BIOTRANSFORMATION OF ALKANES, ALKYLBENZENES AND THEIR DERIVATIVES BY GENETICALLY ENGINEERED *YARROWIA LIPOLYTICA* STRAINS

BY

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TABLE OF CONTENTS

CHAPTER ONE

HYDORCARBON DEGRADATION IN YEASTS

A LITERATURE REVIEW

1.1 Introduction

Hydrocarbons are insoluble hydrophobic molecules composed of carbon-carbon and carbon-hydrogen linkages (Watkinson & Morgan, 1990). They may be straight chained, branched or cyclic (Jones ***et al., 2001;*** Watkinson & Morgan, 1990). Hydrocarbons can range from gases, such as methane and ethane to liquids with a chain length of 40 or more carbons. Hydrocarbons are often products of the petroleum industry. Many of these petroleum based products may cause extensive contamination in both aquatic and terrestrial environments. Hydrocarbons can penetrate from topsoil to subsoil and pose a risk of ground water contamination. They also present a health hazard (Alkasrawi ***et al***., 1999). Contamination is mainly due to oil spills on land, while pumping of ballast waters, effluent from dry docking and the servicing of oil tankers contribute to pollution in the oceans (Zinjarde & Pant, 2002).

As a carbon source ***n***-alkanes enable micro organisms to grow with short generation times and make them highly competitive in aerobic zones of contaminated soil or water habitats (Schmitz ***et al.,*** 2000). A wide variety of yeasts, bacteria and filamentous fungi are capable of utilizing hydrocarbons (Schmitz ***et al.,*** 2000; Watkinson & Morgan, 1990).

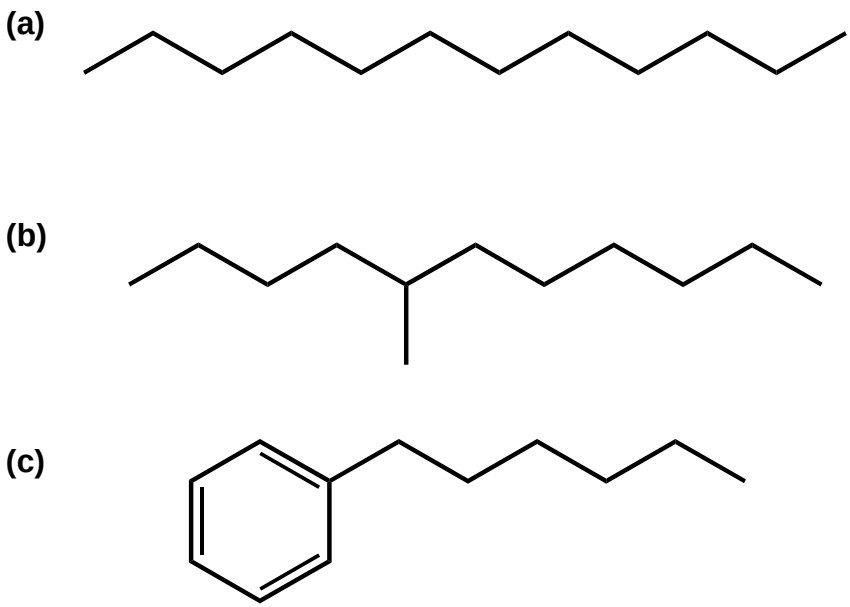


Fig. 1.1: Chemical structures of different classes of hydrocarbons, a) ***n***-alkanes (dodecane), b) branched alkanes (5-methyl undecane) and c) alkyl benzenes (hexylbenzene).

The following discussion will be a brief overview of the degradation pathways involved in hydrocarbon degradation in yeast with special attention to ***Yarrowia lipolytica***. The utilization of different hydrocarbons (Fig. 1.1) including ***n***-alkanes, branched alkanes, and alkylbenzenes will be reviewed. The pathways involved in ***n***-alkane degradation will be discussed in more detail. Some attention will also be given to the application of hydrocarbon degrading yeasts in the industry.

1.2 Hydrocarbon utilization

1.2.1 Straight chained and branched hydrocarbons

Yeasts capable of degrading hydrocarbons include amongst others ***Yarrowia lipolytica***, ***Candida tropicalis***, ***Candida albicans*** and ***Debaryomyces hansenii***. Bacteria capable of degrading hydrocarbons include various ***Pseudomonas*** and ***Rodococcus*** species. These organisms all differ in their substrate spectra. ***Pseudomonas oleovorans*** assimilates ***n-***alkanes with chain lengths

ranging from 6 to 12 carbons, while some yeast and other bacteria are capable of assimilating chain lengths between 6 to 16 carbons or exclusively between chain lengths from 14 to16 carbons (Schmitz ***et al.,*** 2000). ***Penicillium simplicissimum*** YK, a filamentous fungus was shown to degrade ***n***-alkanes ranging in chain length from 20 to 50 carbons (Yamada-Onodera ***et al***., 2002). Competition experiments demonstrated that yeast may be superior to bacteria in sandy soil (Schmitz ***et al***., 2000). There has been specific interest in the fields of substrate uptake and the metabolic processes of these organisms. There has also been interest in their application in biotechnology.

Candida, Pichia, Debaryomyces as well as ***Yarrowia lipolytica*** are not capable of assimilating alkanes, shorter than nine carbons, when these substrates are added as a liquid but these alkanes can be assimilated when supplied in the vapour phase (Mauersberger ***et al***., 1996). Branched alkanes can be utilized by some yeast such as ***C. maltosa*** and ***Y. lipolytica*** and are also incorporated into lipids (Mauersberger ***et al***., 1996). Highly branched compounds are more recalcitrant to biodegradation than simpler compounds. Compounds that are β-branched and quaternary branched seem to be particularly recalcitrant due to the steric hindrance of oxidation enzymes (Watkinson & Morgan, 1990).

1.2.2 Cyclic compounds

Cyclic compounds are major components of the petroleum industry. They are also found in herbicides, insecticides, flavours and fragrances. They also serve as solvents and intermediates in the chemical industry (Cheng ***et al***., 2002). Cycloalkanes are not used as growth substrates by yeast but partial oxidation in the presence of glucose has been witnessed when these substrates were added in non toxic concentrations (Mauersberger ***et al***., 1996). The biological oxidation of cyclic alkanes by bacteria results in the formation of the corresponding dicarboxylic acid, which are further metabolized by the cell (Cheng ***et al***., 2002). The oxidation rates of cycloalkanes and cycloalkanols are only 5-10% that of ***n***-alkanes (Mauersberger ***et al***., 1996).

1.2.3 Alkylbenzenes

Alkylbenzenes are readily used as carbon sources. The primary oxidation takes place via the same enzyme systems used for the oxidation of alkanes and fatty acids (Fig. 1.2). The alkylbenzenes are oxidized to phenylalkanols and phenylalkanoic acids. This results in the formation of either phenylacetic acid for even-numbered and benzoic acid for odd-numbered chains after β-oxidation (Mauersberger ***et al***., 1996).

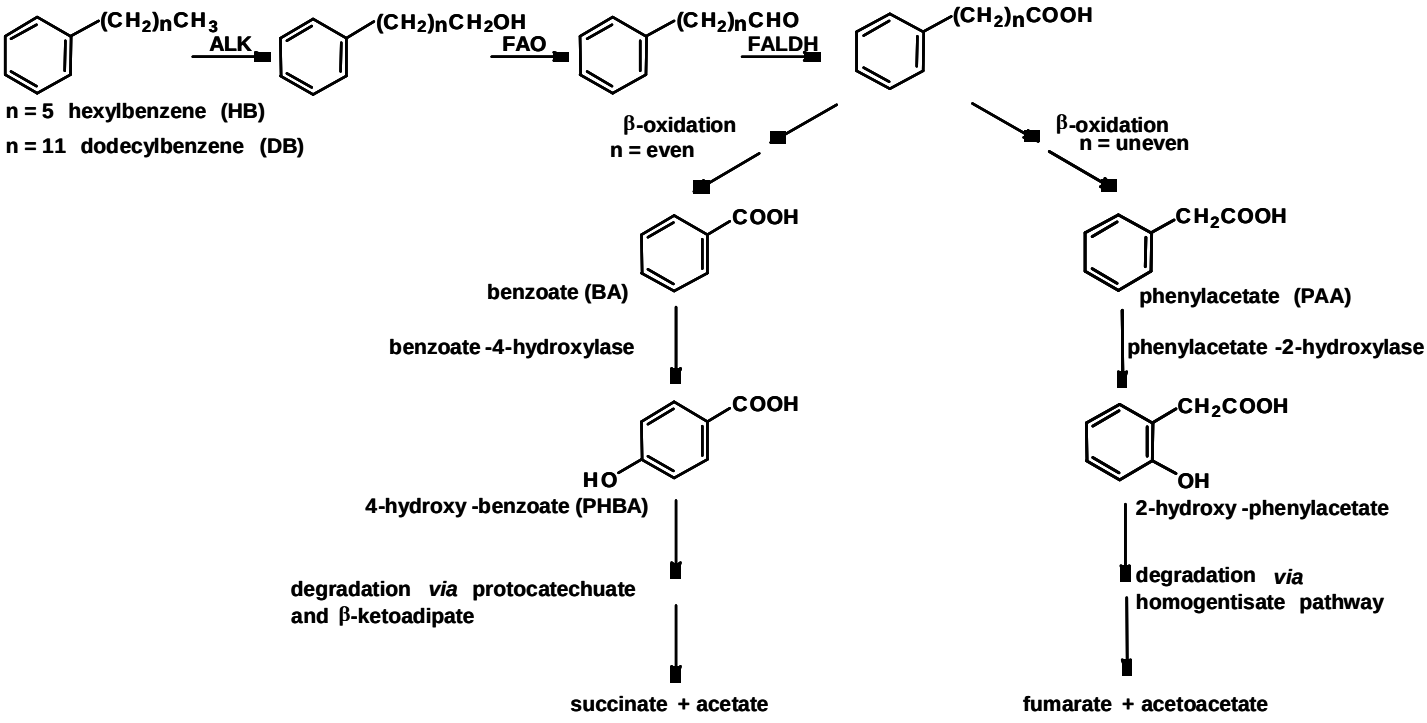


Fig. 1.2: The degradation of alkylbenzenes in yeast. Primary oxidation takes place via the same enzyme systems used for the oxidation of alkanes. The alkylbenzenes are hydroxylated by an alkane hyroxylase (ALK) leading to the formation of a phenylalkanol. Subsequent oxidations by a fatty alcohol oxidase (FAO) and a fatty alcohol dehydrogenase (FALDH) results in the formation of a phenylalkanoic acid that enters β-oxidation. Phenylacetate or benzoate is formed depending on the chain length. Benzoate is degraded via benzoate-4-hydroxylase, protocatechuate and β-ketoadipate while

phenylacetate is degraded via 2-hydroxy-phenylacetate and the homogentisate pathway.

Basidiomycetous yeasts such as ***Rhodotorula graminis*** is capable of degrading benzoate via the formation of 4-hydroxybenzoate (Fig. 1.2). Benzoate ***para*** hydroxylase encoding genes have been cloned from ***Rhodotorula minuta*** and ***Aspergillus niger*** (Van Grocom ***et al***., 1990; Fuji ***et al***., 1997). The 4-hydroxybenzoate is converted by ***Rhodotorula graminis*** into protocatechuate, which is the cleavage substrate, by separate and highly specific NADPH-dependant monooxygenases which are induced by their substrates. 2-Hydroxybenzoate is hydroxylated and decarboxylated by a specific NADPH-dependent monooxygenase into catechol which is further metabolized by the 3-oxoadipate pathway (Middelhoven, 1993).

In filamentous fungi such as ***Aspergillus nidulans***, phenylacetic acid is degraded via 2-hydroxy-phenylacetate and homogentisate which is converted to acetoacetate and fumarate (Fig. 1.2). A gene coding for phenylacetate-2-hydroxylase had been identified in ***Aspergillus nidulans*** (Middelhoven, 1993; Mingot ***et al.***, 1998). Benzoic acid and phenylacetic acid are generally not degraded by ascomycetous yeasts, because they lack the necessary hydroxylases. One exception is ***Zygosaccharomyces bailii***, which produces a benzoate hydroxylase which does not appear to be a microsomal P450 (Mollapour & Piper, 2001).

1.2.4 Phenolic compounds

Phenolic compounds can be degraded by both prokaryotic and eukaryotic microorganisms. The aerobic degradation of these compounds is common and proceeds through catechol. Eukaryotic microorganisms produce catechol from phenol via an epoxide and transdiol using a monooxygenase. Aerobic degradation processes for these compounds are usually preferred due to the lower costs associated with this process (Ruiz-Ordaz ***et al***., 2001). Phenolic compounds can occur naturally in humic acids. They can also originate from petrochemical, drug and chemical industries (Fialova ***et al***., 2004).

Alkylphenols are often produced during the biodegradation of non ionic surfactants such as alkylphenol polyethoxylates (APEOs). While these polyethoxylates are rapidly transformed into metabolites such as nonylphenol, these compounds appear to be recalcitrant to further microbial attack. As consequence they accumulate in the ground water, sediments and sewage sludge (Vallini ***et al***., 2001).

In an experiment using a ***C. maltosa*** strain, 4-(1-nonyl)phenol was broken down. Growth slowed down after seven days, most probably because of toxic intermediates formed in the breakdown. 4-Acetylphenol was the most prominent product found in the culture broth (Corti ***et al***., 1994). Vallini and co-workers proposed the possible breakdown of 4-(1-nonyl)phenol by ***C. aquaetextoris***. They proposed that the substrate would first undergo a hydroxylation at the ω (terminal) position on the alkyl chain. This would be followed by oxidation to its corresponding carboxylic acid which would then enter β-oxidation. This would lead to 4-hydroxy-benzoic acid as terminal product (Vallini ***et al***., 2001).

1.3 *n*-Alkane assimilation

1.3.1 Pathway for degradation

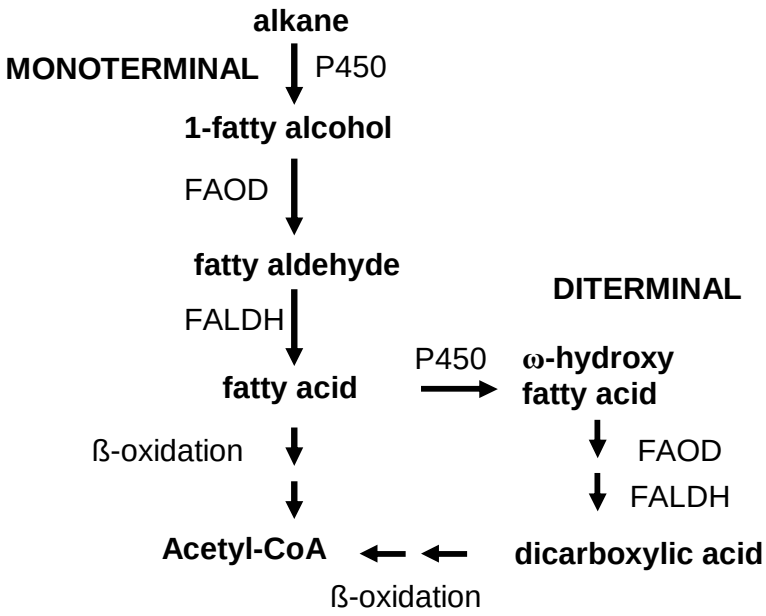


Fig. 1.3: Pathways of alkane degradation in yeasts. Alkanes are hydroxylated by a P450 monooxygenase system resulting in the formation of a fatty alcohol. The 1-fatty alcohol is oxidised to a fatty aldehyde by a fatty alcohol oxidase (FAOD). A fatty aldehyde dehydrogenase (FALDH) converts the fatty aldehyde to fatty acid which may either enter β-oxidation or undergo a second P450 hydroxylation in the diterminal oxidation pathway, resulting in the formation of dicarboxylic acid that may also enter β-oxidation. The acetyl-CoA resulting from β-oxidation is used for the synthesis of tricarboxylic acid cycle intermediates via the glyoxylate cycle.

An important characteristic of alkane assimilation by yeast is the flow of carbon from alkane substrates to synthesis of all cellular carbohydrates via fatty acids. This process is quite different from substrates like carbohydrates (Mauersberger ***et al***., 1996).

The assimilation of alkanes occurs via the monoterminal and diterminal pathways (Fig 1.3). The alkanes need to be taken up and transported into the cell. Unlike carbohydrates that are rich in oxygen and hydrophilic, hydrocarbons are hydrophobic. Because of their hydrophobicity, cells needed to develop specific modifications to facilitate uptake of hydrocarbons into the cell. After uptake the hydrocarbon is transported to the ER (endoplasmic reticulum) where it is oxidized by a cytochrome P450 catalysed terminal hydroxylation to it corresponding fatty alcohol which is subsequently oxidized to the fatty acid. This is followed by the activation of the free fatty acids to their corresponding CoA esters which are subsequently degraded to acetyl-CoA via peroxisomal β-oxidation. Tricarboxylic acid cycle intermediates are synthesized from the acetyl-CoA via the glyoxylate cycle and is used in anabolic pathways for the synthesis of cellular components (Fickers ***et al***., 2005; Mauersberger ***et al***., 1996).

1.3.2 Transport

The utilization of hydrocarbons starts with the uptake of the substrate into the cell. Because of their weak solubility in water, microorganisms had to develop specific adaptations to facilitate uptake of hydrocarbons. There are three ways for hydrocarbons to enter the cell, direct contact through attachment of the cell to large oil drops, through adsorption of submicron oil droplets to the cell surface, or by uptake of pseudosolubilized hydrocarbon in the aqueous phase (Mauersberger ***et al***., 1996). Emulsified hydrocarbons are much smaller than the microorganism and hence the droplets can attach to the cell surface, instead of the cells attaching to the surface of the hydrocarbon drops. In the case where the hydrocarbon is in the larger drop form and the cells attach to the hydrocarbon drop, uptake is presumed to take place through diffusion at the point of contact (Kim ***et al***., 2000). The availability of hydrocarbon surface area for cell attachment can be considered a limiting factor in hydrocarbon uptake (Kim ***et al***., 2000).

The hydrophobicity of cells can also be considered as one of the factors controlling hydrocarbon uptake. Cells grown on alkane are more hydrophobic when compared to cells grown on glucose. This is necessary because cells with higher hydrophobicity have a better chance to adhere to oil droplets (Kim ***et al***., 2000).

The cell wall structures change and there is the formation of special channels (pores) that permit penetration of the hydrocarbons. This is also accompanied by the formation of slime-like outgrowths and increased membrane vesicles (Mauersberger ***et al***., 1996). In experiments using a ***Y. lipolytica*** strain various changes were noted in the cell wall structures. Small extrusions were radially distributed over the cell surface off cells grown on crude oil. These extrusions were not visible in cells grown on glucose. The ratio of cell wall thickness to whole cell diameter also differed in cells grown on crude oil, compared with cells grown on glucose. The cells grown on crude oil had a higher cell wall thickness to whole cell diameter ratio when compared to the cells grown on glucose (Kim ***et al***., 2000).

The passage through the cell wall is also made easier by the excretion of biosurfactants (Mauersberger ***et al***., 1996). Biosurfactants are typically composed of a hydrophilic part and a hydrophobic part. The hydrophilic group usually consists of carbohydrates or peptides and the hydrophobic group is composed of various fatty acids. Biosurfactants can be classified into four categories. These categories include glycolipid, fatty acid, lipopeptide and polymer type, based upon the structure of their hydrophilic part. Biosurfactants are capable of emulsifying the substrate and so extending the interfacial area between the substrate and the microorganism (Kitamoto ***et al***., 2002). An example is the lipopolysaccharide excreted by ***C. tropicalis***. The hydrocarbon droplets are encapsulated in the surfactant micelles which then facilitate assimilation by the cell (Mauersberger ***et al***., 1996; Watkinson & Morgan, 1990). In contrast trehalose lipids are cell wall associated and involved in the cellular adaptation to the presence of ***n***-alkanes. Trehalose lipids render the cell surface hydrophobic, which may facilitate the attachment and passive transport of the substrate into the cell.

After the hydrophobic substrate has successfully entered the cell it is ready to enter the first step in the oxidation pathway.

1.3.3 The Cytochrome P450 monooxygenase system

Cytochrome P450s (***CYP***) are part of a super family of heme-proteins that exhibit an absorption peak at 450nm when carbon monoxide is bound to the reduced form of the enzymes. They can be found throughout nature. These enzymes catalyse the transformation of hydrophobic xenobiotics or endogenous compounds to more hydrophilic compounds by introducing an oxygen atom derived from molecular oxygen (Iida ***et al., 2000)***. P450s in prokaryotes are soluble proteins in contrast to eukaryotic P450s where they are usually bound to the endoplasmic reticulum or inner membrane of the mitochondria (Werck-Reichhart & Feiereisen, 2000). Mammalian P450s are involved in the biosynthesis and metabolism of steroid hormones such as mineral corticoids and glucocorticoids. They are also involved in the detoxification of carcinogens from foods and chemical pollutants. In higher animals these P450s are mainly localized in the liver (Sakaki & Inouye, 2000; Sanglard & Fiechter, 1989). In plants P450s help with the metabolism of chemicals such as herbicides and pesticides (Werck-Reichhart ***et al.,*** 2000).

1.3.3.1 Reaction Cycle of Cytochrome P450

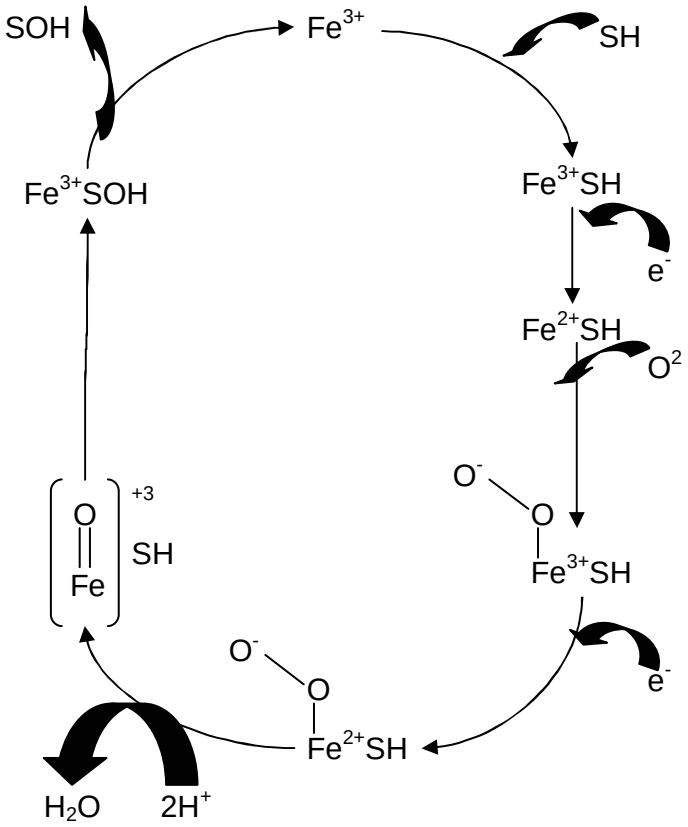


Fig. 1.4: Oxidation cycle of cytochrome P450. SH and SOH indicate the substrate and product respectively (Sakaki and Inouye, 2000).

