hosts rapidly increases (Madzak ***et al***., 2004).

Heterologous CYP450 expression in mammalian hosts

Several mammalian cell lines have been used as hosts for the heterologous expression of CYP450 cDNA (Crespi and Miller, 1999). The main advantage of mammalian systems is that the cells have the machinery for subcellular localization of CYP450 and the compatible electron transport chain. In addition these cells have the ability to carry out further metabolism of the CYP450 products, which is useful in the evaluation of cytotocixity, and mutagenesis tests for drugs (Sakaki and Inuoye, 2000).

Heterologous CYP450 expression in bacterial host

Heterologous expression of CYP450s in bacteria such as ***E. coli*** has become a very useful tool in biomedical research and is widely used in the study of different foreign CYP450s (Hussain and Ward, 2003). Hussain and Ward, (2003) overexpressed cytochrome P450SU1 and P450SU2 from ***Staphylococus. grieseolus*** into ***E. coli*** in order to study the activity of these proteins towards heterologous compounds as well as to investigate their application in biocatalysis. While bacterial expression of these enzymes is relatively easy and inexpensive, bacteria lack the endoplasmic reticulum to which mammalian microsomal CYP450s bind and also the requisite electron transfer partner for microsomal CYP450 activity. This complication has been overcome through the development of the CYP450-CPR bicistronic or biplasmid ***E. coli*** expression systems (Dong and Porter, 1996). The other drawbacks of CYP450 expression in ***E. coli*** are that

the expression levels are quite different among CYP450 species (Inuoye ***et al***, 2000). An elevated level of expression of CYP450-CPR often results in loss of bacterial cell viability and also reduced catalytic activities (Crespi and Miller, 1999). Several authors have cloned mainly the 13-positional isomers of linoleic acid specific hydroperoxide lyase, which do not require co-factors, in bacterial expression systems. Noordermeer ***et al***., (2000) cloned and expressed in ***E. coli*** 13-hydroperoxide lyase (CYP74) from alfalfa (Bourel ***et al***., 2004) while Matsui ***et al***, in 2000 cloned the fatty acid hydroperoxide lyase of tomato fruits in ***E. coli.***

Heterologous P450 expression in yeasts

Yeasts from the genera ***Schizosaccharomyces, Pichia, Saccharomyces and Yarrowia*** have all been used to express foreign CYP450 genes(Mukarami ***et al***., 1990; Nthangeni ***et al***., 2004). While the expression of human CYP450 proteins in ***Schizosaccharomyces pombe*** (Ehlmer ***et al***., 2002) and ***Pichia pastoralis*** (Anderson and Moller, 2002) has been reported, ***S. cerevisiae*** proved to be the yeast most often used for the heterologous expression of human CYP proteins (Nthangeni ***et al***., 2004). Yeasts have been reported to offer an advantage especially when a eukaryotic environment is required for the functional expression of the heterologous gene (Blanquet ***et al***., 2003). However, in most of the reported cases in literature, the recombinant P450 activities were never evaluated in terms of whole cell biotransformations.

The first microbial system to successfully express a mammalian CYP450 was yeast. Rat CYP1A1 was used to produce a recombinant ***Saccharomyces cerevisiae*** using the vector pAAH5, which had an alcohol dehydrogenase promoter (Gonzalez ***et al***., 1995). Liu ***et al***., (1998) used passively immobilized recombinant cells of the flocculent yeast ***Saccharomyces diastaticus*** ATCC60715 carrying the fused genes of rat P4501A1 and yeast NADPH-P450 reductase. The recombinant strain was able to perform the bioconversion of acetanilide to ***p***-acetaminophene (pAAP). When this same strain was used under optimal conditions in a fluidised-bed bioreactor, the final product concentration achieved from 15 mM acetanilide was 68 µM of ***p***-acetaminophene in immobilized cells and the product yield based on the substrate consumed was only 0.45% after 8 hours. The

maximum ***p***-AAP production rate was $6.61X10^{-3}$ nmol/mg-cell/min ($6.61X10^{-1}$ U (g dwt)$^{-1}$ (Liu ***et al***., 1998) (**Table** 2.3).

Expression of plant CYP450, ferulate 5-hydroxylase (F5H) in ***Saccharomyces cerevisiae*** WAT11 has been reported by Jiang and Morgan (2004). These two authors looked at the influence of different parameters such as media design, addition of heme precursors and induction time on the whole cell biocatalytic activities. Under optimal conditions when 1mM substrate was used, the maximum specific activity reached up to 35-µmol/g cell/hr (0.5 U (g. dwt)$^{-1}$.

Müller ***et al***., (1998) compared the efficiency of several yeasts including ***S. cerevisiae*** and ***Y. lipolytica*** as hosts for heterologous protein production. Their results revealed ***Y. lipolytica*** as the most suitable host for foreign protein expression. However, a literature search on the use of ***Y. lipolytica*** to produce recombinant CYP450 only revealed three reported successful attempts. The first attempts involved expression of bovine P45017α (CYP17A cDNA) under the control of the strong ***ICL1*** promoter (Juretzek ***et al***., 2000; Fickers ***et al***., 2005). The CYP450 expressing strains catalyzed the biotransformation of progesterone, pregnenolone and related derivatives to the corresponding 17α-hydroxysteroids (Fickers ***et al***., 2005). More recently, Nthangeni ***et al.,*** (2004) expressed human CYP1A1 in ***Y. lipolytica*** under the control of the promoter of the fatty acyl-CoA oxidase 2 (***POX2***) and isocitrate lyase (***ICL***) promoters. The ***POX2*** promoter is induced by fatty acids. The highest CYP1A1 activity was observed in whole cell biotransformations of hydroxyresorufin into resorufin when multicopies of CYP1A1 were inserted, and ***YlCPR*** was co-expressed (Nthangeni ***et al.,*** 2004).

Table 2.3. Selected examples of activities obtained with recombinant yeast whole cell biocatalysts expressing different CYP450s.

Source of P450	Recombinant host	Hydroxylase activity	Substrate	Product	Ref.
Rat CYP1A1	***S. diastaticus***	$6.61x10^{-3}$µU (g dwt)$^{-1}$	acetanilide-	acetaminophene	Liu ***et al***., 1998).
Human (CYP1A1)	***Y. lipolytica***	1.3-1.8 µU (g dwt)$^{-1}$	hydroxyresorufin	resorufin	Nthangeni et al., 2004
Plant ferulate 5-hydroxylase	***S. cerevisiae***	0.5 U (g dwt$^{)-1}$	Ferulic acid	5-hydroxyferulic acid	Jiang and Morgan, 2004

Where U is defined as 1 µmol of product formed per minute.

2.2.2.5 Protein engineering of CYP450 monooxygenases

Protein engineering involves modification of enzyme by rational design or directed evolution with the aim of altering their properties (activity and substrate range). Rational design of an enzyme through site directed mutagenesis requires a solid basis of structural knowledge and an understanding of its catalytic mechanisms (Urlacher ***et al***., 2004).

The structures that are available for several P450 enzymes, thus allows for the rational design of proteins, which provides important information on the role of the most important amino acids in the binding pocket. It has been shown that mutations of the active site residue Y96 into more hydrophobic residues considerably increased the activity of P450cam towards the oxidation of molecules smaller than camphor, such as alkanes or styrene (Stevenson ***et al***., 1996; Nickerson ***et al***., 1997) or larger molecules such as naphathalene and pyrene (England et al., 1998; Harford-Cross ***et al***., 2000; Urlacher ***et al***., 2004).

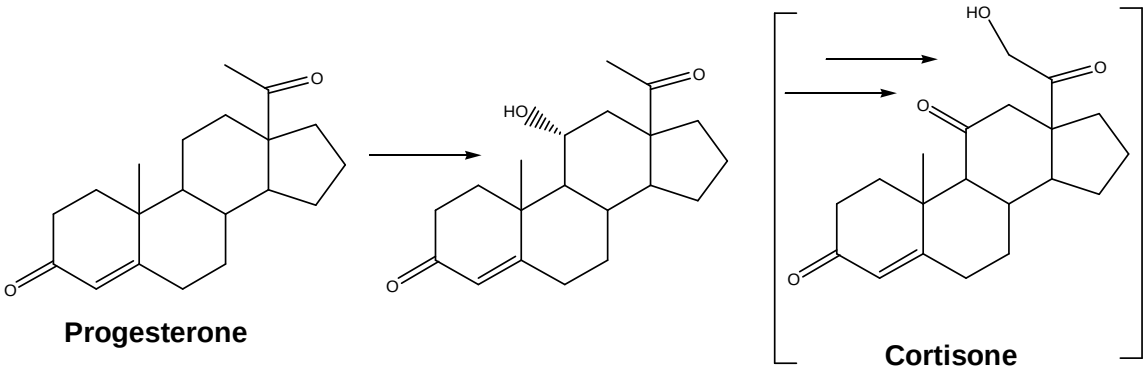
Mutations carried out on amino acid residues, Y51 and R47 of P450 BM-3 led to an alteration of specificity (Cowart ***et al***., 2001; Urlacher ***et al***., 2004). On the other hand three amino acid substitutions in P450 BM3 (R47/Y51F/A264) to create the mutant, F87 W/Y96F/T101L/V247L, led to a 200-fold increase in activity of this enzyme to PAHs such as phenanthrene, fluoranthene and pyrene (Li ***et al***., 2001a; Urlacher ***et al***., 2004). These

examples clearly indicate the synthetic potential of this exciting approach that allows the preparation of 'tailor-made' enzymes (Li ***et al***., 2000).

2.2.2.6 Application of CYP450 hydroxylations

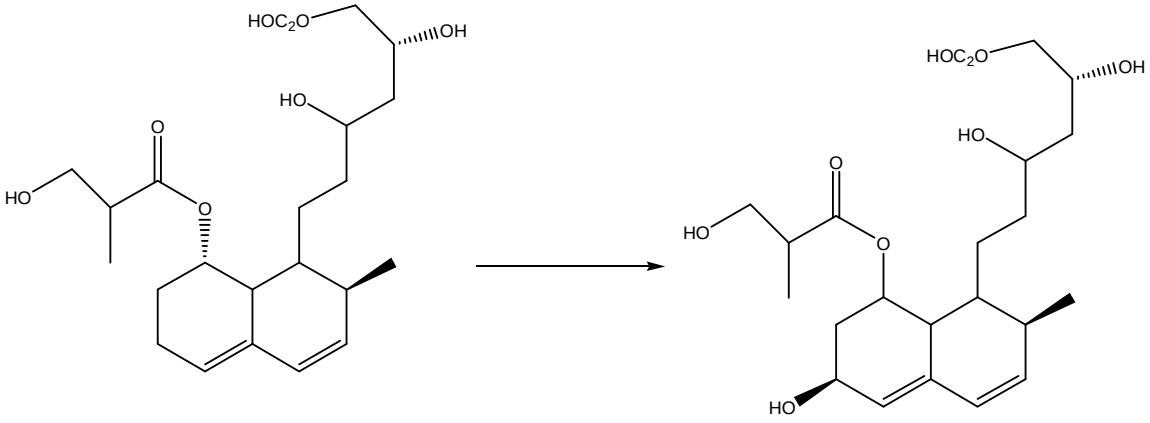
CYP450 hydroxylations have potential uses in the synthesis and discovery of drugs, as well as drug development. It is not a new idea to use enzymes to introduce functional groups (hydroxylation reaction) into drugs that would be difficult to introduce chemically. The introduction of hydroxyls into sterols was done using bacterial fermentations as early as the 1950s; for example, the conversion of progesterone to cortisone through 11α-hydroxylation by ***Rhizopus*** cultures (**Figure** 2.12a) as reported by Guengerich, (2002), was achieved by Upjohn in 1952. A later example is the 6β-hydroxylation of compactin to the hypocholesterolaemic compound pravastatin (**Figure** 2.12b), which is catalyzed by a CYP450 from ***Mucor hiemalis***.

a



Progesterone **Cortisone**

b



Compactine **Pravastin**

Figure 2.12: CYP450 hydroxylations used to synthesis drugs. (**a**), Cortisone and (**b**), pravistatin which are commercial drugs that are prepared by 11α-hydroxylation of progesterone by ***Rhizopus***, and 6β-hydroxylation of compactin by ***Mucor hiemalis***, respectively (Adapted from Guengerich, 2002).

The use of enzymatic hydroxylation for the production of chiral starting materials continues to be an area of interesting study. The hydroxylation of N-benzylpyrrolidine by ***Pseudomonas oleovorans*** Gpo1 and by an ***E. coli*** transformant carrying the hydroxylase gene from ***P. oleovorans*** for the production of (***S***)-N-benzyl-3-hydroxypyrrolidine (**Figure** 2.13 d), which is a starting material for the preparation of a calcium antagonist, has been reported (Holland and Weber, 2000).

Some other industrial chemicals such as alcohols (1-octanol, 2-octanol and perillyl alcohol) (**Figure** 2.13) and dioic acids are also produced as a result of P450 hydroxylation reactions. The first step towards the formation of long chain α,ω dioic acids by members of the yeast genus ***Candida*** is the ω-hydroxylation by a P450 hydroxylase complex (Eschenfeldt ***et al***., 2003). The use of P450-BM3 from ***B. megaterium*** to catalyze hydroxylation reaction in the production of synthetically useful diastereomerically defined vicinal diols has also been reported (Holland and Weber, 2000). Dicarboxylic acids are used for a variety of different applications. Adipic acid and sebacic acid is used in the manufacture of plasticizers. Sebacic acid also serves as a component of engineering grade nylon. The lithium and aluminium salts of azelaic acid are used as lubricants while

its alkaline salts are used as additives in antifreeze mixtures (Endrizzi ***et al***., 1996; Green ***et al.,*** 2000).

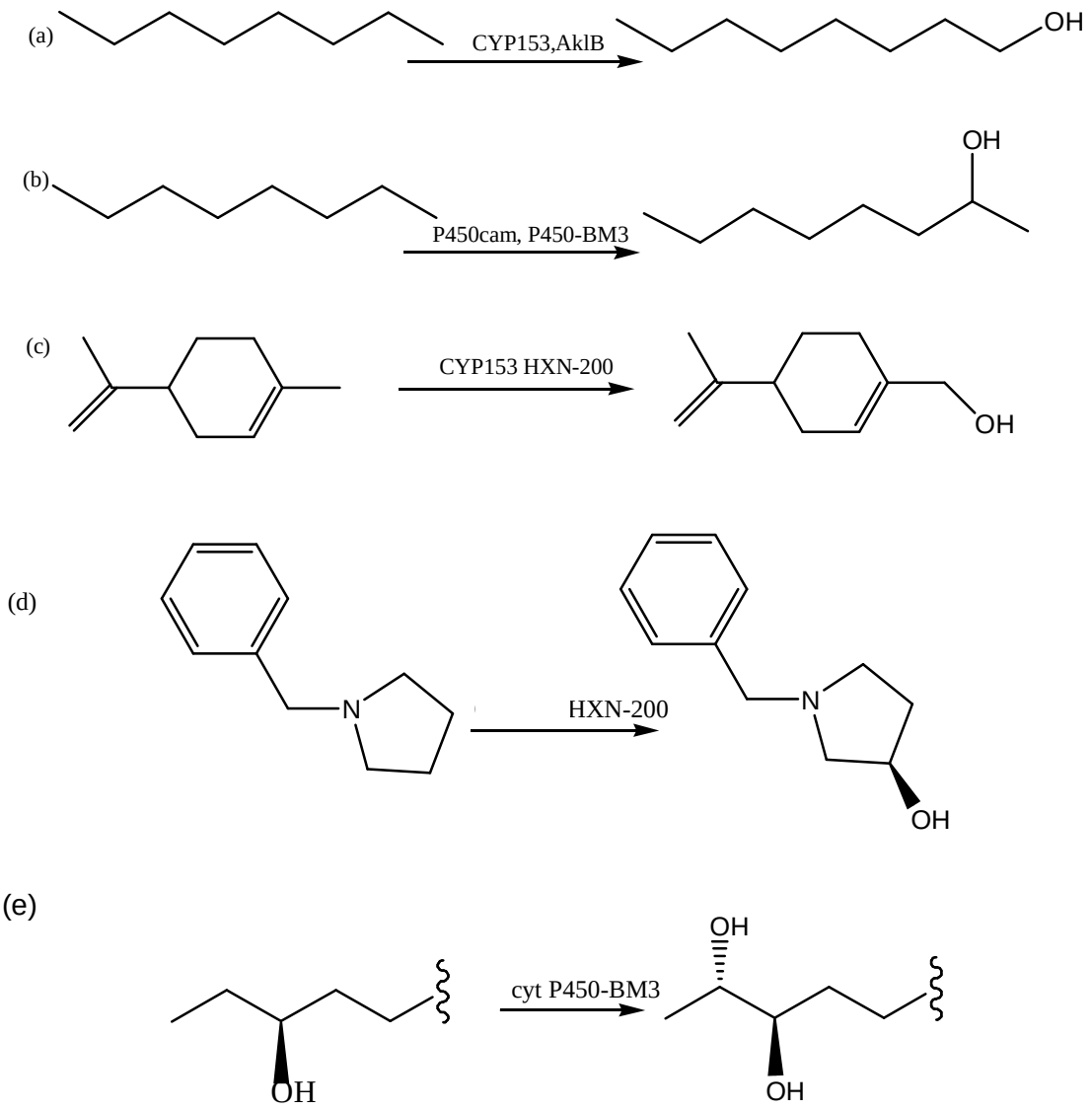


Figure 2.13: Representative reactions catalyzed by CYP450 hydroxylations. **(a)** octane to 1-octanol, **(b)** octane to 2-octanol, **(c)** limonene to perillyl alcohol, **(d)** N-benzyl-pyrrolidine to N-benzyl 3-hydroxy-pyrrolide, **(e)** Hydroxylation of diastereomeric diol. Adapted from Holland and Weber, (2000); Van Beilen and Funhoff, (2005).

CYP450 catalyzed enzymatic hydroxylation reactions are also commonly observed during the degradation of environmental pollutants (Holland and Weber, 2000). For

biodegradation, the hydrocarbon molecules have to be activated to allow further metabolic steps. The first step in the aerobic degradation of alkanes by bacteria, fungi and yeasts is catalyzed by oxygenases. These enzymes will introduce oxygen atoms (hydroxylation) derived from molecular oxygen into the substrate, thus playing an important role in oil bioremediation (van Beilen ***et al***., 2003). The product is made more polar, thus easily susceptible to further degradation processes (Nthangeni ***et al***., 2004), which are critical for bioremediation to occur.

2.3 Conclusions

Hydroxylation of various substrates is catalyzed by different types of enzymes. Amongst these are the heme-containing cytochrome P450 monooxygenases and the non-heme enzymes such as methane monooxygenase, alkane hydroxylase and the fatty acid desaturases (Ayala and Torres, 2004). It is clear that although a lot of information exists on biocatalytic hydroxylation, little is still understood about the impact of genetic modification on these enzymes. And, with the emergence of new hydroxylation genes being isolated, further studies involving their characterization and functionalities need to be carried out.

Recent advances in protein engineering involving enzyme modifications by rational design, direct evolution or site directed mutagenesis have led to an increase in hydroxylating enzymatic activities as well as substrate range (Urlacher ***et al***., 2004). This implies that, in order to evaluate product optimisation as well as substrates specificities, there is need for rigorous studies on recombinant microorganisms.

Even though genetic engineering has opened up many possibilities involving the application of these hydroxylating enzymes, in other cases, the heterologous overexpressions of some oxygenases in recombinant hosts result in lower activities as compared to the wild type strains. The factors limiting in the recombinant hosts still need further evaluation.

Some of the already reported limitations are a result of the differences in the molecular environment of the recombinant host and the wild type strain, which might complicate the stable and functional expression of oxygenases in recombinant strains. The critical factors for the overproduction of the active hydroxylating enzymes include protein folding, protein stability, genetic stability, the ratio of multiple components, cofactor incorporation, the interactions of membrane-associated components of the host membrane, the requirements for additional proteins, and the formation of reactive oxygen species (Bühler and Schmid, 2004).

For the industrial application of these hydroxylating enzymes, critical intrinsic enzyme properties including low enzymatic rates, uncoupling, and multiple oxidation of the substrate has to be considered. Since the specific catalytic rate $\boldsymbol{k}_{cat}$, of most cofactor dependent oxygenases are relatively low compared to hydrolytic enzymes, optimization of the productivity is always a major challenge (Bühler and Schmid, 2004).

CHAPTER THREE

Materials and Methods

3.1 Part A: Basic Methods

3.1.1 Microorganisms

The yeast strains used in this study are summarized in table 3.1. All strains were stored under liquid nitrogen in the MIRCEN yeast culture collection of the University of Free State (UFS), South Africa. For this purpose cultures were frozen in LN broth (see below) containing glycerol (7% v/v). Strains were revived by streaking on YM agar plates (see below) supplemented with vitamin solution.

Table 3.1: Yeast strains used in this study. Footnotes explain the origin of the different strains.

Yeast	**Strain Number**		**Additional *CYP* genes added**			**Additional *CPR* cloned**		***β*-oxidation**
		Hydroxylase activity	**Gene cloned**	**Source**	**Promoter**	**Gene cloned**	**Promoter**	
Y. lipolytica	JMY1057[1]	Acetanilide hydroxylases	*CYP1A1*	Human	*pPOX2*	*YlCPR*	*pPOX2*	Functional
R. retinophila	TVN 295	native alkane / fatty acid hydroxylase	none	n.a.	n.a.	n.a.	n.a.	Functional
Y. lipolytica	W29 (TVN 400)	native alkane / fatty acid hydroxylases	none	n.a.	n.a.	none	n.a.	Functional
Y. lipolytica	TVN399CPR[2]	native alkane / fatty acid hydroxylases	none	n.a	n.a.	*YlCPR*	*pICL*	Functional
Y. lipolytica	TVN 356[2]	native & cloned alkane / fatty acid hydroxylases	*CYP557A1* (Multiple copies)	*R. retinophila*	*pPOX2*	*YlCPR*	*pICL*	Functional
Y. lipolytica	TVN493[3] (Alk 1-4)	native & cloned alkane / fatty acid hydroxylases	*CYP52F1* (Multiple copies)	*Y. lipolytica*	*pPOX2*	*YlCP*	*pICL*	Functional

Y. lipolytica	TVN 497[4] TVN499[4] TVN500[4] TVN501[4] TVN502[4]	native&cloned alkane / fatty acid hydroxylases	*CYP557A1* (Multiple copies)	*R. retinophila*	*pPOX2*	*YlCPR*	*pPOX2*	Disrupted
Y. lipolytica	TVN440[5]	native & cloned alkane / fatty acid hydroxylases	*CYP52F1*	*Y. lipolytica*	*pPOX2*	*YlCPR*	*pPOX2*	Disrupted
Y. lipolytica	TVN442[5] TVN445[5]	native & cloned alkane / fatty acid hydroxylases	none	n.a.	n.a.	*YlCPR*	*pPOX2*	Disrupted
R. minuta	CBS 2177	native benzoate *para* hydroxylase	none	n.a.	n.a.	n.a.	n.a.	Functional
Y. lipolytica	TVN97[2]	native alkane / fatty acid hydroxylases & cloned benzoate *para* hydroxylase	*CYP53B1* (Multiple copies)	*R. minuta*	*pPOX2*			Functional
Y. lipolytica	TVN91[2]	native alkane / fatty acid hydroxylases & cloned benzoate *para* hydroxylase	*CYP53B1* (Multiple copies)	*R. minuta*	*pPOX2*	*YlCPR*	*pICL*	Functional

1. Strains were constructed by Dr. B. Nthangeni in the laboratory of Dr. J.-M Nicaud at the Laborotoire de Microbiologie et Génétique Moléculaire, INRA CNRS INAP-G, UMR2585, Centre de Biotechnologie Agro-Industrielle, 78850 Thiverval-Grignon, France
2. Strains were constructed by Dr. A.N. Shiningavamwe in the Laboratory of Dr. J. Albertyn in the Department of Microbial, Biochemical and Food Biotechnology, UFS, South Africa
3. Strains were constructed by Dr. M.E. Setati in the laboratory of Dr. J. Albertyn in the Department of Microbial, Biochemical and Food Biotechnology, UFS, South Africa
4. Strains were constructed by M.S. Smit in the laboratory of Dr. J.-M Nicaud at the Laborotoire de Microbiologie et Génétique Moléculaire, INRA CNRS INAP-G, UMR2585, Centre de Biotechnologie Agro-Industrielle, 78850 Thiverval-Grignon, France
5. Strains were constructed by M.T. le Dall in the laboratory of Dr. J.-M Nicaud at the Laborotoire de Microbiologie et Génétique Moléculaire, INRA CNRS INAP-G, UMR2585, Centre de Biotechnologie Agro-Industrielle, 78850 Thiverval-Grignon, France

3.1.2 Growth media

YPD broth contained (per liter of distilled water): 10 g yeast extract (Merck), 20 g peptone (Merck) and 20 g glucose (Merck) respectively.

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

YM agar contained (per liter of distilled water): 10 g glucose (Merck), 10 g peptone (Merck), 20 g malt extract (Merck), 2 g yeast extract and 20 g agar (Merck).

YNB broth contained (per liter distilled water): 1.7g YNB without amino acids and ammonium sulphate, 2 g casamino acids, 1 g sodium glutamate, 10 g glucose.

Chemically defined medium A contained (per liter of distilled water): Glucose, 20g; Citric acid, 0.25 g; $(NH_4)_2SO_4$, 5 g; KH_2PO_4 , 5 g; $MgSO_4.7H_2O$, 0.75 g; $CaCl_2.2H_2O$, 0.1 g; NaCl, 0.1 g; Trace elements, 1 ml (according to Du Preez ***et al***., 1983).

Semi-defined medium B contained (per liter of distilled water): all the above (defined medium A); Yeast extract (Difco), (3 g. l^{-1}) added.

3.1.3 Growth conditions

Cultivation in liquid media was, unless otherwise stated, performed in 50 ml YPD broth (see above) in 500 ml Erlenmeyer flasks on a rotary shaker at 180 rpm and 25 ºC. Shake flasks were inoculated with 24 h YPD cultures with a flask angle of amplitude of about 70^o.

3.1.4 Turbidimetric measurements

Culture samples (500 µl) in 1.5 ml micro-centrifuge tubes were vortexed for 5 min. The turbidity of 200 µl samples, suitably diluted in physiological saline solution (NaCl 0.9% w/v) before transfer to a microtitre plate, was measured at 620 nm using a Labsystems iEMS microtitre plate reader MF (Thermo BioAnalysis Company, Helsinki Finland).

3.1.5 Dry weight measurements

Cyclohexane (2 ml) and 5 M NaOH (400 µl) were added to culture samples (4 ml) in test tubes, vortexed for 5 min and then filtered under vacuum through pre-weighed glass fibre

filters (GF52 47MMBX200; Schuell). The biomass on the filter was washed with a mixture of distilled water (4 ml), cyclohexane (2 ml) and 5 M NaOH (400 µl) followed by washing with distilled water (26 ml). The filters were oven dried at 110 °C and cooled in a desiccator before weighing.

3.1.6 Extraction and analysis of substrates and products

Samples (500 µl) were taken at regular intervals and acidified to pH 3 by addition of 3M HCl (50 µl). The samples were extracted twice with ***tert***-butylmethyl ether (Fluka) (300 µl) containing myristic acid (0.1% w/v) (The British Drug Houses) as an internal standard and the phases separated by centrifugation.
For GC analysis, the extracts (50 µl) were transferred to new 1.5 ml micro-centrifuge tubes and methylated with the same volume of trimethylsulfonium hydroxide (TMSH) (Butte, 1983) before GC analysis. Concentrations were determined using pre-determined standard curves. The conversion factors (Table 3.2) were calculated based on 0.1% (w/v) of myristic acid used as an internal standard.

GC analysis was carried out using a Hewlett-Packard 5890 series II gas chromatograph with a Chrompack ® CP wax-CB polar column, 30 m x 0.53 mm x 1 µm. The GC conditions were: H_2 was used as a carrier gas at 5 ml min^{-1} with a split ratio of 1:40. The oven was initially at 120 ºC for 5 min; then increased at 10 ºC min^{-1} to 300 ºC and held for 7 mins; The Flame Ionization Detector (FID) was set at 350 ºC.

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

Glucose analysis was carried out using a Water Breeze HPLC having a Differential Refractive Index Detector with, a Water SUGARPACK I column, 300 x 7.8 mm at 84 ºC. The mobile phase was; dionised water at flow rate of 0.5 ml min^{-1} and the sample volume was 20 µl.

For TLC (Thin Layer Chromatography) analyses, extracted samples (10 µl) were spotted onto Alugram Sil G/UV_{245} TLC plates (Machery-Nagel) containing a fluorescent indicator. The mobile phase for TLC development consisted of: di-***n***-butyl-ether (Merck), formic acid (Merck) and water (90:7:3). The plates were visualized under UV- light (short wave length).

Table 3.2: Summary of substrate/product retention times and the conversion factors used for calculating concentrations

Substrate	**Retention time (min)**	**Conversion factor**
Myristic acid	11.38	1
Benzoic acid	12.37	1.43
p-Hydroxybenzoic acid	5.48	1.43
Phenylacetic acid	7.96	1.39
Dodecane	1.42	1.62
Phenylnonane	9.26	1.03
Stearic acid	15.89	1.60
4-Nonyloxybenzoic acid	19.57	1
4-(2-arboxyethoxy)benzoic acid	23.34	1.3
4-Nitrophenyloctyl ether	20.44	1
4-nitrophenoxybutanoic acid	24.446	1.3
trans-Cinnamic acid	12.20	1.35

3.1.7 Preparation of substrates

Most hydrophobic substrates were prepared as stock solutions before addition to the cultures. The pHs of the stock solutions were adjusted to 8 using NaOH. The substrates which were prepared as a 10 % stock solution were benzoic acid (w/v), 4-phenylbutanoic acid, oleic acid (v/v) and stearic acid (w/v). Acetanilide, 4-nonyloxybenzoic acid and ***trans***-Cinnamic acid were prepared as 5 % (w/v) stock solutions.). In all the cases, 0.1% Tween 80 was added to aid in dispersal. The substrates were stirred continuously until they became homogenous before use.