Figure. 1.4 shows the P450 reaction cycle. The essential step in the oxidation of a substrate by P450 is the addition of one molecular oxygen atom, which is activated by a reduced heme iron, to the substrate. The activation of oxygen is common to all P450s (Urlacher ***et al***., 2004). The activation takes place at the iron-protoporphyrin IX (heme). The heme iron is sixfold coordinated. It has a conserved thiolate residue as the fifth ligand and, in the inactive ferric form, a water molecule as its sixth ligand (Urlacher ***et al***., 2004). The reaction cycle can be divided into six steps: The first step involves the binding of the substrate to the P450 monooxygenase. This step occurs rapidly. Then the ferric enzyme is reduced to a ferrous state by a one electron transfer from

NADPH via the NADPH-dependant P450 reductase (***CPR***) to the P450. Molecular oxygen is then bound, resulting in a ferrous-dioxy species. A second reduction, followed by a proton transfer leads to an iron-hydroperoxo intermediate. The cleavage of the O-O bond releases water and an activated iron-oxo ferryl species. This iron-oxo ferryl oxidises the substrate and the product is subsequently released from the substrate heme pocket (Urlacher ***et al***., 2004; Sakaki & Inouye, 2000).

P450s can be divided into 4 classes depending on how electrons from NAD(P)H are delivered to the catalytic site. Class 1 P450s require a FAD reductase and an iron sulphur redoxin. Class 2 requires a FAD/FMN-containing P450 reductase. Class 3 enzymes do not require an electron donor and class 4 P450s receive electrons directly from NAD(P)H (Werck-Reichhart & Feyereisen, 2000). Fungal ***CYPs*** usually fall into the class 2 category.

P450 Monooxygenases are necessary for the assimilation of hydrocarbons in yeast. They are anchored in the ER where they catalyze the first enzymatic step in assimilation, namely alkane hydroxylation (Sakaki & Inouye, 2000; Sanglard & Fiechter, 1989)**.** The ***P450ALKs*** that are classified into the ***CYP52*** family are responsible for this reaction in yeast (Iida ***et al.,*** 2000). They catalyze the terminal monooxygenation of ***n-***alkanes and convert them to long-chain fatty alcohols. The ***P450ALKs*** can also hydroxylate alkane metabolites in the omega position to form dicarboxylic acids (Sumita ***et al***., 2002).

Candida maltosa contains a multigene family of at least eight structurally related P450 forms (***CYP52***). In a study to determine the substrate specificities it was determined that ***P450ALK1A*** displayed a significant hydroxylation preference for hexadecane and dodecane and seems to be the most important enzyme for the primary hydroxylation of ***n***-alkanes. ***P450ALK5A*** was induced by ***n***-alkanes but could not efficiently hydroxylate the inducing substrates (Zimmer ***et al***., 1996). It may be possible for P450's to catalyse a cascade of sequential mono- and diterminal monooxygenation reactions. The alkanes are oxidised directly by the P450s to fatty acids and

dicarboxylic acids. The study was done ***in vitro*** with ***C. maltosa*** P450 52A3 and an alternative pathway was suggested which includes this P450 oxygenation cascade together with the accepted route of degradation (Scheller ***et al***., 1998).

At least 10 ***CYP52*** genes were isolated from ***Candida tropicalis*** ATCC 20336. Two of the genes isolated, ***CYP52A12*** and ***CYP52D2*** did not seem to have allelic variants. ***CYP52A12*** was shown to be induced by fatty acids and alkanes while the other gene, ***CYP52D2***, was not induced by these substrates (Craft ***et al***., 2003). It was also shown that ***CYP52A13*** and ***CYP52A17*** are strongly induced by oleic acid. When these two P450s were expressed in insect cells in conjunction with the ***C. tropicalis*** P450 reductase, ***CYP52A13*** preferentially hydroxylated oleic acid and other unsaturated acids to ω-hydroxy acids and ***CYP52A17*** hydroxylated oleic acid as well as shorter saturated fatty acids such as myristic acid ($C_{14:0}$) effectively. Both these enzymes were capable of oxidizing ω-hydroxy fatty acids, ultimately generating α,ω-diacids (Craft ***et al***., 2003 & Eschenfeldt ***et al***., 2003).

In another study the existence of different P450 alkane hydroxylase genes were investigated in the halotolerant yeast ***Debaryomyces hansenii***. Four distinct P450alk gene segments and an allelic segment were isolated. Two full length genes, ***DH-ALK1*** and ***DH-ALK2*** were isolated. The proteins had predicted molecular weights of 59,254Da (***DH-ALK1***) and 59,614Da (***DH-ALK2***). Phylogenetic studies done showed that ***DH-ALK1*** and ***DH-ALK2*** constitute new genes located on two distinct branches most related to the gene ***CYP52A3*** (60% deduced aa homology) and least related to the gene ***CYP52C2*** (41% deduced aa homology) both of ***C. maltosa*** (Yadav & Loper, 1999).

At least twelve ***P450ALK*** genes have already been identified in the yeast ***Y. lipolytica*** (Fickers ***et al***., 2005)***.*** ***YlALK***1 and ***YlALK2*** also designated as ***CYP52F1*** and ***CYP52F2,*** were shown to have prominent roles in ***n***-alkane assimilation in ***Yarrowia lipolytica***. It was also found that six of eight ***YlALK*** genes were induced by ***n***-tetradecane. ***YlALK1*** showed the highest

expression in response to ***n***-tetradecane, whereas ***YlALK5*** and ***YlALK6*** were only weakly induced. It was reported that ***YlALK1*** was important in the assimilation of short-chain ***n***-alkanes, such as ***n***-decane, because a mutant with this gene disrupted grew poorly on ***n***-decane. When the ***YlALK2*** was also disrupted poor growth was also observed on ***n***-hexadecane. An explanation may be that ***YlALK***2 is specific for longer molecules while ***YlALK***1 is responsible for the oxidation of a wide variety of ***n***-alkanes (Iida ***et al.,*** 2000).

The transcriptional induction properties of individual P450alk isozymes in response to various species of inducers are diverse (Kogure ***et al***., 2005). Studies on the induction of the ***ALK***1 gene by decane in ***Y. lipolytica*** identified an alkane responsive region (ARR1), consisting of two alkane-responsive elements named ARE1 and ***2***. It had also been revealed that a deficiency in peroxisomes leads to the ***ALK***1 induction being repressed. This involved the ***PEX***10, ***PEX***5 and ***PEX***6 gene products (Fickers ***et al***., 2005; Sumita ***et al***., 2002). In a study using the yeast ***C. maltosa*** two regions were identified (ARE2 and CRE2) on the promoter of the ***ALK***2 gene. ARE2 functions in response to ***n-***alkanes and oleic acid, while CRE2 functions in response to some peroxisome proliferators, unsaturated fatty acids and steroid hormones. The study suggested that these two elements work on two distinct transcriptional induction pathways because of the difference in their inducer chemicals (Kogure ***et al***., 2005).

1.4 Oxidation of fatty alcohols to fatty acids

1.4.1 Fatty Alcohol oxidase and dehydrogenase

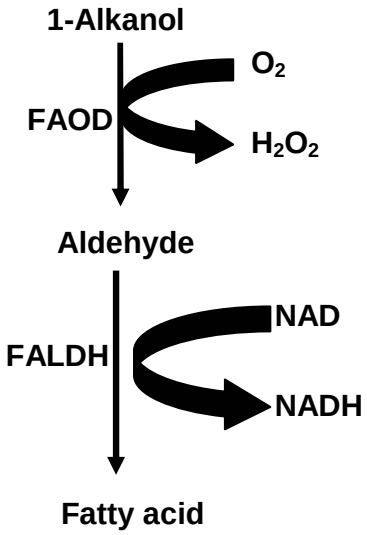


Fig. 1.5: The oxidation of fatty alkanol to fatty acids by fatty alcohol oxidase and fatty aldehyde dehydrogenase.

The next step in the pathway is the oxidation of the alkanol to its corresponding fatty acid. The reaction responsible for the conversion of the alkanol to a fatty aldehyde is catalyzed by an alkane inducible fatty alcohol oxidase and not by a NAD(P) dependant FADH as was previously assumed (Fickers ***et al***., 2005) (Fig. 1.5). Molecular oxygen serves as the electron acceptor of fatty alcohol oxidation and hydrogen peroxide arises from the reaction. A study using a 70-kDa FAOD protein that was purified from alkane grown ***Y. lipolytica*** H222 and then used in experiments on FAOD substrate specificity pointed to the probability of the existence of several FAOD enzymes. They reported that the oxidase had specificity for primary alcohols with a chain length ranging from 10 to 18 carbon atoms. The activity of the oxidase was optimal at a pH 9.3 (Il'chenko ***et al.,*** 1994). The aldehyde is then oxidized to a fatty acid by a membrane bound FALDH. Ueda & Tanaka (1990) found that the long-chain aldehyde dehydrogenase activity in alkane grown

cells of ***C. tropicalis*** ATCC 20336 was almost 10 times higher (98nm/min/mg protein) than glucose grown cells (8nmol/min/mg protein). They also found that the long chain aldehyde dehydrogenase of ***Y. lipolytica*** NRRL Y-6795 oxidises primary aldehydes ranging in chain length from C7-C17 and that the aldehyde dehydrogenase of ***C. tropicalis*** had little to no activity on substrates with a chain length of C15 or greater, although the yeast is able of assimilating chain lengths greater than C15 (Ueda & Tanaka, 1990). After oxidation by FAOD and FALDH the resultant fatty acids enter β-oxidation. (Mauersberger ***et al***., 1996).

1.4.2 β-oxidation

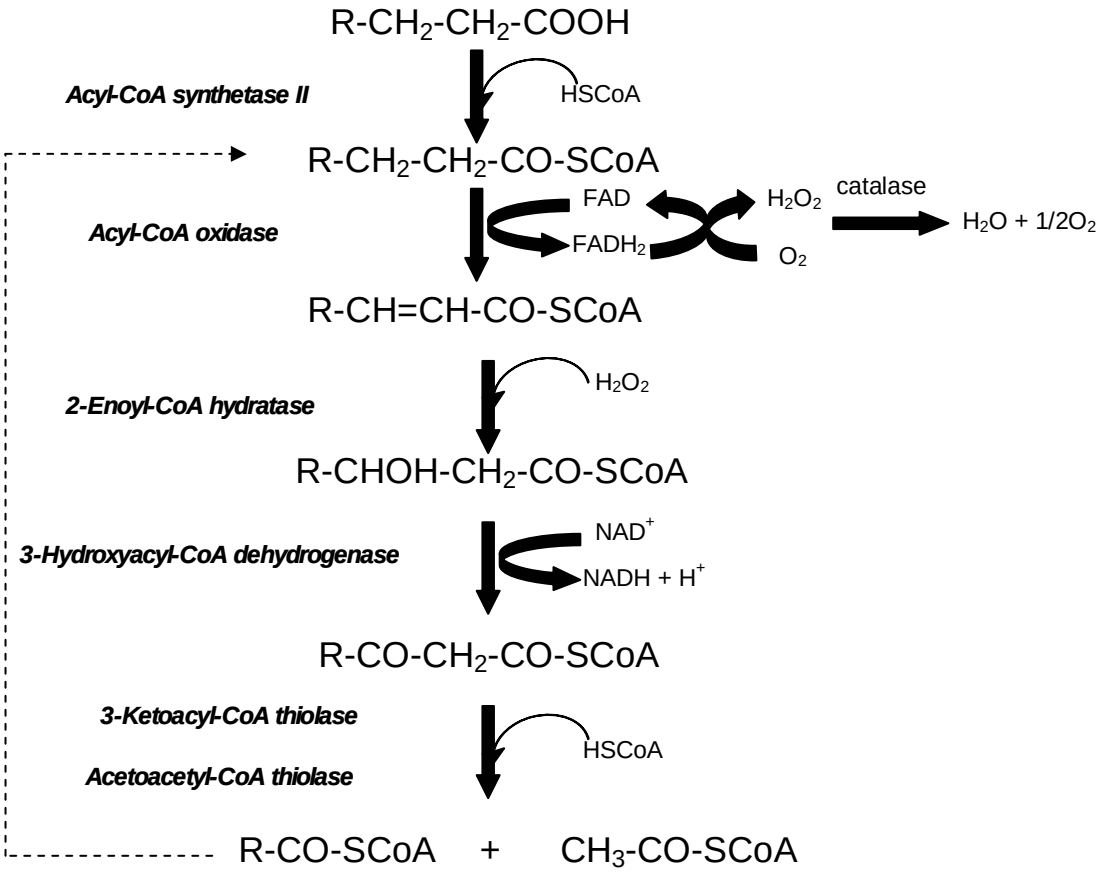


Fig. 1.6: β-oxidation of fatty acids. Fatty acids are converted into acyl-CoA esters which then enter a four step β-oxidation process. Each cycle results in the loss of two carbons (Fickers ***et al***., 2005).

Once inside the cell the fatty acids are energized as acyl-CoA esters (Fig. 1.6). The reaction is catalyzed by acyl-CoA synthetase enzymes. In ***Y. lipolytica*** long-chain specific acyl-CoA synthetases I and II have been detected and studied about 25 years ago. Acyl-CoA synthetase I is distributed among different subcellular fractions and appears to be involved in lipid synthesis, whereas acyl-CoA synthetase II is present in peroxisomes where β-oxidation takes place (Fickers ***et al***., 2005).

β-Oxidation (Fig. 1.6) can be defined as a cyclic degradation process resulting in the shortening of fatty acids by two carbons per cycle. The final reaction releases an acetyl CoA and acyl CoA shortened by two carbons. There are two types of beta oxidation systems, mitochondrial and peroxisomal. In hydrocarbon utilizing yeast β-oxidation is exclusively localized in the peroxisomes in contrast to animal cells where both systems exist. The main mechanistic difference lies in the enzymatic step following fatty acid activation to its CoA ester (Endrizzi ***et al.,*** 1996., Mauersberger ***et al***., 1996).

Mitochondria utilize an acyl-CoA dehydrogenase to convert the acyl-CoA to enoyl-CoA. This enzyme transfers electrons to FAD and then to the electron transport chain. Peroxisomes utilize an acyl CoA oxidase, an octameric flavoprotein, for the conversion of acyl-CoA to enoyl-CoA. The electrons are transferred to oxygen producing hydrogen peroxide (Picataggio ***et al***., 1991). The hydrogen peroxide is then converted to water and oxygen by catalase.

The acyl CoA oxidase is transported into the peroxisomes as a heteropentamer (Titorenko ***et al***., 2002). Some yeast species can contain more than one acyl CoA oxidase encoding gene. ***C. tropicalis*** contains three***, C. maltosa*** contains two and ***Y. lipolytica*** contains six (Picataggio ***et al***., 1991; Titorenko ***et al***., 2002; Fickers ***et al.,*** 2005). The large number of genes in ***Y. lipolytica*** indicates the adaptation of the species to hydrophobic substrates (Fickers ***et al.,*** 2005). Studies have shown that two acyl-CoA oxidases are chain length specific. Aco2p is specific for medium chain substrates while Aco3p is specific for short chain substrates. The others exhibited weak activity toward a variety of different chain lengths (Fickers ***et al.,*** 2005; Mlickova ***et al.,***

2004). The deletion of genes coding for acyl-CoA oxidase does not seem to affect growth when glucose is used as carbon source, however growth on fatty acids like oleic acid is affected. In a study where the acyl-CoA oxidase isozyme function was evaluated, it was found that if only ***POX1*** was left functional no growth was seen on fatty acids. ***POX4*** only partially restored growth. The strain containing the double deletion of ***POX2*** and ***POX3*** grew normally. Thus no one individual ***POX*** gene is absolutely required for β-oxidation of long chain acids (Wang ***et al***., 1999).

The next two reactions involve a hydration reaction handled by a 2-enoyl-CoA hydratase and a dehydrogenation reaction catalysed by 3-hydroxyacyl-CoA dehydrogenase. It has been shown that mitochondrial 2-enoyl-CoA hydratase-1 converts 2-enoyl-CoA esters to (S)-3-hydroxyacyl-CoA esters, whereas the peroxisomal β-oxidation system contains two multifunctional enzymes, perMFE-1 and perMFE-2, which display different stereochemistry. Peroxisomal MFE-1 displays 2-enoyl-CoA hydratase-1 and (S)-3-hydroxyacyl-CoA dehydrogenase activities. Peroxisomal MFE-2 displays 2-enoyl-CoA hydratase-2 and (R)-3-hydroxyacyl-CoA dehydrogenase activities. The (R)-3-hydroxyacyl-CoA dehydrogenase has been described as an integral part of the peroxisomal multifunctional enzyme in ***Candida tropicalis*** and ***Saccharomyces cerevisiae***. In a recent study the two dehydrogenase domains of ***Candida tropicalis*** MFE was expressed in ***E. coli*** and purified. The results demonstrated that the expressed 65kDa protein showed substrate specificity similar to perMFE-2, suggesting a common ancestor for the yeast MFE and mammalian perMFE-2 (Qin ***et al***., 2000).

The final step which involves the thiolytic cleavage of 3-ketoacyl-coenzyme A to acetyl and acyl CoA is handled by a thiolase enzyme. Two types of thiolase enzymes exist. They are classified by the chain length of the substrates that are used for the thiolysis reaction (Yamagami ***et al***., 2001). Acetoacetyl-CoA thiolase is specific for acetoacetyl-CoA while 3-oxoacyl-CoA has broad range chain length specificity for 3-oxoacyl-CoA substrates. 3-Oxoacyl-CoA thiolase reside in the mitochondria as well as the peroxisomes where they are involved in fatty acid metabolism. Acetoacetyl-CoA thiolases are involved in ketone

body metabolism in the mitochondria and in the mevalonate pathway in the cytoplasm.

Acetoacetyl-CoA thiolase in peroxisomes has also been reported. In a recent study a decane inducible peroxisomal acetoacetyl-CoA thiolase from ***Y. lipolytica*** had been cloned by complimenting a $C10^-$ mutant. The enzyme showed a high homology to corresponding thiolase enzymes in ***C. tropicalis*** (50%) and ***S. cereviseae*** (49%) (Yamagami ***et al***., 2001). The acetyl-CoA formed in the β-oxidation pathway then enters the glyoxalate pathway where it is further metabolized (Fickers ***et al.***, 2005).

In whole cell metabolism diterminal oxidation is an initial stage of ***n***-alkane metabolism. Under normal conditions the resultant dicarboxylic acid would enter β-oxidation to be broken down to its CoA esters. Only a small amount of dicarboxylic acid would be accumulated (Chan ***et al***., 1991). This makes full scale biological production of dicarboxylic acids economically unviable when wild type strains are used.

The deletion of the genes coding for acyl-CoA oxidase results in the effective blocking of the β-oxidation pathway. This would direct the metabolic flux towards ω-oxidation with alkanes being more efficiently converted to dicarboxylic acids. This allows for the industrial production of dicarboxylic acids, since blocking β-oxidation prevents the yeast from using the hydrocarbon for growth. Picataggio created ***C. tropicalis*** mutants by partially and fully deleting the genes coding for Acyl-CoA oxidases. They carried their experiment out in bioreactors using both strains. The ***C. tropicalis*** strain that had its β-oxidation fully blocked yielded $140g.l^{-1}$ dioic acid as compared to the ***C. tropicalis*** strain that had its β-oxidation only partially blocked which produced dioic acid to the concentration of $95g.l^{-1}$ (Picataggio ***et al.,*** 1992).

1.5 Applications

1.5.1 Bioremediation

Yeasts capable of using hydrocarbons as carbon source have been used in various bioremediation processes. A ***Y. lipolytica*** strain that was isolated from diesel oil was employed in studies to develop a biosensor. This yeast can be used to detect middle chain length alkanes at temperatures ranging between 5 and 25^0C making it perfect for bioremediation processes in cold climates (Alkasrawi ***et al***., 1999).

Mediterranean countries produce almost all the olive oil sold worldwide. During the oil extraction from olive trituration, a major quantity of an aqueous black liquid waste is generated (Ettayebi ***et al***., 2003). The treatment of olive mill wastewater has become a critical environmental problem for Mediterranean countries. The composition of olive mill wastewater is a stable emulsion constituted by ''vegetation waters'' of the olives, water from the processing, olive pulp and oil (Fickers ***et al***., 2005; Lanciotti ***et al***., 2005). The chemical and biological oxygen demand can reach as high as 100 and 200g O_2 l^{-1} respectively (Ettayebi ***et al***., 2003).

Most of the problems associated with olive mill wastewater can be attributed to the phenolic fraction (Fickers ***et al***., 2005; Lanciotti ***et al***., 2005). The phenolic content can range between 1.5 to 4g.l^{-1} (Ettayebi ***et al***., 2003). The biological treatment of olive mill wastewater are suitable because they lead to the elimination of the toxicity of the olive mill waste water by converting the toxic compounds to useful bioproducts such as biogas and single cell proteins (Ettayebi ***et al***., 2003).

A study using a thermophillic strain of ***C. tropicalis*** showed a reduction 69.7%, 69.2% and 55.3% reduction of chemical oxygen demand, monophenols and polyphenols respectively, after a 24h fermentation cycle. Hexadecane was used as a co-metabolite (Ettayebi ***et al***., 2003). A study using the yeast ***Y.***

lipolytica also showed a marked reduction in the chemical oxygen demand of up to 80% (Fickers ***et al***., 2005; Lanciotti ***et al***., 2005). In an experiment using a marine strain of ***Y. lipolytica*** and using palm mill oil as substrate, the chemical oxygen demand was reduced by 95% (Oswal ***et al***., 2002).

1.5.2 Production of single cell proteins and oils

Since World War II, yeast species such as ***Candida*** and ***Saccharomyces*** spp. has been employed as producer of microbial protein to convert agro-industrial waste, e.g., effluents from paper mills and olive mills, into a valuable protein supplement for animal feeds. The process is generally thought to be an attractive way to both enhance wastewater purification and increase resource utilization (Zheng ***et al***., 2005).

The production of single cell oil (SCO) also has industrial application. It has been shown that production of SCO by ***Y. lipolytica*** was dependant on growth conditions and substrates. Production can be enhanced by ***Teucrium polium L***. aqueous extract (Mlickova ***et al.,*** 2004). In salad oil manufacturing, a considerable amount of fatty acid in raw vegetable oil is separated from glyceride by a water washing process in the refining plant, and discarded as high-strength salad oil manufacturing wastewater. In a study various yeast species were isolated from salad oil manufacturing wastewater. One of the isolates C***. utilis*** was isolated as the sole biomass producer from the experiments. This was due its efficient uptake of the oil by the cells. The cells contained 26% protein, 9% crude lipid, 55% carbohydrate and balanced amino acid composition after cultivation with salad oil as carbon source (Zheng ***et al***., 2005).