3.2 PART B: Biotransformation of hydrocarbon substrates using yeast strains

3.2.1 Acetanilide biotransformation using *Y. lipolytica* strains W29 (wild type) and JMY1057 (overexpressing human *CYP1A1*)

Y. lipolytica strains W29 (wild type) and JMY1057 (overexpressing human ***CYP1A1***) were grown in YPD broth for 24 h to late exponential phase (OD~5), before oleic acid (final concentration 1 % v/v) was added from a stock solution (10% v/v) in one culture while in another culture, ethanol (final concentration of 1% v/v) was added . 12 h later, acetanilide (AA) (Sigma-Aldrich) (0.2% w/v) was added from a stock solution (5% w/v). Samples were taken at regular intervals and extracted following the normal procedure (adjusting pH to 3 by the addition of 3M HCl (50 µl). Samples were also extracted without the addition of acid (pH 8). Organic extracts were analysed with TLC.

In a second experiment, three cultures of ***Y. lipolytica*** W29 were grown as before but without the addition of oleic acid. Two hours prior to substrate addition, ethanol (1 % v/v) was added to one flask, ethanol (1 % v/v) and glucose (0.5 % v/v) were added to a second flask and nothing was added to the third flask. The rest of the experiment was carried out as before.

3.2.2 Biotransformation of 4-nonyloxybenzoic acid and 4-nitrophenyl octyl ether by yeast strains overexpressing *CYP53B1*, *CYP52F1* and *CYP557A1*

Y. lipolytica strains TVN91, TVN356 and TVN493 were grown in YPD broth to late exponential phase (24 h)(OD~5) before oleic acid (final concentration 0.5 % v/v) from a stock solution (10% w/v) was added. In another experiment, ethanol (final concentration of 1 % v/v) was added at the same growth phase instead of oleic acid. Twelve hours later, 4-nonyloxybenzoic acid (Acros) (0.2 % w/v) or 4- nitrophenyl octyl ether (Acros) (0.2 % v/v) (both from stock solutions (5%)) were added to the cultures. Samples were taken at regular intervals for TLC, glucose and GC analyses.

3.2.3 Biotransformation of 4-nonyloxybenzoic acid by yeast strains overexpressing *CYP450* but with oxidation pathway disrupted

Y. lipolytica strains TVN440, TVN442, TVN445, TVN497, TVN498, TVN499, TVN500, TVN501 and TVN502 were grown as above. Ethanol (1% w/v) was added as co-substrate after 24 h growth. Substrate (0.2% w/v) was added 24 h later and again after another 24 h interval.

3.2.4 Biotransformation of phenylnonane and 4-nonyloxybenzoic acid by wild-type strains of *R. retinophila*, *R. minuta* and *Y. lipolytica*

The yeast strains used for this study were; ***R. retinophila***, ***R. minuta*** and ***Y. lipolytica***, W29. The strains were grown in YPD broth. Towards the end of exponential phase, phenylnonane (1% v/v) (Aldrich) and 4-nonyloxybenzoic (0.2% w/v) was added to the culture. 4-Nonyloxybenzoic acid (0.2% v/v) was added a second time after 24 h. Samples were extracted at regular intervals for TLC, glucose and GC analyses.

3.2.5 Isolation and purification of 4-nonyloxybenzoic acid biotransformation products

The three products of 4-nonyloxybenzoic acid biotransormation were purified for GC-MS analysis by TLC. The extracted sample of the biotransformation of 4-nonyloxybenzoic acid biotransformation by TVN 356 after 24 h from substrate addition were loaded on a TLC plate and developed using the previously described solvent system. In order to achieve a complete separation of the bands, the TLC plate was developed twice. The bands were visualized under UV and marked with a pencil. Each of the bands were scraped off into separate microcentrifuge tubes, redissolved in ethyl acetate and centrifuged. The supernatants were transferred into a new microcentrifuge tube. The three fractions were again subjected to TLC analysis to confirm their purity before GC-MS analysis.

3.2.6 Toxicity of benzoic acid to *R. minuta* CBS 2177 and biotransformation by this strain

Pre- cultures (24 h; OD ~ 8) ***of R. minuta*** were used to inoculate main cultures in YPD broth to a starting OD ~ 0.2 at 620 nm. One set of cultures was maintained at a pH of 8 by addition of phosphate buffer (500 mM) to a final concentration of 50 mM while the other set was left unadjusted (pH ~6). After 15 h of growth, (towards the end of the exponential phase (OD~5)), different concentrations (0.1%, 0.2%, 0.5% and1.0% w/v) of BA (from a 10% stock solution) were added. The pH of the adjusted flasks were continuously monitored and maintained at 8 by the addition of 5M NaOH. Samples were taken at regular intervals for GC, glucose, and biomass analyses. At the end of the experiment (96 h of growth), cell viability was monitored by plate counts.

3.2.7 Use of benzoic acid as the only carbon source by *R. minuta* CBS 2177

A YPD pre-culture (24 h) was used to inoculate main cultures in YNB broth. Different concentrations (0.1%-1% w/v) of benzoic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid and 4-chlorobenzoic acid were added as the only carbon source to different cultures.

In another experiment, the main culture contained YPD (glucose 1%) with an additional substrate, benzoic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid or 4-chlorobenzoic

acid (each, 0.1 % (w/v) from a 10% stock solution) added after 24 h (OD ~ 5). Benzoic acid (0.1% w/v) was added a second time after 24 h from the time of first addition.

The pH of the cultures was maintained at 8. Samples were taken at regular intervals for growth measurements.

3.2.8 Toxicity of BA to *Y. lipolytica* TVN91 and biotransformation of BA by this strain when the cloned *CYP53B1* is not induced

Y. lipolytica TVN91 grown for 24 h in YPD broth was used to inoculate main cultures in YPD broth to a starting OD of ~0.2. Towards the end of exponential growth phase (OD~7) (after 24 h of growth), different concentrations of benzoic acid (0.1%, 0.5% and 1.0% from a 10% w/v stock solution) were added to different cultures. The pH was maintained at 8 as before. Samples were taken at regular intervals for GC analyses, biomass determinations and OD measurements. Cell viability was determined at the end of the experiment (160 h).

3.2.9 Toxicity of pHBA to *Y. lipolytica* TVN91 and possible biotransformation by this strain

Y. lipolytica TVN91 was grown in YPD broth as described above. After 24 h of growth (towards the end of the exponential growth phase (OD~7)), pHBA (0.5 % (w/v) was added to the culture broth. The pH was maintained at 8.0. Samples were taken at intervals for GC and glucose analyses as well as biomass measurements.

3.2.10 Optimization of biotransformation of BA by *Y. lipolytica* TVN91

The main cultures (grown in YPD broth) were inoculated to a final OD_{620} of ~ 0.2. The cultures (buffered at pH of 8 using 50 mM phosphate buffer) were incubated on a rotary shaker at 180 rpm and 25 °C.

Stock solutions (10% (w/v) of different inducing substrates and BA (supplemented with Tween 80 (Sigma) (0.1% v/v)) were made. The pH of the stock solutions were adjusted and the contents were continuously stirred until the substrates either dissolved or formed

homogenous suspensions. Different inducing substrates (2% w/v) were added at the end of the exponential growth phase followed by BA (0.5% (w/v) 18 h later (See graphs for details).
A further optimization attempt of biotransformation was done by continuous addition of inducer (stearic acid (1% w/v)) at intervals.

3.2.11 Testing for substrate (BA) transport limitation by *Y. lipolytica* TVN91

Y. lipolytica TVN91 was grown in YPD broth for 24 h after which inducers were added. In one experiment, phenylnonane (1%v/v) (Aldrich) was added as the substrate while in a second experiment, ***trans***-cinnamic acid (0.25% w/v) (Sigma) was added as the substrate. In the first experiment, two different inducers were used. In one experiment dodecane (2% v/v) was added as inducer while in the second stearic acid (2 % w/v) was added as an inducer. Phenylnonane was added to each of the cultures 24 h after inducer addition. An additional experiment was carried out in the same way, but this time using strains, TVN356 and TVN493 respectively.

In a second experiment, all the processes was the same except this time, only oleic acid (2 % v/v) was added as inducer and the substrate used was ***trans***-cinnamic acid (0.25 % w/v).

3.2.12 Selective induction of *POX2* promoter

TVN91 was grown in YPD broth for 24 h after which different acid substrates were added as inducers to switch on the ***POX2*** promoter. The substrates tested included phenylacetic acid (10 g/l), 4-hexylbenzoic acid (5 g/l) (Merck), 4-phenylbutanoic acid (2 g/l) (Aldrich), 4-phenylbutanoic acid (5 g/l) together with ethanol (1%), stearic acid (20 g/l), stearic acid (20 g/l) together with ethanol (1%). The last two substrates were used as controls. Biotransformations were carried out as before.

3.2.13 Use of YNB media for biotransformation of BA

Y. lipolytica was grown in YNB broth (with 10 g/l glucose) with addition of different amounts of casamino acids. To one flask, 2 g/l of casamino acids was added while 4 g/l of cassamino acid was added to the other. After 36 h growth, SA (2% w/v) was added to each of the cultures. BA (0.5 % w/v) was added 24 h later.

3.2.14 Optimization of BA biotransformation under bioreactor conditions

Batch cultivations were carried out using a 2 l bench top bioreactor (Multigen TM New Brunswick Scientific Co. Inc. Edson NJ USA) with a working volume of 1 l. The cultivation parameters were; temperature 27 ºC, dissolved oxygen ≥ 30 %, aeration range 500 to 1000 cc/min, agitation range 400 to 800 rpm and cultivation time of about 200 h. The relative aeration and agitation ranges were continuously adjusted to maintain the dissolved oxygen concentration at ≥ 30 %. The dissolved oxygen in the culture was monitored with a polarographic pO_2 electrode (Mettler, Toledo, Halstead, UK) and maintained at the desired level by increasing the air flow rate and manual adjustment of the stirrer speed within the preset ranges as indicated above. The pH was controlled by titration with 3 M KOH. Dow corning 1520 Silicone antifoam (BDH Laboratory Supplies, Poole, England) was used.

The inoculum was prepared and used to inoculate the bioreactor to a starting OD of 0.2. The temperature was maintained at 27 ºC. The cultivation medium was YPD broth. Stearic acid (2 % w/v) was added as an inducer after an OD of about 10 was reached (20 h of growth) and the substrate added 18 h later. Further stearic acid (40 ml of 10 % stock solution containing 0.5 ml of antifoam) were added after 80 h and 134 h of growth.

3.2.15 Effect of cell concentration on BA biotransformation by TVN91

Cells were harvested after 48 h of oleic acid induction. Different volumes of the harvested wet cells from 20 ml, 15 ml, 10 ml, 5 ml and 2.5 ml culture were subdivided into separate centrifuge tubes (50 ml volume). (The corresponding cell biomass concentrations were;

59.5 g/l, 44.6 g/l, 29.7 g/l, 14.9 g/l and 7.4 g/l respectively). Each of these was washed twice and resuspended in 50 mM phosphate buffer (5 ml). The contents were transferred into Erlenmeyer flasks (100 ml in volume). BA was added and biotransformation followed as before.

3.2.16 Use of resting cells for biotransformation

Resting whole-cell biotransformation was done using strains TVN91 and TVN356. Cells were grown in YPD broth to the end of exponential phase (24 h). Oleic acid (2 % v/v) was added as an inducer. The duration of induction was 24, 48 and 96 hours respectively.

Cells (5 ml) were harvested at each of these times, centrifuged at 5000 rpm for 5 minutes, washed twice with 50 mM phosphate buffer and resuspended in the same volume buffer (5 ml). The resuspended cells (in phosphate buffer) were transferred into Erlenmeyer flasks (100ml volume). Benzoic acid (0.5 % (w/v) and hexylbenzene (1% v/v) were added as substrates for TVN91 and TVN356, respectively. Biotransformation experiments were carried out on a rotary shaker at 180 rpm and 25 ^{o}C.

3.2.17 Effect of cell storage

In one experiment, the harvested cells were stored immediately at 4 ^{o}C and -20 ^{o}C respectively. After 24 h of storage, the samples were thawed, and resuspended in 50 mM phosphate buffer (5 ml). Benzoic acid (0.5% w/v) acid was added as a substrate and biotransformations carried out accordingly.

For control experiment, biotransformation reactions were carrying out immediately without storage.

3.2.18 Effect of oxygen limitation on BA and HB biotransformation by TVN91 and TVN356

The strains used were, ***Y. lipolytica*** TVN91 and TVN356 and the substrates used here were benzoic acid (0.5% v/v) and hexylbenzene acid (1% w/v) respectively. Cells harvested after 36-hour induction on oleic acid (2% v/v) (Fluka) were used. The harvested

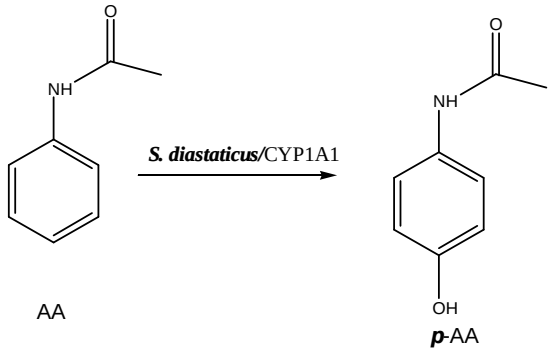
cells (25 ml) were resuspended in 25 ml of buffer (50 mM phosphate) and transferred to Erlenmeyer flasks of different sizes; 50 ml, 100 ml, 250 ml, and 500 ml. BA (0.5 %) was added to cells of TVN91 while, HB (1%) was added to cells of TVN356.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Biotransformation of acetanilide using a *Y. lipolytica* strain overexpressing human *CYP1A1*

The strains used for this study were ***Y. lipolytica*** W29 (wild type) and JMY1057. ***Y. lipolytica*** JMY1057 is a recombinant strain overexpressing human ***CYP1A1*** under the ***POX2*** promoter together with an additional ***YlCPR*** under the same promoter. Using the latter strain, Nthangeni ***et al***. (2004) reported the biotransformation of ethoxyresorufin to hydroxyresorufin with specific activities of up to 1.3-1.8 μU (g dry wt)$^{-1}$. where 1 U is defined as 1 μmol of product formed per minute. These reactions were, however, obtained under analytical activity assay conditions (1 ml reactions carried out in UV cuvettes). It was therefore our aim to use the available strains overexpressing ***CYP1A1*** for larger scale whole cell biotransformation of acetanilide, which is an alternative cheaper substrate of CYP1A1. ***Liu et al***. (1998) showed that when using recombinant cells of ***Sacccharomyces diastaticus*** overexpressing rat ***CYP1A1*** and yeast ***CPR***, acetanilide (AA) was hydroxylated at the ***para*** position to give ***p***-acetaminophene (***p***-AAP) (**Figure** 4.1) as the only product. This reaction was carried out with immobilized cells under optimal conditions in a bioreactor and the final product concentration achieved from 15 mM AA was 68 μM of ***p***-AAP. The product yield based on the substrate consumed was only 0.45% after 8 h.



AA ***p***-AA

Figure 4.1: Biotransformation of acetanilide (AA) to ***p***-acetaminophene using ***S. diastaticus*** overexpressing rat ***CYP1A1*** (Liu ***et al***.,1998).

When we used the recombinant ***Y. lipolytica*** strain JMY1057 as well as the ***Y. lipolytica*** wild type strain W29 to test biotransformation of AA (14.8 mM, 2 g/l) both strains degraded acetanilide. TLC analysis of extracts from extractions performed at pH 3 indicated that all the AA had completely disappeared from both cultures within 5 h (**Figure** 4.2). However, no product could be detected. When extractions were done at pH 8, TLC analysis showed the formation of a product (**Figure** 4.2 b). This product was identified as aniline by comparison with an authentic sample (results not shown). Thus both strains hydrolyzed AA to aniline (**Figure** 4.3), which did not undergo any hydroxylation even after 60 h (results not shown).

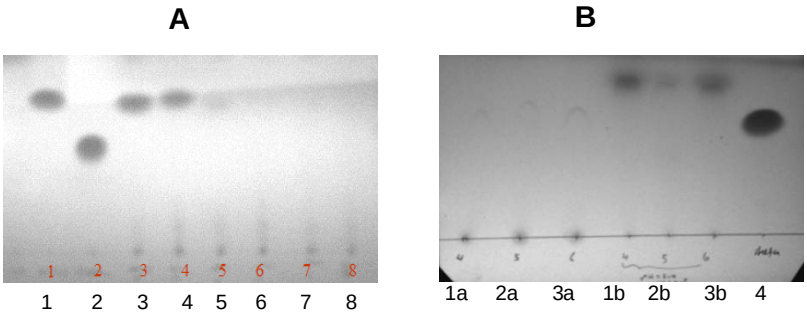


Figure 4.2: TLC plates showing biotransformation of AA by ***Y. lipolytica*** strains JMY1057 (expressing ***CYP1A1***) and W29 (wild type). (**A**) Standards: 1 - AA, 2 - ***p-***AAP, Extracts at pH 3 from: 3 - JMY1057 at 0 h, 4 – W29 after 0 h, 5 – JMY1057 after 5 h, 6 - W29 after 5 h, 7 - JMY1057 after 24 h, 8 - W29 after 24 h. (**B**) Extracts from wild type strain W29 grown in YPD broth with the following additional carbon and energy sources added after 24 h: 1. ethanol (1%), 2. ethanol (1%) + glucose (0.5 %), and 3. none; 4. ***p***-AAP standard. Extractions 1a – 3a done at pH 3. Extractions 1b-3b done at high pH (8.0).

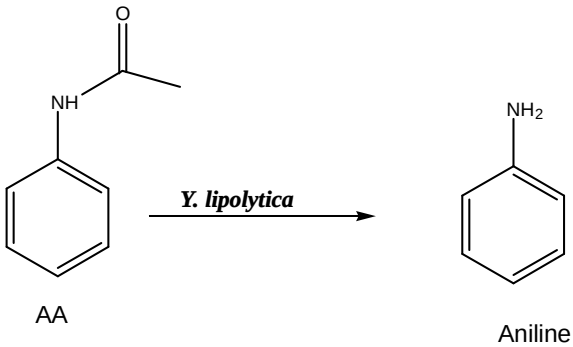


Figure 4.3: Degradation of acetanilide to aniline by ***Y. lipolytica*** strains, JMY1057 and W29

An attempt to repress the production of the hydrolytic enzymes (i.e. lipases or esterases) by addition of ethanol (1% v/v) and glucose (0.5% w/v) to the media prior to substrate addition did not prevent hydrolysis of AA. These results indicate that the production of hydrolytic enzymes, for which ***Y. lipolytica*** is well known (Pignède ***et al***., 2000; Fickers ***et al***., 2005), might in some cases limit its use as a whole cell biocatalyst if the substrate contains ester or amide bonds. This is a limiting factor to consider when planning its use as a host for heterologous expression of some CYP450s.

Being that CYP1A1 have been reported to be responsible for the degradation of poly aromatic hydrocarbons (PAH), the other possible substrates to use to test for activity in the recombinant ***Y. lipolytica*** strains overexpressing ***CYP1A1*** would therefore be pyrene, benzo(a)pyrene, naphthalene and *β*-naphthylamine. Most of these compounds have been reported to be present in tobacco (Smith ***et al***., 2001). The only limitation is that these types of compounds are very hydrophobic and would be difficult to work with in an aqueous environment.

4.2 *Y. lipolytica* strains expressing alkane and fatty acid hydroxylases

At least twelve ***P450ALK*** genes have already been identified in the yeast ***Y. lipolytica*** (Fickers ***et al***., 2005)***.*** The substrate specificities and induction of some of these gene products have already been confirmed. ***YlALK1*** and ***YlALK2***, also designated ***CYP52F1*** and ***CYP52F2***, have been shown to play prominent roles in ***n***-alkane assimilation in this yeast. ***YlALK1*** has been shown to play an important role in the assimilation of short-chain ***n***-alkanes, such as ***n***-decane, because a mutant with this gene disrupted did not grow on ***n***-decane (Iida ***et al.,*** 2000). ***YlALK1*** showed the highest expression in response to ***n***-tetradecane, whereas ***YlALK5*** and ***YlALK6*** were only weakly induced. When ***YlALK1*** and ***YlALK2*** were disrupted, no growth was observed on ***n***-hexadecane. An explanation may be that ***YlALK***2 is specific for longer chain alkanes while ***YlALK***1 is responsible for the oxidation of a wide range of ***n***-alkanes (Iida ***et al.,*** 2000). Expression of the first eight ***YlALK*** genes in ***Nicotiana benthamiana*** leaves showed that ***Yl***ALK1, ***Yl***ALK2, ***Yl***ALK4 and ***Yl***ALK6 have no fatty acid hydroxylase activity, while ***Yl***ALK3, ***Yl***ALK5 and ***Yl***ALK7 had lauric acid ω-hydroxylase activity (Hanley ***et al***., 2003).

CYP557A1 is a gene first isolated in our laboratory from the basidiomycetous yeast, ***R. retinophila*** CBS 8446 which has the ability to grow on dodecane and limonene vapors. Comparison of the protein sequence of CYP557A1 with other CYP450s showed that the deduced protein sequence had less than 40% amino acid identity with any classified CYP450 (Shiningavamwe, 2004). In order to investigate its activity as well as substrate specificity, the gene was overexpressed in ***Y. lipolytica*** (Shiningavamwe, 2004). Preliminary tests, demonstrated the difficulty of testing the activity of the putative fatty acid hydroxylase in ***Y. lipolytica*** due to problems with selective induction and complete degradation of test substrates such as oleic acid.

To construct ***Y. lipolytica*** strains overexpressing ***CYP52F1*** and ***CYP557A1***, a single copy of the ***YlCPR*** (reductase) was first inserted into ***Y. lipolytica*** E150 using a single copy plasmid, JMP21. Multicopies of the ***CYP450s*** were then inserted using the multicopy plasmid, JMP64 (Shiningavamwe, 2004). The overexpressed CYP450s were under the ***POX2*** promoter which is induced by alkanes and fatty acids (Juretzek ***et al***., 2000). The additional copy of the ***YlCPR*** was under ***pICL*** which is induced by alkanes, fatty acids, acetate and ethanol (Juretzek ***et al***., 2000).

Biotransformations were performed using ***Y. lipolytica*** strains, TVN356, TVN493 and TVN91 respectively. TVN356 is a recombinant strain overexpressing ***CYP557A1***. TVN493 overexpresses ***CYP52F1*** (alkane hydroxylase from ***Y. lipolytica***) and TVN91 overexpresses ***CYP53B1*** (benzoate ***para*** hydroxylase ***from R. minuta***). The latter strain was used as a control strain. Ethanol and oleic acid were used to induce expression of the cloned genes.

Previous results by Van Rooyen (2004) illustrated that, when hexylbenzene was used as a substrate with ethanol as an inducer, the endogenous CYP450s were not induced when the two strains, TVN356 and TVN493 were used for biotransformations. This resulted in a significant difference in activity between the strains with cloned alkane or fatty acid hydroxylases and the strain with only endogenous alkane / fatty acid hydroxylases, even though it took longer for product (phenylacetic acid) accumulation to start. The intermediates formed from the transformation of hexylbenzene probably acted as inducers for the ***POX2*** promoter, once the ethanol had been used. The activity in the presence of ethanol was higher than that in the presence

of oleic acid which was expected to also induce the endogenous fatty acid hydroxylases, as an inducer (Van Rooyen, 2004). To further explore these observations, two substrates, 4-nonyloxybenzoic acid and 4-nitrophenyl octyl ether were used for biotransformations in the presence of either ethanol or oleic acid as inducers.

4.2.1 Biotransformation of 4-nitrophenyl octyl ether

Peters ***et al***. (2003) had used 4-nitrophenyl octyl ether as a chromogenic substrate to select CYP102 (P450 BM-3) mutants with alkane hydroxylating activity, while Schwaneberg ***et al***. (1999) had evaluated the activity of CYP102 and a mutant towards ***p***-nitrophenoxycarboxylic acids of various chain lengths. Both groups of researchers proposed reaction schemes, which involved initial hydroxylation at the carbon atom attached to the ***p***-nitrophenoxy moiety to form an unstable hemiacetal, which dissociated into the corresponding aldehyde or ω-oxycarboxylate and nitrophenolate as the end products (**Figure** 4.4). The formation of nitrophenolate was assayed spectrophotometrically at 410 nm.

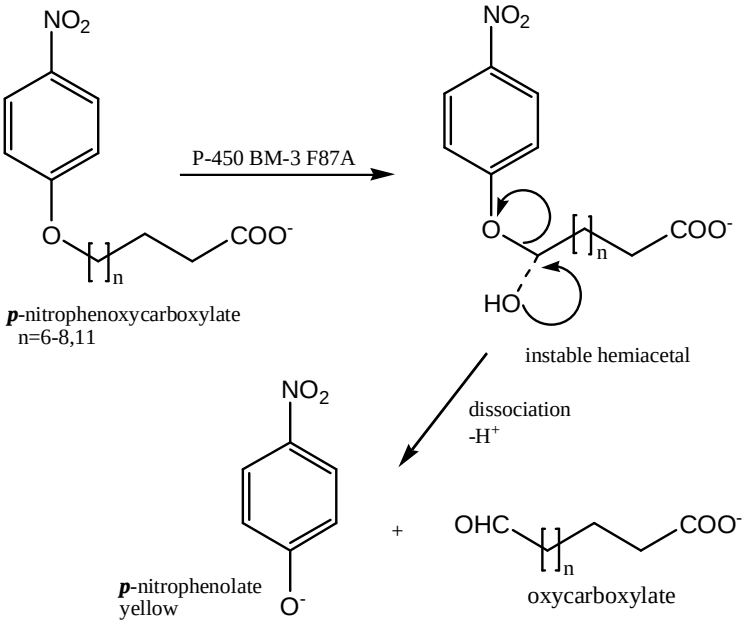


Figure 4.4: The hydrolysis of ***p***-nitrophenoxycarboxylate by P450 BM-3 to form ***p***-nitrophenolate and oxycarboxylate (Schwaneberg ***et al***., 1999).

The biotransformation of 4-nitrophenyl octyl ether using ***Y. lipolytica*** strains TVN91, TVN356 and TVN493, yielded two major products (**Figure** 4.5) which were identified as 4-nitrophenol and ***p***-nitrophenoxybutanoic acid by GC/MS analysis (**Figures** 4.6 and 4.7). Formation of 4-nitrophenol was also confirmed by comparison with an authentic standard, while the mass spectrum of the second product matched that of methyl 4-nitrophenoxybutyrate in the mass spectrum library. Mass spectra data for the 4-nitrophenyl octyl ether and its methylated biotransformation products are summarized in table 4.1. We did not detect any significant amounts of straight chain alkane derivatives (i.e. octanal, octanoic acid, ω-oxyoctanoic acid or ω-hydroxyoctanoic acid) that might have resulted from hemiacetal hydrolysis. The formation of 4-nitrophenol resulted in the characteristic yellow color in the media (Fickers ***et al***., 2003).

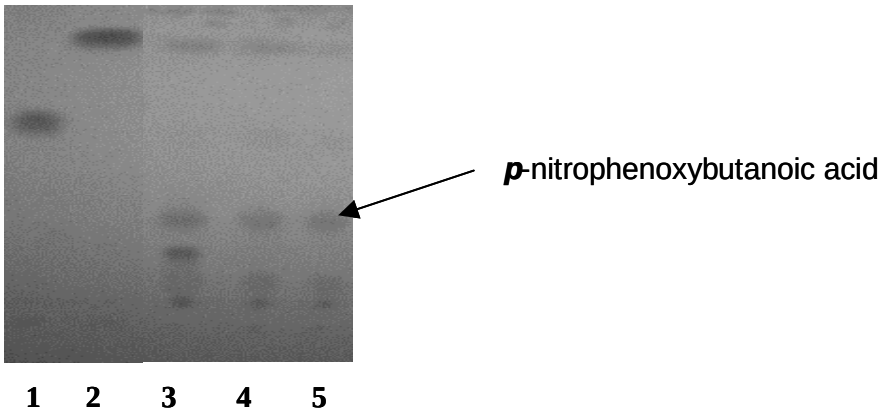


Figure 4.5: TLC plate of product formation from biotransformation of 4- nitrophenyl octyl ether in the presence of ethanol by strains **3**.TVN91, **4**.TVN356 and **5**.TVN493, respectively, 84 h after substrate addition. Line **1**. 4-nitrophenol **2**. 4-nitrophenyl octyl ether.

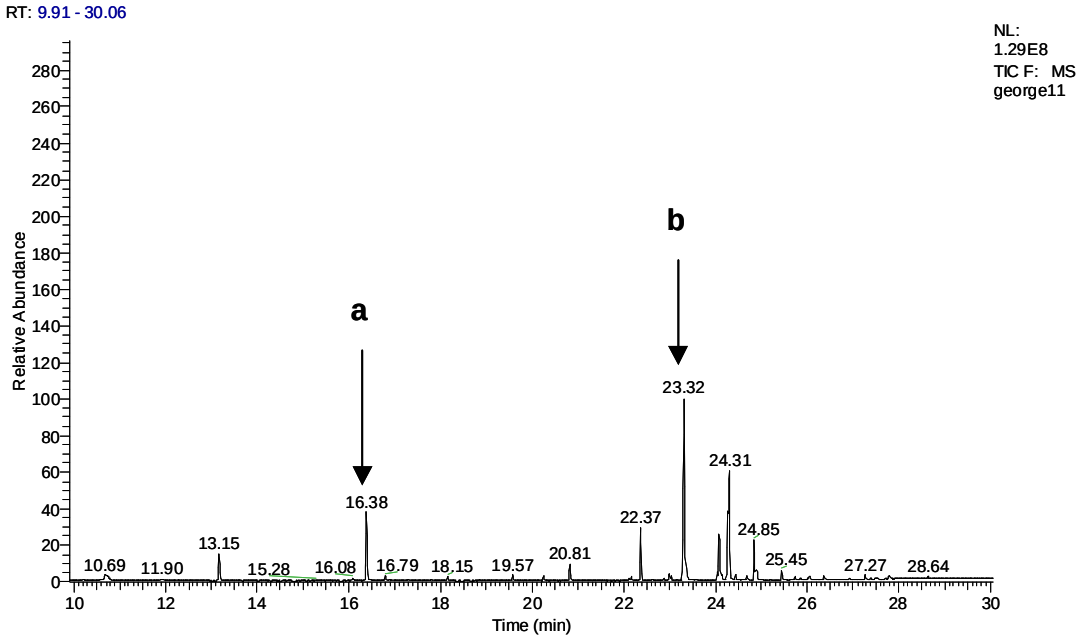


Figure 4.6: The GC spectra of the biotransformation products of 4-nitrophenyl octyl ether by TVN493 after 84 h. The peaks of interest have retention times (**a**) 16.38 min (***p***-nitrophenol) (**b**) 23.32 min (***p***-nitrophenoxybutanoic acid).

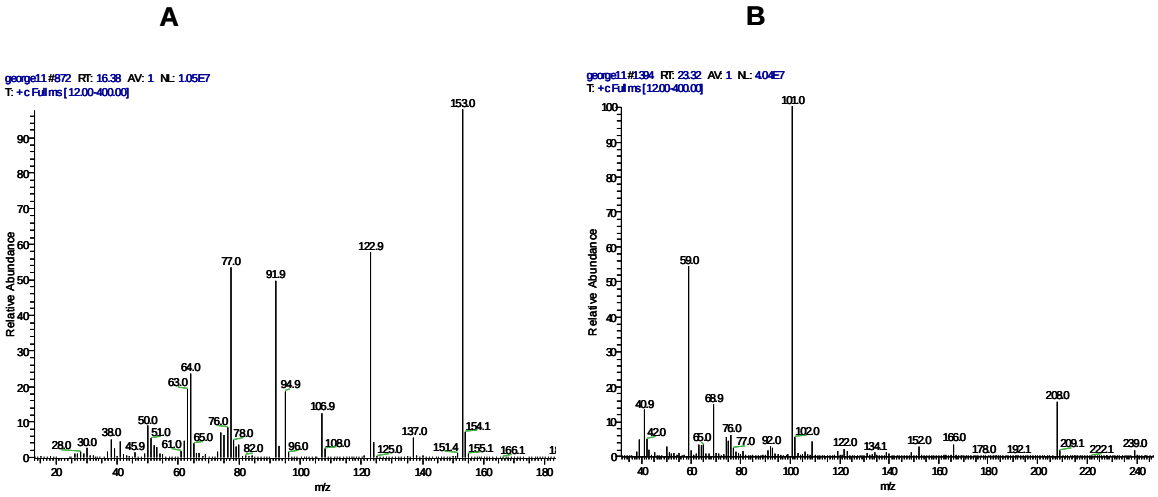


Figure 4.7: The MS spectrum of the biotransformation products formed from 4-nitrophenyl octyl ether by TVN493 after 84h. The methylated products were identified as (**A**) 4-nitro-methoxy benzene and (**B**) Methyl 4-(***p***-nitrophenoxy) butyrate.

Table 4.1: Mass spectra data of methylated biotransformation products of 4-nitrophenyl octyl ether ***m/e*** with relative intensities and assignment in brackets

Name	Molecular formula	*m/e* with relative intensities and assignment in brackets
4-nitrophenyl octyl ether	$C_{14}H_{21}O_3N$	251.1 (45) M^+; 71.0 (100); 221.1 (15) $[M-30]^+$
methyl 4-(***p***-nitrophenoxy) butyrate	$C_{11}H_{13}O_5N$	239.0 (17) M^+; 101.0 (100); 208.0 (10) $[M-31]^+$
4-nitro-methoxy benzene	$C_7H_7O_3N$	153.0 (100)M^+; 122.9 $[M-30]^+$

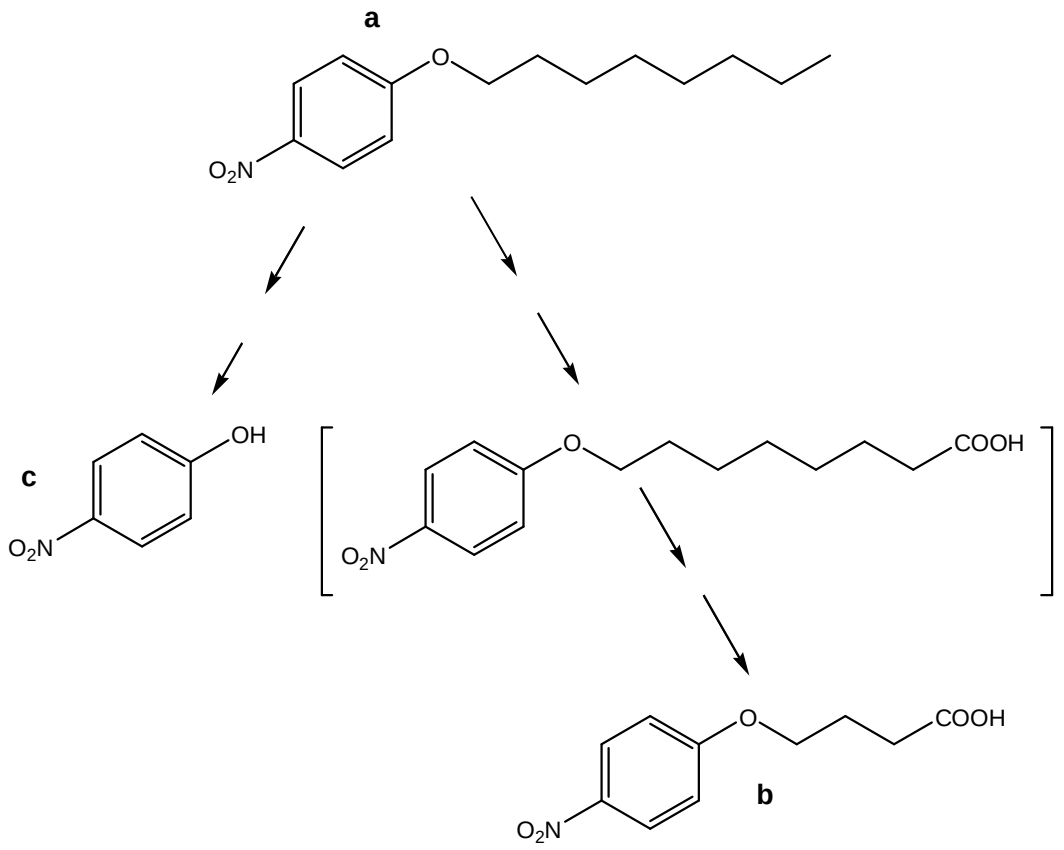


Figure 4.8: Proposed reaction scheme of the biotransformation of (**a**) 4-nitrophenyl octyl ether by ***Y. lipolytica*** strains, TVN91, TVN356 and TVN493. The methylated derivatives of (**b**), ***p***-nitrophenoxybutanoic acid (**c**) 4-nitrophenol, were identified on the GC/MS.

Quantitative GC analysis (**Figures** 4.9) showed that 4-nitrophenol was accumulated earlier or simultaneously with 4-nitrophenoxy butanoic acid. 4-Nitrophenol started accumulating as early as 2 h after substrate addition. This implied that the formation of these two products was in parallel. It was also apparent that 4-nitrophenoxy butanoic acid was not eventually oxidized to 4-nitrophenol, since it did not

disappear from samples when reactions were continued for up to 150 h, but remained at a more or less constant concentration for the last 100 h. Our proposed reaction scheme based on these results for the biotransformation of 4-nitrophenyl octyl ether is shown in Figure 4.8.

Given that each experiment was done only once and that conversion factors for 4-nitrophenyl octyl ether and 4-nitrophenoxybutanoic acid were estimated (based on 0h samples and samples where all substrate had been consumed, respectively) there was not a significant difference in the results obtained with the three strains TVN91, TVN356 and TVN493. It appears as if more substrate had initially been added to the cultures induced with ethanol than to the cultures induced with oleic acid – this might have been an unfortunate experimental error. Accurate determination of substrate concentrations is however difficult when the substrates are as insoluble and hydrophobic as 4-nitrophenyl octyl ether. When ethanol was used as inducer of the cloned ***YlCPR*** the extent of substrate utilization was less than when oleic acid was used as inducer, which led to a higher residual substrate concentration in the media (ca. 2.5 mM). The addition of ethanol also delayed production of 4-nitrophenoxybutanoic acid in two of the three strains although it did not to the same extent affect formation of 4-nitrophenol. When oleic acid was used as an inducer formation of both products started immediately and at approximately the same rate. These results indicate that ethanol suppressed induction of the endogenous and cloned CYP450s, while 4-nitrophenyl octyl ether or its ***p***-nitrophenoxycarboxylic acid products were not as strong inducers of the endogenous or cloned ***CYP*** genes as oleic acid or the alkylbenzenes and their degradation intermediates (Van Rooyen, 2004). The cloned CYP450s apparently had no effect on product formation, since results obtained with the three strains were essentially the same. This indicates that oleic acid as well as the ***p***-nitrophenoxycarboxylic acid products could not selectively induce the cloned CYP450s driven by the ***POX2*** promoter. Thus the background activity of the endogenous CYP450 enzymes could not be distinguished from the activity of the cloned CYP450s.

From, these results, it appears as if there were two distinct hydroxylase activities present in the ***Y. lipolytica*** strains – an activity that hydroxylated the carbon atom attached to the ***p***-nitrophenoxy moiety as well as an activity that hydroxylated the

terminal methyl group. The latter activity was never reported with CYP102. The presence of two activities however limits the use of 4-nitrophenyl octyl ether to study alkane hydroxylase activity in ***Y. lipolytica***. Another limitation of using 4-nitrophenyl octyl ether was the fact that this substrate as well as its transformation products were not as sensitive to TLC and GC analysis as substrates that contained or formed ***p***-hydroxybenzoate (next sections). 4-Nitrophenyl octyl ether is also a relatively expensive substrate (~15.3 US $ per g – from Acros Chemicals, 2005).

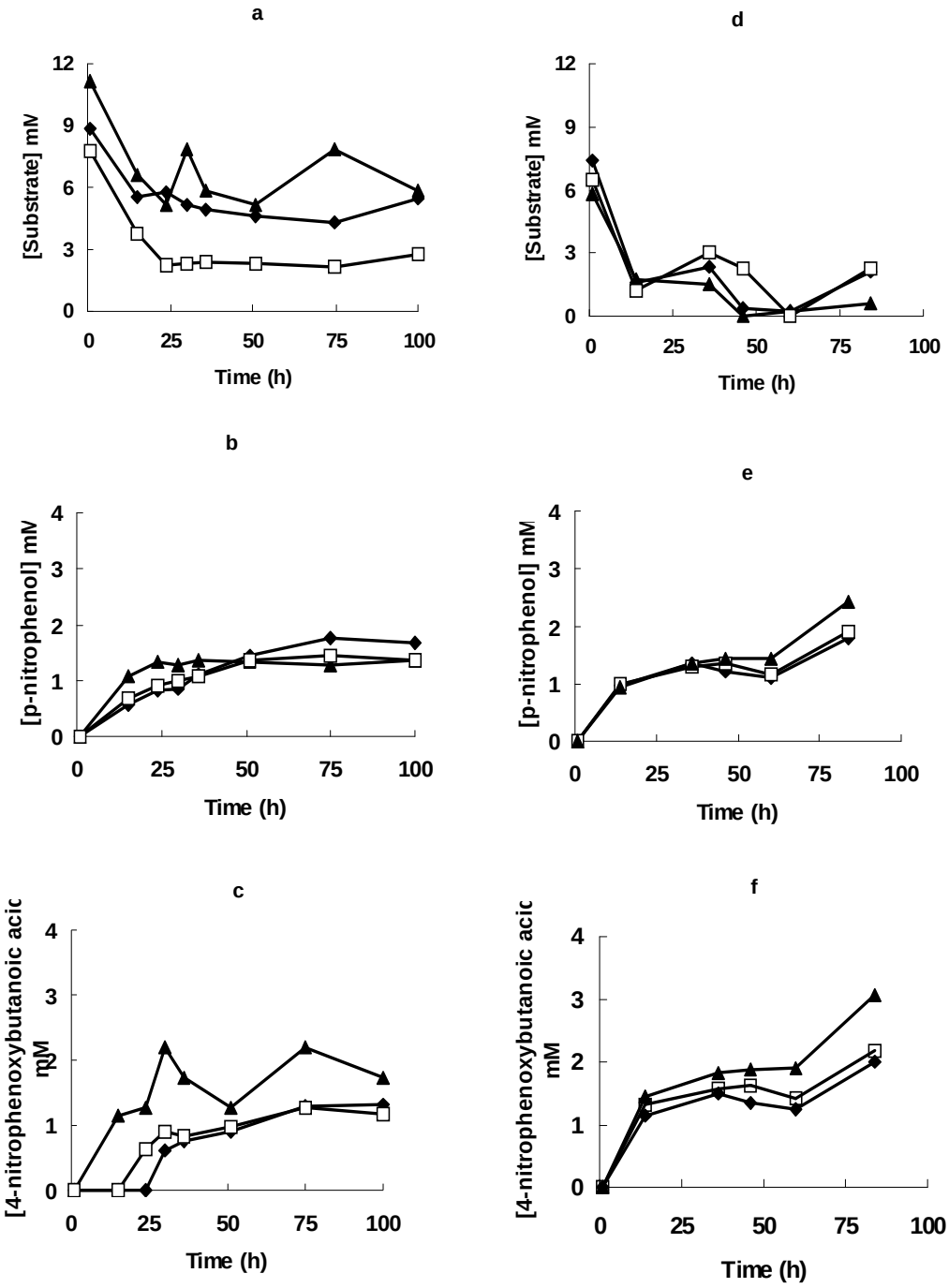


Figure 4.9: Biotransformation of 4-nitrophenyl octyl ether (2 g/l, 7.5 mM) added after 48 h growth. Ethanol (10 g/l) (**a**, **b**, **c**) or oleic acid (5 g/l) (**d**, **e**, **f**) were added as inducers after 24 h growth. Graphs show utilization of 4-nitrophenyl octyl ether (**a**,**d**), formation of 4-nitrophenol (**b**, **e**) and formation of 4-nitrophenoxybutanoic acid (**c**, **f**) by ***Y. lipolytica*** strains TVN493 (♦), TVN356 (□) and TVN91 (▲). Dry biomass measurements after 24 h growth were 13.0; 12.9 and 11.2 g/l, respectively

4.2.2 Biotransformation of 4-nonyloxybenzoic acid using *Y. lipolytica* strains TVN91, TVN356 and TVN493

4-Nonyloxybenzoic acid has a long chain alkane attached to ***p***-hydroxybenzoic acid and could possibly be used as a fatty acid analogue to screen for fatty acid omega hydroxylase activity. There is, however, also the possibility of hydroxylation at the carbon attached to the oxygen to form a hemiacetal as with 4-nitrophenyl octyl ether. Biotransformations were again performed using ***Y. lipolytica*** strains TVN91, TVN356 and TVN493. TVN91 was used as a control strain, since the cloned ***CYP53B1*** does not have alkane hydroxylase activity.

A literature search on the biotransformation of 4-nonyloxybenzoic acid by yeasts did not yield any reported cases. However, Zhang ***et al.*** (2003***)*** reported that as little as 0.033 g/l and 0.333 g/l of 4-nonyloxybenzoic acid were inhibitory to the growth of two bacterial strains, ***Mycobacterium smegmatis*** H37Ra and ***Mycobacterium tuberculosis*** respectively. This substrate was more sensitive to both TLC and GC analysis than 4-nitrophenyl octyl ether.

4.2.2.1 Isolation and characterization of 4-nonyloxybenzoic acid biotransformation products

TLC analysis of extracts from 24 h samples of biotransformations with TVN493 showed the formation of three products. It was noted that after further incubation, the product with the lowest R_f (labeled c) disappeared, indicating product degradation. The product with the highest R_f (labeled a) was provisionally identified as ***para***-hydroxy benzoic acid (pHBA), by comparison with an authentic sample (**Figure** 4.10 A). In order to identify all the products the relevant spots were scraped from TLC plates and the products extracted from the silica (**Figure** 4.10 B). The extracts containing the purified products were submitted to GC-MS analysis (**Figure** 4.11and 4.12).

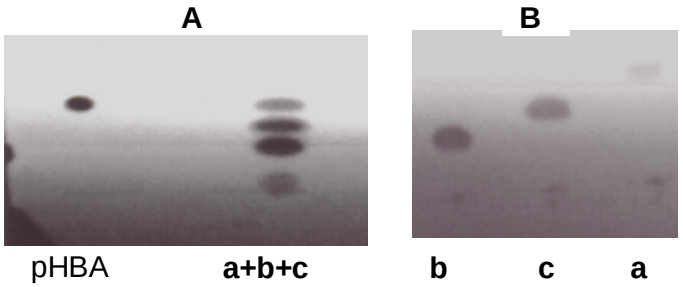


Figure 4.10: TLC plates showing (**A**) the three products formed during the biotransformation of 4-nonyloxybenzoic acid by ***Y. lipolytica*** TVN493 overexpressing ***CYP52F1*** (24h extract) (**B**) the purified products which were individually subjected to GC/MS for characterization. The three products were identified as **a**. ***p***-hydroxybenzoic acid, **b**. 4-(2-carboxyethoxy)benzoic acid, **c**. 4-(8-carboxyoctyloxy)benzoic acid.

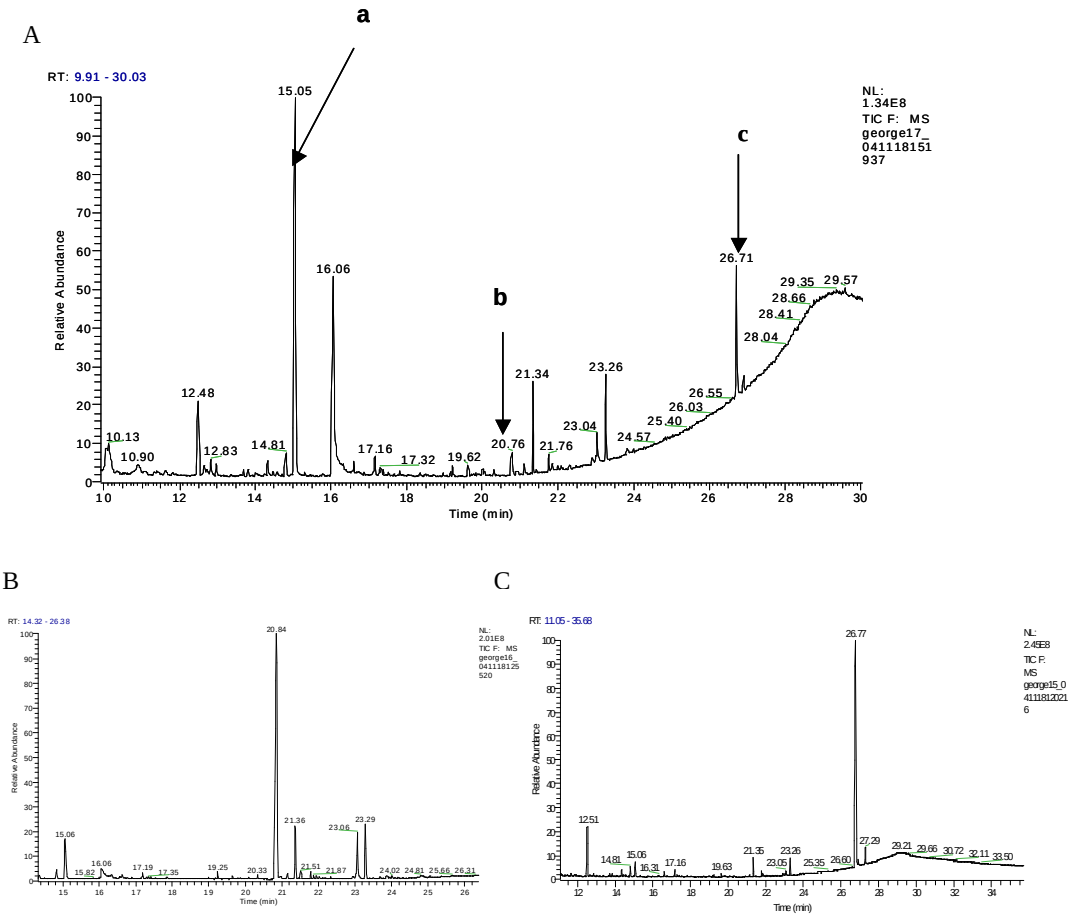


Figure 4.11: GC spectra of (**A)** 24 h extract from biotransformation 4-nonyloxybenzoic acid by TVN493. (**B**) & (**C**) The GC chromatograms of the isolated and partially purified products, which were subjected to GC/MS. The peaks of interest have retention times: 20.84, (**B**) and 26.77, (**C**) respectively.

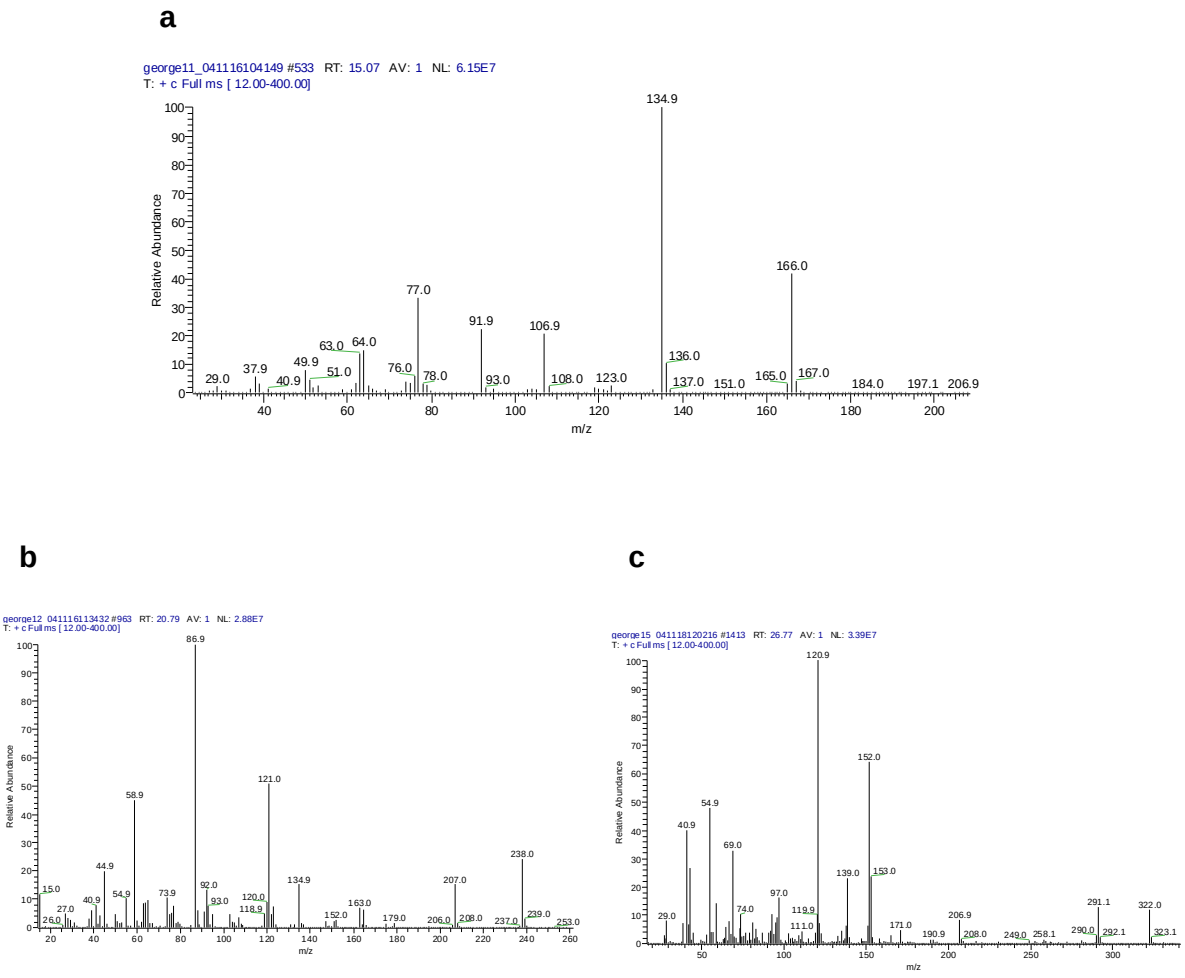


Figure 4.12: Mass spectra of purified methylated products from the biotransformation of 4-nonyloxybenzoic acid by ***Y. lipolytica*** TVN493. The methylated products were identified as (**a**) methyl 4-methoxy benzoate (**b**) Methyl 4-(3-methoxy-3-oxopropoxy) benzoate (**c**) Methyl 4-(9-methoxy-9-oxononyloxy) benzoate.

pHBA was the only isolated compound which could be identified by comparison of the GC-MS data with an authentic standard. It should be noted that the product analysed after TMSH methylation was methyl 4-methoxy benzoate. The methylated compound b was identified as methyl 4-(3-methoxy-3-oxopropoxy) benzoate by comparison of its mass spectrum (**Figure** 4.12 b) with spectra in the mass spectrum library. The mass spectrum of the methylated compound c (**Figure** 4.13 c) did not match any spectrum in the mass spectrum library. The fragmentation pattern and the molecular ion peak of 322 m/z (**Figure** 4.12 c) fitted the methylated intact diacid product, methyl 4-(9-methoxy-9-oxononyloxy)benzoate that could be expected from

terminal hydroxylation of the 4-nonyloxybenzoic acid. Mass spectra data for the methylated nonyloxybenzoate and its methylated biotransformation products are summarized in Table 4.2. A literature search did not yield 4-(2-carboxyethoxy)benzoic acid or 4-(8-carboxyoctyloxy)benzoic acid as products of biotransformation by any organism, thus this could be the first time such compounds have been produced through biotransformation.

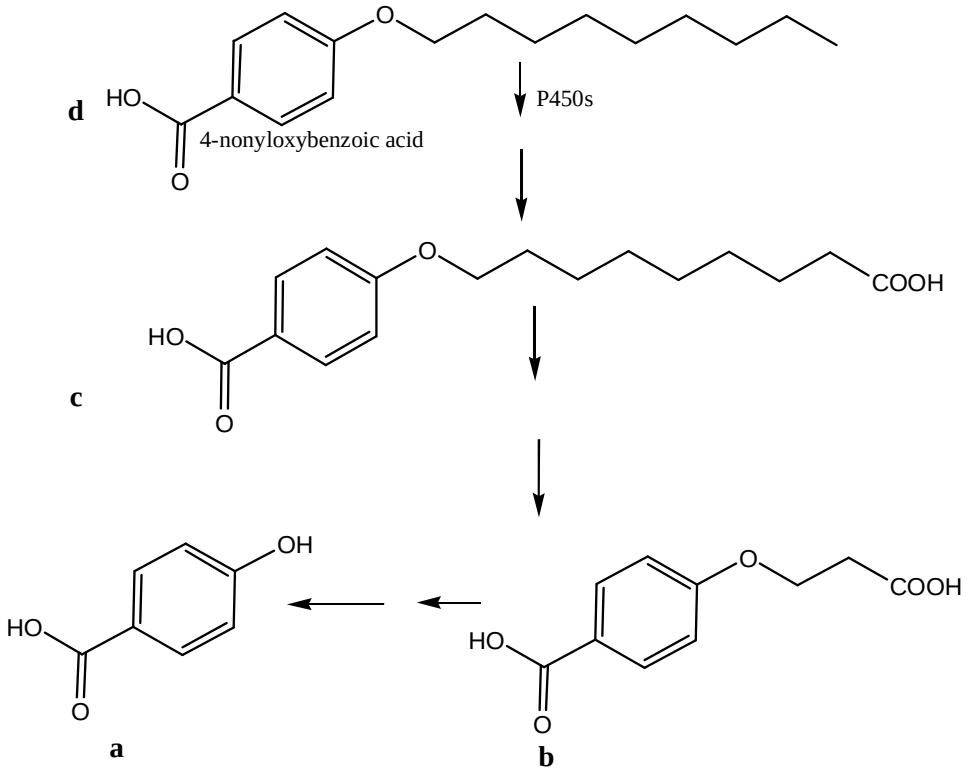


Figure 4.13: Products formed during the biotransformation of 4-nonyloxybenzoic acid

Table 4.2: Mass spectra data of methylated biotransformation products of 4-nonyloxybenzoic acid

Name	Molecular formula	*m/e* with relative intensities and assignment in brackets
4-nonyloxybenzoic acid-methyl ester	$C_{17}H_{26}O_3$	278.2 (25)M^+; 152.0 (100); 247.0 (10) $[M-31]^+$
methyl 4-(9-methoxy-9-oxononyloxy) benzoate	$C_{18}H_{26}O_5$	322.0 (12)M^+; 120.9 (100); 291.1 (10) $[M-31]^+$
methyl 4-(3-methoxy-3-oxopropoxy) benzoate	$C_{12}H_{14}O_5$	238.0 (22)M^+; 89.9 (100); 207.0 (10) $[M-31]^+$
benzoic acid, 4-methoxy-methyl ester	$C_9H_{10}O_3$	166.0 (42)M^+; 134.9 (100)$[M-31]^+$

4.2.2.2 Comparison of 4-nonyloxybenzoic acid biotransformation by TVN91, TVN356 and TVN493

Biomass determination of the three strains TVN91, TVN493 and TVN356 after 24 h growth, before addition of the inducers (ethanol and oleic acid), did not indicate any significant differences in growth. The biomass values were 11.1 g/l, 12.9 g/l and 13.0 g/l dry weight respectively. However, 26 h later (50 h after inoculation) i.e. after addition of inducer and substrate, the biomass differences between the three strains were pronounced with the biomass of TVN493 and TVN356 in both cases approximately 4.5 g/l higher than the biomass of TVN91 (**Table** 4.3). More experiments will be required to explain this observation. One explanation might be that production of CYP53B1 puts the host under stress.