1.5.3 Biosurfactant production

Microorganisms that are capable of growth on hydrocarbons may also be able to produce biosurfactants (Watkinson & Morgan, 1990). Although bacteria are capable of producing biosurfactants, they produce it in relatively low

concentrations. This may be because the cell membrane of prokaryotic cells is damaged by high levels of biosurfactants. Yeasts generally produce biosurfactants in higher concentrations than bacteria because of their rigid cell walls (Kim ***et al***., 1999).

Biosurfactants can provide new possibilities in the food, chemical, pharmaceutical, environmental protection and the energy saving industries. They are non-toxic, biodegradable and biologically active (Kitamoto ***et al***., 1993). Microbiological surfactants can be applied for removing contamination from petroleum polluted soil or waters and for washing petroleum storage tanks (Bednarski ***et al***., 2004). They have various novel properties allowing its use as gelling agents, emulsifiers, stabilizers, flocculants and dispersing agents (Cirigliano & Carman., 1985). Biosurfactants can also be used to control ice particle agglomeration in ice slurry systems. If ice particles are stably dispersed in the slurry system by surfactants, the agglomeration of the particles will be suppressed. This will lead to higher ice packing and higher system efficiency (Kitamoto ***et al***., 2002).

C. antarctica produces mannosylerithritol lipids (glycolipids) that has been shown to have antimicrobial properties. The mannosylerithritol lipids were strongly active against gram-positive bacteria and weakly active against gram-negative bacteria (Kitamoto ***et al***., 1993). In a study using media supplemented with oil refinery waste both ***C. antarctica*** and ***C. apicola*** produced glycolipids as biosurfactant in concentrations ranging from 7.3-13.4 and from 6.6 to 10.5g/l respectively (Bednarski ***et al***., 2004). ***Y. lipolytica*** is capable of producing a biosurfactant called liposan when grown on hexadecane as carbon source. Liposan is composed of approximately 83% carbohydrate and 17% protein. The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine and galacturonic acid (Cirigliano & Carman, 1985).

1.5.4 Lactone production

Products formed from the biotransformation of various hydrocarbons have industrial application in various fields. Lactones are mainly used in the food industry because of their fruity aroma. The microbial production of lactones provides a natural alternative because these compounds can then be given a natural label. Production involves the biotransformation of hydroxyl fatty acids.

One of the most important lactones for flavour application is γ-decalactone. It had been reported that the world-wide production of γ-decalactone was 10t in 1997. Companies that just required a simple production process are now looking for a way to lower manufacturing costs (Wache ***et al***., 2003). Lactones can be produced using ***Y. lipolytica*** as organism and castor oil as substrate. The pathway involves the β-oxidation of ricinoleyl-CoA until 4-hydroxydecanoic acid is formed, which lactonises to yield γ-decalactone. Toxicity of the lactone may influence yield. The mechanisms involved in the toxicity shows that the carbon lateral chain of the lactone interacts with membranes. This increases their fluidity and decreases their integrity (Fickers ***et al***., 2005).

The rate limiting step in the production of lactones is considered to be the step catalysed by acyl-CoA oxidase. By using mutant yeast strains that had one or more of the genes coding for acyl-CoA oxidase disrupted it was possible to optimize the production of lactones. The ***POX3*** gene coding for the short chain specific acyl-CoA oxidase is responsible for the degradation of lactone and genetic constructs have been made to remove its activity from yeasts (Groguenin ***et al***., 2004). In a study using a ***Y. lipolytica*** strain that was altered by deleting the genes coding for Aco2p, Aco3p and Aco5p it was found that lactone production was better (150mg/l) when compared with the wild type strain (71mg/l) (Waché ***et al***., 2002). Lactone production in the mutant strain was however much slower, taking 4 days. The wild type only took 12h to accumulate lactone (Waché ***et al***., 2002). Another study used two mutant ***Y. lipolytica*** strains to try and elucidate the role of other ***POX*** genes in lactone

production. They found that the ***Y. lipolytica*** MTLY36-P (Δ***pox***3 Δ***pox***5) strain lacking 2 functional acyl-CoA oxidases showed comparable results to the wild type strain, producing 150mg/l after 24h decreasing to 50-60mg/l after 40hrs. Another ***Y. lipolytica*** MTLY40-2P strain also had the Aco4p encoding gene (***POX***4) deleted in addition to the others showed rapid lactone production (300mg/l) after 20h with no significant decrease after 230h. This implied that Aco4p is not only involved in the regulation of other acyl-CoA oxidases, but also exhibited activity on a broad spectrum of straight chain acyl-CoA oxidases (Groguenin ***et al***., 2004).

1.5.5 Dicarboxylic acid production

Dicarboxylic acids are used for a variety of different applications. Adipic acid and sebacic acid is used in the manufacture of plasticicers. 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). α,ω-Tridecanedioic acid is used in the production of ethylene brassylate, a synthetic musk manufactured from ***n-***tridecane (Lui ***et al***., 2003). Cyclic esters derived from ω-hydroxyfatty acids find application as flavours, fragrances and solvents (Endrizzi ***et al***., 1996; Green ***et al.,*** 2000). Dicarboxylic acids are not readily available from petrochemicals if the carbon chain length exceeds 13. Fabritius and co-workers reported the formation of (Z), (Z)-octadeca-6, 9-dienedioic acid (6.4g/l) and (Z), (Z)-3-hydroxy-octadeca-9, 12-dienedioic acid (6.9g/l) when using a ***C. tropicalis*** mutant in batch and fed batch fermentation (Fabritius ***et al***., 1997).

Study on dicarboxylic acid formation includes the recovery of mutants and the optimization of fermentation processes. Usually, high dicarboxylic acid producing mutants of ***Candida tropicalis*** are used to achieve high-level dicarboxylic acid production from ***n***-alkanes, parrafins, and fatty acids as substrates. Engineered strains of ***Candida tropicalis*** that lack acyl-CoA oxidase can more efficiently convert ***n***-alkanes and fatty acids or their derivatives to their corresponding dioic acids (Liu ***et al***., 2004). In a study

using ***Candida tropicalis*** which had their β-oxidation fully blocked as well as ***Candida tropicalis*** strains that only had a partially blocked β-oxidation i.e. both ***POX*** 4 or ***POX*** 5 genes were deleted respectively, it was found that the partially blocked strains converted 21wt% of the substrate to dioic acid in the ***POX*** 4 deleted case and 35wt% of the substrate in the ***POX*** 5 deleted case. In both cases short chain derivates were also accumulated. In contrast, the ***Candida tropicalis*** strain with β-oxidation completely disrupted (i.e. all ***POX*** genes disrupted) converted 80wt% of the substrate dodecane exclusively to dodecanedioic acid (Picataggio ***et al***., 1992). In another study a ***Candida tropicalis*** strain with the ***POX4*** gene deleted was used to determine the effect of the disruption of this gene on dicarboxylic acid production. They showed that the deletion of the ***POX4*** gene does not necessarily lead to high dicarboxylic acid formation. They overexpressed the ***POX4*** gene to investigate whether dicarboxylic acid production could be repressed in this way. Although the acyl-CoA activity increased to 0.13µmol/min/mg protein, the strain produced 15.3g/l dicarboxylic acid. This was comparable to their strain containing the empty plasmid which produced 14.4g/l dicarboxylic acid. The strain containing the ***POX***4 deletion produced 17.6g/l dicarboxylic acid (Hara ***et al***., 2001). The overexpression of the ***CYP52*** genes together with its reductase in a β-oxidation blocked ***Candida tropicalis*** strain for the production of dicarboxylic acid was also studied. The strain demonstrated a 30% higher productivity in fermentations than the strain that was only deficient in β-oxidation accumulating a final concentration of 150g/l after 92h (Picataggio ***et al***., 1992).

Various process parameters, including pH influence dioic acid production. Green reported optimal production of dioic acid at pH 5-6 for ***Candida cloacae*** while Piccataggio reported dioic acid production by ***C. tropicalis*** at a pH of 8 (Green ***et al***., 2000; Picataggio ***et al***., 1992). In another study, also using a mutant strain of ***Candida tropicalis*** and using tridecane as substrate, it was found that a pH of between 7.8 and 8 was ideal for high dioic acid production. It was observed that lower concentrations of dioic acid (60g/l) was formed when the pH rised above 8.2 or was below 7.2 (Liu ***et al***., 2004). The

intracellular pH may also play an important role in the production of dioic acids. Also using ***C. tropicalis*** and tridecane as substrate Lui ***et al*** (2003) measured the intracellular pH at regular intervals. Dioic acid was produced optimally at a pH_i of 6.55 (Liu ***et al***., 2003). Pristane is sometimes added to the media. This acts as co solvent and makes substrate more accessible for conversion to dioic acids (Green ***et al***., 2000). Glucose also affects the production of dioic acids. Green reported low dioic acid production with high levels of glucose because high levels of glucose probably repress cytochrome P450/reductase activity. When glucose levels are depleted, dioic acid production increases (Green ***et al***., 2000; Picataggio ***et al***., 1992)**.** It had also been reported that when working with bacterial cells treating the cells with solvents and detergents may give improved conversions (Chan ***et al***., 1991). The oxygen supply plays an important role in aerobic fermentation for the production of dioic acids. An insufficient oxygen supply can lead to suboptimal productivities as well as products of low quality. Various different methods have been tried to increase oxygen supply including modifying reactor designs, using oxygen vectors, increasing oxygen composition or using pure oxygen in the inlet gas (Jiao ***et al***., 2001). These methods retain the basic limitation of gas-liquid oxygen transport. Using ***Candida tropicalis*** CT1-12 Jiao ***et al***., (2001) showed that by adding H_2O_2 oxygen transfer is improved and this leads to an increased product yield. The H_2O_2 is converted to oxygen and water by enzyme catalase available from the culture itself. The oxygen molecule in liquid phase ready for consumption by the cells and the gas-liquid oxygen transport resistance is nullified. They also found that the enhanced dicarboxylic acid production was not only due to the addition of H_2O_2 but that the cytochrome P450 system was also induced. The brassylic acid concentration was increased from 18g/l to 23g/l after 96h by adding 1ml of a 2mM H_2O_2 solution to 50ml bioconversion medium every 3h in shake flasks. The dioic acid yield was improved by 14.7% by regular feeding of H_2O_2, maintaining H_2O_2 concentration at 2mM in a 22L bioreactor.

1.6 Conclusion

It is clear that although a lot of information exists on the degradation of hydrocarbons but that very little is still understood about the different systems involved in the degradation of hydrocarbons. The products that these yeasts are capable of producing from these compounds are varied and can find application in different field of biotechnology including bioremediation, biosurfactant production, production of flavour compounds and the production of single cell proteins. Genetic engineering opens up many possibilities with regard to the optimization of yeast to accumulate different products from hydrocarbons especially with information becoming available from genomic sequencing projects.

CHAPTER TWO

INTRODUCTION TO PRESENT STUDY

The hydrocarbon utilizing yeast ***Yarrowia lipolytica*** has been used in studies in various fields including peroxisome biogenesis, dimorphism and hydrocarbon degradation (Fickers ***et al***., 2005). ***Y. lipolytica*** is a naturally dimorphic fungus, capable of forming yeast cells, pseudohyphae and septated hyphae (Barth & Gaillardin, 1996). In the 1960's interest in this yeast was awakened due to its ability to grow on hydrocarbons and the fact that alkane grown ***Y. lipolytica*** could be used in the production of single cell proteins as well as organic acids such as citric acid (Barth & Gaillardin, 1996). The large scale production of citric acid and single cell proteins by ***Y. lipolytica*** led to the accumulation of large amounts of data on its behaviour in large scale fermentors (Barth & Gaillardin, 1996). ***Y. lipolytica*** strains can be isolated from dairy products such as cheese and yoghurt as well as from meat and other products, rich in lipids. It is regarded as non pathogenic and has been classified as Generally Regarded As Safe (GRAS) by the American Food and Drug Administration (FDA) for citric acid production (Fickers ***et al.,*** 2005)***. Y. lipolytica*** is strictly aerobic and most of its strains can grow in temperatures up to 34^{o}C. The first genetic engineering systems for ***Y.lipolytica*** became available in the 1980s. More recently the complete genome sequence of ***Y. lipolytica*** E150 was determined through the Génolevures Consortium (Fickers ***et al.,*** 2005). A large number of genetically engineered strains, which can be useful for studies on hydrophobic substrate degradation, also became available.

It is often difficult to study the systems involved in the breakdown of hydrophobic substrates in yeasts such as ***Y. lipolytica***. The hydrophobic nature of the substrates makes it difficult to measure consumption. Alkanes are broken down with no or very little product formed in the process, making it difficult to follow product formation. Although some work has been done on the accumulation of dicarboxylic acids in ***C. tropicalis*** with disrupted β-oxidation, very little work had been done on ***Y. lipolytica***. Alkylbenzenes are

degraded to phenylacetate for even numbered chains or benzoate for odd numbered chains (Mauersberger ***et al***., 1996). These products are not further degraded by ***Y***. ***lipolytica*** even with intact β-oxidation, making it possible to follow product formation. It was the aim of this project to investigate the biotransformation of alkanes, alkylbenzenes and their derivatives by different groups of genetically engineered ***Y. lipolytica*** strains to gain more clarity on the systems involved. The strains studied comprised of three groups: (i) ***Y. lipolytica*** with β-oxidation disrupted, (ii) ***Y. lipolytica*** strains with β-oxidation intact and overexpressing both ***CPR*** and ***CYP*** genes that encode cytochrome reductase and a ***Y. lipolytica*** alkane hydroxylase respectively; and (iii) ***Y. lipolytica*** strains with disrupted β-oxidation overexpressing both ***CPR*** and ***CYP*** genes. It has been shown that higher hydroxylase activity in ***Y. lipolytica*** is achieved if the ***YlCPR*** is co-expressed with a ***CYP*** gene (Nthangeni ***et al***., 2004). The different ***CYP*** genes that were co-expressed with the ***YlCPR*** included: ***CYP52F1,*** also designated ***YlALK1,*** coding for a confirmed alkane hydroxylase from ***Y. lipolytica*** with specificity towards both shorter and long chain molecules; ***CYP52F2***, also designated ***YlALK2,*** coding for a confirmed alkane hydroxylase from ***Y. lipolytica*** with specificity towards longer chain molecules; ***CYP557A1*** a putative fatty acid hydroxylase isolated from ***Rhodotorula retinophila*** (Shiningavamwe, 2004).

All strains with β-oxidation disrupted were constructed by M.T. Le Dall and Prof M.S. Smit in the laboratory of Dr. J.-M Nicaud from the Laboratoire de Microbiologie et Génétique Moléculaire, Centre de Biotechnologie Agro-Industrielle, Thiverval-Grignon, France. Strains with β-oxidation intact overexpressing ***CPR*** and ***CYP*** genes were constructed by Dr. A.N. Shiningavamwe and Dr. M.E. Setati in the laboratory of Dr. J. Albertyn in the Department of Microbial Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa.

The first β-oxidation deficient strain constructed from ***Y. lipolytica*** Po1d by deletion of the ***POX2***, ***POX3***, ***POX4*** and ***POX5*** genes had been ***Y. lipolytica*** MTLY37 (Wang ***et al***., 1999). This strain had previously been shown to

accumulate dioic acids from ***n***-alkanes (Smit ***et al***., 2005) and was also used at the beginning of this study.

Two strains, ***Y. lipolytica*** E150 and ***Y. lipolytica*** MTLY66 were used in the construction of the different ***Y. lipolytica*** strains that contained cloned ***CPR*** and ***CYP*** genes. All strains with intact β-oxidation were derived from ***Y. lipolytica*** E150, a laboratory strain from the French inbreeding line (Madzak ***et al***., 2004). This strain contains zeta sequences for homologous recombination of vectors containing zeta elements (Madzak ***et al***., 2004). Strains with disrupted β-oxidation were derived from the ura^-, leu^- auxotroph, ***Y. lipolytica*** MTLY66. This strain had been derived from ***Y. lipolytica*** Po1d, by deletion of the ***POX2***, ***POX3***, ***POX4*** and ***POX5*** genes. The deletions were done in such a way that the ura^- and leu^- markers were recovered (J.M. Nicaud and M.T. Le Dall, personal communication)***. Y. lipolytica*** Po1d is also an ura^-, leu^- auxotroph which had been derived from ***Y. lipolytica*** W29, also a strain from the French inbreeding line. Strains derived from W29 do not contain the zeta sequences of the retrotransposon ***Ylt1*** (Juretzek ***et al***., 2001***;*** Madzak ***et al***., 2002; Pignede ***et al***., 2000)***.*** The presence of the zeta sequences allow for potential targeting sites for homologous integration of vectors containing zeta elements into the yeast genome. A higher number of transformants can be expected when using a strain containing the zeta sequence, than using a strain that does not possess zeta sequences (Pignede ***et al***., 2000).

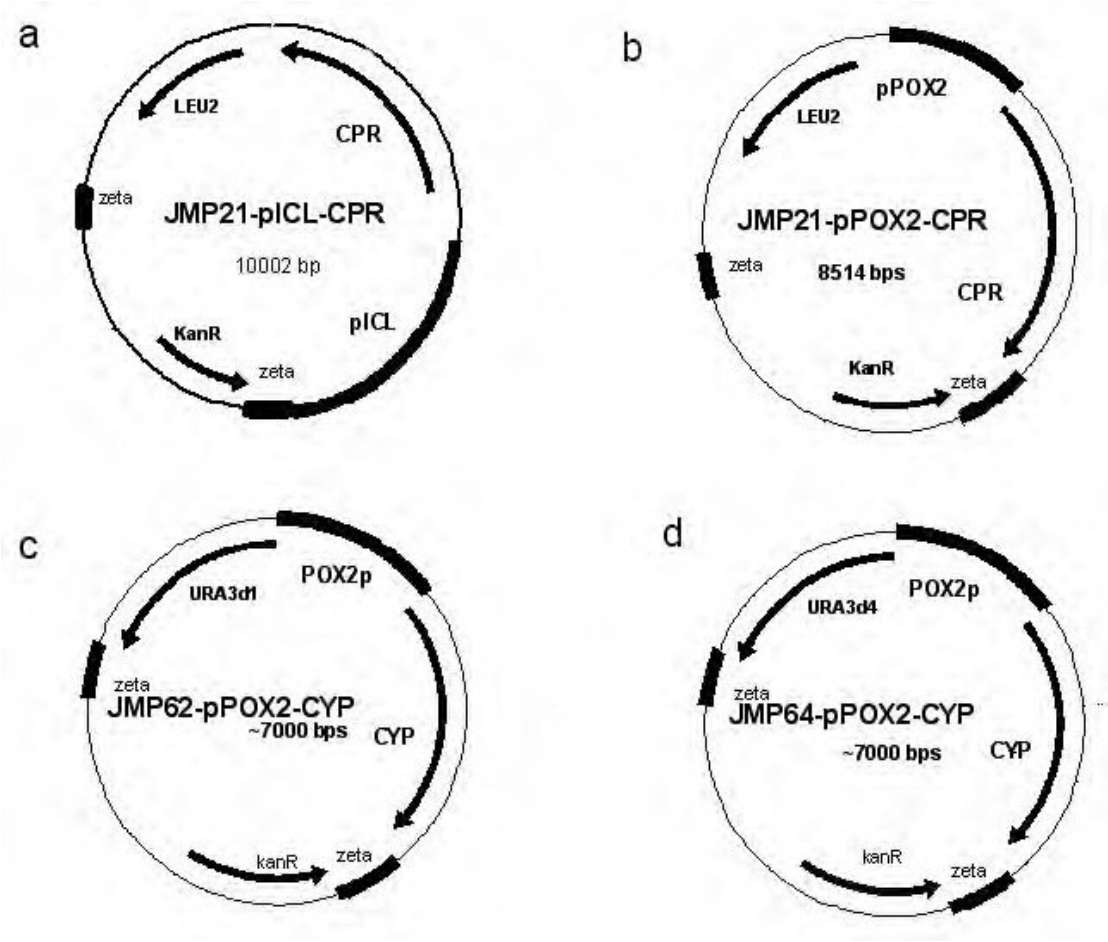


Fig 2.1: Vectors used for construction of ***Y. lipolytica*** strains. Vectors were obtained from the laboratory of J-M. Nicaud from the Laboratoire de Microbiologie et Génétique Moléculaire, Centre de Biotechnologie Agro-Industrielle, Thiverval-Grignon, France.

Four different vectors were used to introduce the genes encoding the reductase and CYP450s into the yeast strains (Fig 2.1). The JMP21 vector with the selective ***LEU2*** marker was used to insert an extra copy of the reductase into ***Y. lipolytica*** E150 under the control the ***ICL1*** promotor, while the JMP21 vector used for insertion into ***Y. lipolytica*** MTLY66 inserted the ***CPR*** under the control of the ***POX2*** promotor. Although transformation with JMP21 is supposed to give single copy integration, because it contains a non defective ***LEU2*** marker, it has been shown that it occasionally yields strains with two copies of the ***CPR*** gene inserted (Nthangeni ***et al***., 2004). Multiple copies of the ***CYP*** genes under the control of the ***POX2*** promotor were introduced into ***Y. lipolytica*** E150 with the JMP64 vector. The vector contained

a defective ura3d4 marker to ensure multiple integrations as well as zeta elements for homologous integration into ***Y. lipolytica*** E150. A single copy of the ***CYP52F1*** gene under control of the ***POX2*** promotor was inserted into ***Y. lipolytica*** MTLY66 with the JMP62 vector. The vector contained the non-defective ura3d1 marker for single copy integration and zeta sequences that in this case gave non homologous integration into ***Y. lipolytica*** MTLY66. The ***ICL1*** promoter can be induced by acetate, ethanol and fatty acids and alkanes and not completely repressed by glucose while the ***POX2*** promotor is induced by alkanes and fatty acids (Juretzek ***et al***., 2000; Juretzek ***et al***., 2001).