The results illustrated that in the presence of ethanol (1%) as an inducer, 4-nonyloxybenzoic acid (7.57 mM, 2 g/l) was completely utilized in both the experimental strains, TVN356 and TVN493, within only 36 h from the time of addition. However, when the control strain TVN91 was used, it took up to 50 h (i.e. 15 h later) before the substrate was completely depleted. When oleic acid was used as an inducer, the substrate (4-nonyloxybenzoic acid) was depleted within only 14 h with all the strains (**Figure** 4.14).

It is evident from Figure 4.14 that the rate of substrate consumption (degradation) was faster than product formation. This is difficult to explain. One explanation might be that the 4-nonyloxybenzoic acid was transiently incorporated into the lipid (triacylglycerol) stores. Lipid analysis should be done to clarify this issue. However, given this situation substrate disappearance cannot be used to estimate hydroxylase activity.

Figure 4.14 also shows that the dioic acid products (4-(8-carboxyoctyloxy) benzoic acid and 4-(2-carboxyethoxy)benzoic acid), only accumulated transiently and were evidently oxidized to yield pHBA (**Figure** 4.14). Specific hydroxylase activities were therefore evaluated in terms of total product formation (**Figure** 4.14 d&h). Specific activities were calculated using the biomass values obtained after 24 h and 50 h growth.

A critical analysis of the total hydroxylase activities did not reveal significant differences in activities between the control strain, TVN91 and the experimental strains, TVN356 and TVN493. The slight differences in specific activities could be attributed to the differences in dry biomass values between the three strains as well as experimental error in determining concentrations of substrate and products, especially when dealing with such insoluble substrates as 4-nonyloxybenzoic acid. Furthermore approximated conversion factors (based on 0h samples and the conversion factor determined for hexadecanedioic acid), were used to calculate the concentrations of the substrate and the dioic acid products. It was thus difficult to distinguish the hydroxylase activities due to the overexpressed ***CYP*** genes from the activity due to the endogenous ***CYPs,*** but it was evident that the maximum specific hydroxylase activities for all the strains were in the order of 0.12 to 0.18 U (g. dwt)$^{-1}$. In this experiment ethanol also delayed the formation of hydroxylated products as was seen in the case when 4-nitrophenyl octyl ether was used as substrate.

Table 4.3: Hydroxylase activities in terms of total product formation from 4-nonyloxybenzoic acid by recombinant ***Y. lipolytica*** strains when ethanol (5 g/l) and oleic acid (10 g/l) were used as inducers

Strain	Biomass (24h) (g/l)	Biomass (50h) (g/l)	Total volumetric hydroxylase activity U (µmol /min/l)	Total specific hydroxylase activity U (g. dwt)$^{-1}$. (biomass after 24 h of growth)	Total specific hydroxylase activity U (g. dwt)$^{-1}$ (biomass after 50 h of growth)	Period of maximum activity (h after substrate addition)
	Inducer: Ethanol					
TVN91	11.2	11.9	1.82	0.16	0.17	24-51
TVN356	12.9	16.8	2.41	0.18	0.14	4-36
TVN493	13.0	16.1	2.39	0.18	0.15	24-51
	Inducer: Oleic acid					
TVN91	11.2	12.0	1.83	0.16	0.15	0-36
TVN356	12.9	17.8	2.26	0.17	0.12	0-36
TVN493	13.0	17.4	1.96	0.17	0.12	0-36

NB: where U refers to the amount of product in µmol formed per minute

Conversion factors: The ratio of substrate against the internal standard at time 0 h was divided by concentration of substrate (4-nonyloxybenzoic acid) added, to derive a constant factor used for calculations

Since, no authentic standards for the two , dioic acid products, (4-(8-carboxyoctyloxy) benzoic acid and 4-(2-carboxyethoxy)benzoic acid) were available, the conversion factor derived from hexadecanedioic acid standard curve was used for calculations.

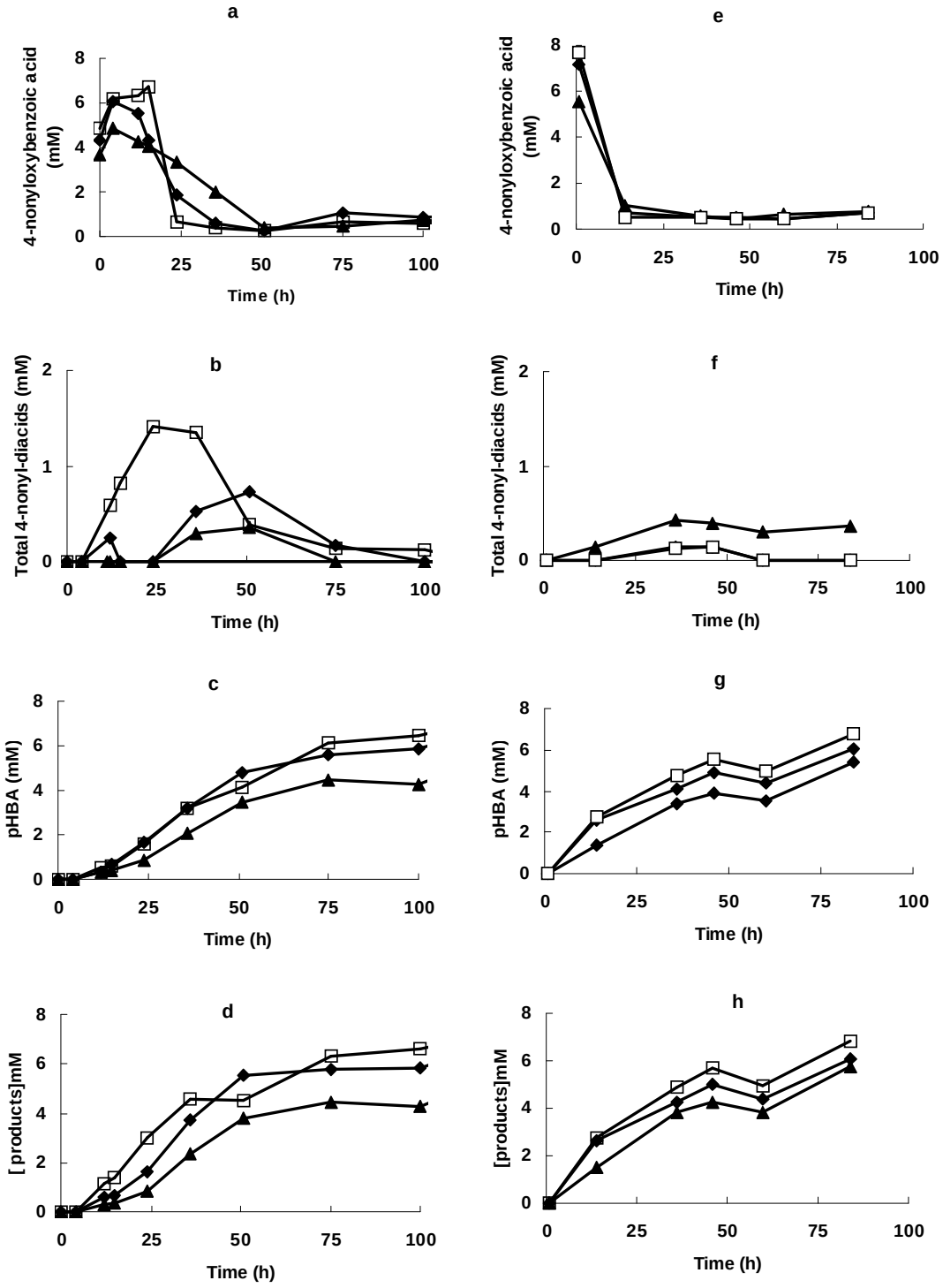


Figure 4.14: Biotransformation of 4-nonyloxybenzoic acid when ethanol (10 g/l) (**a**, **b**, **c, d**) or oleic acid (5 g/l) (**e**, **f**, **g**, **h**) was used as inducer. ***Y. lipolytica*** strains tested were TVN493 (♦), TVN356 (□) and TVN91 (▲). Graphs show utilization of 4-nonyloxybenzoic acid (**a**, **e**), formation of 4-nonyl diacids (**b**, **f**), formation of PHBA (**c**, **g**) and total product formation (**d**, **h**).

4.2.2.3 Biotransformation of 4-nonyloxybenzoic acid by strains with partially disrupted β-oxidation overexpressing CYP450s

In order to investigate possible production of the intact 4-(8-carboxyoctyloxy) benzoic acid, we tested biotransformaton of 4-nonyloxybenzoic acid by strains overexpressing ***CYP450*** genes but with the *β*-oxidation pathway partially disrupted. Smit ***et al***. (2005) have previously accumulated intact dicarboxylic acids from alkanes and fatty acids using an acyl-CoA oxidase deficient mutant of ***Y. lipolytica*** with four of the six ***POX*** genes ***(POX2, POX3, POX4 and POX5***), coding for the acyl-CoA oxidases, disrupted***.*** When dodecane (23 g/l) was added to this ***POX*** deleted strain it accumulated dodecanedioic acid (2 g/l).

Three types of strains were available for this experiment. The first type comprised recombinant ***Y. lipolytica*** strains TVN442 and TVN445 with the acyl-CoA oxidase encoding genes ***POX2***, ***POX3***, ***POX4*** and ***POX5*** deleted and overexpressing ***YlCPR*** driven by the ***POX2*** promoter. The second type was strain TVN440 which was the same as the previous strains but additionally overexpressing ***CYP52F1***. The third type, strains TVN497, TVN498, TVN499, TVN500, TVN501 and TVN502, was also the same as the first type but additionally overexpressing ***CYP557A1.*** The cloned ***CYP*** genes were also driven by the ***POX2*** promoter. The ***YlCPR*** and ***CYP52F1*** genes were cloned using vectors that normally give integration of one or two copies of the cloned gene (Nthangeni ***et al***., 2004), while the ***CYP557A1*** gene was cloned with a zeta based vector that gives multiple integration of the cloned gene (3-39 copies of the cloned gene inserted) because it has a defective ***ura3d4*** allele (Le Dall ***et al***., 1994; Juretzek ***et al***., 2001). It was reported that transformants with less than 10 copies of the vector inserted displayed a decreased growth rate (Juretzek ***et al***., 2001).

Biomass determination of the eight strains used for this experiment after 24 h growth (before addition of inducer), 48 h growth (before addition of substrate) and 132 h growth (at end of experiment) indicated significant differences in growth (**Figure** 4.16). Consequently, this also implied a concomitant difference in residual glucose, which had not been identified for this experiment, has been shown to repress induction of the ***CYP*** genes as well as the ***POX2*** promoter (Green ***et al***., 2000; Juretzek ***et al***., 2001). It was noted that TVN445 had the least biomass at all the time intervals. The reasons for the variation in growth of the strains used might be due to differences in the

number of integrations of the ***zeta*** based vector carrying the cloned ***CYP450*** gene and the defective ***ura3d4*** allele (Juretzek ***et al***., 2001). The results seemed to indicate that, strains with fewer or no cloned CYP genes had the highest activities. This implied that the resultant hydroxylase activity could have been due to the endogenous enzymes and that; they were competing for the available reductase.

It is evident from the TLC results that, the rate of substrate consumption was, in most of the strains, very rapid during the initial 12 h. The only strains, which did not consume all the substrates in 12 h, were TVN445 and TVN498. This could have been due to their slow growth, which might have led to more residual glucose preventing induction of the endogenous and cloned CYP450s. Because the substrate was consumed so quickly additional substrate (0.2% w/v) was added to all strains after 25 h. After the second substrate addition, only three strains TVN442, TVN499 and TVN500 again consumed all the substrate.

These results also showed that TVN445 was the only strain, which did not produce significant amounts of products. It was also the strain with the least amount of biomass at the end of the experiment. It was also noted that TVN442 (no cloned ***CYP***, but with ***CPR***) was the only strain that produced the shortened product (4-(2-carboxyethoxy) benzoic acid). All the other strains including TVN440, TVN445, TVN497, TVN498, TVN499, TVN500, TVN501 and TVN502 accumulated the intact diacid product (4-(8-carboxyoctyloxy) benzoic acid). From the TLC results it appeared that the two strains, TVN499 and TVN500, produced comparatively higher amounts of the intact diacid product, (4-(8-carboxyoctyloxy) benzoic acid) from 4-nonyloxybenzoic acid). Due to the variation in many parameters such as dry biomass, residual glucose and differences in genotype of these strains, the exact reasons why these two strains produced the most product still needs further investigations. All these differences affect the cell physiology and thus product formation.

Quantification through GC analysis was unfortunately not possible at the time when this experiment was done, due to a technical problem with the GC apparatus. However, GC/MS analysis of selected samples confirmed the TLC results that substrate was completely converted to the respective dioic acid products, with no significant amounts of other products detected (**Figure** 4.15).

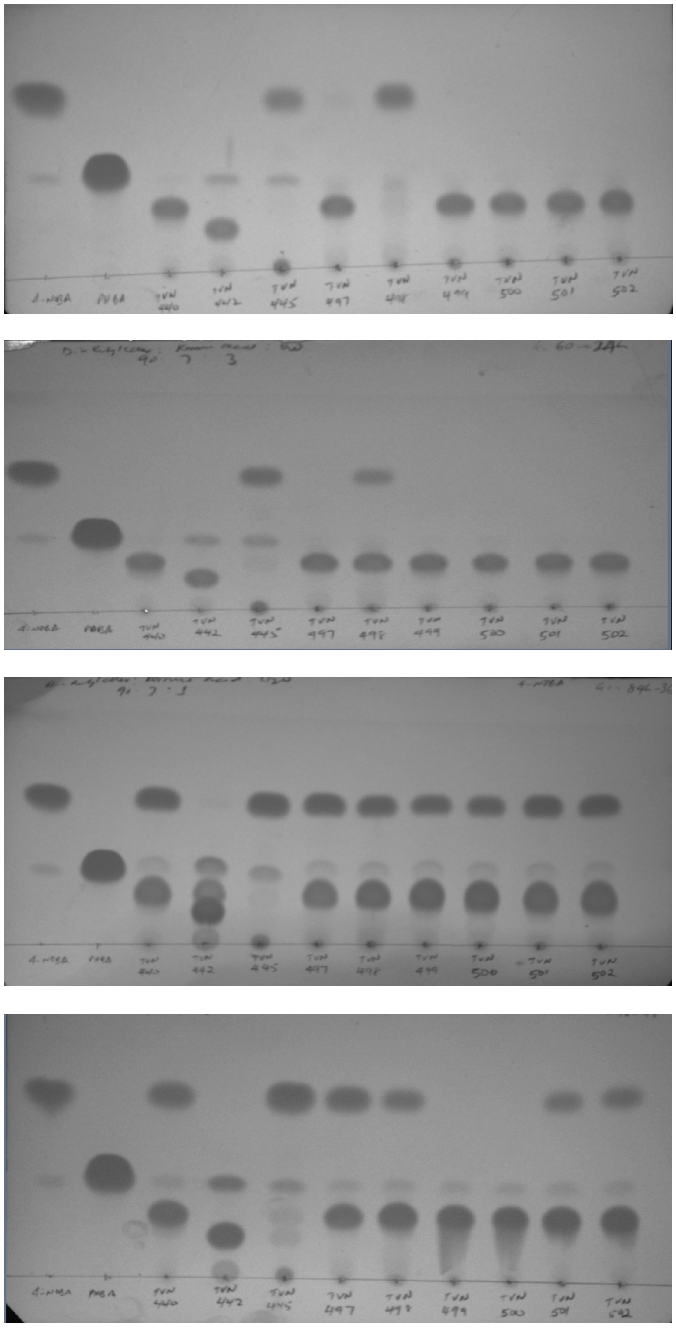


Figure 4.15: TLC plates showing the effect of using ***β***-oxidation partially blocked strains on the accumulation of the intact 4-nonyloxybenzoic acid, diacid derivative. Additional substrate was added after 25 h. The spots are as follows 1-Substrate, 2 -pHBA, 3-TVN440, 4-TVN442, 5-TVN445, 6-TVN 497, 7-TVN498, 8-TVN499, 9-TVN500, 10-TVN501, 11- TVN502.

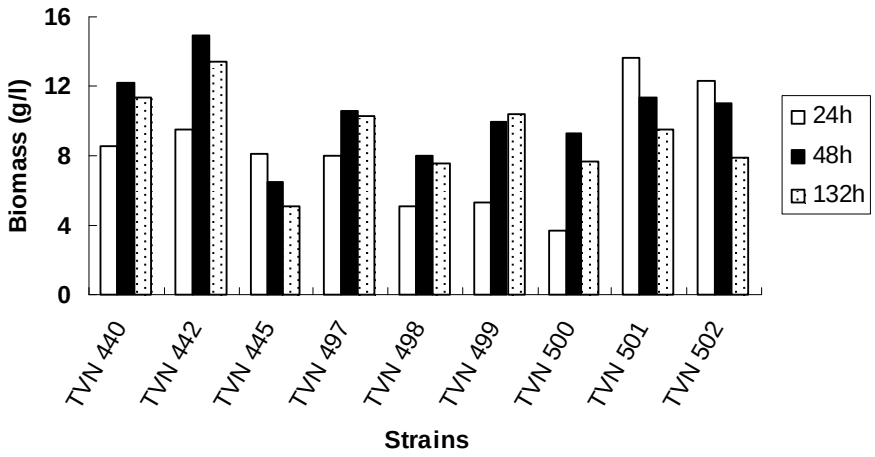


Figure 4.16: Biomass values for ***Y. lipolytica*** strains with β-oxidation pathway disrupted grown on YPD in the presence of 4-nonyloxybenzoic acid.

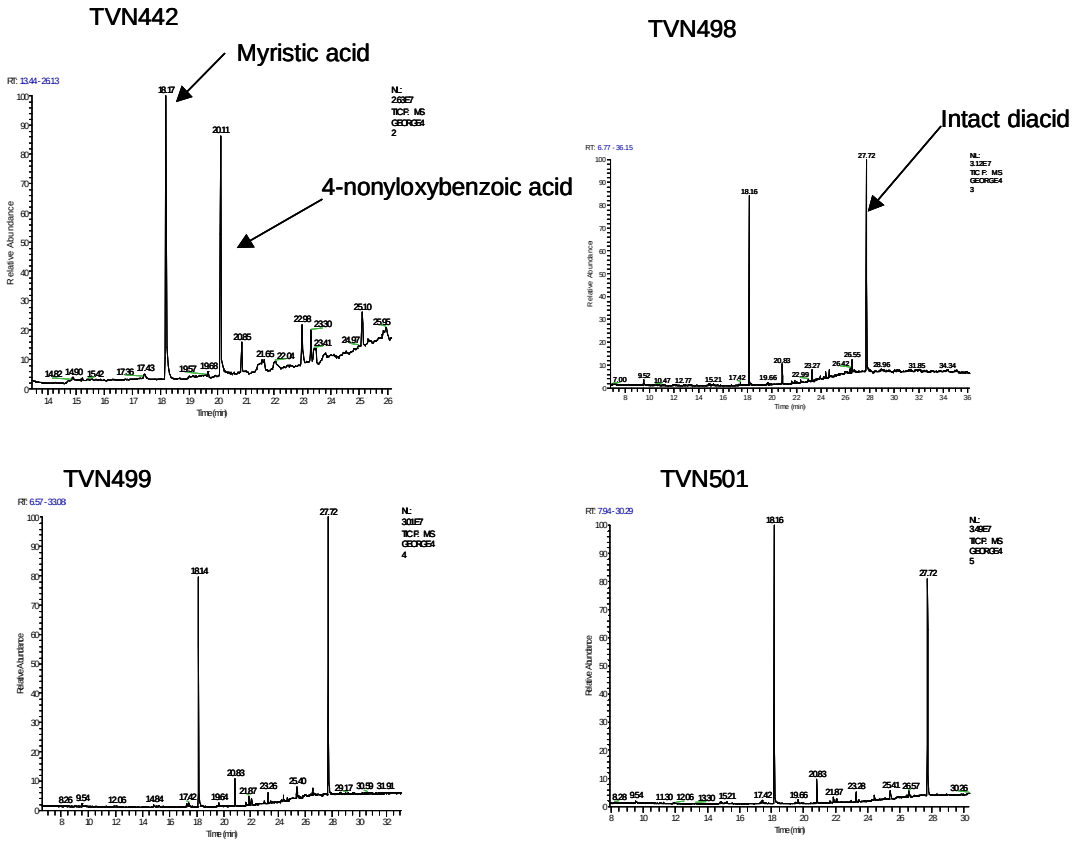


Figure 4.17: GC spectra of biotransformation intermediates on 4- nonyloxybenzoic acid by ***Y. lipolytica*** strains TVN442, TVN498, TVN499 and TVN501 after 72h. The peaks of interest are marked.

4.2.2.4 Biotransformation of 4-nonyloxybenzoic acid using wild type strains

In order to investigate the biotransformation of 4-nonyloxybenzoic acid by wild type yeasts, three strains were used. These strains were ***R. minuta*** (from which the ***CYP53B1*** gene was cloned), ***R. retinophila*** (from which the ***CYP557A1*** gene was cloned) and ***Y. lipolytica,*** W29 (wild type from which the β-oxidation disrupted strains had been derived). Comparison of their dry biomass values after 36 h of growth demonstrated significant differences especially between the ***Y. lipolytica*** W29 and the two ***Rhodotorula*** spp. After 36 h of growth, ***Y. lipolytica*** had a higher biomass (12.1 g/l) than ***R. minuta*** (8.9 g/l) and ***R. retinophila*** (8.3 g/l) respectively.

The initial rate of substrate consumption was very rapid in all the strains. However, it was observed that the two ***Rhodotorula*** strains did not consume all the 4-nonyloxybenzoic acid, while ***Y. lipolytica*** W29 did. In the case of the two ***Rhodotorula*** strains, there was a rapid consumption of the substrate within the initial 36h from ca. 7.5 mM to ca 2.5 mM after which, the residual substrate concentration levelled off (**Figure** 4.19). On the other hand, ***Y. lipolytica*** W29 consumed the substrate rapidly for the first 24 h followed by a gradual decline to disappearance after 60h. It was also noted that ***R. retinophila*** did not only initially consume the substrate at a faster rate than ***R. minuta,*** but also formed detectable amounts of the products (pHBA and the diacid intermediates), while no products were detected with ***R. minuta***. This made sense considering that it is from ***R. retinophila*** that the ***CYP557A1*** was cloned.

It is still not clear why the rate of substrate consumption was initially so rapid in all the strains even though we did not detect concomitant accumulation of intermediate products and specifically with ***R. minuta*** no products were detected with GC analysis***.*** One of the reasons why no or very little products were observed with the two ***Rhodotorula*** strains could have been that, as soon as pHBA was formed it was immediately hydroxylated and degraded. However, although ***R. retinophila*** has been reported to grow on alkanes and therefore might be able to degrade the alkyl chain of 4-nonyoxybenzoic acid, we have not been able to demonstrate degradation of benzoic acid by ***R. retinophila*** (Ramorobi, unpublished results). ***R. minuta*** on the other hand is not known as an alkane degrading yeast although it is well known for the degradation

of various aromatic compounds such as BA and pHBA. The initial apparent substrate concentration might be the result of incorporation in to lipids, as mentioned above.

Comparison of the total hydroxylase activity of the wild-type strain ***Y. lipolytica*** W29 with the activities of the recombinant strains TVN91, TVN356 and TVN493 revealed that the wild type ***Y. lipolytica*** had the highest specific total hydroxylase activity. The activity of the wild-type strain was 0.38 U $(g.dwt)^{-1}$ while the recombinant ***Y. lipolytica*** strains had total specific activities in the range 0.12-0.18 U $(g.\ dwt)^{-1}$. This further illustrates, that the resultant activity was as a result of the endogenous CYP450s. The total specific hydroxylase activity of the wild type ***R. retinophila*** was as low as only 0.06 U $(g.dwt)^{-1}$.

This result together with the results obtained with the β-oxidation disrupted strains, which indicated that the strains with no or fewer cloned CYP genes had the highest activity seems to indicate that the 4-nonyloxybenzoic acid hydroxylase activity can be ascribed to endogenous enzymes. The heterelogous expression of foreign CYP450s therefore apparently reduces these activities; probably by displacing the native enzymes from the CPR (also see results obtained with phenylnonane section 4.5.1-4.5.3).

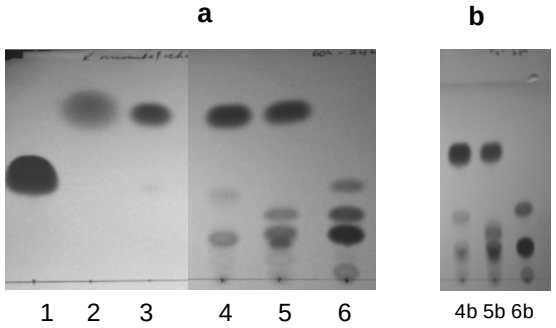


Figure 4.18: TLC plates showing biotransformation of 4- nonyloxybenzoic acid by wild type strains. Lines **1**. pHBA **2**. BA **3**. 4-nonyloxybenzoic acid **4**. ***R. minuta,*** **5**. ***R. retinophila***, **6**.***Y. lipolytica*** W29**.** (**4-6**: biotransformation after 24h). **4b**. ***R. minuta***, **5b**. ***R.retinophila***, **6b**-***Y. lipolytica*** W29. (**4b-6b**: biotransformation after 60h). The substrate was added after 36h of growth.

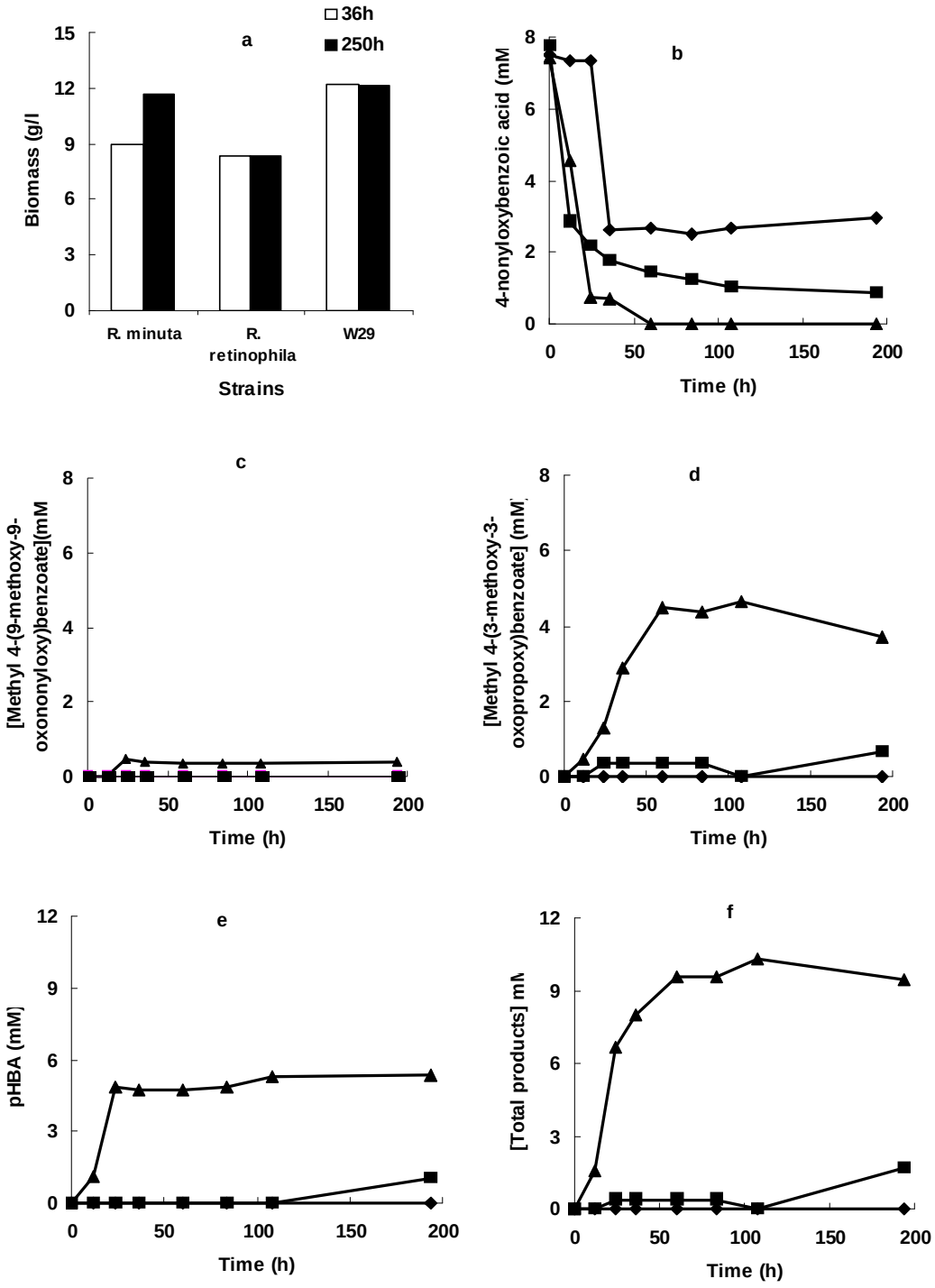


Figure 4.19: Biotransformation of 4-nonyloxybenzoic acid by wild type strains***, R. minuta*** (♦)***, R. retinophila*** (■) and ***Y. lipolytica*** W29 (▲), grown on YPD broth. (**a**) biomass values of the strains grown in YPD (**b**) degradation of 4-nonyloxybenzoic acid. Formation of (c) (4-(8-carboxyoctyloxy) benzoic acid, (d) 4-(2-carboxyethoxy)benzoic acid, (e) pHBA, (f) Total products by the three strains

4.3 Biotransformation reactions using strains with benzoate *para*-hydroxylase activity

R. minuta is a basidiomycetous yeast, which can grow on a variety of aromatic compounds ***via*** benzoic acid (BA). ***CYP53B1*** has been isolated from ***R. minuta*** and shown to encode a bifunctional enzyme with isobutene-forming and benzoate-4-hydroxylase activities (Fujii ***et al***., 1997). From its cDNA sequence, it was deduced to have 527 amino acids with a calculated molecular weight of 59136. It was found to share 48% amino acid sequence identity with CYP53A1 from ***Aspergillus niger***. Benzoate-***para*** hydroxylase (CYP53A1) was first purified from ***Aspergillus niger,*** and was shown to be capable of degrading benzoate. It is due to this protein that ***R. minuta*** is capable of degrading various aromatic compounds through the *β*–ketoadipate pathway (Fujii ***et al***., 1997).

Shiningavamwe ***et al***. (2006) cloned and overexpressed ***CYP53B1*** from ***R. minuta*** in ***Y. lipolytica***. Preliminary screening results gave an indication that the recombinant strain TVN91 had benzoate ***para***-hydroxylase activity, but the activity was not accurately quantified or optimized. As a follow up, our aim was to quantify and optimize benzoate ***para***-hydroxylase activity in the recombinant ***Y. lipolytica*** strain TVN91 and to determine factors limiting the hydroxylase activity of such a heterologously expressed CYP450.

In order to compare the CYP53B1 activity in the wild type yeast strain with that of the recombinant strains, a set of experiments was first done with the wild-type ***R. minuta*** to determine toxicity of benzoic acid as well as benzoate ***para***-hydroxylase activity. Faber ***et al***. (2001) had determined the substrate specificity of benzoate-***para*** hydroxylase from ***Aspergillus niger*** and showed that this enzyme could hydroxylate benzoic acid as well as some of its chlorinated derivatives. These researchers revealed that several single substituted benzoate derivatives such as 2-chlorobenzoate and 3-chlorobenzoate were hydroxylated at the ***para***-position by benzoate ***para*** hydroxylase. Further work by Sun ***et al***. (2000) revealed that a strain of ***Rhodotorula rubra*** Y-1529 initially degraded 2-chlorocinnamic acid to 2-chlorobenzoic acid before hydroxylation at the ***para***-position to form 2-chloro, 4-hydroxybenzoic acid. Based on the results from these previous researchers, we decided to also investigate hydroxylation of 2-

chlorobenzoic acid (2-ClBA), 3-chlorobenzoic acid (3-ClBA) and 4-chlorobenzoic acid (4-ClBA) by the wild-type ***R. minuta*** as well as the recombinant ***Y. lipolytica*** TVN91.

Strains used for this study included ***R. minuta*** CBS 2177, ***Y. lipolytica*** TVN91 (overexpressing ***CYP53B1*** and the ***YlCPR***) and ***Y. lipolytica*** TVN97 (overexpressing only the ***CYP53B1***). The initial experiments involved biotransformations using the wild type ***R. minuta*** strain.

4.3.1 Toxicity of benzoic acid to *R. minuta*

BA (0.5 % (w/v)) was added to two cultures of ***R. minuta*** CBS 2177 grown for 15 h in YPD broth. In the case of the first culture the pH was left unadjusted and measured to be pH 6, while the pH of the second culture was adjusted to pH 8 by the addition of 50 mM phosphate buffer. At pH 6, the addition of BA at early stationary phase led to a significant reduction in final growth. The biomass was only 5.0 g dry weight /l after 36 h of growth, with a concomitant reduction in glucose consumption (**Figure** 4.20). When the pH was maintained at 8, the growth continued until all the glucose was utilized reaching a biomass of 9.32 g dry weight/l after 36 h.

In a second experiment, different concentrations of BA (0.1-1.0%) were added after 15 h of growth to two ***R. minuta*** cultures grown in YPD broth. In one culture, the pH was left unadjusted, while the pH of the second culture was adjusted to pH 8 as before. After a further 36 h of growth, plate counts were performed to determine the viable cell counts (**Figure** 4.21). The control cultures (no BA added) gave higher viable cell counts compared to the cultures to which BA had been added. It confirmed that BA had a significant inhibitory effect on cell viability, especially at the lower pH. It was also noted that even at pH 8, there was also inhibition of growth of ***R. minuta*** by BA, especially at higher concentrations. The reason why there was an apparent increase in viability with increase in BA concentration could not be completely be explained. This could have been due to experimental errors, especially with viable counts which were only done in duplicate.

At low pH, substrates such as BA (pK_a 4.19) exist essentially in the undissociated state, a form in which it is a potent growth inhibitor. At pH 6, the amount of undissociated benzoic acid (0.6 mM) was much great than that at pH 8 (0.06

mM).The undissociated BA readily diffuses across the cell membrane only to dissociate and generate protons and anions in the higher pH environment of the cytosol. The anions generated might accumulate and generate high turgor pressure, leading to oxidative stress. The protons released might in turn acidify the cytosol, thus inhibiting many metabolic functions (Krebs ***et al***., 1983; Piper ***et al***., 2001). On the contrary at high pH values, the BA is essentially completely dissociated, posing a smaller threat and may even provide a potential carbon source.

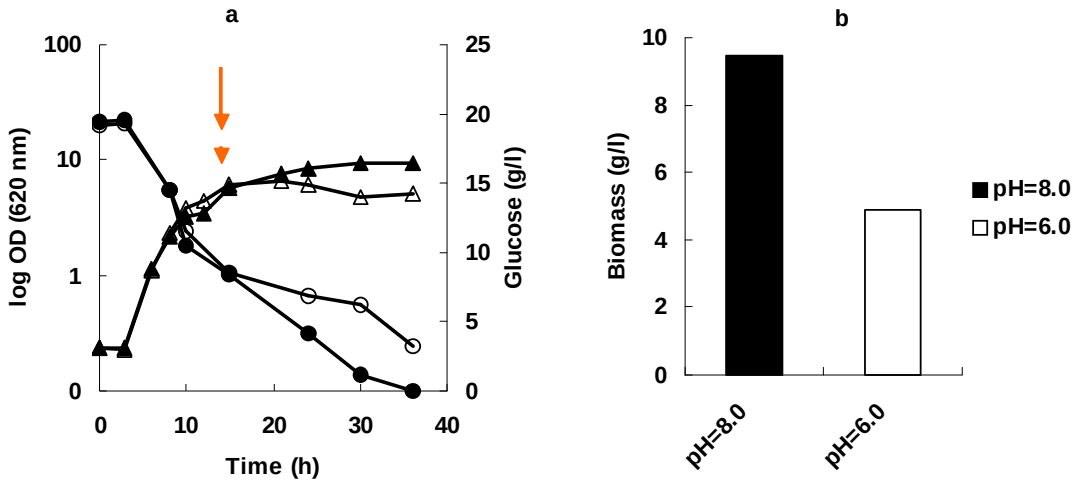


Figure 4.20: (**a**) Growth of ***R. minuta*** on YPD and 0.5% BA at two different pH values; (Δ) pH 6, (▲) pH 8 and the glucose uptake, (○), pH 6, (●) pH 8. The arrow indicates the time of BA addition. (**b**) The total biomass of ***R. minuta*** grown in YPD broth with 0.5% BA added to cultures at pH of 6 and 8 after 36 h.

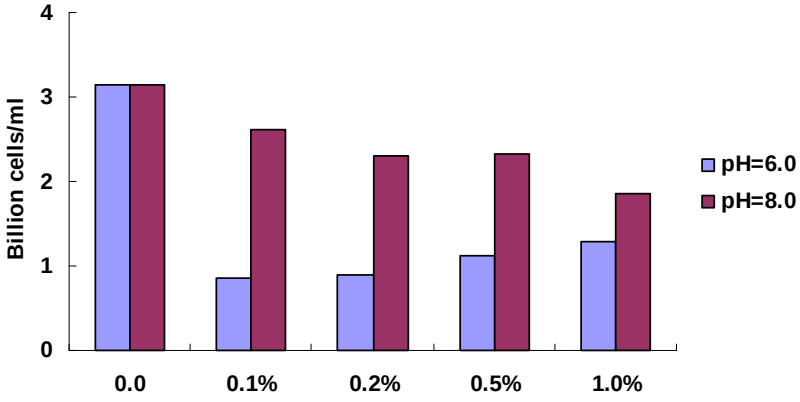


Figure 4.21: Viable cell counts to demonstrate the effect of media pH and substrate concentration on the toxicity of BA to ***R. minuta.*** The counts were done 36 h after BA addition.

4.3.2 Growth of *R. minuta* on BA

In this experiment, BA (0.1% w/v) and glucose (0.1% w/v) were added as the only carbon sources to two cultures of ***R. minuta*** grown in YNB broth. The control culture was grown in YNB broth without a carbon source. From the growth data, it was evident that BA, at such a low concentration, supported growth almost as well as glucose (**Figure** 4.22a).

In another experiment, BA (0.1% w/v) was added at intervals, and the fate of BA was followed. The results demonstrated that BA was initially hydroxylated to form pHBA, which underwent further degradation (**Figure** 4.22b).

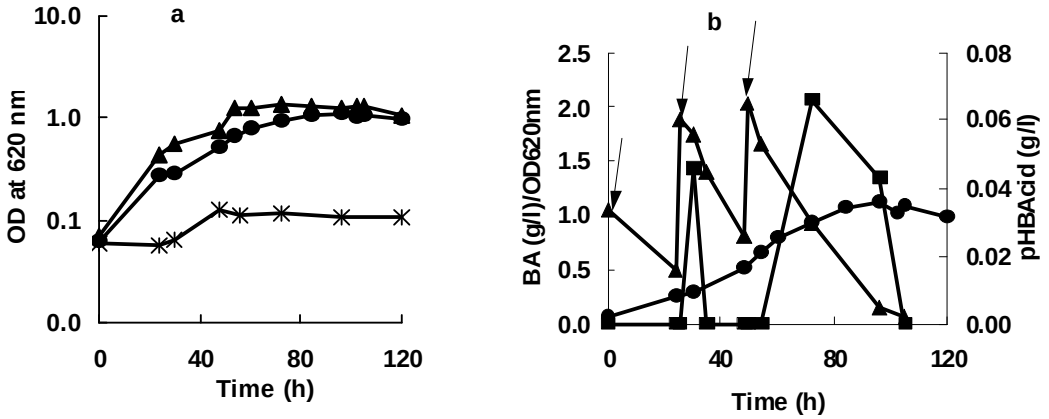


Figure 4.22: (**a**). Comparative growth of ***R. minuta*** in YNB with different carbon sources; (●) 0.1% BA; (▲) 0.1% glucose, (∗) no carbon source added (**b**). (▲) BA utilization, (■) pHBA accumulation (●) Growth ($OD_{620\ nm}$). ***R. minuta*** was grown in YNB and BA added at intervals as a carbon source. The arrows indicate the times of BA addition.

4.3.3 Biotransformation of BA using *R. minuta*

In order to later compare benzoate ***para***-hydroxylase activity of the wild-type ***R. minuta*** with activity of the recombinant ***Y. lipolytica*** overexpressing ***CYP53B1,*** a biotransformation experiment was performed using the wild type strain under conditions similar to the conditions used in biotransformation experiments with recombinant ***Y. lipolytica*** strains with the pH adjusted to 8. BA (0.5 % w/v) was added to a culture of ***R. minuta*** grown in YPD broth for 36 h. Within only 24 h, after BA addition, the substrate was completely consumed. BA was degraded ***via*** the formation

of pHBA, which underwent further degradation. Although pHBA was completely degraded by ***R. minuta,*** it still accumulated within 24 h up to 1.8 g/l (13 mM) pHBA. The maximum specific rate of pHBA accumulation was determined to be 0.017 g. (g dwt) h^{-1} while the maximum specific rate at which pHBA was consumed was 0.004 g. (g dwt) h^{-1}. If the sum of the rates of pHBA accumulation and pHBA consumption is taken as a measure of the hydroxylase activity, then the maximum specific benzoate ***para***-hydroxylase activity was at least 0.021 g. (g dwt) h^{-1}. This translates to 2.54 U (g dwt)$^{-1.}$

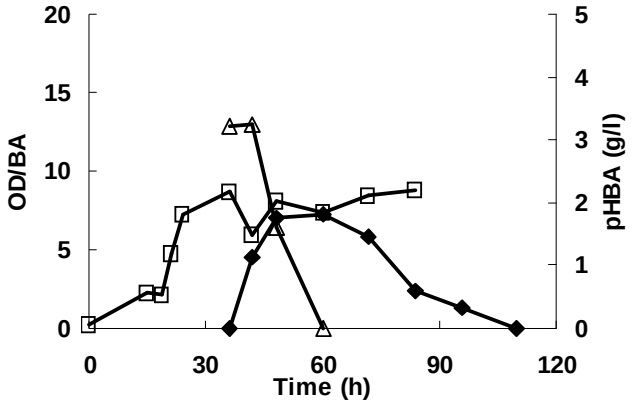


Figure 4.23: Biotransformation of BA by ***R. minuta*** grown in YPD broth with 5 g/l BA added after 36 h; (∆) BA (♦) pHBA (□) OD at 620 nm. The pH of the medium was maintained at 8. The dry weight at 36 h was 9.4 g/l

4.3.4 Biotransformation of chlorobenzoates using *R. minuta*

In order to test if the wild type strain could also hydroxylate chlorobenzoates, experiments were initially carried out to establish the toxicity of these substrates. All these experiments were done at pH 8.

In order to investigate the toxicity of the various chlorobenzoates, 0.1% (w/v) each of BA, 4-ClBA, 3-ClBA, and 2-ClBA were added to cultures of ***R. minuta*** grown for 36 h in YPD broth containing 1 % w/v glucose. To establish whether the substrates added served as additional carbon sources two controls were included. An equivalent amount of glucose (0.1 % w/v) was added to one culture, while nothing was added to the other. Dry biomass was determined at intervals and viable cell counts were determined after 48 h (**Figure** 4.24). Based on these measurements, the order of

toxicity was determined to be: 4-ClBA > 3-ClBA > 2ClBA >BA. The final biomass results confirmed that BA was used as carbon source [BA, biomass of 8.5 g/l, negative control (no additional substrate added), biomass of 7.8 g/l and positive control (additional glucose added), biomass of 8.7 g/l]. In the case of the chlorobenzoates the final biomass was in all cases lower than the negative control [the highest 2-ClBA was 7.22 g/l]. When grown in YNB media with the chlorinated benzoates (0.1%) as the sole carbon source, it was apparent that these compounds could not be degraded in the absence of an alternative carbon source (results not shown). Inhibition of growth resulted in very little biomass.

In a biotransformation experiment, the chlorobenzoates (1.0% w/v (63.9 mM); 0.5% w/v (31.9 mM); 0.1 % w/v (6.3 mM)) were added to cultures of ***R. minuta*** grown for 36 h in YPD broth with 2 % w/v glucose. The results indicated that even a concentration of 1.0% (w/v) of 2-ClBA was degraded by ***R. minuta***. However, the residual 2-ClBA concentration remained at 1.1 g/l and it appeared that at a concentration of 0.1% (w/v), the 2-ClBA was not degraded, since the 2-ClBA concentration apparently remained constant at 1 g/l. These results might be due to a problem with the GC analysis with an interfering compound having the same retention time or it might be that the 2-ClBA is not degraded (bound by the monooxygenase) when the concentration drops below 1.1 g/l. When 3-ClBA was used as the test substrate, it was only degraded at a concentration of 0.1% w/v (6.3 mM). Higher concentration (0.5% and 1.0%) were not degraded at all (**Figure** 4.25 a and b). 4-ClBA being toxic, was not degraded at all under similar conditions (results not shown). Previous research by Katayama ***et al***., (1994) also reported the co-metabolic degradation of chlorophenols by a strain of ***R. glutinis*** in the presence of glucose. The amount of chlorophenols that were degraded in the presence of glucose was only ca. 0.47 g/l (3.7 mM). Sun ***et al***. (2000) reported the degradation of 2-chlorocinnamic acid (0.025 mM) by ***R. rubra*** grown in YPD broth via the formation of 2-ClBA, which was further metabolized.

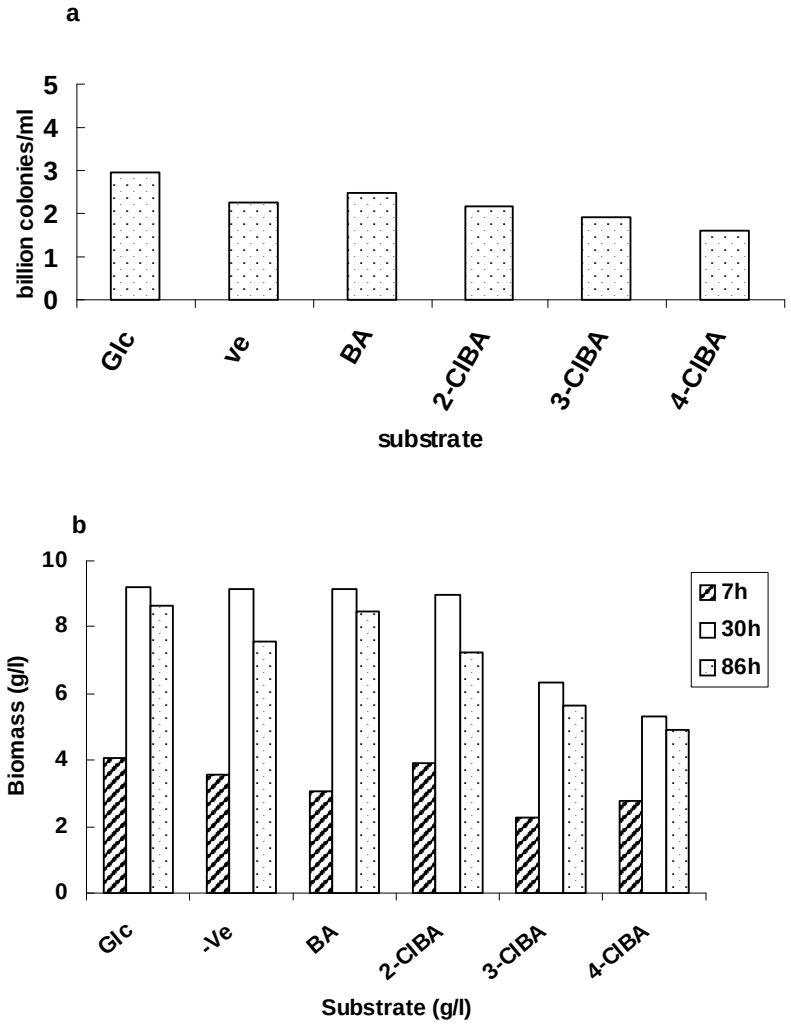


Figure 4.24: (**a**) Viable cell counts of ***R. minuta*** after 48 h growth in YPD (1% glucose) and 0.1% each of different carbon sources. (**b**) Biomass in samples taken at various times after addition of test compounds. pH of the media was maintained at 8 in all cases.

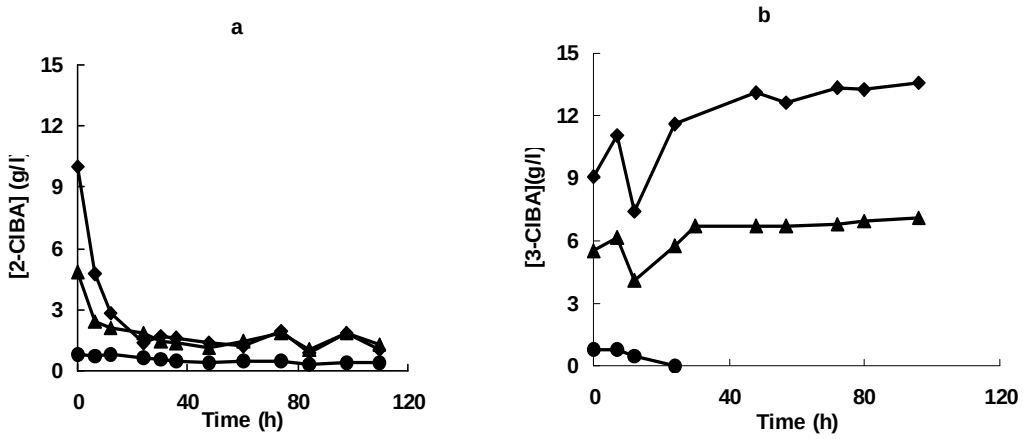


Figure 4.25: Degradation of (**a**) 2-ClBA and (**b**) 3-ClBA by ***R. minuta*** grown in YPD broth for 36 h before addition of different concentrations of the test substrates; (●), 0.1% (w/v), (▲), 0.5% (w/v) and (♦), 1.0% (w/v).

4.4 Biotransformations using recombinant *Y. lipolytica* strains overexpressing *CYP53B1*

Heterologous expressions of CYP450s in foreign hosts usually require the co-expression of a compatible CPR. In order for the heterologous expression system to be functional the CYP450 and the CPR should be in the correct ratio (Dong and Porter, 1996; Backes and Kelly, 2003). In order to investigate this theory, two strains with multicopy integration of the ***CYP*** gene were used. The first strain was, TVN91, (overexpressing both ***CYP53B1*** and ***YlCPR***) while second strain used was TVN97, (overexpressing only the ***CYP53B1***). To construct ***Y. lipolytica*** strain TVN91, the vector containing the ***CYP53B1*** cDNA from ***R. minuta*** was cloned into ***Y. lipolytica*** E150 by using the multicopy shuttle vector, JMP64 (Shiningavamwe ***et al***., 2006). The ***CYP53B1*** gene was introduced under the control of the ***POX2*** promoter. The clone with the highest activity (labeled TVN97) was subsequently transformed with JMP21-pICL-YlCPR containing ***YlCPR*** under the control of the ***ICL*** promoter.

Biotransformation experiments were carried out using 0.5 % (w/v) BA. pH was adjusted to 8 with 50 mM phosphate buffer just before substrate addition. In this experiment, the culture (***Y. lipolytica*** TVN91) was initially grown in YPD broth for 24 h

after which inducer was added. The substrate was added between 18 and 24 h after the inducer.

4.4.1 Effect of CPR on CYP450 activity

Since it has been shown that the CYP:CPR ratio is important for optimal CYP450 activity (Truan ***et al***., 1993), and that in the recombinant system, the ***Y. lipolytica*** CPR may be a limiting factor (Nthangeni ***et al***., 2004), studies were done to determine the extent of this limitation on biotransformation of BA by the ***Y. lipolytica*** strains overexpressing CYP53B1.

Investigation on the effect of CPR on the CYP450 activity was carried out using the strains, TVN91 (overexpressing both ***CYP53B1*** and ***YlCPR***) and TVN97 (overexpressing only the ***CYP450***, but without the ***YlCPR***). After 48 h growth (24h after oleic acid (OA) addition), TVN97 had a dry biomass of only 8.1 g/l while TVN91 had a biomass yield of 16.7 g/l. The reason why TVN91 had a better growth was because it had only a single auxotrophy for histidine while TVN97 still had double auxotrophy for both leucine and histidine, since it had not been transformed with an empty JMP21 vector. Since the growth of these two strains were significantly different, this resulted in differences in glucose as well inducer utilization (**Figure** 4.26).

Although the concentration of OA added was 20 g/l, less than 3 g/l of OA was recovered from both cultures within 1 h after addition. Similar results were always obtained when OA was used as inducer. This can probably be ascribed to a combination of poor mixing/sampling (an unavoidable problem with such hydrophobic substrates) and rapid accumulation of OA into the cells (Mlíčková ***et al***., 2004).

In the case of TVN91, pHBA (0.12 g/l) was detected 24 h after substrate addition (**Figure** 4.26), while there was a long delay before pHBA was detected in TVN97 (40h). The highest concentration of pHBA detected with TVN91 was 0.36 g/l after 180 h growth while for TVN97 only 0.06 g/l pHBA was detected after the same duration (**Table** 4.4). Even though, both the volumetric and specific benzoate ***para***-hydroxylase activities were higher in the case of TVN91, it was impossible to conclusively determine and quantify the exact influence of CPR limitation in this case due to the variation in growth, residual glucose, and relative rate of inducer uptake. This is because all the

above-mentioned factors affected the induction of ***CYP53B1*** (under control of ***pPOX2***) as well as the endogenous ***CYP450***s and ***YlCPR***. In order to carry out this investigation accurately, it would have been better to use a control strain overexpressing ***CYP53B1*** and also transformed with empty JMP21-pICL vector. This would alleviate the leucine auxotrophy and therefore improve growth. Since TVN91 gave better growth and maximum product formation (**Figure** 4.26), all further experiments were carried out using only this strain.

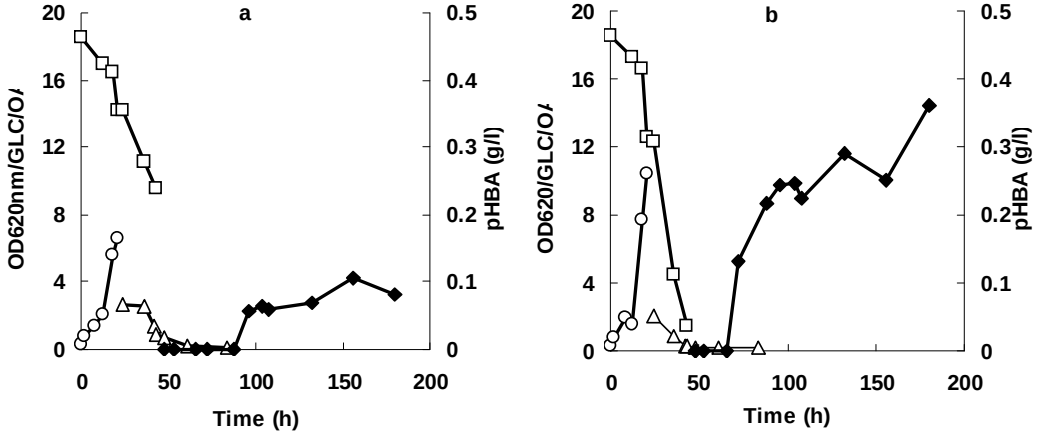


Figure 4.26: Biotransformation of BA (5g/l added after 48 h) by ***Y. lipolytica*** strains (**a**) TVN 97 (**b**) TVN91 grown on YPD broth with oleic acid (1% v/v) added after 24 h. Glucose (g/l) open squares. OD 620 nm (open circles). Oleic acid (open triangles) and pHBA (g/l)(closed diamonds

Table 4.4: Specific activity in terms of product formation illustrating the effect of ***CPR*** limitation

Strain	**Biomass (g/l)**		**Maximum activity**			**Maximum product (g/l)**
	at 24 h growth	**at 48 h growth**	**Period (h)**	**Volumetric activity (g (l.h)$^{-1}$**	**Specific activity U. (g.dwt)$^{-1}$.**	
TVN97	5.8	8.1	40-48	0.0070	0.08	0.06 g/l
TVN91	8.2	16.7	40-48	0.0089	0.05	0.36 g/l

NB: Specific activity was calculated based on the biomass at 48 h. Specific activities are given in U (g.dwt)$^{-1}$.The periods sued were time after substrate addition.

4.4.2 Effect of substrate limitation and product toxicity

In order to investigate the possibility of substrate limitation, different concentrations of BA (0.1%, 0.5%, 1.0% and 2.0% (w/v)) were added to cultures of TVN91 grown in YPD broth containing 2% (w/v) glucose. The substrate was added, 24 h after the inducer (2% oleic acid). Biotransformation reactions were then monitored through TLC analysis.

In a second experiment, toxicity of both BA and pHBA to ***Y. lipolytica*** was investigated by the addition of different concentrations (0.1%, 0.5%, 1.0% and 2.0%) to cultures grown in YPD broth for 36 h. Growth and dry biomass were monitored to determine the toxicity of these two substrates. GC analysis was also done of samples taken from the culture that had received pHBA in order to confirm that ***Y. lipolytica*** TVN91 did not degrade pHBA.

Growth results indicated that both of BA and pHBA up to 2 % w/v were not toxic to ***Y. lipolytica*** (results not shown). When BA was added at concentrations of 0.5% w/v, it was still detected in the culture, 72 h after substrate addition, at a lower concentration of 0.1% w/v BA was no longer detected after the same duration (**Figure** 4.27). Since the product concentration on TLC was apparently the same at BA concentrations of 0.5 % w/v and higher, it was concluded that only at a concentration 0.1% did the substrate become limiting after 72 h. In all further experiments BA was therefore added at concentration of 0.5 % w/v.

Even though it has been suggested by Eppink ***et al***. (1997), that other ascomycetous yeasts such as ***Candida parapsilopsis*** completely degrade pHBA through an initial decarboxylation step, our results showed that ***Y. lipolytica*** (an ascomycetous yeast) did not degrade pHBA since GC analysis showed that the pHBA concentration never decreased when it was added at a concentration of 0.1% and higher (results not shown).