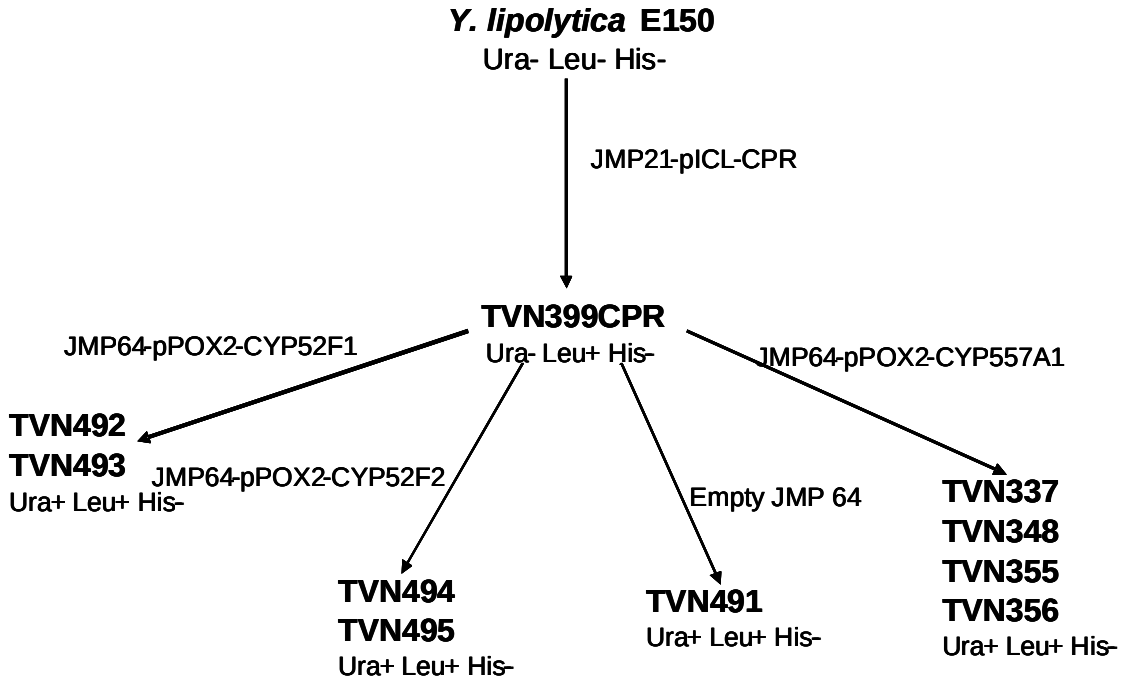


Fig 2.2: Transformation of strains with intact β-oxidation. Diagram (a) depicts the transformation with the ***CPR*** and additional ***ALK*** genes.

To construct the β-oxidation functional ***Y. lipolytica*** strains overexpressing ***CYP52F1***, ***CYP52F2*** and ***CYP557A1***, coding for alkane hydroxylase and a putative fatty acid hydroxylase respectively, a single copy of the reductase was first inserted into ***Y. lipolytica*** E150 with the JMP21 vector (Fig 2.2). Multiple copies of the CYP450s were then inserted using the JMP64 vector.

TVN491 only contained the reductase gene together with an empty JMP64 vector.

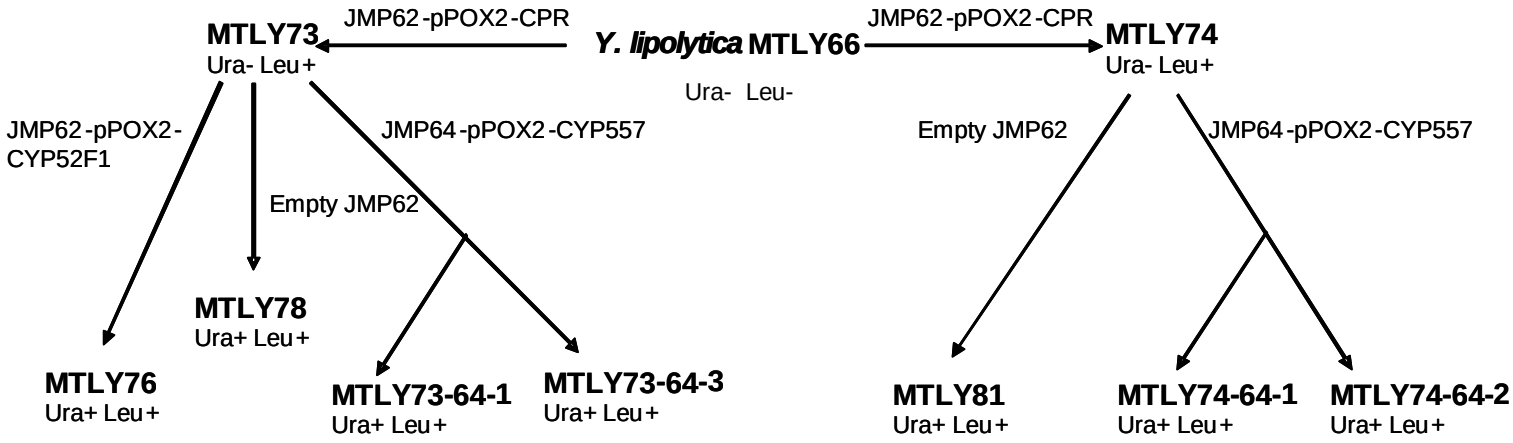


Fig 3.3: Transformation of strains with β-oxidation disrupted.

The strains with deficient β-oxidation and overexpressed CYP450s were constructed in the same manner (Fig 3.3). The strains overexpressing ***CYP52F1*** was transformed with the JMP62 vector for single copy integration and the strains overexpressing ***CYP557A1*** was transformed with the JMP64 vector for multicopy integration.

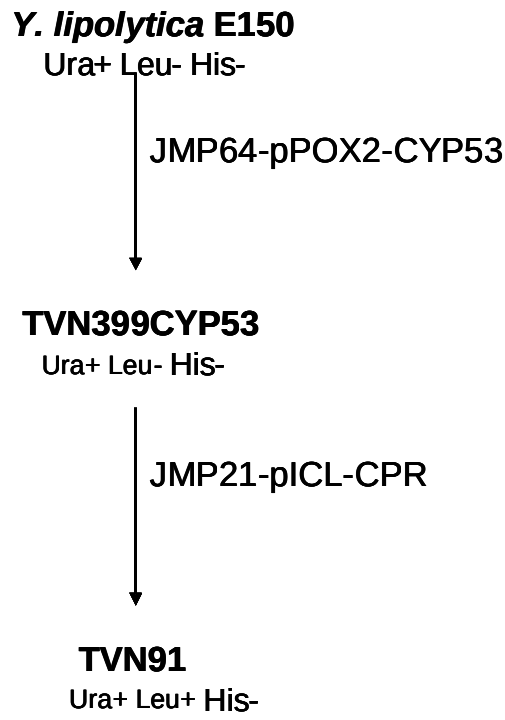


Fig. 3.4: The insertion the ***CYP***53 gene together with a ***CPR*** gene.

A control strain, TVN91 had been constructed by first inserting the ***CYP53*** gene, coding for benzoate ***para*** hydroxylase from ***Rhodotorula minuta***, using the JMP64 vector for multicopy integration into ***Y. lipolytica*** E150 (Fig 3.4). A single copy of the reductase under the control of the ***ICL1*** promoter was then inserted using the JMP21 vector.

Questions that we aimed to investigate using these strains included:

1. Whether mono acids would accumulate from substrates such as undecene and hexylbenzene that can not form dioic acids.
2. Whether branched alkanes would be hydroxylated and whether the product formed would be a dioic acid or possibly a mono acid.
3. Whether overexpression of ***CPR*** and P450s had an effect on alkane hydroxylation in strains with β-oxidation intact or disrupted.
4. Whether it would be possible to distinguish between different cloned P450s using an alkylbenzene as substrate.
5. Whether the time of substrate or inducer addition is important for the activation of the hydroxylase system.

6. Whether an additional co-substrate/ energy source is important for hydroxylase activity.

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 in LN broth (see below) containing glycerol (7% v/v) under liquid N_2 in the MIRCEN yeast culture collection of the University of the Free State, South Africa. Strains were revived by streaking out on YM plates (see below) supplemented with a vitamin solution (1% v/v).

LN broth contained (per 300ml distilled water): 12g glucose, 3g tryptone and 3g yeast nitrogen base (YNB) containing amino acids and ammonium phosphate. YM solid media contained (per liter distilled water): 10g peptone, 10g glucose, 20g malt extract, 3g yeast extract and 20g agar.

Table 3.1: Strains used in this study:

	Additional *CYP* genes inserted			
Strain	***CYP* gene cloned**	**Source**	**Hydroxylase Activity**	β-oxidation
TVN399CPR[1]	no ***CYP***	n.a.	n.a.	Functional
TVN324(CPR) [2]	no ***CYP***	n.a	n.a.	Functional
TVN91[2]	***CYP53***	***Rhodotorula minuta***	benzoate ***para***-hydroxylase	Functional
TVN493[1]	***CYP52F1***	***Yarrowia***	alkane	Functional

		lipolytica	hydroxylase	
TVN495[1]	***CYP52F2***	***Yarrowia lipolytica***	alkane hydroxylase	Functional
MTLY 81(74) [3] (TVN445)	no ***CYP***	n.a.	n.a.	Disrupted
MTLY 78(73) [3] (TVN442)	no ***CYP***	n.a.	n.a.	Disrupted
MTLY 76(73) [3] (TVN440)	***CYP52F1*** (single copy)	***Y. lipolytica***	alkane hydroxylase	Disrupted
TVN337[2] TVN348[2] TVN355[2] TVN356[2]	***CYP557A1*** (multiple copies)	***R. retinophila***	putative fatty acid hydroxylase	Functional
73-64-1[4] (TVN497) 73-64-3[4] (TVN499) 74-64-1[4] (TVN501) 74-64-2[4] (TVN502)	***CYP557A1*** (multiple copies)	***R. retinophila***	putative fatty acid hydroxylase	Disrupted
MTLY 37	no CYP	n.a.	n.a.	Disrupted

1. Strains were constructed by Dr. M.E. Setati in the laboratory of Dr. J. Albertyn in the Department of Microbial, Biochemical and Food biotechnology, University of the Free State, P.O Box 339, Bloemfontein, 9300, South Africa.
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, University of the Free State, P.O Box 339, Bloemfontein, 9300, South Africa.
3. Strains were constructed by M.T. Le Dall in the laboratory of Dr. J.-M Nicaud at the Laboratoire de Microbiologie et Génétique Moléculaire,

INRA CNRS INAP-G, UMR2585, Centre de Biotechnologie Agro-Industrielle, 78850 Thiverval-Grignon, France.

4. Strains were constructed by Prof. M.S. Smit in the laboratory of Dr. J.-M Nicaud at the Laboratoire de Microbiologie et Génétique Moléculaire, INRA CNRS INAP-G, UMR2585, Centre de Biotechnologie Agro-Industrielle, 78850 Thiverval-Grignon, France.

3.1.2 Growth conditions

Cultivation in liquid media was, unless stated otherwise, performed with 25ml YP broth (see below) in 250ml normal Erlenmeyer flasks for pre cultures and 50ml YP broth in 500ml normal Erlenmeyer flasks in the case of main cultures on a rotary shaker at 180rpm at 25^0C. Shake-flasks were inoculated with 24h YPD_2 cultures.

YP broth contained (per liter distilled water): 10g yeast extract (Merck) and 10g peptone (Merck). YPD, YPD_2 and YPD_4 contained per liter 10, 20 and 40g glucose, respectively. YP_2D_2 contained per liter 20g peptone and 20g glucose.

3.1.3 Turbidemetric measurements

Culture samples (500µl) were added to 200µl 5M NaOH in 1.5ml microcentrifuge tubes, vortexed for 5min and centrifuged at 10 000 x g for 10 min. The supernatants were discarded and the pellets were resuspended in physiological saline (500µl). The turbidity of samples (200µl) suitably diluted before transfer to a microtitre plate, were measured at 620nm using a Labsystems iEMS reader MF (Thermo BioAnalysis company, Helsinki Finland). Samples were not washed with cyclohexane because the residual hydrocarbon still present in the samples did not appear to be problematic.

3.1.4 Dry weight measurements

Cyclohexane (2ml) and 5M NaOH (400µl) were added to samples (4ml) of broth in test tubes, vortexed for 5min and then filtered under vacuum through dried and pre-weighed glass fibre filters (GF52 47MM BX200; Schleicher and Schuell). The biomass on the filter was washed with a mixture of distilled water (4ml), cyclohexane (2ml) and 5 M NaOH (400µl) followed by washing with distilled water (26ml). Biomass on filters were dried overnight in an oven (100^0C), and then cooled in a dessicator before weighing.

3.1.5 Extraction and analysis

Samples (500µl) were taken at regular intervals and acidified to a pH of 3 by addition of 1M HCL (50µl). The samples were extracted twice with 300µl Tertiary-butyl-methyl-ether (Fluka) containing myristic acid (0.1% w/v)
(The British Drughouses) as internal standard and the phases separated by centrifugation (10 000 x g for 10min). The extracts were combined. Samples of the extracts (50µl) were transferred to new 1.5ml microcentrifuge tubes and methylated with trimethylsulfonium hydroxide (50µl).

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

GC-MS (Gas Chromatography-Mass Spectography) analysis of methylated samples was carried out on a Finnigan Trace Ultra and the MS analysis on a Finnigan DSQ run on full scam. The GC was equipped with a HP 5 (Hewlett Packard) column measuring 60m x 0.32mm x 0.25µm. The carrier mode used was defined as constant flow with a split ratio of 1:40. The inlet temperature was 235^oC. The initial oven temperature was 70^oC held for 3

minutes. The temperature was increased by 10^{o}C per minute until the final oven temperature of 300^{o}C was reached and held for 20 minutes.

Glucose concentration was determined using HPLC (High Pressure Liquid Chromatography). Samples (1ml) were taken at regular intervals and filtered using a 0.45µm nylon filter (Uniflo). The analysis was carried out on a Waters Breeze with differential refractive index detector HPLC system, using a Waters sugarpack1 300x7.8mm at 84^{o}C. Mobile phase was deionised water at 84^{0}C at 0.5ml/min. Filtered samples (20µl) were injected into the system. Quantification was done to a pre-determined standard curve of glucose.

For TLC (Thin Layer Chromatography) analysis samples were spotted onto Alugram Sil G/UV$_{245}$ TLC plates (Machery-Nagel) containing a fluorescent indicator. Plates were developed using a mobile phase consisting of Di-n-butyl-ether (Merck), formic acid (Merck) and water in a 90:7:3 ratio. Plates were visualized under short wavelength UV-light or stained using a Bromocresol green stain consisting of 0.1g Bromocresolgreen (Fluka), Ethanol (500ml) and 0.1M NaOH (5ml). Before staining with bromocresolgreen, plates were heated in an oven at 110^{o}C for 10-15 minutes. After staining plates were again heated at 110^{o}C until spots developed.

3.2 Part B:

3.2.1 Biotransformation of dodecane by the β-oxidation disrupted Y*. lipolytica* MTLY37 strain

To determine the effect glucose concentration and time of substrate addition, might have on the production of dioic acid, ***Y. lipolytica*** MTLY37 (β-oxidation disrupted) was cultured in YPD broth containing either 2% or 4% glucose. After 24h and 48h dodecane (3% v/v) was added as substrate. No buffer was added to the media and pH was adjusted to 8 with 1M NaOH and then measured periodically and maintained. The optical density was measured

every 12h for 48h and then every 24h up to 144h. Samples were taken at regular intervals for GC analysis.

3.2.2 Biotransformation of undecane, undecene, 5-methyl-undecane and hexylbenzene by *Y. lipolytica* MTLY37

To investigate the possible biotransformation of the compounds undecane, undecene, 5-methyl-undecane and hexylbenzene, 24h old pre cultures (1% v/v) incubated in YPD_2 broth was inoculated into Erlenmeyer flasks containing YPD_4 broth. The flasks were incubated for 48h on a rotary shaker at 25^{o}C after which the different substrates (500µl) were added to each flask. Samples to monitor growth and for extraction was taken at regular intervals. Samples for dry weight measurements were taken at the end of the experiment. Analysis was done with GC.

3.2.3 Possible toxic effect of alkylbenzenes in the β-oxidation disrupted strain *Y. lipolytica* MTLY37

To test the effect of toxicity of different chain lengths of alkylbenzenes the ***Y. lipolytica*** strain MTLY37 was used. Four different alkylbenzenes namely hexylbenzene, dodecylbenzene, ethylbenzene and butylbenzene was used in the study. The yeast was grown in YP broth supplemented with 4% (w/v) glucose. The alkylbenzenes were added in concentrations of 0.1, 0.5 and 1% (v/v) when the yeast reached mid exponential growth phase, approximately after 15h. Growth was followed and biomass was done at end of experiment after 144h.

3.2.4 Determination of time of addition of inducer and substrate in strains expressing *CPR* and *CYPs*

To determine the best time for the addition of the inducer and the addition of substrate ***Y. lipolytica*** TVN493 was incubated for 24h in pre culture consisting of YPD_2 broth. The main culture consisting of YP_2D_2 broth was inoculated to an OD of 0.2. The growth was measured periodically using optical density and samples were taken for glucose analysis.

3.2.5 Biotransformation of various substrates by *Y. lipolytica* strains containing additional *CPR* and *CYP* genes

A basic method was devised for all subsequent experiments based on the results of the previous experiments. Pre cultures consisting of YPD_2 broth were incubated for 36h on a rotary shaker at 25^{o}C. Erlenmeyer flasks containing YP_2D_2 broth buffered with 50mM phosphate buffer, pH8 (1% v/v) were inoculated from the pre culture flasks to an optical density of 0.2. Various inducers (500µl in the case of liquids and 0.5g in the case of solids) were added to each flask after 24h of incubation. After a further 18h of incubation the different substrates (500µl in the case of liquids and 0.5g in the case of solids) were added to each flask. When 4-hexylbenzoic acid was used as substrate only 50µl was added due to possible toxicity of the substrate towards the yeast strain. Samples were taken at regular intervals for turbidity measurements, analysis of glucose, product formation, substrate utilization and dry weight determination.

3.2.5.1 Biotransformation of hexylbenzene by *Y. lipolytica* strains with functional β-oxidation and additional *CPR* and *CYP* genes

Yeast strains used in the study included ***YlALK***1 and ***ALK***2 strains, ***Y. lipolytica*** TVN399CPR and Y. lipolytica TVN91. Hexylbenzene was used as substrate. Oleic acid was used as inducer.

3.2.5.2 The effect of different inducers on the biotransformation of hexylbenzene by *Y. lipolytica* strains with functional β-oxidation and additional *CPR* and *CYP* genes

The strains used to conduct the experiment include ***Y. lipolytica*** TVN91 and TVN493. Hexylbenzene was used as substrate. Compounds used as possible inducers included dodecane, oleic acid, ethanol, glucose and stearic acid. One flask contained no inducer.

3.2.5.3 The biotransformation of hexylbenzene by *Y. lipolytica* strains with functional and disrupted β-oxidation containing additional *CPR* and *CYP* genes

The yeast strains TVN493, MTLY78 and MTLY76 were selected to compare transformation of hexylbenzene by strains with functional β-oxidation (TVN493) and disrupted β-oxidation (MTLY78 and 76). Stearic acid and ethanol were both used as inducers in the study.

3.2.5.4 Biotransformation of 5-methylundecane by *Y. lipolytica* strains with intact and disrupted β-oxidation containing additional *CPR* and *CYP52F1* genes

The Yeast strains TVN493, MTLY78 and MTLY76 was selected for use in this study. Stearic acid, oleic acid and ethanol were used as inducers in the study. 5-Methylundecane was added to all flasks as substrate.

3.2.5.5 Biotransformation of decylbenzene by *Y. lipolytica* strains with intact and disrupted β-oxidation containing additional *CPR* and *CYP* genes

The following ***Y. lipolytica*** strains were used TVN493, TVN495, TVN91, TVN356, TVN348, MTLY73-64-3, MTLY73-64-1, MTLY76 and MTLY78. Ethanol was used as inducer and decylbenzene was used as substrate. Ethanol and decylbenzene was added at the same time after 24h of incubation.

3.2.5.6 Biotransformation of 4-hexylbenzoic acid by *Y. lipolytica* strains with disrupted β-oxidation containing additional *CPR* and *CYP557A1* genes

The yeast strains ***Y. lipolytica*** MTLY73-64-1, MTLY73-64-3, MTLY78 and MTLY81 were used to investigate the transformation of 4-hexylbenzoic acid (0.1% v/v). Ethanol (1% v/v) was added as inducer.

3.2.5.7 Biotransformation of stearic acid by *Y. lipolytica* strains with disrupted β-oxidation containing additional *CPR* and *CYP557A1* genes

The yeast strains ***Y. lipolytica*** MTLY73-64-3 and MTLY78 were used to investigate the transformation of stearic acid (1% w/v). Ethanol (1% v/v) was added as inducer.

CHAPTER FOUR
RESULTS

4.1 Biotransformation of undecane, undecene, 5-methyl-undecane and hexylbenzene by the β-oxidation disrupted strain *Y. lipolytica* MTLY37

The blocking of the β-oxidation pathway results in the formation of dioic acids from ***n***-alkanes (Picataggio ***et al***., 1992). There are no reports in the literature on the biotransformation of substrates such as 1-alkenes and hexylbenzenes that can not be hydroxylated at both ends, by yeasts with disrupted β-oxidation. In a first series of experiments ***Y. lipolytica*** MTLY37, a quadruple ***POX2-POX3-POX4-POX5***-deleted mutant, was used for the biotransformation of dodecane, undecane, undecene, 5-methyl undecane and hexylbenzene. The substrates (1% v/v ≈ 40 – 50mM) were added to cultures which had been grown for 48h in YPD_4 broth. After 48h these cultures had reached stationary phase and the biomass was ca. 17 – 20g dry wt l^{-1}. Dodecanedioic acid accumulated from dodecane (Fig. 4.1). In the case of undecane and 5-methyl undecane we did not have the corresponding dioic acids available as standards, but acid products with GC retention times (16.6 and 17.0min, respectively), similar to that of dodecanedioic acid (19.4min), accumulated (Fig. 4.2 and Fig. 4.3). We assumed that these products were the corresponding dioic acids. In the case of undecene no acid product accumulated to a comparable extent. In all three cases the substrates disappeared with time, despite the fact that ***Y. lipolytica*** MTLY37 is a β-oxidation disrupted strain that is according to Wang ***et al***., (1999) unable to grow on oleic acid.

If hexylbenzene was to be degraded via β-oxidation, phenylacetic acid would accumulate. If it was only hydroxylated and oxidized to the corresponding in tact monocarboxylic acid, phenylhexanoic acid was expected to accumulate.

We had a standard of phenylacetic acid, but no standard of phenylhexanoic acid. Very little phenylacetic acid accumulated, but a product with a GC retention time (14.1min) just shorter than that of myristic acid (tetradecanoic acid) (14.5min) accumulated (Fig. 4.4). The hexylbenzene did not disappear from the culture.