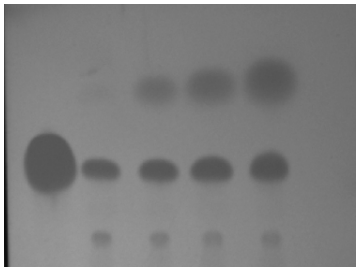


Figure 4.27: TLC plate showing biotransformation after 72 h of different concentrations of BA using TVN91 grown in YPD broth with 2% glucose for 24 h before oleic acid (2% w/v) addition. The different concentrations of BA were added 24 h after oleic acid addition.

4.4.3 Effect of product inhibition on biotransformation

The possibility of product (pHBA) inhibition, which has been reported to be a problem with CYP450 based hydroxylations (Duetz ***et al***., 2001), was investigated. TVN91 was grown in YPD broth for 36 h prior to inducer (2 % OA) addition. Product inhibition was investigated by then adding pHBA (0.5 g/l) 5 h prior to BA addition. The control experiment was carried out in the same way, but in this case pHBA was not added to the culture.

In this experiment TVN91 did not grow as well as in previous experiments. Dry weights of samples taken immediately before the addition of inducers were approximately 7 g/l. After 56 h growth, dry biomass was 9 g/l. The reason for variations in growth between different experiments was not elucidated. A possible explanation might be that a complex growth medium was used and that different batches of yeast extract and peptone were used over time.

When the benzoate ***para***-hydroxylase activities were compared for the two cultures, the amounts of additional pHBA formed were similar in both cases and the addition of pHBA to the one culture had no effect on hydroxylase activity (**Figure** 4.28a & b). Product inhibition was thus not the reason why hydroxylation activities could not be maintained.

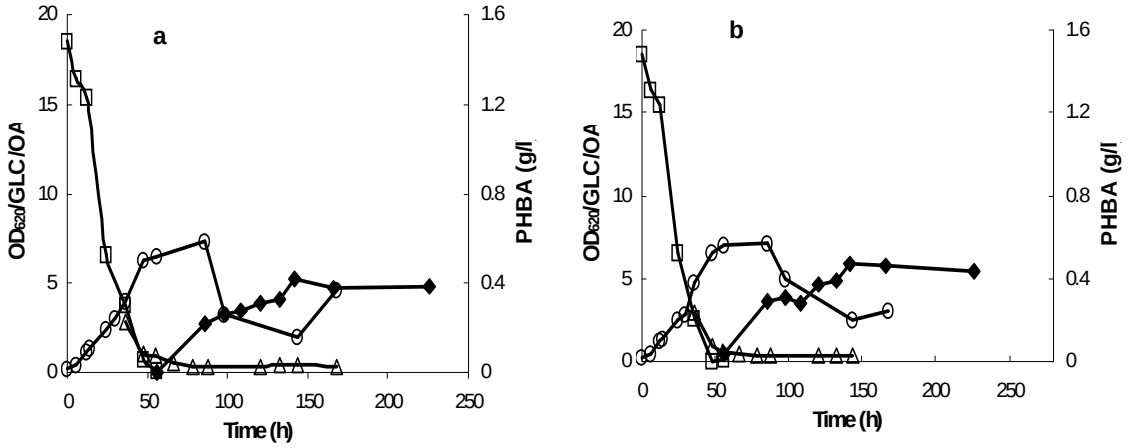


Figure 4.28: Biotransformation of BA (5 g/l added after 54 h) by ***Y. lipolytica*** strain TVN91 grown in YPD broth with (**a**) 2% OA added after 36 h. (**b**) 2% OA and 0.05% pHBA added after 49 h. Glucose (g/l) open triangles. OD 620 nm (open circles). Oleic acid (open triangles) and pHBA (g/l) (closed diamonds). Note that in **b** only additional pHBA formed is shown, not total pHBA.

4.4.4 Optimization using different inducers in shake flasks

In the previous experiments where OA was used as inducer the production of pHBA started to level off within 50 h of BA addition and production stopped within 96 h. The pHBA concentration then remained constant at ca. 0.4 g/l. In an attempt to optimize biotransformation of BA using the recombinant yeast strain TVN91, several induction conditions were tested (**Figure** 4.28 a&b, 4.29 a-d). Juretzek ***et al***. (2000) reported that different inducers such as acetate, ethanol, fatty acids and alkanes have varying effects on the ***ICL*** and ***POX2*** promoters. Since the ***CYP53B1*** gene was under the control of the ***POX2*** promoter and the ***YlCPR*** was under the control of the ***IC***L promoter, the effects of different inducers on the benzoate ***para***-hydroxylase activity was investigated. TVN91 was grown in YPD broth with inducers added after 36 h and BA 18 h later.

Nthangeni ***et al***.(2004) used YPDH medium containing both glucose (1%) and olive oil (2.5% v/v) to monitor induction of CYP1A1 activity driven by ***pPOX2***. Therefore olive oil (2 % v/v), was first tested as inducer. The results showed that production of pHBA still started to level off within 50 h of BA addition and production still stopped

within 96 h and the maximum pHBA concentration still did not exceed 0.4 g/l (**Figure** 4.28a).

The biomass concentration in the sample, taken immediately before the addition of olive oil was still 7 g/l. After 56 h of growth (i.e. 20 h after the addition of the inducer) the dry biomass was approximately 11 g/l. Although the concentration of the olive oil, added was ca. 20 g/l, only 11 g/l OA was recovered immediately after olive oil addition. Olive oil is hydrolyzed to yield mainly OA (Menendez ***et al***., 2005). Within 12 h after olive oil addition the olive oil concentration had dropped to less than 1 g/l.

In the experiments, where OA and olive oil were used as inducers, the average volumetric production rates of approximately 6 mg $(l.h)^{-1}$ was obtained during the first 42 h after substrate addition. The hydroxylation rate was apparently not constant, but rapid during the first 30 h and slower later. This may be due to the fact that the inducers (olive oil/OA) were consumed so rapidly. We therefore tested stearic acid (SA) as an inducer (**Figure** 4.29 b-d).

When SA was added as an inducer, the dry biomass was approximately 20 g/l after 56 h of growth (i.e. 20 h after addition of inducers). Although the concentration of inducer added was 20 g/l, only 12 g/l was recovered within 1 h after SA addition, due to the reasons explained above. SA as an inducer increased the volumetric hydroxylation activity to 7 mg $(l.h)^{-1}$ (**Figure** 4.29 b). A volumetric activity of 7 mg $(l.h)^{-1}$ translated to a specific activity of 0.04 U $(g\ dwt)^{-1}$ (where 1 U is defined as 1 µmol of product formed per minute). This hydroxylation activity seemed to be more constant and appeared to correlate with the slower utilization of SA, which is a solid and is much less soluble than OA and olive oil. The bioconversion stopped 88 h after BA addition when there was no more SA in the media. A maximum pHBA concentration of 1.1 g/l was obtained. In the case of cultures induced with olive oil and OA, less biomass was obtained so that specific activities were higher, i.e. 0.07 and 0.08 U $(g\ dwt)^{-1}$, respectively. It is possible that the dry biomass was overestimated and specific activity thus underestimated in the case of cultures to which SA had been added, because SA is very insoluble and very difficult to wash from the filters despite washing the filters with cyclohexane and 0.5 M NaOH.

The addition of ethanol together with SA delayed the onset of hydroxylation activity by 36 h, probably until all the ethanol was used up (**Figure** 4.29c). However, the level of hydroxylation activity (7 mg $(l.h)^{-1}$) remained the same as with SA alone. When SA was added repeatedly, the same hydroxylation activity (7 mg $(l.h)^{-1}$) was maintained for 200 h, and a final product concentration of almost 1.6 g/l (32% conversion) was reached (**Figure** 4.29d).

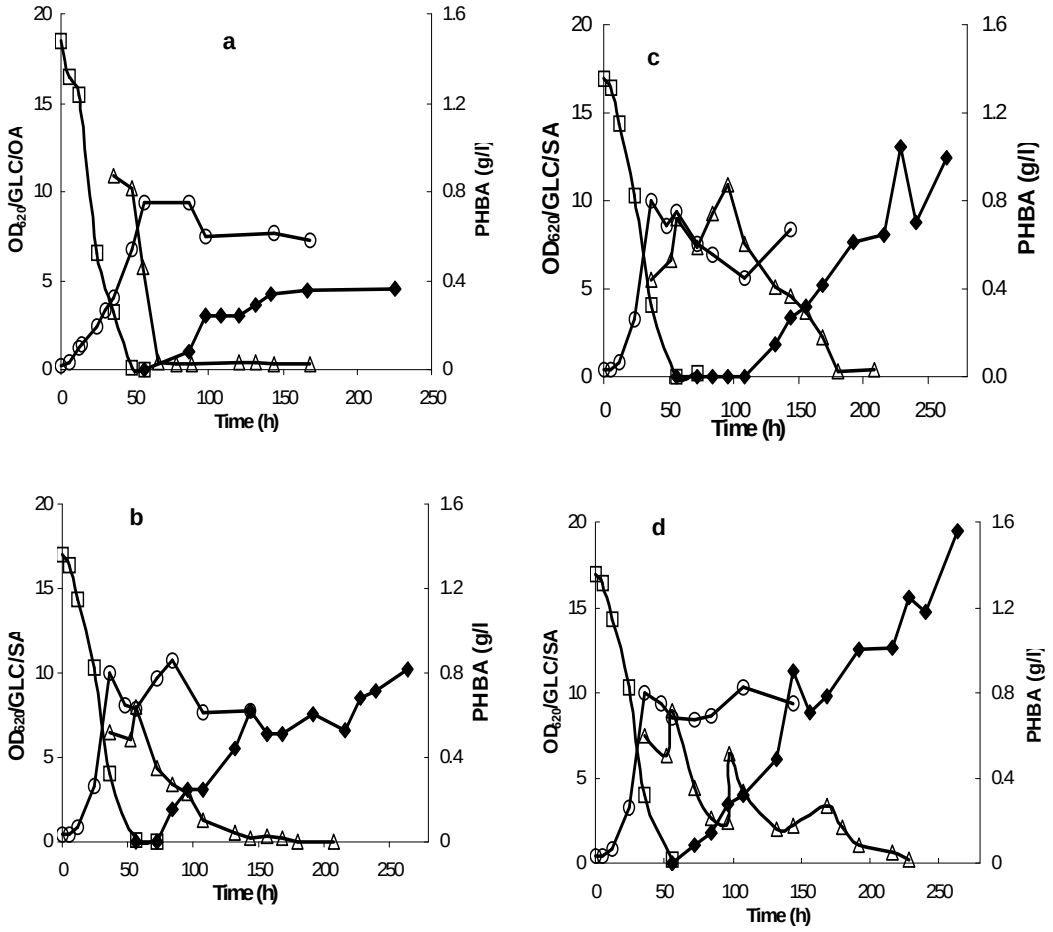


Figure 4.29: Biotransformation of BA (5 g/l added after 54 h) by ***Y. lipolytica*** TVN91 grown in YPD broth with different additions made after 36 h: **a**. 20 ml/l olive oil, **b**. 2% SA, **c**. 2% SA and 2% ethanol, **d**. 2% SA added followed by 0.5% every 56 h. Glucose (g/l) (open squares), OD 620 nm, (open circles), fatty acid (g/l) (open triangles), pHBA (g/l) (closed diamonds).

4.5 Biotransformation of phenylnonane

Two questions that still remained were (i) whether transport of BA into the cell limited the hydroxylation rate and (ii) how cloned and endogenous CYP450s affected each others activity. It has been reported that biotransformation of alkybenzenes with

odd numbered side chain by yeasts lead to the formation of benzoic acid as the major product (Mauersberger ***et al***., 1996). It has been demonstrated (Van Rooyen, 2004) that the endogenous alkane hydroxylases (CYP52s) of ***Y. lipolytica*** as well as the overexpressed ***CYP52F1*** and ***CYP557A1*** in TVN493 and TVN356, respectively, hydroxylate even-chain length alkylbenzenes. These strains transformed hexylbenzene and decylbenzene to phenylacetic acid (PAA). Based on this information, we decided that phenylnonane (PN) would be a good substrate to investigate the two questions posed above. We expected PN to be degraded by the yeast to form BA intracellularly before further hydroxylation by the overexpressed benzoate ***para***-hydroxylase.

4.5.1 Biotransformation of phenylnonane by Y. lipolytica TVN91 overexpressing CYP53B1

In order to investigate the possibility of substrate transport limitation, biotransformation was carried out using TVN91. This strain was initially grown in YPD broth for 24 h before the addition of inducers. In one experiment dodecane (2% v/v) was added as an inducer while in the second stearic acid (2% w/v) was added as an inducer. PN (1 % w/v) was added to each of the cultures 24 h after inducer addition.

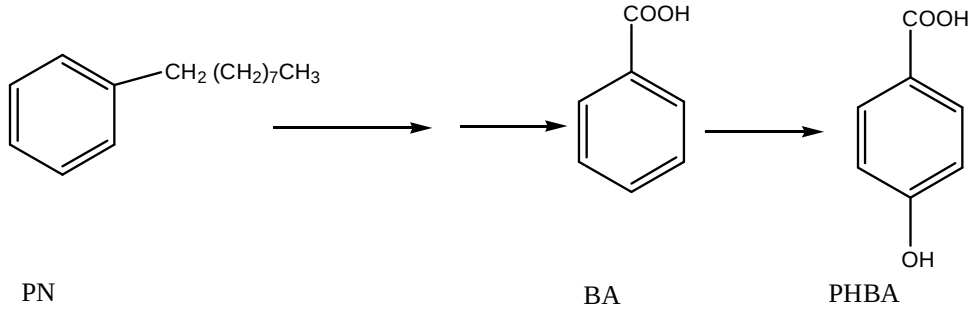


Figure 4.30: Scheme illustrating the biotransformation of phenylnonane by ***Y. lipolytica*** TVN91

Biotransformation of PN by TVN91 resulted in accumulation of BA and pHBA as the two major products (**Figure** 4.30). As expected, BA was initially formed after which it was hydroxylated to pHBA. This hydroxylation was definitely due to the hydroxylating activity of the overexpressed ***CYP53B1*** in TVN91, since no pHBA was formed in strains without ***CYP53B1*** (see next section). The dry biomass concentration

in samples taken immediately before the addition of inducers was 13 g/l. With both SA and dodecane as inducers, the rate of BA accumulation was greater than the rate at which pHBA was accumulated.

The use of dodecane as an inducer resulted in higher volumetric and specific rates of product accumulation than OA. The volumetric rate of BA accumulation in the presence of dodecane was 27 mg. $(l.h)^{-1}$ while the rate at which pHBA was accumulated was only 9.0 mg. $(l.h)^{-1}$. These translated to specific activities of 0.28 U $(g\ dwt)^{-1}$ and 0.09 U $(g\ dwt)^{-1}$ respectively. On the other hand, when SA was used as an inducer, the volumetric rate of BA formation was 12 mg $(l.h)^{-1}$ while the rate at which pHBA was formed was 3.9 mg $(l.h)^{-1}$. These translated to specific activities of 0.13 U $(g\ dwt)^{-1}$ and 0.04 U $(g\ dwt)^{-1}$ respectively. The rates of BA hydroxylation were not significantly different from the rates obtained when BA was added as substrate, indicating that transport of BA into the cell was probably not a limiting factor. This interpretation was supported by results obtained using CA as substrate (see section 4.6).

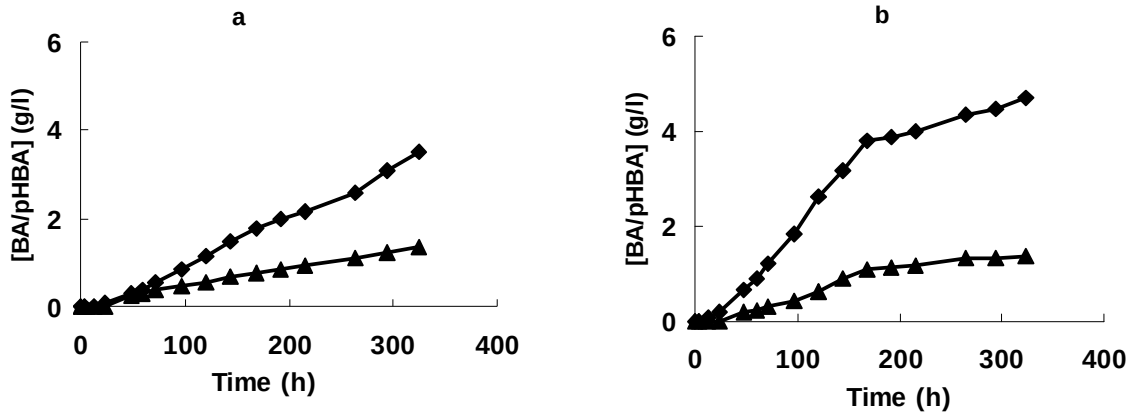


Figure 4.31: Biotransformation of PN (10 g/l; 40 mM) using TVN91 in the presence of (**a**) stearic acid as inducer (**b**) dodecane as inducer. BA (♦), pHBA (▲)

Table 4.5: Activity in terms of products formation from PN by TVN91, illustrating the effect of using different inducers

Inducer	**Biomass (24h) g/l**	**Product**	**Maximum activity**			**Maximum product (g/l)**
			Period (h)	**Volumetric activity (g (l.h)$^{-1}$**	**Specific activity U (g.dwt)$^{-1}$.**	
SA	13	BA	24-168	0.0120	0.12	3.5
SA	13	pHBA	48-298	0.0039	0.03	1.3
Dodecane	13	BA	72-168	0.0270	0.28	4.7
Dodecane	13	pHBA	96-168	0.0093	0.08	1.4

4.5.2 Comparison of hydroxylase activities in strains overexpressing different *CYP450s*

In order to investigate how the cloned and endogenous CYP450s affected each other's activity, two additional strains TVN356 (overexpressing CYP557A1) and TVN493 (overexpressing CYP52F1) were also tested for PN biotransformation in the same experiment described above.

To determine the effect of the cloned genes in the three strains, the rates of inducer and substrate utilization as well that of product formation by these strains were compared. Even though, the added concentrations of both the inducers were 20 g/l only 15 g/l of SA was recovered 1 h after addition and less than 8 g/l dodecane. The results demonstrated that, dodecane was utilized much faster than SA in all the strains (**Figure** 4.32). There was residual SA in all the cultures even after 200 h from its addition while, dodecane was completely utilized after only 120 h in cultures of TVN493 and TVN356. The residual concentration of dodecane in the culture of TVN91 at 120 h was approximately 3 g/l. The rate of dodecane utilization was much faster in the two strains, TVN356 and TVN493 respectively compared to the rate with TVN91. The same trend was observed with the utilization of SA. In this case, the rate of SA utilization appeared faster withTVN356 while TVN91 appeared to have the lowest rate of SA utilization.

The results further demonstrated significant differences in the rates of PN utilization in the three strains. Once more, the rates of substrates utilization in the presence of SA as an inducer by the two strains, TVN356 and TVN493 were higher than that of TVN91. For the two strains, TVN356 and TVN493, the rate of substrate utilization was more rapid. In both cases, the substrate was completely utilized after 150 h, while in the case of TVN91, the rate of substrate utilization was much slower and the substrate remained for a longer duration, being utilized only after 300 h. The same trend was observed when dodecane was used as an inducer, although PN disappeared more quickly from the culture of TVN91.

BA was the major product formed in all the cultures, but small amounts of PAA was also formed (max conc. 0.55 g/l after 168 h). In the TVN91 cultures pHBA was also formed as discussed above. In order to compare the total "alkane" hydroxylase activity in the three strains, concentrations of the three products were added together. The rates of total product formation correlated well with the above reported rates of inducer and substrate utilizations. When SA was used as an inducer, the rate of product formation was faster with the two strains TVN356 and TVN493. In this case, there was a constant rate of product formation until 180 h, when all the substrate was utilized. The rate of product formation with TVN91 was much lower though it was maintained for a longer time up to 300 h, when almost all the substrate was again utilized. The volumetric rate of product formation with TVN356 and TVN493 were 54 mg $(l.h)^{-1}$ and 52 mg $(l.h)^{-1}$ respectively while that with TVN91 was only 18 mg $(l.h)^{-1}$. This translated to specific activities of 0.56 U, 0.59 U and 0.18 U respectively (**Table** 4.6). In the case when dodecane was used as an inducer, the volumetric rates of product formation were 63 mg $(l.h)^{-1}$ and 66 mg $(l.h)^{-1}$ for TVN356 and TVN 493, and only 39 mg $(l.h)^{-1}$ for TVN91. This translated to specific activities of 0.70 U, 0.77 U (g $dwt)^{-1}$ and 0.33 U (g $dwt)^{-1}$ respectively (where 1 U is defined as 1 µmol of product formed per minute). These results seemed to demonstrate an increase in the hydroxylation activity from the synergistic activity of the overexpressed ***CYP557A1*** and ***CYP52F1*** genes together with the endogenous ***CYP450s***. The overexpression of ***CYP53B1*** appeared to reduce the "alkane" hydroxylation activity in ***Y. lipolytica.*** However, conclusive results would only have been possible if we used a control strain without any ***CYP450*** overexpressed but still overexpresses the ***YlCPR***. This type of strain was unfortunately not available at the time.

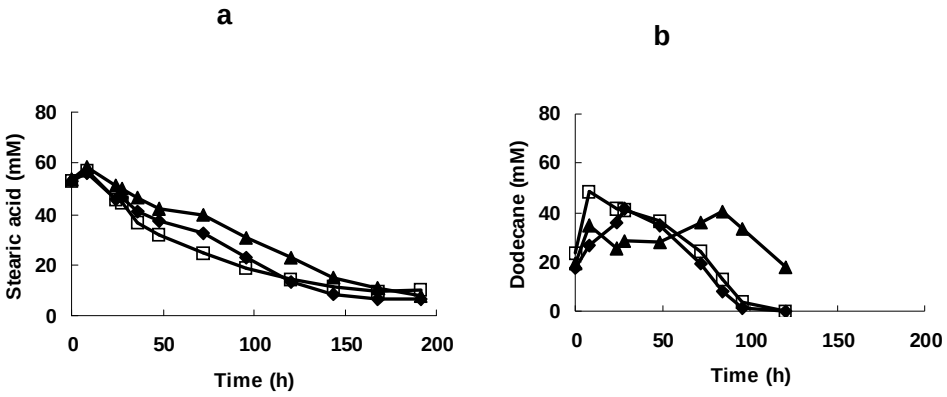


Figure 4.32: Utilization of inducers, (**a**) SA (**b**) dodecane by ***Y. lipolytica*** strains, (♦) TVN 493, (□), TVN356 and (▲) TVN91

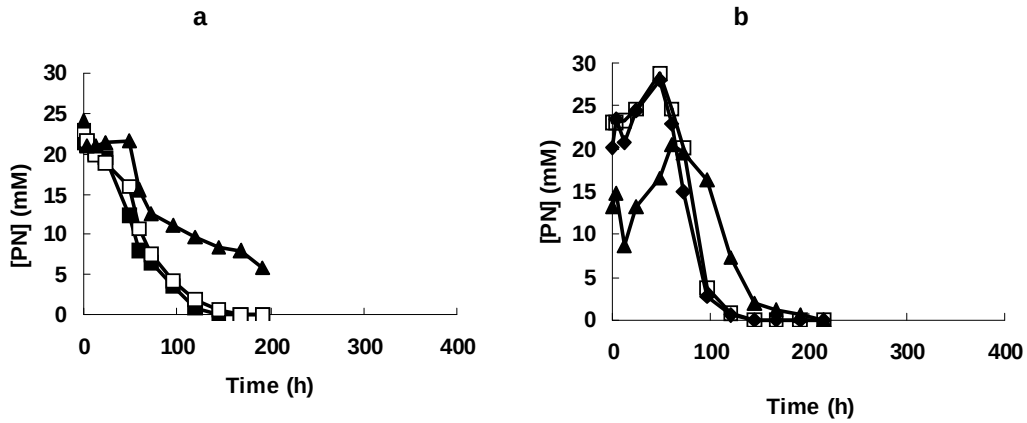


Figure 4.33: Utilization of PN when (**a**) SA (**b**) dodecane was used as an inducer by ***Y. lipolytica*** strains, (♦) TVN 493, (□), TVN356 and (▲) TVN91

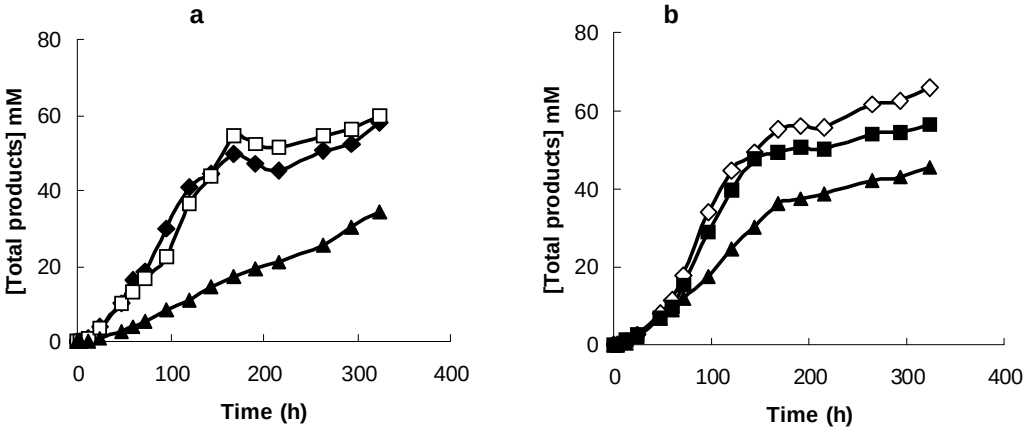


Figure 4.34: Total product formation from biotransformation of PN (1% v/v) in the presence of (**a**) SA (**b**) dodecane as inducers with ***Y. lipolytica*** strains, (♦) TVN493 (□) TVN356 and (▲) TVN91

Table 4.6: Total specific activities in terms of products formation from PN by TVN91, TVN356 and TVN493

Strain	Biomass (24 h) (g/l)	Inducer	Specific activity (g (g.dwt) h^{-1})				
			PAA	BA	pHBA	Total activity	Total specific activity (U (g dwt)$^{-1}$
TVN91	11.1	SA	0.0003	0.0010	0.0003	0.0014	0.18
	11.1	Dodecane	0.0003	0.0021	0.0007	0.0024	0.32
TVN356	12.8	SA	0.0005	0.0037	0	0.0041	0.56
	12.8	Dodecane	0.0005	0.0046	0	0.0052	0.70
TVN493	13.0	SA	0.0004	0.0040	0	0.0044	0.59
	13.0	Dodecane	0.0005	0.0052	0	0.0057	0.77

NB: where 1 U is defined as 1 µmol of product formed per minute

4.5.3 *Biotransformation of phenylnonane using wild type strains*

In order to compare the hydroxylase activity in the wild type yeast strains with the activity in the recombinant ***Y. lipolytica*** strains, a further biotransformation experiment was carried out using three wild type strains; ***Y. lipolytica*** W29, ***R. retinophila*** TVN295 and ***R. minuta*** CBS 2177***.*** These strains were initially grown in YPD broth for 36 h before the addition of phenylnonane (10 g/l) to each of the cultures.

The biomass yield for ***R. minuta*** and ***R. retinophila*** was approximately 8 g/l after 36 h of growth, while ***Y. lipolytica*** W29 had a biomass yield of 12.2 g/l. The added concentration of PN was 10 g/l and 8 g/l of PN was recovered 1 h after addition from the culture with ***Y. lipolytica*** W29. The concentration of PN recovered from cultures with ***R. minuta*** and ***R. retinophila*** were only 2.6 g/l, and 1.4 g/l respectively. The PN concentration in all three cultures remained more or less constant. The reason why lower concentrations of substrate were recovered from the ***Rhodotorula*** cultures was probably due to poor sampling rather than substrate utilization, since very little product could be detected and the PN concentration did not decrease. Higher substrate recovery from the culture of ***Y. lipolytica*** W29 could be attributed to the fact that ***Y. lipolytica*** is well adapted for the uptake of such hydrophobic substrates (Mauersberger ***et al***., 2001; Fickers ***et al***., 2005). This yeast has the ability to produce biosurfactants, which aid in the dispersal of such substrates in the media. The ***Rhodotorula*** spp.

apparently lacks this ability, thus the substrate was poorly dispersed in the media. Due to this, there was poor sampling, which resulted in the poor substrate recovery.

Biotransformation of PN by ***Y. lipolytica*** W29 resulted in accumulation of BA (1.32 g/l; 10.23 mM) and PAA (0.19 g/l; 1.5 mM) as the two main products within 200 h after substrate addition (**Figure** 4.36). The volumetric rate at which BA was accumulated with W29 was for a short time between 12 and 24 h 33 mg $(l.h)^{-1}$ while the rate at which PAA was accumulated by the same strain was only 8.4 mg $(l.h)^{-1}$. These translated to specific activities of 0.37 U $(g\ dwt)^{-1}$ and 0.084 U $(g\ dwt)^{-1}$ respectively. For this short period the alkane hydroxylase activity in ***Y. lipolytica*** W29 was thus in the same order as the activity in TVN91, but it then leveled off to only 1.3 g/l while in TVN91 the higher activity was maintained for over 150 h. A notable difference between the experiments with the recombinant strains and the wild type strains was that no inducer (neither SA nor dodecane) was added to the wild type strain. From this we thus know that PN induces the endogenous alkane hydroxylases. However, SA and dodecane apparently also served as energy sources to maintain the hydroxylase activity for longer. This hypothesis requires further investigation.

R. retinophila accumulated only PAA. The rate of PAA accumulation by ***R. retinophila*** was constant for a longer duration giving a maximum product of 1.03 g/l (7.8 mM) after 200 h. The volumetric rate of PAA accumulation by this strain was 5.3 mg $(l.h)^{-1}$, which translated to specific activity of 0.08 U $(g\ dwt)^{-1}$. ***R. minuta*** did not accumulate any detectable product. The reason why ***R. retinophila*** accumulated only PAA and ***R. minuta*** did not accumulate any detectable product could be ascribed to the fact that as soon as BA was formed, it was immediately hydroxylated to pHBA, which underwent further degradation. The catabolism of BA is a well-established pathway in ***R. minuta***.

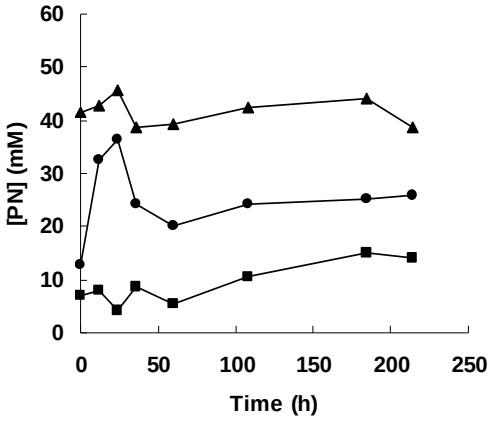


Figure 4.35: Degradation of PN (1 % w/v) by wild type strains, (▲) ***Y. lipolytica*** W29, (♦) ***R. minuta*** and (■) ***R. retinophila***. PN was added after 36 h of growth in YPD broth.

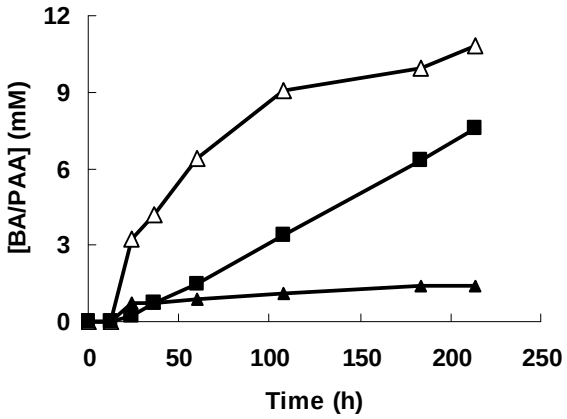


Figure 4.36: Formation of BA (open symbols) and PAA (closed symbol) from PN (1% w/v) by wild type strains, (∆) and (▲) ***Y. lipolytica*** W29 and (■) ***R. retinophila***. PN was added after 36 h of growth in YPD broth. Graph only show biotransformation period

Table 4.7: Total specific activities in terms of products formation from PN by wild type strains W29 and ***R. retinophila***

Strain	**Biomass (36h) g/l**	**Product**	**Maximum activity**			**Maximum product (g/l)**
			Period (h)	**Volumetric activity (g (l.h)$^{-1}$**	**Specific activity U (g.dwt)$^{-1}$.**	
W29	12.2	BA	12-24	0.0331	0.036	1.32
W29	12.2	PAA	12-24	0.0084	0.086	0.19
R. retinophila	8.0	PAA	60-184	0.0053	0.086	1.03

4.6 Formation of pHBA from *trans*-cinnamic acid by TVN91

Further investigation on transport limitation was carried out using ***trans***-cinnamic acid (CA) as the substrate for biotransformation reactions. TVN91 was initially grown in YPD broth for 24 h before the addition of oleic acid as the inducer. 24 h later, ***trans***-cinnamic acid (0.25 % w/v) was added to the culture.

The dry biomass after growth of TVN91 in YPD broth for 24 h was determined to be 11 g/l. Biotransformation results demonstrated that CA was initially degraded to BA after which it was hydroxylated to pHBA (**Figure** 4.37). The formation of pHBA only started 24 h after BA formation. There was a constant rate of BA formation until 90 h, when all the substrate was utilized. The results also demonstrated that the rate of pHBA formation was constant for the first 90 h before leveling off. The rate of BA accumulation from CA was much faster than the rate of pHBA accumulation (**Figure** 4.38). These results (**Table** 4.8) further demonstrated that the rates of alkane hydroxylase activity (benzoate ***para***- hydroxylase) was still in the same order as the activity when TVN91 was used for the biotransformation of both BA and PN as substrates. This confirmed that substrate uptake was not limiting.

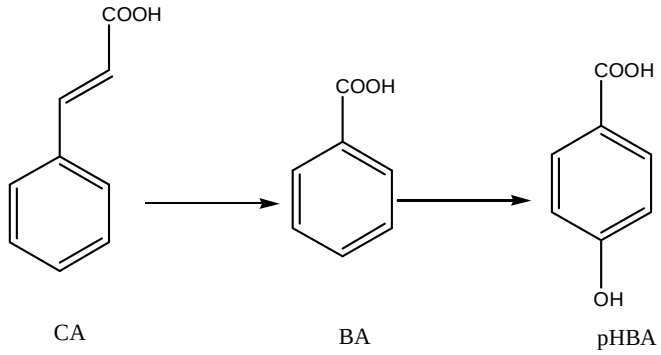


Figure 4.37: Proposed reaction scheme for the biotransformation of CA by ***Y. lipolytica*** TVN91

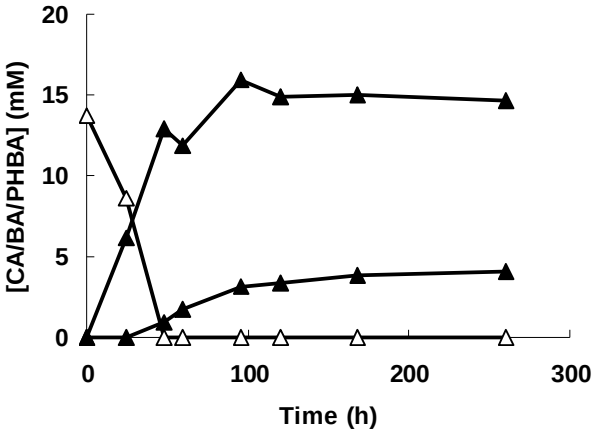


Figure 4.38: Biotransformation of CA using TVN91 in the presence of OA as inducer. (□) CA, (♦) BA, (▲) pHBA. Graph only shows the biotransformation period

Table 4.8: Total specific activities of biotransformation products from CA using TVN91

Strain	**Biomass (24h) g/l**	**Product**	**Maximum activity**			**Maximum product (g/l)**
			Period (h)	**Volumetric activity (g (l.h)$^{-1}$**	**Specific activity U (g.dwt)$^{-1}$**	
TVN91	11.1	BA	0-48	0.0329	0.409	1.79
TVN91	11.1	pHBA	48-96	0.0061	0.073	0.56

4.7 Attempts at selective induction of *POX2* promoter driving the *CYP53B1* gene in TVN91

Due to the fact that we were not able to selectively induce only cloned ***CYP450*** genes, under the control of the ***POX2*** promoter, the interpretation of the results was very difficult. We therefore tried to test for acid substrates we thought might selectively induce only the ***POX2*** promoter without any background activities. The aim was to eliminate the apparent competition for reductase or reducing equivalents between the overexpressed ***CYP450*** genes and the endogenous ***CYP450s***.

TVN91 was initially grown in YPD broth as before but in this case, different acid substrates were added as inducers to switch on the ***POX2*** promoter. The substrates tested included, phenylacetic acid (10 g/l), 4-hexylbenzoic acid (5g/l) and 4-phenylbutanoic acid (2 g/l).

In the first experiment 4-hexylbenzoic acid (5 g/l) was added as an inducer followed by substrate addition 24 h later. To first exclude the possibility of toxicity 4-hexylbenzoic acid was added together with SA to one flask while in the control flask, only SA was included as an inducer. pHBA did not accumulate at all even 72 h after substrate addition in the culture where 4-hexybenzoic was added together with SA. When only SA was added as an inducer, pHBA accumulation was observed (**Figure** 4.39a). It thus appeared that 4-hexylbenzoic acid was toxic at 5 g/l. This was also demonstrated by a reduction in growth (results not shown). The formation of a lot of fatty layer, which did not pellet even after high-speed centrifugation (10,000 rpm) was observed in cultures grown in the presence of 4-hexylbenzoic acid. If this fatty material had affected biomass measurements it would have resulted in an apparent higher biomass.

In a second experiment, phenylbutanoic acid was tested as inducer. Because we did not know whether phenylbutanoic acid would also induce p***ICL*** driving expression of the ***YlCPR,*** ethanol was also added to two of the cultures (**Figure** 4.39b). The cultures to which phenylbutanoic acid, phenylbutanoic acid and ethanol and SA and ethanol had been added all three produced very little pHBA after 24 h compared to the culture to which only SA had been added. We know from previous experiments that addition of ethanol together with SA resulted in a delay in the onset of the hydroxylation activity during the initial 24 h after substrate addition. Addition of phenylacetic acid (10 g/l) alone as an inducer also resulted in the accumulation of only small amounts of pHBA (results not shown). The results therefore demonstrated that phenylacetic acid, 4-hexylbenzoic acid and 4-phenylbutanoic acid apparently were poor inducers of the ***POX2*** promoter, although the need to add ethanol for induction of the cloned ***YlCPR*** complicated the experiments and their interpretation. For now it is thus not possible to detect the proposed advantage that selective induction of only the cloned ***CYP53B1*** should offer.

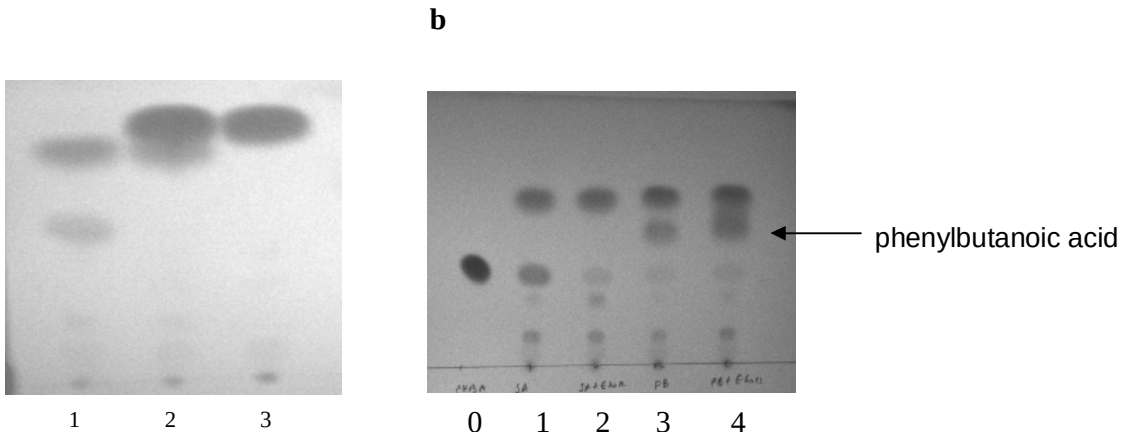


Figure 4.39: Biotransformation of BA in the presence of various inducers. (**a**) Biotransformation of BA in the presence of 4-hexylbenzoic acid as inducer, 48 h after BA addition: 1. SA (Control), 2. 4-HBA, 3. 4-HBA standard. (**b**) Biotransformation of BA after the addition of phenybutanoic acid as inducer. Extracts of samples taken 24 h after BA addition. 0. pHBA standard, Inducers were: 1. SA, 2. SA+ethanol, 3. phenylbutanoic acid, 4. phenylbutanoic acid+ethanol

4.8 Biotransformation of BA in a bioreactor condition

In order to investigate whether the benzoate-***para*** hydroxylase activity of ***Y. lipolytica*** TVN91 could be increased under more controlled conditions with better aeration and mixing, a bioreactor experiment was carried out. The culture was grown in YPD broth so that these results could be compared with shake flask experiments. The biomass yields of TVN91 when grown in a bioreactor were 20 g/l, 12.2 g/l and 14 g/l after 16 h, 134 h and 278 h of growth respectively. The biomass after 16 h growth was higher than the biomass achieved with any shake flask culture, but then decreased to approximately the same values. From the results (**Figure** 4.40), it was evident that even under bioreactor conditions; the maximum pHBA achieved was about 1.5 g/l, 180 h after BA addition. The volumetric rate of pHBA accumulation in the bioreactor experiment was 13 mg $(l.h)^{-1}$ while the same rate in shake flask experiments was approximately, 6-7 mg $(l.h)^{-1}$. This rate in the bioreactor experiment translated to a specific activity of 0.089 U $(g\ dwt)^{-1}$ (0.0007 g. $(g.dwt)\ h^{-1}$) if the biomass was taken as 20 g/l. This specific activity was within the same range as the maximum activities achieved in shake flask experiments with OA as inducer. However, the maximum activities were obtained for only very short periods. With SA added repeatedly as inducer in shake flask experiments the volumetric rates and specific activities were 7

mg $(l.h)^{-1}$ and 0.04 U $(g\ dwt)^{-1}$. Thus the activities obtained in the bioreactor were about double that obtained in the corresponding shake flask experiment, but the final product concentration was still the same. The onset of product formation was also much earlier in the bioreactor experiments than in shake flask experiments. This was probably due to better mixing, which caused a rapid inducer (SA) uptake from the culture broth.

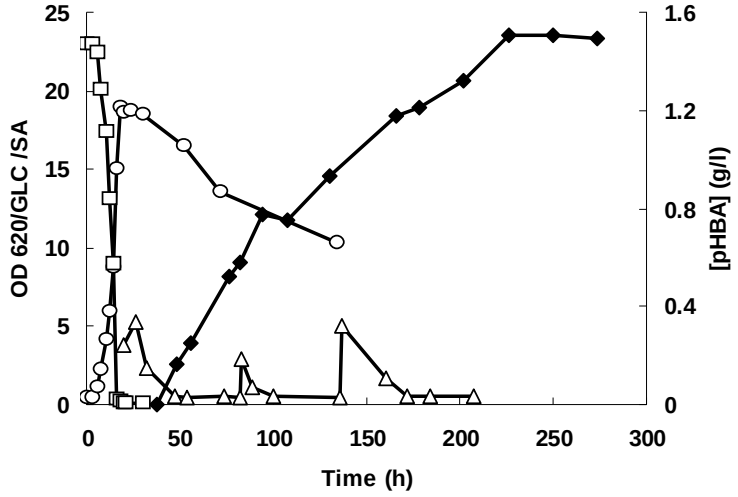


Figure 4.40: Biotransformation of BA (5 g/l) in a bioreactor by TVN91 grown in YPD broth with SA added after 20 h, 80 h and 134 h of growth. BA was added after 38 h of growth.
(○) $OD_{620\ nm}$, (□) Glucose (g/l), (♦), pHBA (g/l), (Δ), SA (g/l).

Even though bioreactors have various advantages such as better aeration, mixing, and ease of sampling, there were some drawbacks experienced when ***Y. lipolytica*** TVN91 was used for the biotransformation of BA. The addition of stearic acid, after 20 h growth resulted in excessive foaming (**Figure** 4.41) even though antifoam was included in the growth media. Thus 0.02% v/v of antifoam was added together with the subsequent inducer additions to minimize the excessive foaming.

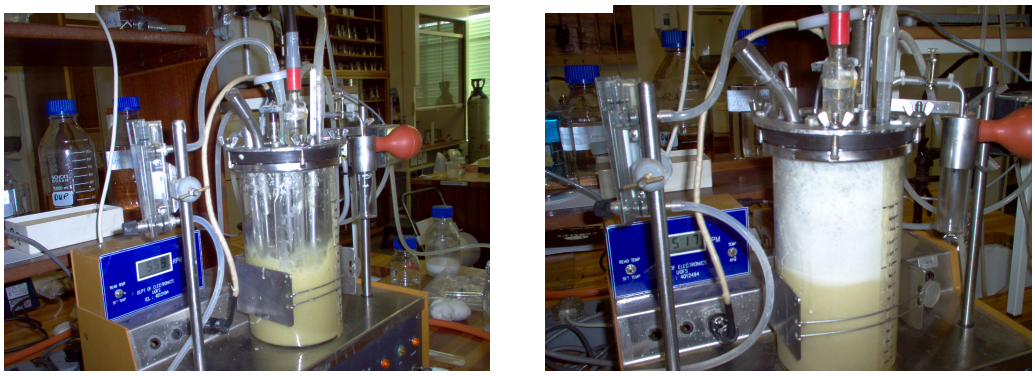


Figure 4.41: A biotransformation experiment with ***Y. lipolytica*** TVN91 in a bioreactor. The bioreactor (**a**) before addition of SA and (**b**) foaming immediately after the addition of SA

4.9 Growth of recombinant *Y. lipolytica* TVN91 on chemically defined media

In an effort to get more reproducible growth chemically defined media were tested. Chemically defined media are generally also preferred for bioreactor studies.

In a first experiment, the recombinant yeast, TVN91 was grown in a chemically defined medium (according to Du Preez ***et al***., 1983) with and without the addition of yeast extract (3 g/l). In the control experiment, TVN91 was grown in YPD broth. In the chemically defined, there was poor growth. This was demonstrated by a low maximum specific growth rate, μ_{max} value of only 0.1 h^{-1} and a low OD measurement of 0.6 (**Figure** 4.42) after 36 h. The addition of yeast extract (YE) to the chemically defined medium still led to the same maximum specific growth rate of 0.1 h^{-1} but an improved final growth giving an OD of 3.2 after 36 h. However, growth was still not nearly as good as in YPD broth. When the same strain was grown in YPD broth, the maximum specific growth rate, μ_{max} was 0.5 h^{-1} and the final OD of 8 after 36 h of growth. It should be kept in mind that the peptone in the YPD medium supplied additional carbon. Growth onnYPD was similar as in previous experiments.

In a second experiment TVN91 was grown in YNB broth (with glucose (10 g/l)) and casamino acids (2 g/l and 4 g/l). The biomass produced in the YNB cultures were again much less than in the control YPD culture. In the culture with 2 g/l casamino acids (c/a) the biomass was measured to be 2.8 g/l after 48 h, while in the culture with 4 g/l casamino acids it was 3.5 g/l. In the YPD culture it was 15 g/l after 48 h. It should

however be kept in mind that the available carbon in the YNB cultures had been much less than in the YPD culture. Since TVN91 is auxotrophic for histidine (Barth and Gaillardin, 1996), the addition of c/a, which contains histidine, resulted in an increase in biomass due to external histidine supplementation. When biotransformation of BA was carried out with the culture to which 2 g/l c/a had been added, there was no pHBA accumulation. However, the addition of 4 g/l c/a resulted in a significant improvement of pHBA production. The volumetric activity for pHBA accumulation was 1.9 mg $(l.h)^{-1}$, which translated to a specific activity of 0.07 U $(g\ dwt)^{-1}$. The rates were calculated based on the gradients of progress curves between two time points. This result demonstrated that it should in future be possible to develop a chemically defined medium for the expression of CYP450s. The exact effect of the addition of casamino acids on biotransformation or CYP450 expression needs to be further investigated and the effect of specific amino acids should also be investigated.

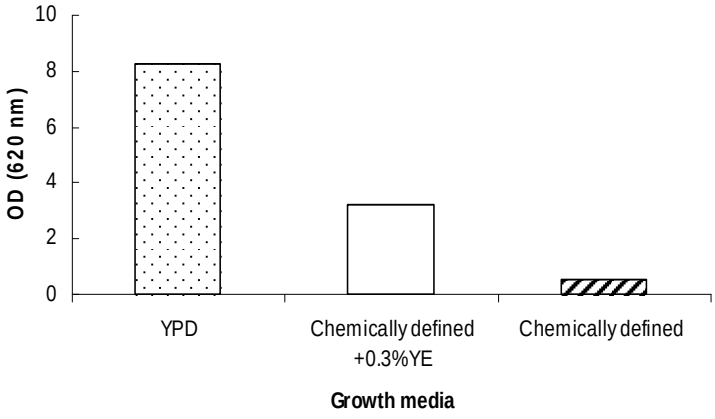


Figure 4.42: Comparative growth of ***Y. lipolytica*** TVN91 as determined by OD values on different media as indicated after 36 h of incubation at 25 ^{o}C

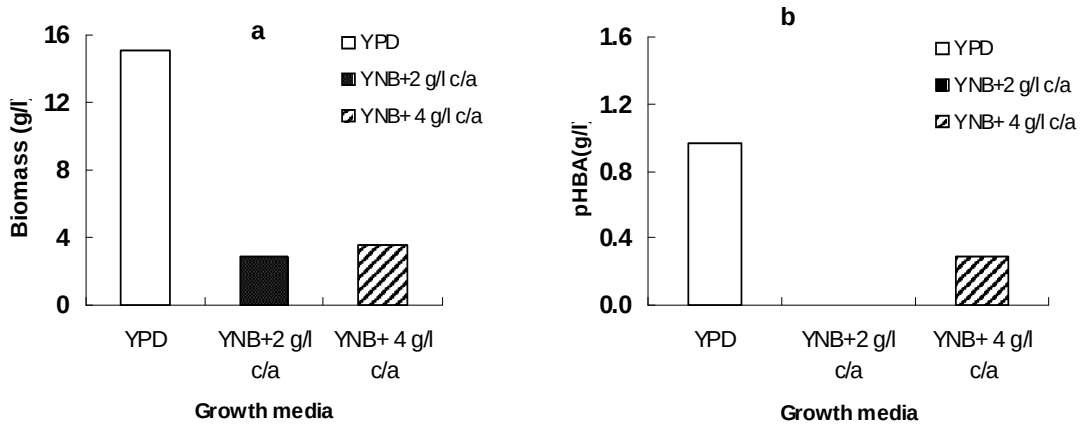


Figure 4.43: Comparative (**a**) growth of ***Y. lipolytica*** TVN91 in different growth media after 48 h and, (**b**) the corresponding biotransformation 168 h after substrate (BA) addition.

4.10 Use of harvested cells to perform CYP450 catalyzed reactions

In order to study in future the reaction kinetics of the CYP450s overexpressed ***in Y. lipolytica***, experiments were carried out to investigate the possibility of using harvested cells for biotransformation reactions. TVN91 was therefore grown in YPD broth for 24 h after which oleic acid (2%) was added as inducer. The harvesting of cells after 48 h induction was carried out as described in the materials and methods, section 3.216. The harvested cells were used to perform biotransformation reactions.

From an initial early attempt, it was apparent that harsh treatments such as high-speed centrifugation (10,000 rpm) for 10 minutes and vigorous vortexing (highest speed) during the cell harvesting procedure resulted in a loss of benzoate-***para*** hydroxylase activity (results not shown). However, in a second later attempt where the cells were harvested more carefully (centrifugation at 4,500 rpm for 5 minutes and gentle vortexing), the cells maintained their benzoate ***para***-hydroxylase activity (**Figures** 4.44).

One of the main advantages observed when biotransformation experiments were done using harvested cells as opposed to growing cells was that the hydroxylase activity was maintained and constant for a longer duration. This was not observed when biotransformation was carried out with growing cells using the same inducer (oleic acid) (**Figure** 4.44). It also made the calculation of reaction rates (enzyme activities) from gradients of progress curves easier and more reliable.

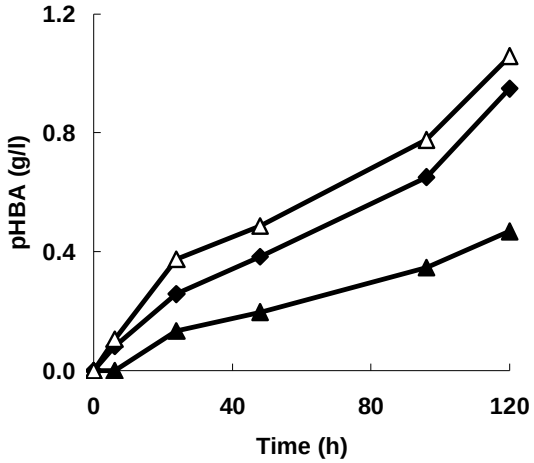


Figure 4.44: Biotransformation of BA (5g/l) to form pHBA using harvested cells of TVN91 grown in YPD and induced with oleic acid (20 g/l). Different cell concentrations used for biotransformation of BA (0.5%) were: (▲) 14. 87 g/l. (Δ) 29.74 g/l. (♦) 44.61 g/l

In this experiment we also evaluated the effect that cell concentration had on ***CYP450*** activity. The results demonstrated that biomass concentrations of more than 30 g/l resulted in lower specific hydroxylase activities, while at biomass concentrations of less than 30 g/l the specific activity was not influenced by the biomass concentration (**Figure** 4.45). In the previous experiments where cultures were used without harvesting the biomass concentrations never exceeded 20 g/l. Thus specific activities were never influenced by a too high biomass concentration. One of the reasons for the low specific activities at high biomass concentrations could be attributed to oxygen limitation as a result of a higher oxygen demand. However, higher biomass concentrations still resulted in higher volumetric activities. Thus although an increase in biomass concentration from 29 g/l to 59 g/l resulted in an 18 % reduction in specific activity it resulted in a 60 % improvement in volumetric activity.

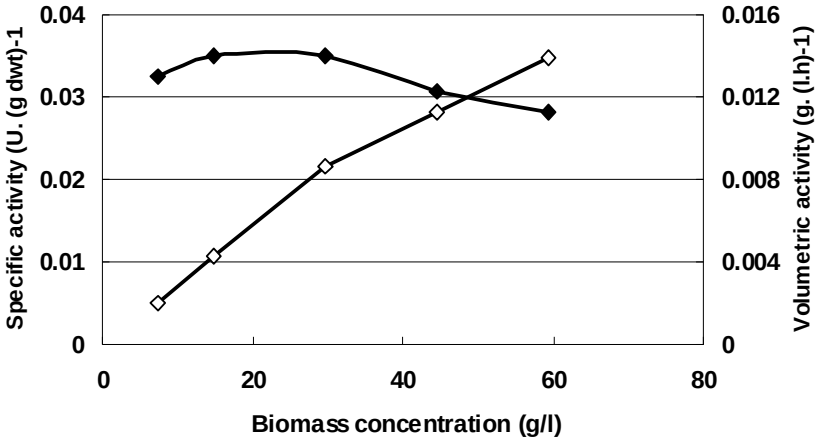


Figure 4.45: Graph illustrating the correlation of biomass cell concentration and, (♦) benzoate ***para***-hydroxylase activity, (◊) benzoate ***para***-hydroxylase volumetric activity. TVN91 cells harvested after 48 h induction with oleic acid were used for the biotransformation of BA (0.5 % w/v)

In order to further explore the effect of oxygen limitation on the CYP450 biotransformation activity, different sizes of Erlenmeyer flasks were used with a constant culture volume. In a shake flask, ratio of the flask volume to culture volume represents the combined effects of aeration and mixing. This experiment was done using two strains, TVN91 and TVN356. The substrates used were BA and hexylbenzene (HB) respectively. It should be noted that the harvested cells in this experiment had almost the same activity with those obtained in previous experiments. The hydroxylase activities observed with TVN356 in the biotransformation of HB was much higher than the hydroxylase activities observed with TVN91 on BA, because endogenous CYP450s also take part in the hydroxylation of HB. With both strains the results demonstrated that, when the flask volume/culture volume ratio was high, higher specific activities were observed. In all previous experiments, the culture volume to flask ratio had been 10:1. Thus same culture volumes in larger flasks (double volume) could have improved specific activity by ca. 30%. A higher ratio implied higher surface area, better aeration as well as better mixing. However, the fact that similar shaped graphs were obtained with both strains that had completely different levels of activity towards the two different substrates probably indicate that oxygen limitation at lower flask volume/culture volume ratios more likely resulted from cellular respiration (van Beilen ***et al***., 2003) rather than from the oxygen demand of the CYP450 reactions.

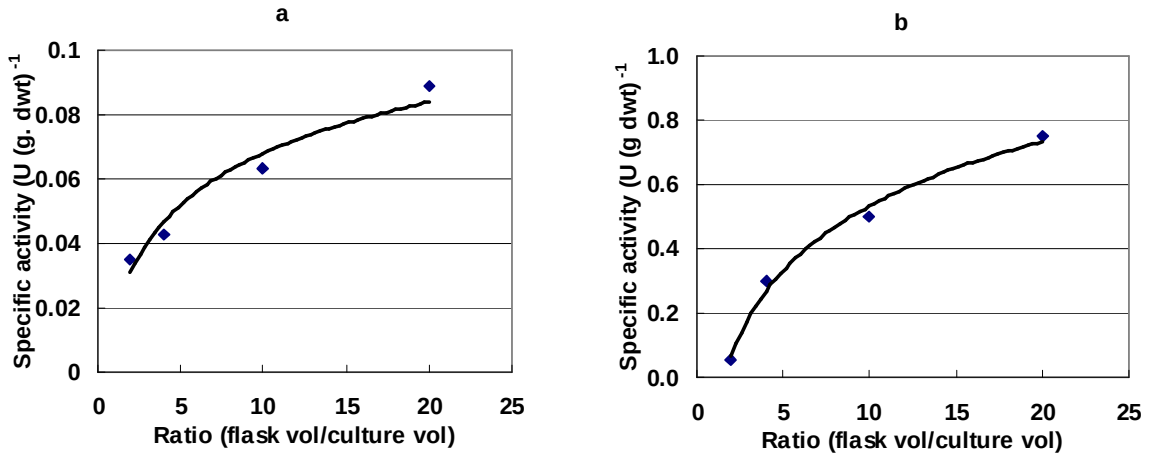


Figure 4.46: Correlation of flask volume/culture volume ratio and specific activity. Harvested cells (biomass 14.87 g/l) of: (**a**) TVN91 was used for biotransformation of BA, (**b**) TVN356 were used for the biotransformation of HB (1 % v/v).