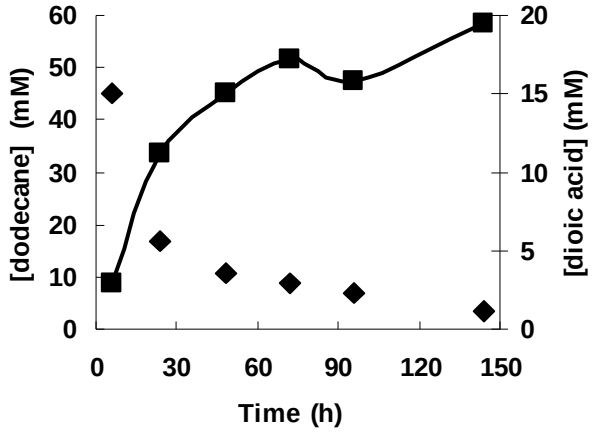


Fig. 4.1: The biotransformation of dodecane (132mM, 30g/l) (♦) by ***Y. lipolytica*** MTLY37 and the formation of the corresponding dioic acid (■). Time 0h indicates the moment when the substrate was added.

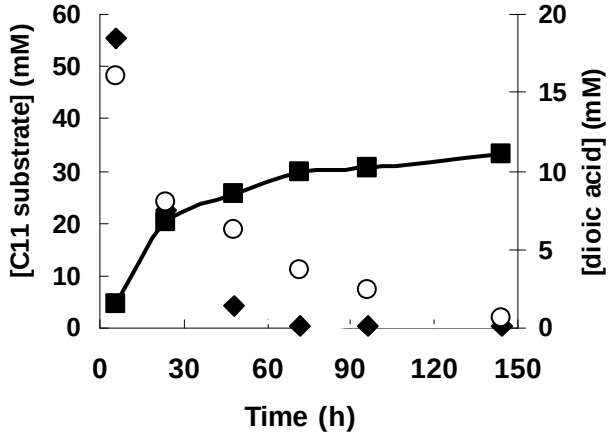


Fig. 4.2: The biotransformation of undecane (58mM, 10g/l) (○) and undecene (58mM, 10g/l) (♦) by ***Y. lipolytica*** MTLY37 and the formation of corresponding dioic acid (■) from undecane. Time 0h indicates the moment when the substrate was added.

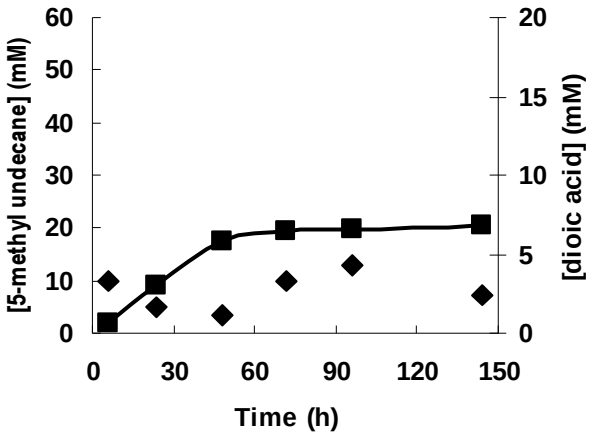


Fig. 4.3: Biotransformation of 5-methyl undecane (58mM, 10g/l) (♦) by ***Y. lipolytica*** MTLY37 and the formation of the corresponding dioic acid (■). Time 0h indicates the moment when the substrate was added.

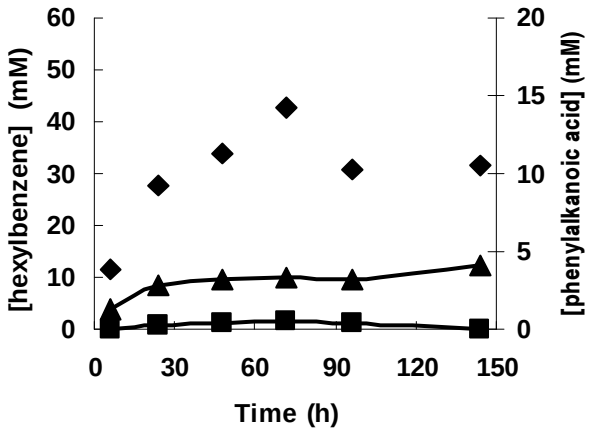


Fig. 4.4: The biotransformation of hexylbenzene (52mM, 10g/l) (♦) by ***Y. lipolytica*** MTLY37 and the formation of phenylhexanoic acid (▲) and phenylacetate (■). Time 0h indicates the moment when the substrate was added.

4.2 Effect of glucose concentration and time of substrate addition on the biotransformation of dodecane by *Y. lipoytica* MTLY37

In this experiment ***Y. lipolytica*** MLTY37 was grown in YPD_2 and YPD_4 broth containing 2% w/v and 4% w/v glucose, respectively. Dodecane (3% v/v = 131mM) was added after 24h and 48h. OD measurements showed that biomass production in the culture with 2% w/v glucose reached a maximum after ca. 24h (OD_{620} 11), while it peaked after ca. 36h in the culture containing 4% glucose (OD_{620} 16) (results not shown). pH was carefully controlled at 7.5 – 8 by the addition of 1M NaOH.

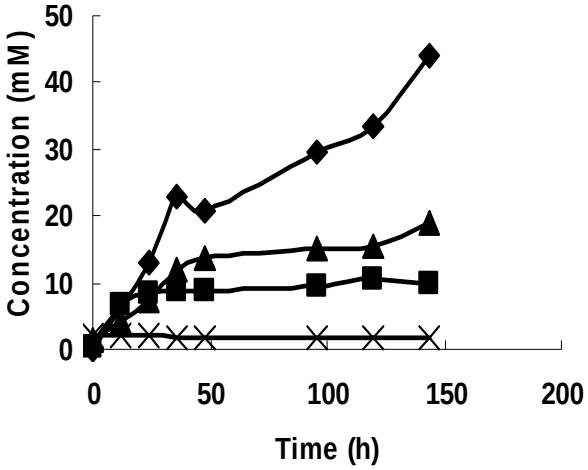


Fig. 4.5: The formation of dicarboxylic acid by ***Y. lipolytica*** MTLY37 with different substrate addition intervals and different glucose concentrations. The graph shows substrate addition after 24h with 4% glucose in media (♦), substrate addition after 24h with 2% glucose in media (■), substrate addition after 48h with 4% glucose in media (▲) and substrate addition after 48h with 2% glucose in media (x). Time 0h indicates the moment when the substrate was added.

In all four cases dioic acid was accumulated in varying concentrations (Fig. 4.5). The highest concentration of dioic acid (44mM) accumulated in the flask with 4% glucose when substrate was added after 24h, while the lowest concentration of dioic acid (1.8mM) was accumulated in the flask with 2% w/v

glucose and substrate added after 48h (Fig. 4.5). In the YPD_2 cultures dodecanedioic acid was apparently consumed, since dodecane concentrations progressively decreased over time, as observed in previous experiments.

The results suggest that glucose concentration and the time of substrate addition plays an important role in the accumulation of dioic acids in the β-oxidation blocked ***Y. lipolytica*** MTLY37. It appears that the optimal glucose concentration for this strain is 4% w/v and the best time to add the substrate is after 24h while there was probably still some glucose left in the media.

4.3 Possible toxic effect of alkylbenzenes in the β-oxidation disrupted strain *Y. lipolytica* MTLY37

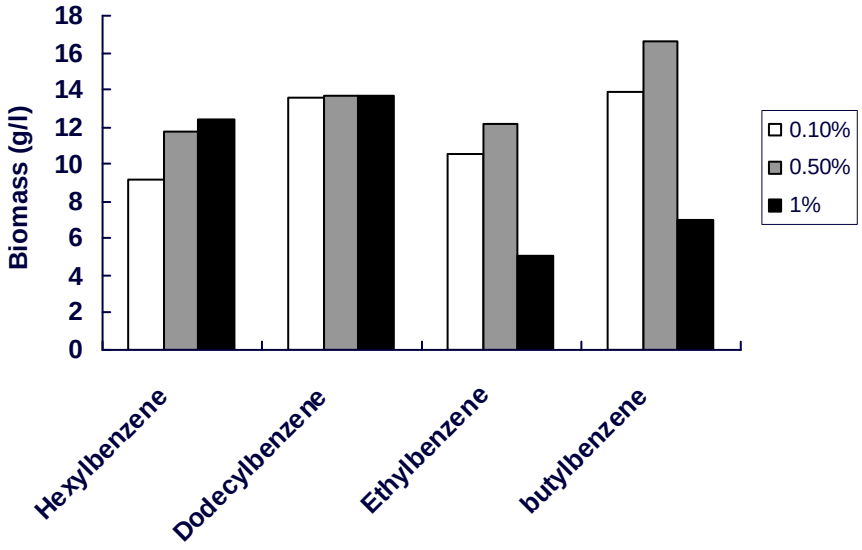


Fig. 4.6: Biomass of ***Y. lipolytica*** MTLY37 after 144h with different concentrations of alkylbenzenes added to YPD_4 cultures after 15h.

Although it had been shown that yeast are capable of utilizing alkylbenzenes, it had also been shown to have an inhibitory effect on growth in the yeast ***C.***

maltosa (Mauersberger ***et al***., 1996). To determine the ability of ***Y. lipolytica*** to tolerate alkylbenzenes, four alkylbenzenes of different chain lengths were added at different concentrations to YPD_4 cultures grown for 15h.

The biomass measurements indicated that both ethylbenzene and butylbenzene are toxic at 1% (v/v) with very little biomass formed, (5.1g dry wt l^{-1} and 7g dry wt l^{-1} respectively) compared to other cultures (12 – 16g dry wt l^{-1}) (Fig 4.6). At concentrations of 1% (v/v) hexylbenzene or dodecylbenzene had no significant effect. Based on these results it was concluded that hexylbenzene is not toxic and that toxicity was probably not the reason why so little phenylhexanoic acid and no phenylacetic acid had been formed from hexylbenzene by ***Y. lipolytica*** MTLY37. It was also decided that hexylbenzene will be a good substrate to use in further experiments.

4.4 Determination of time of addition of inducer and substrate in strains expressing *CYP*s

According to literature glucose represses the ***POX2*** promoter while the ***ICL1*** promoter is only weakly induced in the presence of glucose (Juretzek ***et al***., 2000). If the ***POX2*** promotor is used for the expression of ***CYP*** genes, it is necessary to wait for the glucose in the media to be sufficiently depleted. Once the glucose had been depleted to a level, where it no longer suppresses the ***POX2*** promotor, the inducers could be added. To ensure that the inducer and the substrate was added at the appropriate time, the growth of the yeast strain ***Y. lipolytica*** TVN493, containing additional copies of the ***YlALK1*** gene, was followed spectrophotometrically and the glucose utilization of the strain was followed by HPLC analysis.

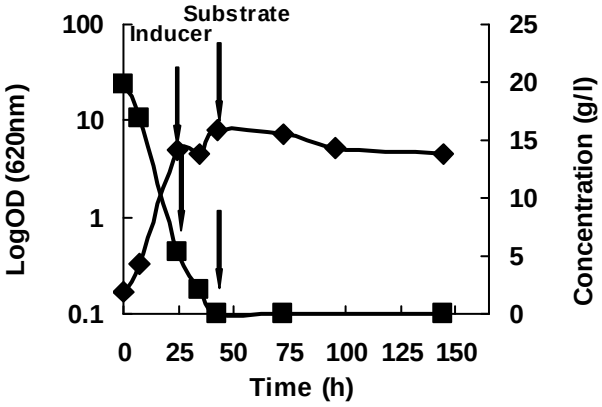


Fig. 4.7: Growth of ***Y. lipolytica*** TVN493 (♦) and glucose utilization ***Y. lipolytica*** TVN493 (■). Time 0h indicates time of inoculation.

From the results it appeared that the glucose level dropped to approximately 5g dry wt l^{-1} after 24h and glucose was fully utilized after 48h (Fig. 4.7). It was decided to add inducer after 24h when the glucose had not yet been fully depleted, but the concentration was sufficiently low so as not to cause any inhibition of the promoters. The substrate could then be added approximately 18 to 24h later when the glucose had been fully utilized and enough time had passed for induction to take place. After 48h growth on YP_2D_2 medium ***Y. lipolytica*** TVN493 normally produced 10 - 12g dry wt biomass $.l^{-1}$.

4.5 Production of phenylacetic acid by *Y. lipolytica* strains with functional β-oxidation and overexpressed *CYP52* genes

The overexpression of the ***CYP52*** genes in ***Y. lipolytica*** may also result in higher production of dioic acids and other compounds, formed from the utilization of hydrocarbons by this yeast. To validate this theory in strains with intact β-oxidation, two strains of ***Y. lipoltyica***, TVN493 expressing ***CYP52F1*** (***YlALK1***) and TVN495 expressing ***CYP52F2*** (***YlALK2***) were used. Two other strains, ***Y. lipolytica*** TVN91 expressing ***CYP53*** and ***Y. lipolytica*** TVN491 expressing only the ***CPR*** gene were included in the study as control strains.

Using hexylbenzene (1% v/v ≈ 52mM) as substrate the production of phenylacetic acid, was monitored.

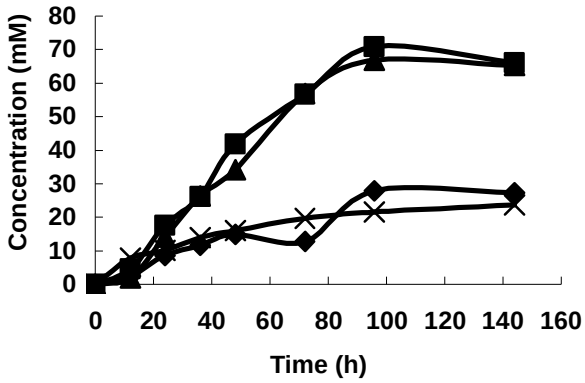


Fig 4.8: Production of phenylacetic acid by ***Y. lipolytica*** strains TVN491 (♦), TVN493 (■), TVN495 (▲) and TVN91 (x), with hexylbenzene (52mM, 10g/l) as substrate and oleic acid (36mM, 10g/l) as inducer. Time 0h indicates the moment when the substrate was added.

Results indicate that both ***Y. lipolytica*** strains with overexpressed ***ALK*** genes produced close to the same amount of phenylacetic acid, in the region of 65 to 66mM (Fig 4.8). Although it has been shown that ***CYP52F1*** functions to assimilate shorter chain molecules, and ***CYP52F2*** for the assimilation of longer chain molecules, the effect could not be observed in this experiment (Iida ***et al***., 2000). The reason for this being although ***CYP52F1*** is required for assimilation of shorter chain molecules, it is also capable of assimilating, longer molecules. The native ***ALK*** genes existing in the yeast strain probably also remained active contributing to the total activity of the ***ALK*** genes. Thus the other two yeast strains included in the study, TVN491 and TVN91 also produced phenylacetic acid, although at lower concentrations of 23 and 27mM respectively.

TVN91 was included in the study because instead of an overexpressed ***ALK*** gene, the gene coding for benzoate ***para*** hydroxylase was overexpressed in this yeast. As expected the yeast did not transform hexylbenzene as efficiently as the strains with ***ALK*** genes overexpressed.

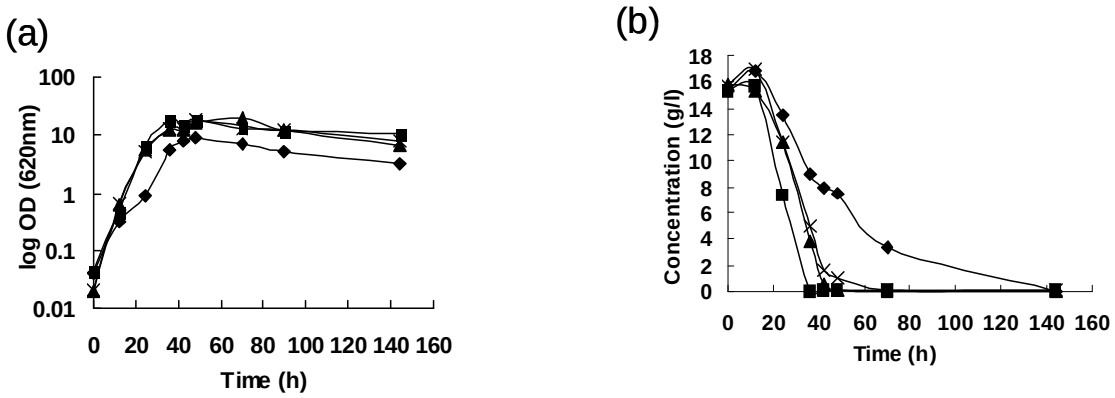


Fig. 4.9: Growth (a) and glucose utilization (b) of ***Y. lipolytica*** strains TVN491 (♦), TVN493 (▲), TVN495 (x) and TVN91 (■). Time 0h indicates time of inoculation.

TVN491 was used as a control strain since it only contained the additional ***CPR*** gene. Although it did not produce high amounts of phenylacetic acid, the results may be deceptive. TVN491 (Fig. 4.9) grew slower than the other yeasts used in the study and also consumed glucose at a much slower rate. It might be that the phenylacetic acid production in this strain could have been slightly higher if it had the same growth rate and glucose utilization rate as the other strains.

4.6 The effect of different inducers in the production of phenylacetic acid in *Y. lipolytica* strains with functional β-oxidation and overexpressed *CYP52* genes

The ***ICL1*** and ***POX2*** promoters used in the expression of the ***CPR*** and ***CYP52*** genes as well as the promoters of the native ***ALK*** genes can be induced or

repressed by different compounds. To study the effect inducers (or repressors) have, ***Y. lipolytica*** TVN493 and ***Y. lipolytica*** TVN91 was tested using 5 different induction conditions. Hexylbenzene was used as substrate.

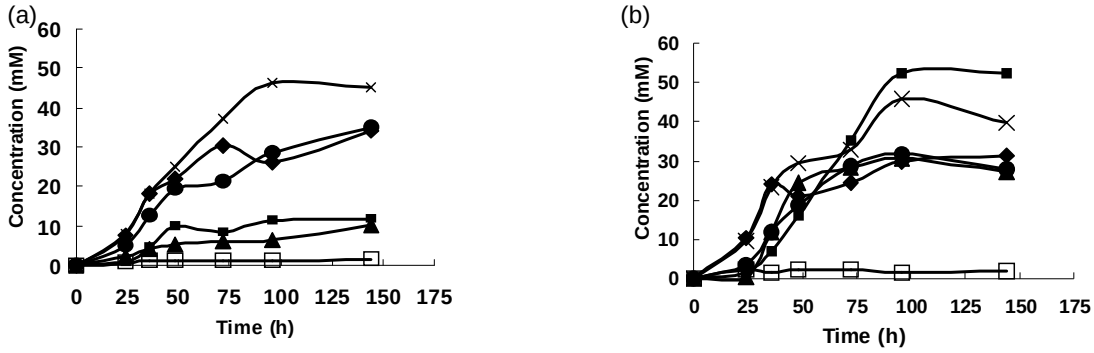


Fig. 4.10: Formation of phenylacetic acid by ***Y. lipolytica*** TVN91 (a) and TVN493 (b) with possible inducers oleic acid (36mM, 10g/l) (♦), ethanol (217mM, 10g/l) (■), glucose (55mM, 10g/l) (▲), dodecane (58mM, 10g/l) (x), stearic acid (35mM, 10g/l) (●) and no inducer (□).Time 0h indicates the moment when the substrate was added.

In ***Y. lipolytica*** TVN493 (Fig. 4.10), phenylacetic acid production was highest (52mM after 144h) with ethanol as inducer. It took longer for product accumulation to start, probably because ethanol acted as inducer of the ***ICL1*** promotor driving expression of the ***CPR*** gene, but repressed the ***POX2*** promoter that was driving the ***CYP*** gene expression (Juretzek ***et al***., 2001; Juretzek ***et al***., 2000). The intermediates formed from the transformation of the hexylbenzene probably acted as inducers for the ***POX2*** promotor, once the ethanol had been utilized. In the strain TVN91, the same high production of phenylacetic acid with ethanol as inducer was not seen. In this strain the highest concentration of phenylacetic acid produced with ethanol as inducer was 11.8mM after 144h. This strain overexpresses the ***CYP53*** gene, which has no alkane hydroxylase activity. However, native ***ALK*** genes remain active.

A possible explanation for the high production in the TVN493 strain and the low production in the TVN91 strain may be that while ethanol induces the ***ICL1*** promoter, hexylbenzene and its degradation products does not induce the native ***ALK*** genes in TVN91 to the same extent as it induces the ***POX2*** promoter., When dodecane was used as inducer high concentrations of phenylacetic acid was produced in both the TVN91 and TVN493 strains with 45mM and 39mM formed respectively after 144h. Dodecane activates both the ***ICL1*** and ***POX2*** promoters as well as the ***ALK*** promotors (Juretzek ***et al***., 2001; Juretzek ***et al***., 2000). The concentration of phenylacetic acid produced when the fatty acids, oleic and stearic acid were used as inducers were 34mM and 35mM after 144h in TVN91 and 31mM and 27.9mM after 144h in TVN493. As had been reported these two fatty acids induce the ***POX2*** promotor as well as the ***ALK*** promoters (Juretzek ***et al***., 2001; Juretzek ***et al***., 2000). This result was different from the result obtained in the previous experiment, possibly indicating that the exact moment of addition of inducers and substrates can be critical for the induction of the cloned genes.

Although it had been reported that glucose not induce or repress the ***ICL1*** promotor, phenylacetic acid production was close to the same level (27mM after 144h) as when fatty acids were used as inducers in the TVN493 strain (Juretzek ***et al***.,2001; Juretzek ***et al***., 2000). Glucose represses the ***POX2*** promoter, and thus delayed the onset of phenylacetic acid production. In ***Y. lipolytica*** TVN91 phenylacetic acid production was low (10.4mM after 144h) when glucose was used as “inducer” again because hexylbenzene and its degradation products could not induce the native ***ALK*** genes. In both cases the phenylacetic acid production was very low when no inducer was added, indicating that additional energy supplied by the ethanol and glucose was necessary for expression and activity of the alkane hydroxylases.

Given the above results it was decided that ethanol was the best inducer/co-substrate to use in future experiments, since it allowed for the best distinction between cloned and endogenous alkane hydroxylases.

4.7 The biotransformation of hexylbenzene by *Y. lipolytica* TVN493, MTLY78 and MTLY76

After evaluating the biotransformation of hydrocarbons in a strain with blocked β-oxidation and in strains that had a functional β-oxidation system but additional ***CPR*** and ***CYP52*** genes, the possibility of hydrocarbon biotransformation in ***Y. lipolytica*** strains that had a blocked β-oxidation system as well as additional ***CPR*** and ***CYP52*** genes, was investigated. The ***Y. lipolytica*** strain MTLY76 (β-oxidation blocked, with additional ***CPR*** and ***CYP52*** genes) was used together with ***Y. lipolytica*** TVN493 (functional β-oxidation with additional ***CPR*** and ***CYP52*** genes) and ***Y. lipolytica*** MTLY78 (β-oxidation blocked, with additional ***CPR***) as control strains. The substrate used to investigate this strain was hexylbenzene. Ethanol and stearic acid were used as inducers, because the ***CPR*** gene in the strains MTLY76 and MTLY78 were also under the ***POX2*** promoter.