An experiment was also done to determine the effect of different induction times and storage of cells on the benzoate ***para***-hydroxylase activity of TVN91. After addition of OA as inducer, the cultures were left to grow in the presence of OA for between 24-96 h. Cells were harvested after different induction times. The specific activity immediately after harvesting when cells were harvested after 24 h induction was significantly higher than the activity of the cells harvested after 48 and 96 h, but these cells apparently lost activity when they were stored at 4^0C and -20^0C for 24 h. Storage of cells for 24 h at both 4^0C and -20^0C did not result in significant loss of specific activity when the cells were harvested after 48 or 96 h induction (**Figure** 4.47). It should be noted that these activities are based on single activity determinations and the experiment should be repeated to confirm the results. However, it is quite likely that activity after 24 h induction might be higher than after longer times. A limitation in reducing equivalents as well as energy supply in cells harvested later could result in lower specific hydroxylase activities. It is expected that, after a long duration of cell growth, most of the available energy goes into maintenance of critical cellular functions especially when the inducer and the carbon source (glucose) is depleted. These factors have adverse effects on the microbial cell physiology and might also affect the stability of the enzyme in harvested cells.

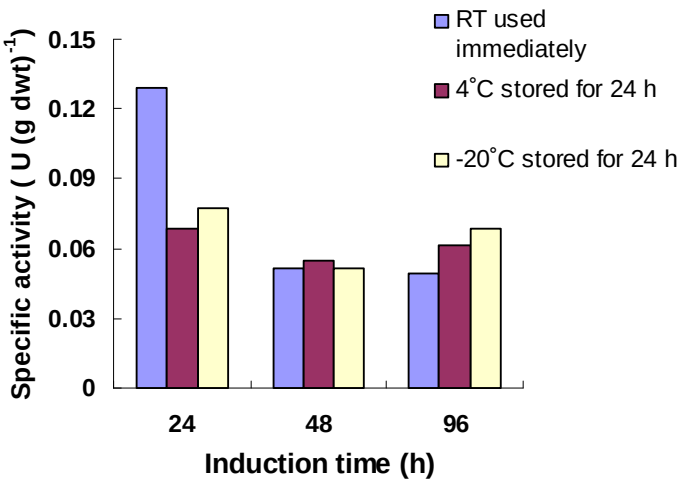


Figure 4.47: Correlation between the specific activity and induction time when TVN91 cells stored at 4˚C and -20 ˚C for 24 h, 48 h and 96 h were used for biotransformation of BA

Even though, the results of biotransformations using harvested cells looked promising, further and more confirmatory experiments still need to be done in this regard to ascertain the repeatability of these results. The preliminary results already demonstrated the possibility of using harvested cells for successful CYP450 catalyzed biotransformation reactions. This therefore opens up new frontiers for possible kinetic studies on these CYP450s.

CHAPTER FIVE

5.0 GENERAL DISCUSSIONS

In order to accumulate various useful intermediate hydrocarbon products by yeasts, two main genetic modification approaches can be followed. These include (i) disruption of critical metabolic pathways by deletion of genes coding for enzymes involved in these pathways and (ii) overexpression of various enzymes involved in the rate limiting steps.

A recent evaluation of several yeasts has revealed that ***Y. lipolytica*** is a highly attractive alternative host for secretion and expression cloning (Müller ***et al***., 1998; Juretzek ***et al***., 2001). The main reasons why ***Y. lipolytica*** might also work very well for ***CYP450*** expressions include the ability of this yeast to utilize various hydrophobic substrates, which are in most cases substrates for ***CYP450*** biotransformation reactions. The recent development of genetic tools for ***Y. lipolytica*** such as several strong and regulated promoters has also enabled it to be used as a host for the overexpression of foreign CYP450 proteins (Juretzet ***et al***., 2001). Large-scale utilization of ***Y. lipolytica*** for production of citric acid, or single cell proteins has also over the years permitted the accumulation of extensive data on its behavior in large-scale bioreactors (Madzak ***et al***., 2004). This yeast is also considered as non-pathogenic, and has been classified as GRAS (generally regarded as safe) by the FDA for citric acid production (American Food and Drug Administration) (Madzak ***et al***., 2004). However, a possible limitation of this yeast with regard to the heterologous expression of CYP450s is the presence of endogenous ***CYP450s***, which can lead to background CYP450 activities. This therefore led us to the major question: Can the beneficial properties of ***Y. lipolytica*** for hydroxylation of hydrophobic substrates by cloned CYP450s be used without interference from the endogenous CYP450s?

A literature search on successful overexpression of ***CYP450s*** in ***Y. lipolytica*** yielded only three cases which included the overexpression of ***CYP17A***, ***CYP74*** and ***CYP1A1*** respectively. A further four ***CYP450s*** had been cloned into ***Y. lipolytica*** in our laboratory. In this project, the use of ***Y. lipolytica*** as a heterelogous host was evaluated through biotransformation of various substrates using strains overexpressing (i) ***CYP1A1*** coding for polyaromatic hydrocarbon hydroxylase, (ii) ***CYP52F1*** coding for alkane hydroxylase, (iii) ***CYP557A1*** coding for putative fatty acid hydroxylase, and (iv) ***CYP53B1*** coding for benzoate ***para-*** hydroxylase. Factors limiting the use of ***Y. lipolytica*** as a host were also investigated.

In order to determine the activities of the overexpressed ***CYP450s*** in the ***Y. lipolytica*** strains and to distinguish between the cloned and endogenous ***CYP450s***, we identified and tested a number of substrates for biotransformation reactions. The substrates used were, acetanilide, 4-nitrophenyl octyl ether, 4-nonyloxybenzoic acid, benzoic acid, phenylnonane and ***trans***-cinnamic acid. Important considerations when selecting substrates for such studies included easy detection on TLC as well as ease of GC and GC-MS analyses. Biotransformations of all these substrates, except AA, were expected to lead to the accumulation of various acidic products. At slightly alkaline pH organic acids form anions that are more soluble in an aqueous medium. This was expected to facilitate sampling. All these substrates also contain aromatic rings, which make them easily detectable when viewing TLC plates with UV indicator under UV light. Methyl esters of these low molecular weight acids were also easy to quantitate with GC analyses. Cost will mainly become a consideration for large-scale experiments. The relative prices of the substrates used are given in Table 5.1. While benzoic acid, acetanilide and ***trans***-cinnamic acid were relatively cheap substrates, 4-nitrophenyl octyl ether was expensive and was found to be less sensitive to TLC, GC and GC-MS analyses. This substrate has been used in other studies because it yielded ***p***-nitrophenol, which can be assayed colorimetrically by measuring absorbance at 420 nm. However, in our

experiments, intermediates accumulated that were not completely transformed into ***p***-nitrophenol while ***p***-nitrophenol was also formed directly, probably by endogenous CYP450s. Because all intermediates were not converted to ***p***-nitrophenol and ***p***-nitrophenol was formed ***via*** more than one route it excluded its use for colorimetric assays. 4-Nonyloxybenzoic acid was slightly cheaper and more sensitive to TLC and GC analyses.

Table 5.1. Prices of the substrates used for biotransformation reactions

Substrate	**Price per g/ml (US $)**	**Supplier**
Acetanilide	1.9	Sigma- Aldrich
4-Nonyloxybenzoic acid	7.3	Acros
4-Nitrophenyl octyl ether	15.5	Acros
Benzoic acid	3.0	Merck
Hexylbenzene	1.9	Sigma-Aldrich
Phenylnonane	6.9	Sigma- Aldrich
trans-Cinnamic acid	5.4	Sigma- Aldrich

The idea to use ***Y. lipolytica*** JMY1057 overexpressing ***CYP1A1*** for the biotransformation of AA had to be discarded because this strain as well as a wild type strain used as control hydrolyzed AA to aniline within only 24 h. These results indicated, that the production of hydrolytic enzymes, for which ***Y. lipolytica*** is well known (Pigdène ***et al***., 2000; Fickers ***et al***., 2004), might in some cases limit its use as a whole cell biocatalyst if the substrate contains ester or amide bonds. Thus, the use of the two substrates, AA and 4-nitrophenyl octyl ether was abandoned early due to problems caused by the pronounced interfering wild type activity.

The substrates which were used for detailed studies were 4-nonyloxybenzoic acid, PN and BA. It was demonstrated that 4-nonyloxybenzoic acid and PN were substrates for the endogenous CYP450s while BA and pHBA were not transformed by the wild type ***Y. lipolytica*** strains. Benzoate ***para***-hydroxylase activity was easily confirmed in ***Y. lipolytica*** TVN91 overexpressing ***CYP53B1*** from ***R. minuta***. A large number of biotransformation experiments were

done using the available strains. However interpretation and comparison of results were unfortunately often limited by a number of factors, which became apparent as work progressed. These factors included:

(i) Variations in growth between strains and between the different experiments were often observed and OD and biomass measurements did not always correlate (**Table** 5.2). In some cases the biomass also dropped significantly in aging cultures. This made the calculation of specific activities unreliable, because one did not know which biomass values to use and biomass could not be determined at all times. Earlier work by us (Mokgoro, 2003) and others had indicated that OD measurements could also not be used as a reliable measure of growth, especially not after the addition of hydrophobic substrates. Venter ***et al***. (2004) had also found that ***Y. lipolytica*** in some instances accumulate lipids which contribute to the biomass. It was also very difficult to get rid of SA when washing cells for biomass determinations. In some cases where biomass was unusually high the specific activities might therefore have been underestimated. Thus, in future specific activities should perhaps rather be expressed in terms of protein concentration.

Table 5.2: A summary of OD 620 nm and biomass measurements from different comparable experiments.

Experiment	Strain	Time (h)	OD $_{620\ nm}$	Biomass (g/l)
1	TVN91	48	10.3	15.9
2	TVN91	48	12.1	15.1
3	TVN91	24	11.5	13.0
		240	10.1	10.0
3	TVN493	24	9.9	11.1
		240	13.8	13.9
3	TVN356	24	8.8	12.9
		240	12.4	10.9

(ii) Lack of suitable control strains that had been transformed with empty vectors to alleviate leucine (CPR negative strains) or uracil (CYP negative strains) auxotrophy limited interpretation of results. In some cases control strains were available, but they still did not grow well enough, probably because enough copies of the empty vector had not been introduced (Juretzek ***et al***., 2001). The strains derived from ***Y. lipolytica*** E150, such as TVN91, TVN97, TVN356, TVN493 remained auxotrophic for histidine. This might have contributed to the variations in growth.

(iii) The insolubility and hydrophobic nature of inducers and substrates made it impossible to accurately follow the consumption of these compounds.

(iv) There were no standards available for some products such as 4-(8-carboxyoctyloxy) benzoic acid and 4-(2-carboxyethoxy) benzoic acid. This could have resulted in inaccurate determination of concentrations.

Despite the limitations mentioned above, biotransformation experiments with 4-nonyloxybenzoic acid, BA and PN yielded some useful and interesting results.

It was demonstrated that 4-nonyloxybenzoic acid was hydroxylated and oxidised by endogenous ***CYP450s*** in ***Y. lipolytica*** as well as ***R. retinophila***. From the results it could not be clearly determined if CYP52F1 or CYP557A1 accepted 4-nonyloxybenzoic acid as a substrate, since activity in the wild-type ***Y. lipolytica*** was higher than in the recombinant strains overexpressing the genes coding for this alkane and fatty acid hydroxylase. *β*-Oxidation of the diacid product from 4-nonyloxybenzoic acid hydroxylation was relatively slow so that the intact diacid, 4-(8-carboxyoctyloxy)benzoic acid could be detected even in cultures with fully functional *β*-oxidation. It was further demonstrated that in most strains with partially disrupted *β*-oxidation (***POX2, POX3, POX4 and POX5*** genes were deleted while ***POX1*** and ***POX6*** genes remained), this potentially new diacid

product was accumulated as the major product. In strains with functional β-oxidation the intermediate diacid product, 4-(2-carboxyethoxy)benzoic acid was also accumulated as well as pHBA. In one β-oxidation disrupted strain, TVN442, this intermediate product was accumulated as the major product. Based on these results, it appeared as if different fatty acyl-CoA oxidases are responsible for β-oxidation of different chain length diacids and that the levels of residual glucose regulated the induction of these different fatty Acyl-CoA oxidases. The two diacid products, 4-(8-carboxyoctyloxy)benzoic acid and 4-(2-carboxyethoxy)benzoic acid have not been previously reported as microbial biotransformation products and might have interesting properties for industrial applications such as polymer production.

Biotransformation of BA and PN did not yield any new products with industrial potential but offered the possibility to study the activity of the cloned ***CYP450s*** and the factors influencing the activity of the heterologously expressed CYP450. Hydroxylase activities obtained with BA, PN, CA and 4-nonyloxybenzoic acid as well as previous results by other researchers are summarized in Table 5.2.

It is evident from table 5.2 that benzoate ***para***-hydroxylase activity in the ***Y. lipolytica*** strain overexpressing ***CYP53B1*** was much lower than the activity in the wild type ***R. minuta*** strain from which the gene was cloned. This activity was also much lower than the alkane hydroxylase activity of the different ***Y. lipolytica*** strains towards PN and 4-nonyloxybenzoic acid and was also lower than the hydroxylase activity obtained with ***S. cerevisiae*** overexpressing a plant ferulate-5-hydroxylase. A number of reasons for this lower activity were considered. The possibilities that were excluded by our experiments were:

(i) Substrate concentration and substrate transport limiting activity. It was demonstrated that BA concentrations of 0.1 % w/v and lower might be limiting, but that at the concentration of 0.5 % w/v used in most experiments it was not

limiting. There was also always still residual substrate even at the end of the experiment when 0.5 % w/v BA was added (200 h after BA addition). When biotransformation was carried out using both PN and CA, BA was accumulated. The fact that the benzoate ***para***-hydroxylase activities were not higher when PN and CA were used as substrates, demonstrated that substrate transport was not limiting, since with these substrates BA was formed inside the cell.

(ii) Product inhibition. When pHBA was added to the culture 5 h prior to substrate addition, the activity still remained the same as in the case when only BA was added. This demonstrated that product inhibition was not limiting.

(iii) Cell concentration. In experiments with whole cultures the biomass concentrations ranged between 13 g/l – 20 g/l. An experiment with harvested cells demonstrated that even when the cell concentration was increased to ca. 30 g/l it did not adversely affect the specific activity. Cell concentrations of 50 g/l and higher reduced the specific activity by ca. 20 %, but it still improved the volumetric activity by ca. 60%

The remaining explanation for the relatively low benzoate ***para***-hydroxylase activity observed with the recombinant ***Y. lipolytica*** strain, that could not be disproved, is induction or activity of the endogenous ***CYP450s.*** It is expected that if the endogenous ***CYP450s*** are also induced, this might lead to competition with the cloned CYP450 for association with ***CPR.*** Furthermore, if the endogenous CYP450s are actively hydroxylating the inducers such as OA or SA, it will additionally be competing with the cloned CYP450 for NADPH. One other factor which could have contributed to the low activity in the recombinant ***Y. lipolytica*** strain could have been inefficient coupling of the ***CYP53B1*** from ***R. minuta*** with the ***CPR*** from ***Y. lipolytica.*** To investigate this phenomenon, different ***CPRs*** would have to be cloned in recombinant strains overexpressing ***CYP53B1***, and the activities determined in each case.

Options for determining (excluding) the effect of endogenous CYP450***s*** would be:

(i) The selective induction of the p***POX2*** and p***ICL*** in the available strains, without inducing the endogenous CYP450s. Attempts to accomplish this have so far been unsuccessful. The complicating factor in this case is that in most strains there are different promoters driving the ***CPR*** and ***CYP450.*** In this regard, it is still not clear if enough ***CPR*** will be formed when the endogenous ***CYP450s*** are not induced. This is because only a single additional copy (perhaps two copies) of the ***YlCPR*** were cloned.

(ii) Construction of new strains with ***CYP450*** and ***CPR*** genes under control of a promoter, which is induced by substrates which do not induce the endogenous ***CYP450s.*** Again the question in this case will be whether enough CPR will be formed***. pICL*** might be a useful promoter for such studies since it is induced by both ethanol and fatty acids. Ethanol does not induce the endogenous CYP450s while fatty acids do (Juretzek ***et al***., 2000; Juretzek ***et al***., 2001).

The possibility of harvesting cells can at least get rid of any residual inducer so that the possibility of inducer being a substrate for endogenous ***CYP450s*** (as mentioned above) is excluded. An interesting observation which is also an additional advantage of using harvested cells was that the activity induced with OA could be maintained at a constant level for much longer in harvested cells. When biotransformation was done using growing cells, there was a need for continuous addition of the inducer (SA) in order to maintain the hydroxylase activity over a longer duration. This observation is still difficult to explain. A bioreactor experiment as well as an experiment with harvested cells indicated that the specific activity could be improved by at least 18 % by improved mixing and aeration.

The use of a bioreactor as well as the possibilities of using chemically defined medium for biomass production and using harvested cells for biotransformations open possibilities for better controlled experiments. Since it was demonstrated that it is possible to obtain ***CYP450*** activity in cells grown in a chemically defined media (YNB) supplemented with yeast extract and casamino acids, further investigations need to be done to determine the exact influence of various amino acids on the hydroxylase activity. Bioreactor studies and further optimization studies were initially postponed in the hope that more and better genetically engineered strains would become available through related projects. Such strains are currently still not available.

Table 5.3. Selected cases showing specific hydroxylase activities in both wild type and some recombinant systems

Host	Gene	Source	Volumetric activity (mg. $(l.h)^{-1}$	Specific activity (U $(g\ dwt)^{-1}$	Substrate	Products	Reference
Y. lipolytica TVN91	***CYP53B1***	*"*	106-109	0.15-0.17	4-nonyloxybenzoic acid	pHBA, 4-(8-carboxyoctyloxy) benzoic acid, 4-(2-carboxyethoxy) benzoic acid	This work
Y. lipolytica TVN356	CYP557A	***R. retinophila***	132-144	0.12-0.18	4-nonyloxybenzoic acid		This work
Y. lipolytica TVN493	CYP52F1	***Y. lipolytica***	127-139	0.12-0.18	4-nonyloxybenzoic acid		This work
Y. lipolytica W29	Wild type	Wild type	277	0.38	4-nonyloxybenzoic acid		This work
R.retinophila TVN295	TVN295	Wild type	27.8	0.06	4-nonyloxybenzoic acid		This work
R. minuta CBS 8446	***CYP53B1***	Wild type	144-178	2.54	BA	pHBA	This work
Y. lipolytica TVN91 ***using whole cells in shake flasks***	***CYP53B1***	***R. minuta***	6-7	0.04-0.08	BA	pHBA	This work
Y. lipolytica TVN91 ***using whole cells in bioreactor***	***CYP53B1***	***R. minuta***	13	0.09	BA	pHBA	This work
Y. lipolytica TVN91 ***using harvested cells in shake flasks***	***CYP53B1***	***R. minuta***	4-13	0.03-0.08	BA	pHBA	This work
Y. lipolytica TVN91	***CYP53B1***	*"*	6	0.06	CA (BA)	pHBA	This work
Y. lipolytica TVN91	***CYP53B1***	*"*	4-9	0.04-0.09	PN (BA)	pHBA	This work
Y. lipolytica TVN91	***CYP53B1***	*"*	18-39	0.18-0.33	PN	PAA, BA, pHBA	This work
Y. lipolytica TVN356	CYP557A	***R. retinophila***	54-63	0.56-0.70	PN	PAA	This work
Y. lipolytica TVN493	***CYP52F1***	***Y. lipolytica***	52-66	0.59-0.77	PN	PAA,BA	This work
Y. lipolytica	Human***CYP1A1***	Human	n.d	$1.3\text{-}1.8 \times 10^{-3}$	ethoxyresorfurin	resorufin	Nthangeni ***et al***., 2004
S. diastaticus	Rat ***CYP1A1***	Rat	n.d	6.61×10^{-6}	acetanilide	acetaminophene	Liu ***et al***., 1998
S. cerevisiae	Plant-ferulate-5 hydroxylase	Plant	n.d	0.535	ferulic acid	5-hydroxyferulic acid	Jiang and Morgan, 2004

CHAPTER SIX

6.0 CONCLUSIONS

The recent development of genetic tools for ***Y. lipolytica*** such as several strong and regulated promoters has enabled it to be used as a host for the overexpression of foreign ***CYP450s*** (Juretzet ***et al***., 2001). However, a possible limitation in this yeast is the presence of endogenous ***CYP450s*** which usually leads to background activities.

In this project, the use of ***Y. lipolytica*** as a heterologous host was evaluated through biotransformation of various substrates using the available strains overexpressing different ***CYP450s***. Factors that might limit the use of ***Y. lipolytica*** as a host were also investigated. The results indicated, that the production of hydrolytic enzymes, for which ***Y. lipolytica*** is well known (Pigdène ***et al***., 2000; Fickers ***et al***., 2004), might in some cases limit its use as a whole cell biocatalyst when using substrates such as AA which contain ester or amide bonds.

It was demonstrated that substrates such as 4-nonyloxybenzoic acid and PN may act both as inducers as well as substrates of the endogenous ***CYP450s*** in ***Y. lipolytica.*** Although it seemed possible that PN could be a substrate of the overexpressed CYP450s i.e. CYP52F1 and CYP557A1, it was unlikely that the same was true for 4-nonyloxybenzoic acid. Biotransformation of 4-nonyloxybenzoic acid by ***Y. lipolytica*** strains led to the accumulation of two new hydroxylated diacid products, namely, 4-(8-carboxyoctyloxy)benzoic acid and 4-(2-carboxyethoxy)benzoic acid***.*** These two products have not been previously reported as microbial biotransformation products and might have interesting properties for polymer production.

The expression of ***CYP53B1*** from ***R. minuta***, which encodes a benzoate ***para***-hydroxylase, served as a useful tool for the study of fundamental questions relating to CYP450 expression in ***Y. lipolytica*** as well

as for optimization of whole cell hydroxylations, since BA and pHBA are not transformed by wild type ***Y. lipolytica.*** Both BA and pHBA were not toxic to ***Y. lipolytica*** at pH 8. These two compounds are cheap and easy to detect on TLC under UV light. The compounds are also easy to assay by GC analysis. Thus, BA could be a possible substrate for large scale process optimization. The hydroxylation of BA to pHBA in the recombinant ***Y. lipolytica*** strain, TVN91 was only due to the activity of the overexpressed ***CYP53B1.*** Using this strain for biotransformation studies, it was demonstrated that, the benzoate ***para***-hydroxylase activity in TVN91 was much lower than the activity in the wild type ***R. minuta*** strain from which the gene was cloned. This activity was also much lower than the alkane hydroxylase activity of different ***Y. lipolytica*** strains towards PN and 4-nonyloxybenzoic acid. Through various experiments, it was further demonstrated that, the benzoate ***para***-hydroylase activity in the recombinant strain, TVN91 was not limited by substrate transport or product inhibition. The possibility to obtain ***CYP450*** activity in cells grown in a chemically defined media (YNB) supplemented with yeast extract and casamino acids was demonstrated although the exact influence of various amino acids in the hydroxylase activity still needs further investigation.

It was also established that it is possible to use harvested cells of the recombinant ***Y. lipolytica*** strains overexpressing (***CYP557A1*** and ***CYP53B1***) to perform hydroxylation reactions without significant loss in activity. When cells in growth medium were used for biotransformations, continuous induction was necessary while this was not the case with harvested cells. When biotransformation reactions were carried out using harvested cells it was demonstrated that the specific hydroxylase activity could be improved by better mixing and aeration, while the volumetric activity could be improved by using higher cell biomass concentration.

However, the main limitation in using ***Y. lipolytica*** for the expression of ***CYP450*** was the inability to selectively induce the overexpressed ***CYP450*** without background activity due to the endogenous ***CYP450s***. Therefore selective expression of the cloned ***CYP450s*** is still desired though not yet possible. The results obtained in this study thus opens the way for further

development and optimization of ***Y. lipolytica*** expression systems for heterologous expression of CYP450s.

Recommendations for future research based on the findings of this work would include overexpression of the ***CYP450s*** under different promoters such as p***ICL*** which would eliminate the background activities of the endogenous genes. The effect of additional copies of the ***CPR*** on the CYP450 activity could also be evaluated through multicopy integrations of the ***CPR.*** Whole cell biotransformations could then be complemented CO difference spectra to determine total CYP450 content and CPR activity assays using cytochrome C and NADPH. Measuring growth and reporting biotransformation activities in terms of protein content should also be considered.

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SUMMARY

Biohydroxylation reactions are catalyzed by various types of hydroxylating enzymes (Ayala and Torres, 2004) which include dioxygenases, lipooxygenases as well as CYP450 monooxygenases. These particular hydroxylation reactions have several advantages over chemical synthesis. Several microorganisms including yeasts have the ability to hydroxylate various substrates. The exploitation of microbial hydroxylations for the production of industrially useful products such as pharmaceuticals is a more recent development (Holland ***et al***., 2000).

Yeasts from the genera ***Schizosaccharomyces, Pichia, Saccharomyces and Yarrowia*** have all been used to express foreign ***CYP450*** genes (Mukarami ***et al***., 1990; Nthangeni ***et al***., 2004) since they offer an advantage especially when a eukaryotic environment is required for the functional expression of the heterologous gene (Blanquet ***et al***., 2003). A recent evaluation of several yeasts revealed that ***Y. lipolytica*** is, a highly attractive alternative host for secretion and expression cloning (Muller ***et al***., 1998; Juretzek ***et al***., 2001). However, a literature search on successful expression of CYP450s in ***Y. lipolytica*** yielded only six cases. Three of these were done in our laboratory. In most of the reported cases, the recombinant CYP450 activities were never evaluated in terms of whole cell biotransformations.

It was therefore the aim of this study to evaluate ***Y. lipolytica*** as a recombinant whole-cell biocatalyst for hydroxylation reactions by using available ***Y. lipolytica*** strains overexpressing different ***CYP450s*** which were (i) ***CYP1A1*** coding for polyaromatic hydrocarbon hydroxylase (ii) ***CYP53B1*** coding for benzoate ***para***-hydroxylase (iii) ***CYP52F1*** coding for alkane hydroxylase and (iv) ***CYP557A1*** coding for putative fatty acid hydroxylase. Hydroxylase activities of the genetically engineered strains were compared with activities in wild type

yeasts expressing the relevant CYP450s. A variety of substrates used for biotransformation reactions included, acetanilide, benzoic acid , phenylnonane, ***trans***-cinnamic acid, 4-nitrophenyl octyl ether and 4-nonyloxybenzoic acid.

Experiments using ***Y. lipolytica*** overexpressing ***CYP1A1*** illustrated the limitation of using ***Y. lipolytica*** for the biotransformation of substrates such as AA since the endogenous enzymes degraded this substrate within only 12 h after substrate addition.

In an attempt to distinguish the activities of the putative fatty acid hydroxylase and the alkane hydroxylase overexpressed in ***Y. lipolytica*** from the endogenous CYP450s, 4-nitrophenyl octyl ether, 4-nonyloxybenzoic acid and phenylnonane were used as substrates. 4-nitrophenyl octyl ether proved to be expensive and less sensitive to TLC, GC and GC-MS analyses. It has been used in other studies because it yielded ***p***-nitrophenol which can be assayed colourimetrically by measuring absorbance at 420 nm. However, in our experiments, intermediates accumulated that were not completely transformed into ***p***-nitrophenol.

Further biotransformation experiments were carried out using 4-nonyloxybenzoic acid as the substrate. Biotransformation experiments were done using ***Y. lipolytica*** strains with intact and partially disrupted *β*-oxidation pathway overexpressing CYP52F1 and CYP557A1. Additional experiments were carried out using wild type ***Y. lipolytica*** W29, ***R. minuta*** and ***R. retinophila*** strains. The results demonstrated that, the wild type ***Y. lipolytica*** W29 demonstrated the highest specific hydroxylase activity when 4-nonyloxybenzoic acid was used as the substrate. The main limitation here was the inability to selectively induce the overexpressed ***CYP450*** genes alone without the background endogenous CYP450 activity.

Due to the limitations above, the next strain used was ***Y. lipolytica*** TVN91 (overexpressing benzoate-***para*** hydroxylase from ***R. minuta***). In this case, the host strain lacked the specific ***CYP450*** to perform the same hydroxylation reaction. The substrate used here was BA. Different growth and induction conditions were evaluated to optimize benzoate-***para***-hydroxylase activities. A comparison of the hydroxylase activities indicated that the activity of the recombinant ***Y. lipolytica*** strain overexpressing the ***CYP53B1*** was about 30 times slower than that of the wild type ***R. minuta*** from which the gene was cloned. Continuous addition of stearic acid resulted in the best activity with ***Y. lipolytica*** TVN91, because the hydroxylase activity was maintained for a longer duration. When PN and CA were used to evaluate substrate transport limitation, the results demonstrated that substrate transport was not limiting and the specific hydroxylase activity was not increased. PN was initially converted to BA before hydroxylation to form pHBA. These results further demonstrated that hydroxylase activity of PN was much faster than that of BA.

The results from the bioreactor study demonstrated that an improved aeration and mixing led to an increase in the benzoate ***para***-hydroxylase activity. The possibility of using a chemically defined media (YNB) supplemented with yeast extract and casamino acid for biotransformation was also demonstrated.

The results of this study also demonstrated that it is possible to use harvested recombinant cells for biotransformation without significant loss of activity. This makes it possible to study in detail the kinetics of the overexpressed CYP450s. It was, however apparent that the hydroxylase activities were significantly increased by both aeration and cell concentration.

Key Words: ***Yarrowia lipolytica***, ***Rhodotorula minuta***, heterologous expression, benzoic acid, benzoate ***para***-hydroxylase, alkane hydroxylase, biotransformation, fatty acid ω-hydroxylase, CYP450 monooxygenase.