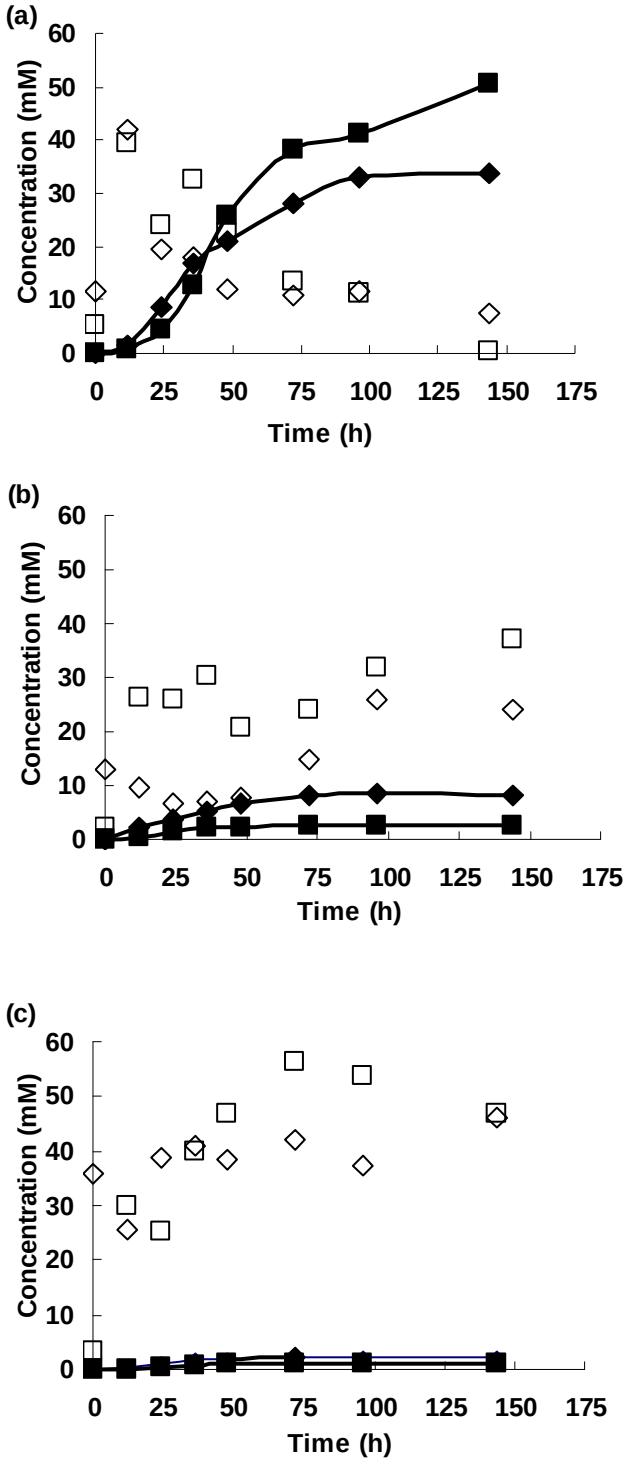


Fig. 4.11: Biotransformation of hexylbenzene (52mM, 10g/l) by ***Y. lipolytica*** TVN493 (a), MTLY78 (b) and MTLY76 (c). Utilization of hexylbenzene (◊, □) and formation of phenylacetic acid (♦, ■) for stearic acid (35mM, 10g/l)

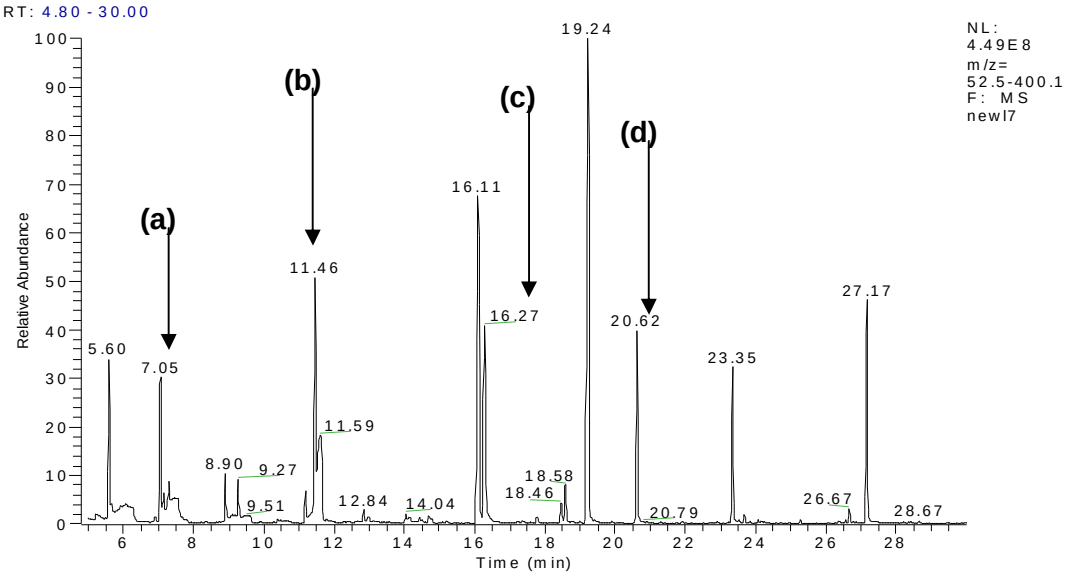
(diamonds) and ethanol (217mM, 10g/l) (squares), as inducers. Time 0h indicates the moment when the substrate was added.

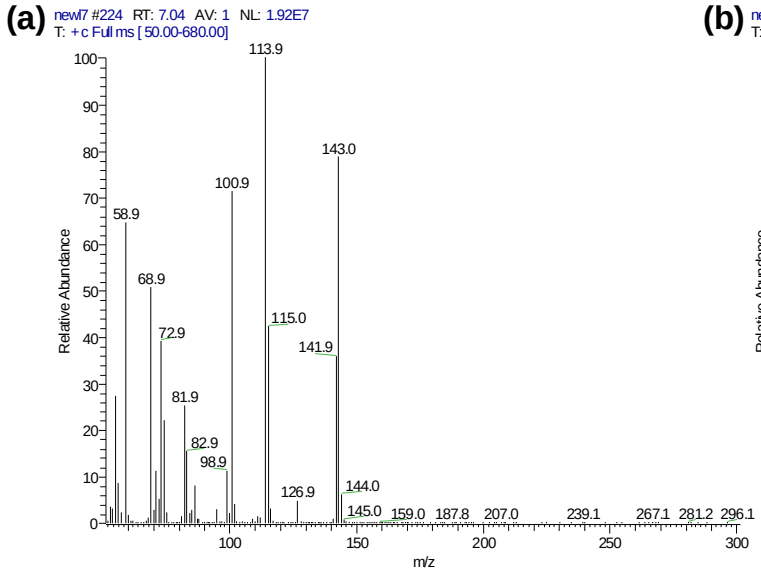
Y. lipolytica TVN493 produced 50.5mM phenylacetic acid after 144h when ethanol was used as inducer and 33.5mM phenylacetic acid after 144h when stearic acid was used as inducer (Fig. 4.11). These results were in accordance with the previous results where ethanol proved to be the better inducer even though phenylacetic acid accumulation takes slightly longer.

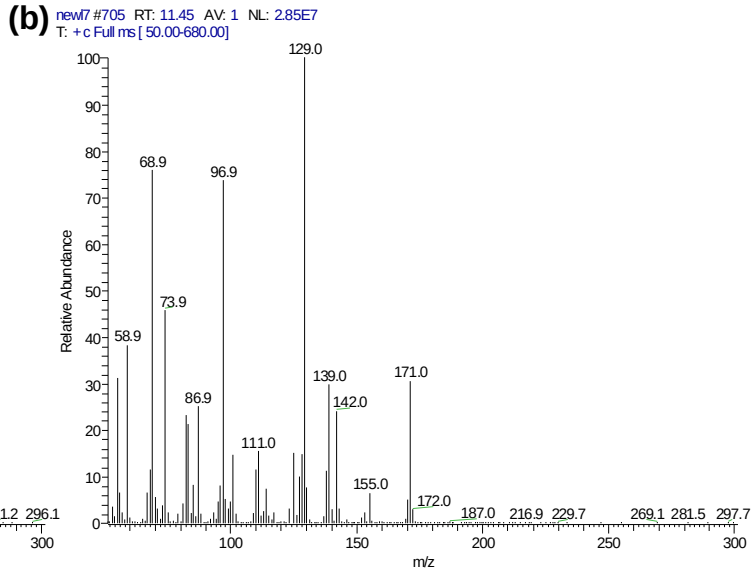
In the case of the strains with disrupted β-oxidation we had expected the accumulation of phenylhexanoic acid, as previously observed with MLTY37. However, no phenylhexanoic acid could be observed with GC and GC-MS. However, phenylacetic acid accumulated, although not in great amounts. ***Y. lipolytica*** MTLY78 produced 2.5mM and 8.3mM of phenylacetic acid after 144h with ethanol and stearic acid as respective inducers. This strain has no ***ALK*** gene overexpressed, also the ***CPR*** gene was under the control of the ***POX2*** promotor, not the ***ICL1*** promotor as in the previous cases. This may explain the slightly better performance of stearic acid as inducer as it induced the ***POX2*** promotor as well as activating the native ***ALK*** genes. In ***Y. lipolytica*** MTLY76 the phenylacetic acid production was also weak in both cases. In this strain only a single copy of the ***ALK1*** gene was inserted and both the ***CPR*** and ***ALK1*** genes were under the control of the ***POX2*** promotor. The type of inducer did not seem to play a role in the strain and the expression of only a single additional copy of the ***ALK*** gene was not enough to accumulate a reasonable amount of product.

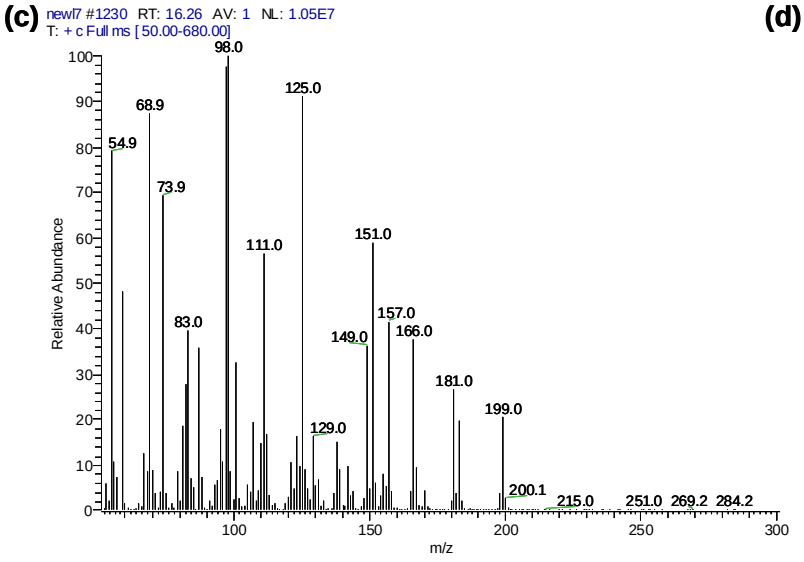
4.8 Biotransformation of 5-methyl undecane by strains with intact and disrupted β-oxidation expressing *CYP52F1*

In the next experiment 5-methyl undecane was used as substrate and the study was carried out using ethanol as the only inducer. ***Y. lipolytica*** TVN493, MTLY78 and MTLY76 were again tested.


RT: 4.80 - 30.00
NL:
4.49E8
m/z=
52.5-400.1
F: MS
newl7
Relative Abundance
Time (min)
(a)
(b)
(c)
(d)
5.60
7.05
8.90
9.27
9.51
11.46
11.59
12.84
14.04
16.11
16.27
18.46
18.58
19.24
20.62
20.79
23.35
26.67
27.17
28.67


(a) newl7 #224 RT: 7.04 AV: 1 NL: 1.92E7
T: + c Full ms [50.00-680.00]
Relative Abundance
m/z
113.9
143.0
100.9
58.9
68.9
115.0
72.9
141.9
81.9
82.9
98.9
126.9
144.0
145.0
159.0
187.8
207.0
239.1
267.1
281.2
296.1


(b) newl7 #705 RT: 11.45 AV: 1 NL: 2.85E7
T: + c Full ms [50.00-680.00]
Relative Abundance
m/z
129.0
68.9
96.9
73.9
58.9
139.0
171.0
142.0
86.9
111.0
155.0
172.0
187.0
216.9
229.7
269.1
281.5
297.7


(c) newl7 #1230 RT: 16.26 AV: 1 NL: 1.05E7
T: + c Full ms [50.00-680.00]
Relative Abundance
m/z
98.0
125.0
68.9
54.9
73.9
151.0
111.0
157.0
83.0
166.0
149.0
181.0
199.0
129.0
200.1
215.0
251.0
269.2
284.2

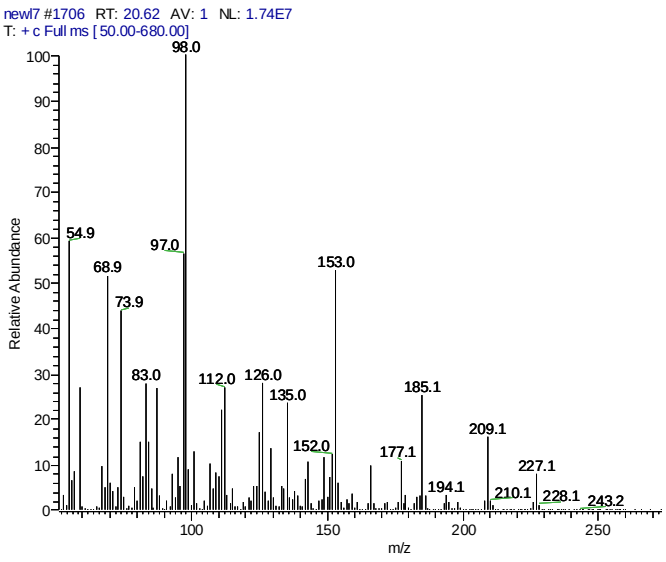

(d) newl7 #1706 RT: 20.62 AV: 1 NL: 1.74E7
T: + c Full ms [50.00-680.00]
Relative Abundance
m/z
98.0
54.9
97.0
68.9
153.0
73.9
83.0
112.0
126.0
135.0
185.1
209.1
152.0
177.1
227.1
194.1
210.1
228.1
243.2

Fig. 4.12: Gas chromatogram and mass spectra of the different breakdown products formed during the transformation of 5-methyl undecane. Products identified were (a) 3-methylpentanedioic acid, (b) 3-methylheptanedioic acid, (c) 5-methylnonanedioic acid and (d) 5-methylundecanedioic acid.

In order to identify the products from the biotransformation of 5-methyl undecane, samples were subjected to GC-MS analysis. The peaks of interest were identified by comparison to known compounds in the database. It was found that all three strains accumulated the full length 5-methylundecanedioic acid as product (Fig. 4.12 (d)). This product then underwent β-oxidation where it was shortened by 2 carbons forming symmetrical 5-methylnonanedioic acid, which also accumulated (Fig. 4.12 (c)). The 5-methylnonanedioic acid underwent another round of β-oxidation forming 3-methylheptanedioic as product (Fig. 4.12 (b)). Finally the 3-methylheptane dioic acid underwent β-oxidation forming a 3-methylpentanedioic acid as product (Fig. 4.12 (a)). Thus all yeast strains were capable of accumulating the intermediaries as well as the full length 5-methyl undecane dioic acid.

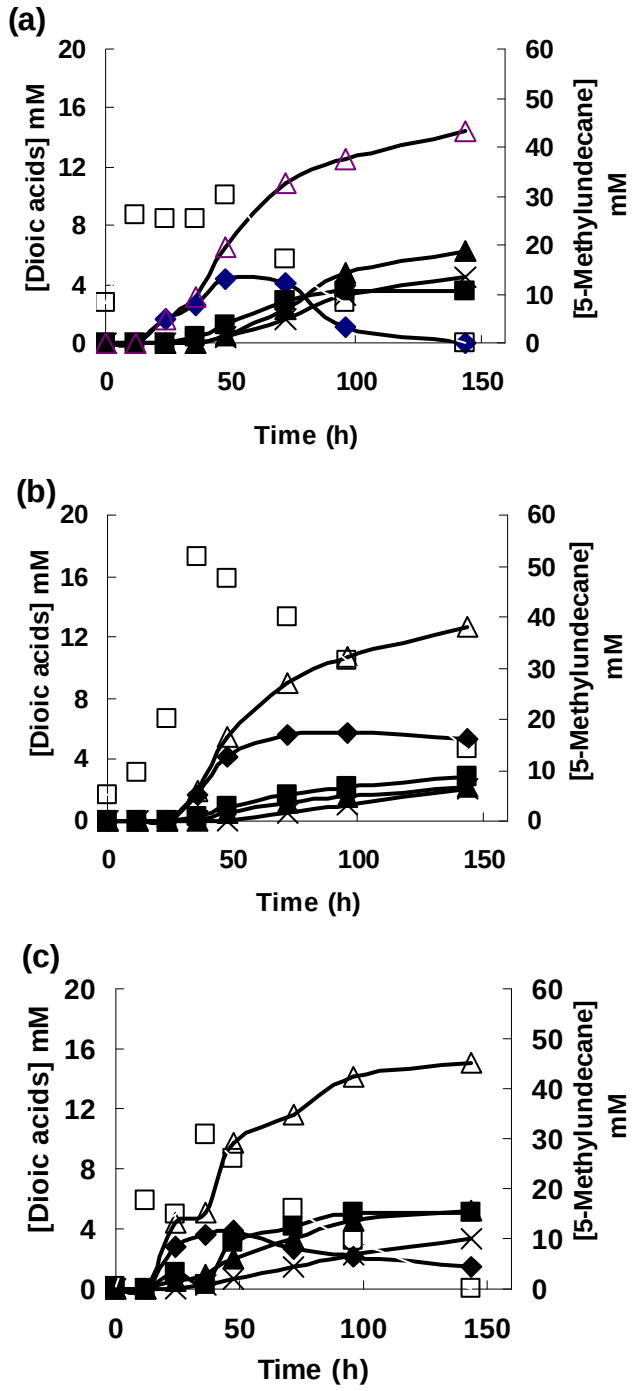


Fig. 4.13: The formation of 5-methylundecanedioic acid (♦), 5-methylnonanedioic acid (■), 3-methylheptanedioic acid (▲), and 3-methylpentane dioic acid (x) by ***Y. lipolytica*** TVN493 (a), MTLY76 (b) and MTLY78 (c) from 5-methyl undecane (□) (58mM, 10g/l), as substrate. Ethanol (217mM, 10g/l) was used as inducer. Total dioic acid (△) is also shown. Time 0h indicates the moment when the substrate was added.

The results indicated that with ethanol as inducer the highest concentration of 5-methylundecanedioic acid was produced by ***Y. lipolytica*** MTLY76 (5.7mM after 96h). ***Y. lipolytica*** TVN493 produced 4.3mM 5-methylundecanedioic acid after 48h and MTLY78 produced 3.8mM 5-methylundecanedioic acid after 48h (Fig. 4.13). In both ***Y. lipolytica*** TVN493 and MTLY78 the highest concentration 5-methylundecanedioic acid was reached after 48h. After 48h the dioic acid was broken down by both these strains and after 144h all the 5-methyl undecane had also been degraded. Exactly how the branched 3-methylpentanedioic acid is degraded is not clear.

Y. lipolytica TVN493 has a fully functional β-oxidation system in addition to multiple copies of the ***ALK***1 gene. In the case of dodecane no dioic acid was accumulated from strains with intact β-oxidation. The additional ***CYP52*** genes thus probably resulted in a more active P450 monooxygenase system, as can be seen from the accumulation of some 5-methylundecanedioic acid. The branching of this molecule probably also slows down degradation, allowing accumulation of some intact dioic acid.

In the ***Y. lipolytica*** MTLY78 strain the β-oxidation system was disrupted but no ***ALK*** genes were expressed in this yeast. The accumulation of dioic acids in these strains may be the result of hydroxylase activity of the native ***CYP*** genes and the blocked β-oxidation system. Similar to the ***ALK1*** strain the full length and both intermediary dioic acids were formed. However 5-mehylundecanedioic acid was partially and not totally broken down as was the case in the TVN493 strain. The partial breakdown of the 5-methyl undecanedioic acid suggests, as previously observed, that the β-oxidation system may still be functional at some level and not fully blocked as was thought.

The insertion of a single copy of the ***ALK1 (CYP52F1)*** gene into the β-oxidation disrupted ***Y. lipolytica*** MTLY76 strain caused better accumulation of the full length 5-methylundecanedioic acid, with the production of the intermediary dioic acids at the same level as in the other two strains. Although there was a higher accumulation of 5-methylundecanedioic acid in this strain

some breakdown was also observed after 96h. This also suggested that the β-oxidation might not be sufficiently blocked.

4.9 Comparison of effectiveness of inducers for the biotransformation of 5-methyl undecane by *Y. lipolytica* MTLY76 with disrupted β-oxidation expressing a single copy of *CYP52F1*

Since the expression of the ***CPR*** and ***CYP52*** genes was done using only the ***POX2*** promoter in ***Y. lipolytica*** MTLY76 and not both the ***ICL1*** and ***POX***2 promoters as was the case with the ***Y. lipolytica*** TVN493 strain, it was decided to investigate the effect of other inducers on the biotransformation of 5-methyl undecane. Stearic acid, oleic acid and ethanol were tested.

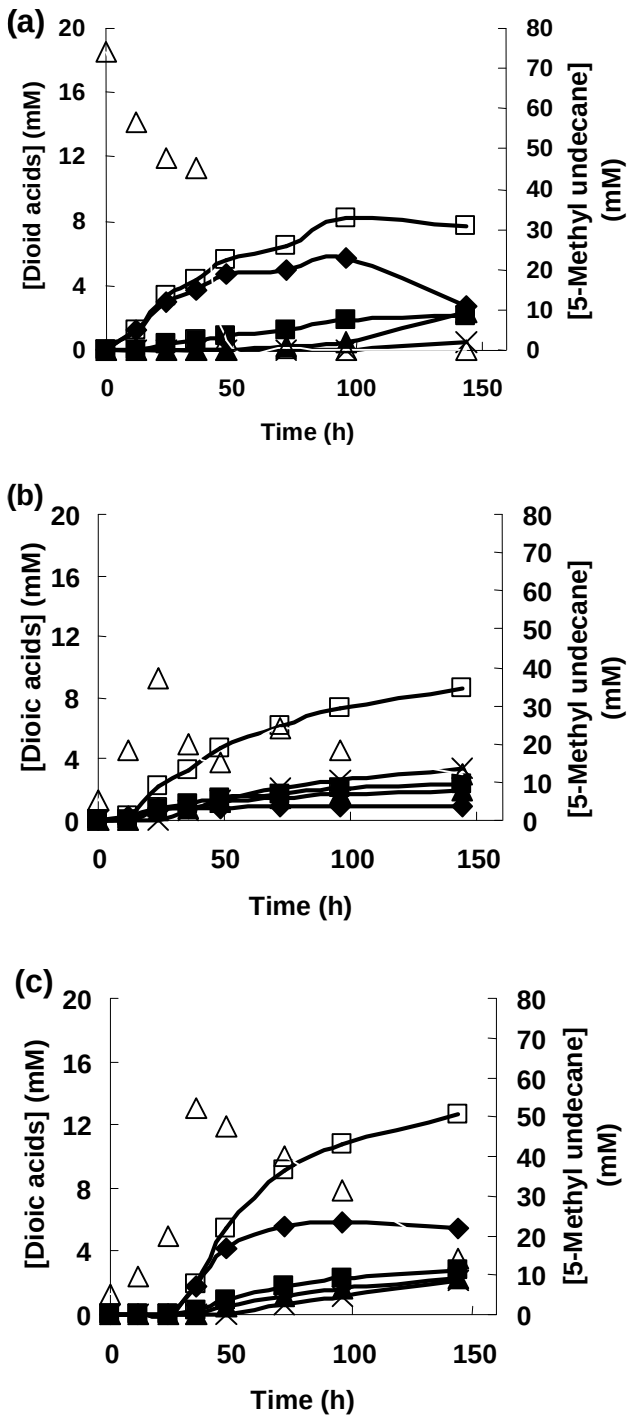


Fig. 4.14: Formation of 5-methylundecanedioic acid (♦), 5-methylnonanedioic acid (■), 3-methylheptanedioic acid (▲), and 3-methylpentanedioic acid (x) from 5-methylundecane (∆) (58mM, 10g/l) by ***Y. lipolytica*** MTLY76. Stearic acid (35mM, 10g/l) (a), oleic acid (36mM, 10g/l) (b) and ethanol (217mM,

10g/l) (c) were used as inducers. Total dioic acid (□) is also shown. Time 0h indicates the moment when the substrate was added.

Similar concentrations of 5-methylundecanedioic acid were produced when stearic acid (4.7mM after 96h) or ethanol (5.4mM after 144h) was used as inducer (Fig.4.14). C11 dioic acid production was weak when oleic acid was used as inducer with only 0.8mM C11 dioic produced after 144h (Fig. 4.14). This is lower than the concentration of the intermediate dioic acids produced. The 5-Methyl undecane was also not fully utilized when oleic acid was used as inducer but was finished in the cases where stearic acid or ethanol was used as inducers/co-substrates (Fig. 4.14). There is a possibility that the oleic acid was used up to quickly to induce the ***POX2*** promoter (Results not shown). Ethanol should in this case not induce the cloned ***CPR*** gene, because it is under the control of the ***POX2*** promoter in this strain, while stearic acid and oleic acid should induce both the inserted ***CPR*** and ***ALK*** genes. Only a single copy of the ***ALK*** gene was inserted and it may not be possible to clearly distinguish between native ***ALK*** gene activity and the activity of the inserted gene. The 5-methyl undecane may in fact act as the inducer of the cloned ***CPR***, ***ALK*** and native genes of this strain. This could make it difficult to explain the effect that ethanol has on this strain.

4.10 The biotransformation of decylbenzene by *Y. lipolytica* strains expressing additional copies of the *CYP52F1*, *CYP52F2* and *CYP 557A1* genes

CYP557A1 is a putative fatty acid hydroxylase which had been cloned from ***Rhodotorula retinophila***. Conclusive results regarding the functional expression of this gene in ***Y. lipolytica*** could not previously be obtained. In this experiment strains with intact β-oxidation as well as strains with disrupted β-oxidation were tested. Decylbenzene rather that hexylbenzene was used as substrate, because we thought that the chance of accumulating the intact

phenylalkanoic acid would be better. We expected the longer chain phenylalkanoic acid (C16 acid) to be less toxic than the shorter chain phenylalkanoic acid (C12 acid).

Y. lipolytica TVN493, TVN495, TVN356 TVN348 and TVN91 al had intact β-oxidation. Multiple copies of the ***CYP557A1*** gene had been cloned into the strains TVN356 and TVN348 while TVN493 and TVN495 contained multiple copies of the ***CYP52F1*** and ***CYP52F2*** genes, respectively. TVN348 with the ***CYP557A1*** gene was slightly faster at phenylacetic acid production than the other three strains (Fig. 4.15). TVN91, which was used as negative control strain, was the slowest at producing phenylacetic acid, although it also eventually gave 100% conversion (Fig. 4.15). When specific rates were calculated taking into account differences in biomass production (dry biomass at 48h growth varied between 9 and 14g dry wt l^{-1}) differences were even clearer (Fig.4.16). These results gave a strong indication that the cloned ***CYP557A1*** genes were functionally expressed in ***Y. lipolytica***.

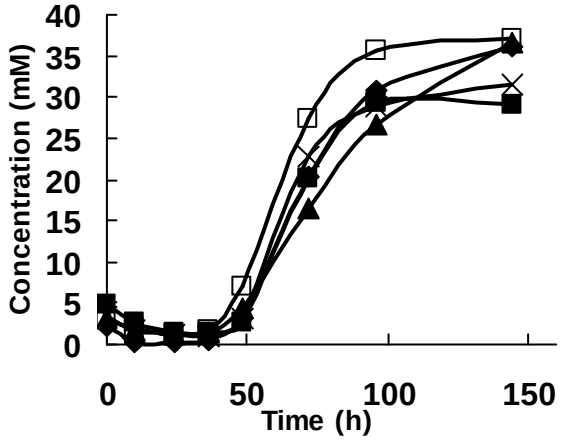


Fig. 4.15: The biotransformation of decylbenzene (39mM, 10g/l) by ***Y. lipolytica*** strains TVN495 (♦), TVN493 (■), TVN91 (▲), TVN356 (x) and TVN348 (□). Phenylacetic acid was formed by all ***Y. lipolytica*** strains. Time 0h indicates the moment when the substrate was added.

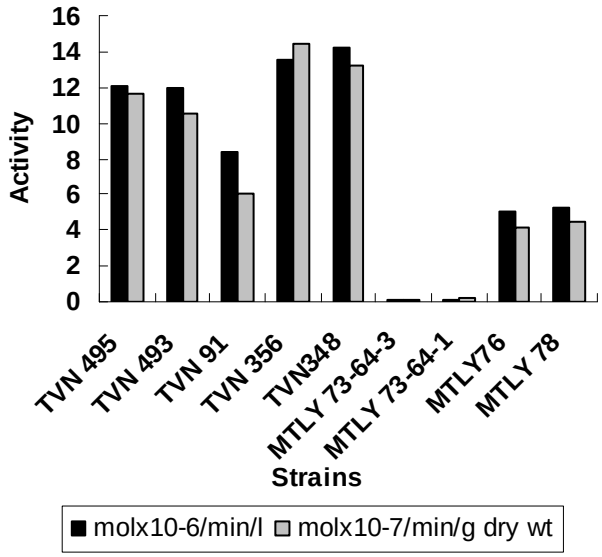


Fig. 4.16: Volumetric activity and specific activity for the formation of phenylacetic acid from decylbenzene (39mM, 10g/l) by ***Y. lipolytica*** strains.

When the strains with disrupted β-oxidation were tested, the results were very different. In this case the previously used strains MTLY76 (additional ***CYP52F1*** and ***CPR*** genes) and MTLY78 (additional ***CPR*** gene) gave as before accumulation of phenylacetic acid (Fig. 4.17). Almost complete conversion was achieved, although the rates were significantly slower than in the case of the strains with intact β-oxidation (Fig. 4.15). However the strains into which the ***CYP557A1*** gene had been cloned (MTLY73-64-1 and MTLY73-64-3) produced almost no phenylacetic acid. These strains had also given significantly less growth than the other two strains (biomass after 48h 6.5 and 9.5g dry wt l^{-1} vs. 12g dry wt l^{-1}).

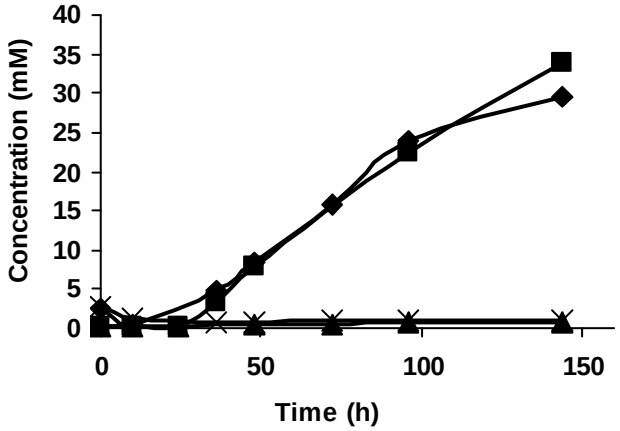


Fig. 4.17: The biotransforamtion of decylbenzene (39 mM, 10g/l) by ***Y. lipolytica*** strains MTLY78 (♦), MTLY76 (■), MTLY73-64-3 (x) and MTLY73-64-1 (▲).Time 0h indicates the moment when the substrate was added.

4.11 Biotransformation of 4-hexylbenzoic acid by *Y. lipolytica* strains with disrupted β-oxidation expressing *CYP557A1*

After the contradicting results obtained with decylbenzene transformation by the strains expressing the ***CYP557A1*** gene several ***Y. lipolytica*** strains with disrupted β-oxidation and additional ***CYP557A1*** and ***CPR*** genes were used for the biotransformation of 4-hexylbenzoic acid. The control strains included ***Y. lipolytica*** MTLY78 and MTLY81. These strains had been derived from two different strains MTLY73 and MTLY74, which both had received additional copies of the ***CPR*** gene, but no additional ***CYP*** genes. The test strains MTLY73-64-1, MTLY73-64-3, MTLY74-64-1 and MTLY74-64-2 had also been derived from MTLY73 and MTLY74 by the insertion of multiple copies of the ***CYP557A1*** gene. Because we did not know whether the 4-hexylbenzoic acid would be toxic only 0.1% v/v (4.9mM) was added to the cultures.

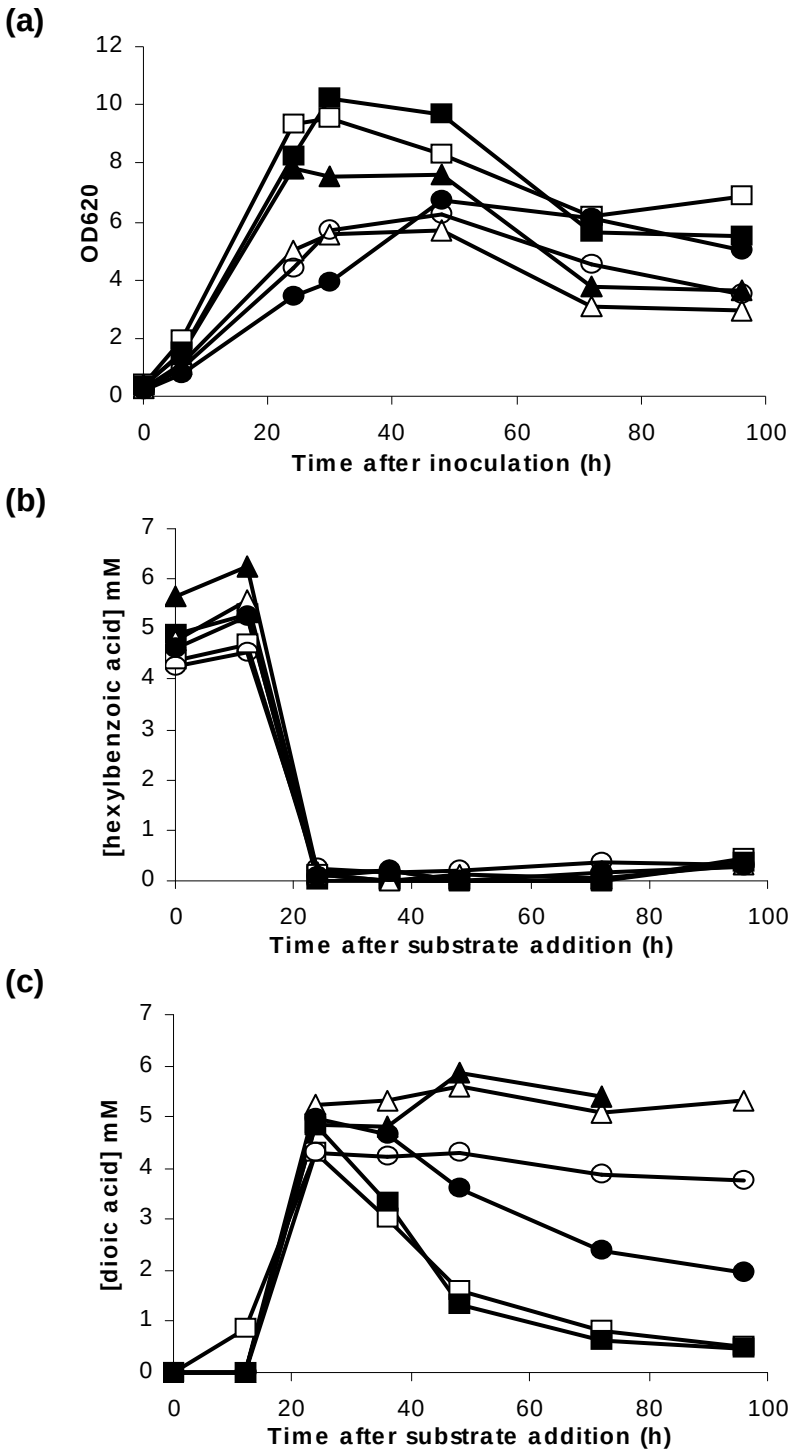


Fig. 4.18: Growth (A), hexylbenzoic acid (4.9mM, 1g/l) utilization (B) and production of the intact dioic acid (C) by ***Y. lipolytica*** MTLY78 (□), MTLY81 (■), MTLY73-64-1 (△), MTLY73-64-3 (○), MTLY74-64-1 (▲) and MTLY74-64-2 (●).

When looking at growth, the strains without the ***CYP557A1*** gene grew faster and produced more biomass. This made interpretation of the results difficult, since it can be argued that the growth influenced glucose and ethanol consumption and thus the influence that these compounds had on the ***POX2*** promoter***. Y lipolytica*** MTLY73-64-1 and MTLY74-64-1 produced a maximum of 5.5 to 5.8mM of the intact dioic acid after 48h (Fig 4.18) although the substrate was fully utilized, after 24h. In MTLY73-64-1, MTLY73-64-3 and MTLY74-64-1 the dioic acid concentration remained constant after 48h. Only in MTLY74-64-2 was the dioic acid slowly consumed. In ***Y. lipolytica*** MTLY78 and MTLY81 which had no additional ***CYP*** genes the trend was similar with the maximum concentration of dioic acid production (4.3mM) being reached after 24h and the substrate being fully utilized at the same time. However in these strains the product was broken down almost immediately and after 144h almost no product was left. This once again suggested that the β-oxidation system in these strain were functional. It is not clear why β-oxidation of the dioic acid product was so limited in the strains with the cloned ***CYP557A1*** gene, since these strains had been derived from the same parental strains (MTLY73 and MTLY74).

4.12 Biotransformation of stearic acid by strains with disrupted β-oxidation expressing *CYP557A1*

The biotransformation of stearic acid by ***Y. lipolytica*** strains with disrupted β-oxidation and additional ***CPR*** and ***CYP557A1*** genes were also investigated. The same strains were used as for the biotransformation of hexylbenzoic acid i.e. ***Y. lipolytica*** MTLY78, MTLY81, MTLY73-64-1, MTLY73-64-3, MTLY74-64-1 and MTLY74-64-2. As observed in the previous experiment, the strains without the ***CYP557A1*** gene grew faster and produced more biomass, making interpretation of the results difficult (Fig. 4.19).

When looking at stearic acid consumption and the total dioic acid production it can be seen that the two strains without the CYP557A1 genes consumed all

the stearic acid without producing any dioic acid (Fig. 4.19). The stearic acid was apparently used for biomass production, since these strains showed an increase in biomass after stearic acid addition. The strains with the CYP557A1 genes had produced after 144h between 9 and 14mM dioic acids, which was just more than half of the amount of stearic acid consumed (Fig. 4.19).The 35mM stearic acid added was in fact a ratio mixture of the C18 acid (stearic acid) and the C16 acid (palmitic acid). 13mM Stearic acid remained after 144h and 6mM palimitic acid remained after 144h. The strains not expressing ***CYP557A1*** also totally consumed the palmitic acid. The strains with the ***CYP557A1*** genes produced C16 and C18 dioic acid at approximately the same rates and in three cases the concentrations after 144h were ca. 6mM each, but the concentrations of these two dioic acids were still increasing (Fig. 4.20). All the strains with the ***CYP557A1*** gene also accumulated C14 dioic acid, but production levelled off after ca. 50h and the concentrations remained at less than 1.8mM (Fig. 4.20).

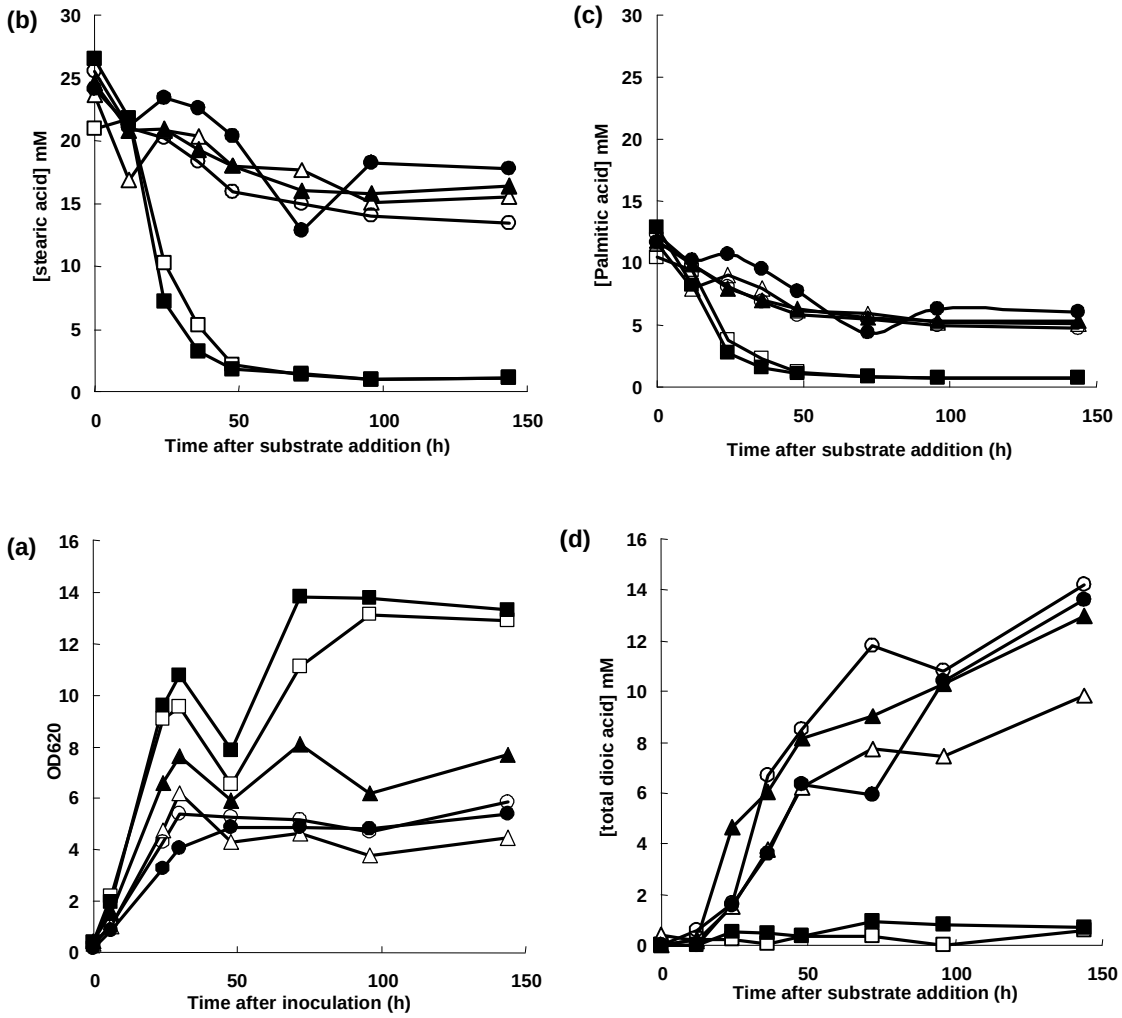


Fig. 4.19: Growth (A), stearic acid utilization (B), palmitic acid utilization (C), and total dioic acid production (D) by ***Y. lipolytica*** MTLY78 (□), MTLY81 (■), MTLY73-64-1 (△), MTLY73-64-3 (○), MTLY74-64-1 (▲) and MTLY74-64-2 (●).

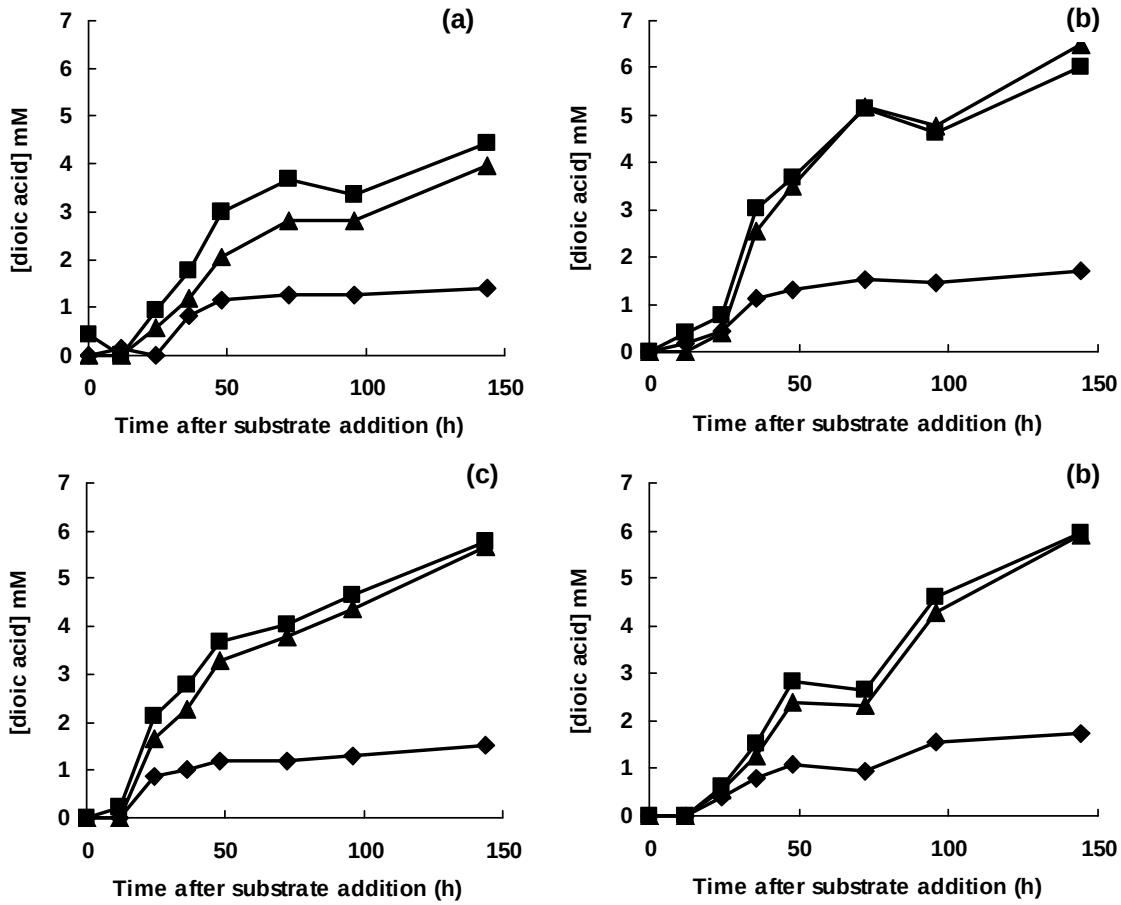


Fig. 4.20: Production of C18 (▲), C16 (■) and C14 (♦) dioic acid by ***Y. lipolytica*** MTLY73-64-1 (A), MTLY73-64-3 (B), MTLY74-64-1 (C) and MTLY74-64-2 (D) using stearic acid (35mM, 10g/l) as substrate and ethanol (217mM, 10g/l) as co-substrate. Time 0h indicates the moment when the substrate was added.

CHAPTER FIVE
DISCUSSION

The results from this study will be discussed under the different questions, which we initially posed and new questions that arose.

5.1 Will strains with disrupted β-oxidation accumulate mono carboxylic acids from hydrocarbons that can not form dioic acids?

The first β-oxidation disrupted strain ***Y. lipolytica*** MTLY37 with ***POX2, POX3, POX4*** and ***POX5*** genes disrupted were initially used to investigate the biotransformation of undecene and hexylbenzene (Section 4.1). It was expected that these substrates would not yield dioic acids, since one end is blocked for hydroxylation by the presence of double bonds. Undecane and dodecane were included as positive controls and expected to yield dioic acids. pH was checked at the end of each biotransformation period to ensure that pH remained constant.

Dodecanedioic acid and undecanedioic acid were produced from the corresponding alkanes, as anticipated. However, yields were less than 100% although the substrate disappeared completely in both cases. The yield of dodecanedioic acid (ca. 45%) was more than the yield of undecanedioic acid (ca. 20%). In the case of undecene the substrate also disappeared but no product could be identified on GC chromatograms. These results confirmed previous results (Smit ***et al***., 2005) which had indicated that β-oxidation is still active in ***Y. lipolytica*** MTLY37, despite the fact that Wang ***et al***., (1999) had found that this strain was unable to grow on oleic acid.

In the case of hexylbenzene the substrate concentration remained constant (ca. 30 – 42mM; 52mM added), within experimental error. Degradation of

hexylbenzene should have led to the accumulation phenylacetic acid, but very little phenylacetic acid (0.4mM) was formed. A small amount (4mM) of a product, which could have been phenylhexanoic acid, the intact mono carboxylic acid expected from hexylbenzene, was formed. Unfortunately this product was, at the time, not positively identified with GC-MS. We investigated the possibility that hexylbenzene might be toxic to ***Y. lipolytica*** MTLY37, but found that this was not the case (Section 4.3).

Later experiments with strains derived from ***Y. lipolytica*** E150, with intact β-oxidation, showed that 100% conversion of 52mM hexylbenzene to phenylacetic acid was achieved with strains containing additional ***CPR*** and ***CYP*** genes (Section 4.5). In the case of the control strain TVN91, which contained an additional ***CYP*** gene which was not an alkane hydroxylase, complete conversion was only observed after the native alkane hydroxylases had been induced with dodecane. A relatively low level of conversion (20%-22%) was observed in this strain when additional ethanol or glucose was added to the culture. In the strains with additional ***CYP52F1*** genes inserted (TVN493) 100 % conversion was achieved when ethanol was added to the culture, indicating that the degradation products from the hexylbenzene activated the ***POX2*** promoter. The ethanol induced the ***ICL1*** promoter driving expression of the cloned ***CPR*** gene. GC chomatograms of extracts from these cultures showed no significant peaks, which could be ascribed to phenylalkanoic acids other than phenyl acetic acid.

When strains with disrupted β-oxidation containing additional copies of the ***YlCPR*** (MTLY78) or the ***YlCPR*** and ***CYP52F1*** genes (MTLY76) were tested for the biotransformation of hexylbenzene using both ethanol and stearic acid for induction of the cloned genes (driven by the ***POX2*** promoter) almost no phenylacetic acid was formed and hexylbenzene concentrations remained constant (Section. 4.7). Again GC chromatograms showed no other phenylhexanoic acid or other phenylalkanoic acids.

To exclude the possibility that toxicity of relatively short-chain phenylalkanoic acids prevented their accumulation, biotransformation of decylbenzene by these strains was also investigated (Section. 4.10). In this case strains with multiple copies of a cloned putative fatty acid hydroxylase (***CYP557A1***) were also included. The strains with only an additional copy of the ***YlCPR*** (MTLY78) and the ***YlCPR*** with ***CYP52F1*** (MLTY76) accumulated in this case some phenylacetic acid (33mM; 84% conversion), but again no other phenylalkanoic acids could be observed. The strains with multiple copies of the ***CYP557A1*** gene accumulated no products. The reason for this was not clear, since strains with intact β-oxidation containing multiple copies of this gene showed slightly higher rates of hexylbenzene transformation than strains with additional copies of the ***CYP52F1*** and ***CYP52F2*** genes. Beta-oxidation disrupted strains containing the cloned ***CYP557A1*** gene also very efficiently converted 4-hexylbenzoic acid and stearic acid to the corresponding dicarboxylic acids, without subsequently degrading the dioic acids. Strains containing only the cloned ***YlCPR*** gene (MTLY78 and MTLY81) also converted 4-hexylbenzoic acid and stearic acid to the corresponding dicarboxylic acids, but eventually degraded the dioic acids.

Results obtained with both undecene and alkylbenzenes therefore at this stage very strongly indicate that ***Y. lipolytica*** does not accumulate monocarboxylic acids – even when alkane hydroxylating genes are overexpressed in β-oxidation disrupted strains which accumulate dioic acids from 4-hexylbenzoic acid and stearic acid.

5.2 Which products will be formed from a branched-chain hydrocarbon, 5-methyl undecane, by strains with disrupted and intact β-oxidation containing additional *YlCPR* and *CYP52F1* genes?

Unfortunately the only branched-chain alkane, which we had available for testing, was 5-methyl undecane. This compound had been specially synthesized by researchers at SASOL.

Initially biotransformation of this compound was tested with the β-oxidation deficient strain MTLY37 (Section 4.1). Later the strains TVN493 (intact β-oxidation additional ***CPR*** and ***CYP52F1*** genes), MTLY78 (disrupted β-oxidation, additional ***CPR***) and MTLY76 (disrupted β-oxidation, additional ***CPR*** and ***CYP52F1*** gene) were also used to investigate biotransformation of this compound (Section 4.8). Accumulation of the intact dioic acid, 5-methylundecanedioic acid could be observed in all cases. The strains MTLY37 and MTLY76 accumulated within 36h after the onset of transformation maximum concentrations of 6.8 and 5.7mM, which remained more or less constant for the duration of the experiment (144h). In the later experiment GC-MS was used to confirm the identity of the 5-methylundecanedioic acid and to detect its degradation products. This showed that all three the strains used in the later experiment degraded the initial product sequentially to 5-methylnonanedioic acid, 3-methylheptanedioic acid and 3-methylpentanedioic acid. In the case of TVN493 and MTLY78 both the substrate and the initial dioic acid product were completely degraded. In the case of MTLY76 only the culture, which had received ethanol as inducer/co-substrate did not eventually degrade the initial dioic acid.

These results indicate that it is possible for ***Y. lipolytica*** to hydroxylate the branched alkane, 5-methyl undecane at both ends to form the corresponding dioic acid. All strains tested were capable of accumulating the full length dioic acid. No mono acid was accumulated from 5-methyl undecane by any of the strains tested.

5.3 Will overexpression of additional *YlCPR* and *CYP52* genes in strains with disrupted and intact β-oxidation have an effect on hydrocarbon hydroxylation?

Picataggio ***et al***., (1992) tested a strain of ***C. tropicalis*** (AR40), a β-oxidation disrupted strain with additional copies of the ***CPR*** and ***CYP*** genes, for the conversion of methyl myristate to tetradecanedioic acid. ***C. tropicalis*** AR40

showed an increase in productivity of about 30% when compared with the parental strain H5343. They concluded that the improvement was due solely to the amplification of the ***CPR*** gene and not the ***P450alk1*** gene, since the ***P450alk1*** gene was not successfully expressed. However, a strain that successfully expressed the ***P450alk1*** gene, had ω-hydroxylase activity and fermentation productivity that was comparable to strain AR40 (Picataggio ***et al***., 1992).

It is difficult to determine hydroxylase activity in strains with intact β-oxidation when using ***n***-alkanes as substrates, because no product accumulates and sampling is complicated because alkanes are not water soluble. We wanted to evaluate the effect that expression of additional ***CPR*** and ***CYP*** genes had on hydroxylase activity of strains with functional and disrupted β-oxidation. However, even chain length alkylbenzenes are degraded to phenylacetic acid, which can not be degraded by ***Y. lipolytica.*** At neutral and basic pH phenyl acetate is soluble in water, so that sampling is not a problem. Thus determining the rate of phenylacetate formation from alkylbenzenes gives a measure of hydroxylase activity, when other intermediates are not accumulated.

To evaluate the effect of expression of additional ***YlCPR*** and ***CYP52*** genes on hydroxylase activity in strains with functional β-oxidation, ***Y. lipolytica*** TVN493 (expressing additional ***CPR*** and ***CYP52F1*** genes, β-oxidation functional) and ***Y. lipolytica*** TVN91 (expressing additional ***CPR*** and ***CYP53*** genes, β-oxidation functional) were used in biotransformation studies with hexylbenzene as substrate under different induction conditions (Section 4.6). The ***CYP53*** gene codes for a benzoate ***para***-hydroxylase that can not hydroxylate alkanes, thus in the strain TVN91 only the native alkane hydroxylases will hydroxylate hexylbenzene. Different induction conditions were tested in order to distinguish between the native and cloned hydroxylases. It was expected that dodecane, oleic acid and stearic acid will induce the native alkane and/or fatty acid hydroxylases as well as the cloned hydroxylases driven by the POX2 promoter and the ***CPR*** driven by the ***ICL1*** promoter. Ethanol was expected to only induce the ***CPR*** driven by the ***ICL1***

promoter, while glucose was expected to at least partially repress induction of the native and cloned ***CPR*** and ***CYP*** genes.

Maximum volumetric reactions rates were calculated to facilitate comparison of the results (Table 5.1). Specific rates could not be used, because ODs and biomass varied during the period of the biotransformation and was also influenced by the inducer/co-substrate that was added. It is known that fatty acids accumulate inside the cells thus giving deceptively high biomass measurements (Fickers ***et al***., 2005; Zheng ***et al***., 2005). Only ***Y. lipolytica*** TVN493 gave 100% conversion of the hexylbenzene (i.e. accumulated 52mM phenylacetate) when ethanol was used as co-substrate. The maximum reaction rate achieved with ethanol as inducer/co-substrate (13µmol.min^{-1}.l^{-1}) was however, not as high as when oleic acid and dodecane were used as inducers (19µmol.min^{-1}.l^{-1}). The onset of hydroxylase activity was also later when ethanol was used as inducer/co-substrate (36h after substrate addition) than when oleic acid, stearic acid and dodecane were used (24h after substrate addition). ***Y. lipolytica*** TVN91 only accumulated 12mM phenylacetate (23% conversion) when ethanol was added as inducer/co-substrate. The hydroxylation rate was only (6µmol.min^{-1}.l^{-1}). With oleic acid and dodecane as inducers TVN91 achieved hydroxylation rates that were only slightly lower than that of TVN493 (15 and 14µmol.min^{-1}.l^{-1}). We concluded that ethanol induced the ***ICL1*** promoter driving the expression of the ***CPR*** gene. The breakdown products from hexylbenzene apparently induced the ***POX2*** promotor driving the expression of the ***CYP*** genes, while not inducing the native ***ALK*** genes to the same extent. Dodecane and oleic acid induced, as expected, the cloned and native hydroxylases. The relatively small difference in maximum reaction rates achieved with TVN493 and TVN91 indicate that the native alkane hydroxylases are very efficient and that additional alkane hydroxylases can not greatly improve the activity, probably because there are other limiting factors.

It is not clear why 100% conversion could only be achieved with TVN493 when ethanol was used as inducer, while only 60 - 65% conversions were achieved with both strains when dodecane, oleic acid and stearic acid were

used as inducers. Although the latter compounds were added as inducers, they can also serve as substrates for the P450s. This could have led to competition between these substrates and the alkylbenzenes which may have resulted in the lower than expected conversion rates.

In the case of the strains with disrupted β-oxidation hexylbenzene transformation was very slow (1–2µmol.min^{-1}.l^{-1}) (see above). The accumulation of dioic acids from substrates such as 5-methyl undecane, 4-hexylbenzoic acid and stearic acid could also not be used for comparisons, because β-oxidation was not completely abolished in the strains that were tested (MTLY78, MTLY76 and MTLY81). When taking total dioic acid accumulation from 5-methyl undecane as a measure of hydroxylase activity approximately the same maximum activity (5 – 6µmol.min^{-1}.l^{-1}) was observed for both MTLY78 (additional ***CPR***) and MTLY76 (additional ***CPR*** and CYP52F1) (Section 4.8).

Table 5.1: Hydroxylase activity of ***Y. lipolytica*** strains expressing ***CPR*** and ***CYP52*** genes.

Strain	Inducer	Substrate	Period of maximum activity (h)	Maximum activity (µmol.min-1.l-1)
TVN91	Oleic acid	hexylbenzene	24-36	15
	Ethanol	hexylbenzene	24-48	6
	Glucose	hexylbenzene	24-48	2
	Dodecane	hexylbenzene	24-36	14
	Stearic acid	hexylbenzene	24-48	10
TVN493	Oleic acid	hexylbenzene	24-36	19
	Ethanol	hexylbenzene	36-72	13
	Glucose	hexylbenzene	24-48	17
	Dodecane	hexylbenzene	24-36	19
	Stearic acid	hexylbenzene	24-36	11
MTLY37	na	4% glucose	24-36	14
MTLY76	Ethanol	Hexylbenzene	12-24	1
MTLY76	Stearic acid	Hexylbenzene	12-36	1
MTLY78	Ethanol	Hexylbenzene	12-36	1
MTLY78	Stearic acid	Hexylbenzene	12-48	2
TVN493	Ethanol	5-methyl undecane	36-48	5
MTLY76	Ethanol	5-methyl undecane	36-48	5

MTLY78	Ethanol	5-methyl undecane	36-48	6

We concluded that the expression of ***CPR*** and ***CYP52*** genes in ***Y. lipolytica*** only had a slight effect on the hydroxylation of hydrocarbons. The insertion of a single copy of the CYP52F1 gene was not enough to see a visible effect in the strain with disrupted β-oxidation, MTLY76.

5.4 Will it be possible to confirm functional expression of a putative fatty acid hydroxylase, *CYP557A1,* in *Y. lipolytica*?

The ***CYP557A1*** gene codes for a putative fatty acid hydroxylase from ***R. retinophila*** (Shiningavamwe, 2004). In order to establish whether this gene was functionally expressed in ***Y. lipolytica*** biotransformations were done using decylbenzene as substrate. Strains expressing ***CYP52F1***, ***CYP52F2*** and ***CYP53*** were included for comparison. Strains with functional β-oxidation as well as strains with disrupted β-oxidation were tested (Section 4.10). Ethanol was used as inducer/co-substrate.

Y. lipolytica TVN356 (expressing ***CPR*** and multiple copies of the ***CYP557A1*** gene, functional β-oxidation) accumulated 35mM phenylacetate which is a 100% conversion. The strain expressing ***CPR*** and ***CYP52F2*** also with functional β-oxidation, also achieved a 100% conversion (35mM). When comparing the rates of total dioic acid formation of strains expressing ***CYP*** 557 to strains expressing ***CYP52*** it can be seen that the rate of the strains TVN356 and TVN348 was slightly higher than the strains expressing the CYP52s (14µmol.min^{-1}.l^{-1} and 12µmol.min^{-1}.l^{-1} respectively). All the strains with additional alkane or fatty acid hydroxylase encoding genes showed significantly higher activities than the control strain TVN91 with additional benzoate ***para***-hydroxylase genes (5 – 6µmol.min^{-1}.l^{-1}) (Table 5.2). These results pointed to the ***CYP557*** genes being actively expressed in ***Y. lipolytica***.

In the strains ***Y. lipolytica*** MTLY73-64-1 and MTLY73-64-3 expressing CYP***557*** and with a disrupted β-oxidation system practically no activity could be observed, while the control strains MTLY78 and MTLY76 showed low activity (5 – 6µmol.min^{-1}.l^{-1}). Growth in the CYP557A1 strains was also weaker than in the other strains and made it difficult to conclusively say that ***CYP557*** was not actively expressed in ***Y. lipolytica***.

Table 5.2: The rate of dioic acid production in different ***Y. lipolytica*** strains with decylbenzene as substrate.

Strain	Inducer	Substrate	Time frame (h)	Activity (µmol/min/l)
TVN495	Ethanol	Decylbenzene	48-72	12
TVN493	Ethanol	Decylbenzene	48-73	12
TVN91	Ethanol	Decylbenzene	48-74	8
TVN356	Ethanol	Decylbenzene	48-75	14
TVN348	Ethanol	Decylbenzene	48-76	14
MTLY73-64-3	Ethanol	Decylbenzene	Na	0
MTLY73-64-1	Ethanol	Decylbenzene	Na	0
MTLY76	Ethanol	Decylbenzene	36-48	6
MTLY78	Ethanol	Decylbenzene	48-96	5

When these strains were tested with 4-hexylbenzoic acid as substrate the rate of dioic acid production (5 – 7µmol.min^{-1}.l^{-1}) was similar in all the strains tested (i.e. strains expressing ***CYP557*** as well as MTLY78 and MTLY81 which only contained additional ***CPR*** genes). When stearic acid was used as substrate only the strains with additional copies of the CYP557A1 gene accumulated dioc acid with rates in the order of (2 - 3µmol.min^{-1}.l^{-1}). These strains showed relatively little β-oxidation, accumulating octadecanedioic acid and hexadecanedioic acid with a combined yield of ca. 40%.

Table. 5.3: Activity of ***Y. lipolytica*** strains using 4-hexylbenzoic acid as substrate

Strain	Inducer	Substrate	Time frame (h)	Activity (µmol/min/l)
MTLY 78	Ethanol	4-Hexylbenzoic acid	12-24	5
MTLY 81	Ethanol	4-Hexylbenzoic acid	12-24	7
MTLY 73-64-1	Ethanol	4-Hexylbenzoic acid	12-24	7
MTLY 73-64-3	Ethanol	4-Hexylbenzoic acid	12-24	6

It appears quite likely that ***CYP557A1*** was functionally expressed in both the strains with functional β-oxidation as well as the strains with disrupted β-oxidation. However, the ***POX2*** promoter is not a good choice for such studies, since it is difficult to induce the ***POX2*** promoter without also inducing the native ***CYP*** genes. The ***ICL1*** promoter will probably be a better promoter for such studies, since ethanol delayed induction of the native alkane and fatty acid hydroxylase encoding genes.

5.5 How important is time of substrate or inducer addition in the activation of the hydroxylase system?

Results from this study seem to indicate that time of addition of substrate or inducers is important for the hydroxylase system. In the biotransformation of dodecane by MTLY37 (β-oxidation disrupted without additional ***CPR*** or ***CYP*** genes) a much higher rate of dioic acid formation and higher yield of dioic acid was obtained when the substrate was added after 24h (14µmol.min^{-1}.l^{-1} and 44% yield with 4% glucose in the medium) rather than after 48h (6µmol.min^{-1}.l^{-1} and 14% yield with 4% glucose in the medium) (Section 4.2). This phenomenon might also explain why the addition of dodecane after 24h growth induced the native hydroxylase system, but addition of hexylbenzene after 36h did not do it to the same extent (Section 4.5). Much higher native hydroxylase activity was observed with the control strain TVN91 in the experiment with decylbenzene as substrate (Section 4.10). In this experiment ethanol and decylbenzene were added at the same time after 24h growth,

while in all other experiment substrates were added 12h after the inducers/ co-substrates (i.e. after 36h growth). In this case the difference in levels of native hydroxylase activity when compared with hexylbenzene can thus either be ascribed to the different substrates or to the different times at which the substrates were added. Time of substrate addition might be very important in the optimization of dioic acid production when hydroxylation will depend on the native hydroxylases. This question thus deserves further investigation.

5.6 How important is an additional co-substrate / energy source for hydroxylase activity?

Indications are that an additional co-substrate possibly serving as energy source is necessary for hydroxylase activity. Biotransformation of dodecane to dioic acid gave much better results when the medium contained 4% glucose rather than 2% glucose (i.e. hydroxylation rate 14µmol.min^{-1}.l^{-1} and 44% yield with 4% glucose vs. 9µmol.min^{-1}.l^{-1} and 7% yield with 2% glucose). Optical density measurements showed that biomass peaked after 24h (OD_{620} 11) in the presence of 2% glucose while the biomass peaked after 36h (OD_{620} 16) in the presence of 4% glucose (Section 4.2). In the experiment with TVN91 and TVN493 and hexylbenzene as substrate (Section 4.5), phenyl acetic acid production was significantly better in the cultures where 1% glucose was added after 24h compared with cultures where nothing was added, even though glucose should at least partially repress the alkane hydroxylases (Juretzek ***et al***., 2000; Juretzek ***et al***., 2001). It is still not clear why the cloned alkane hydroxylases performed so much better when ethanol was added as inducer. Ethanol delayed induction of both the native and cloned hydroxylases, but activity of the cloned hydroxylases was maintained for much longer (period of maximum activity 36 – 72h). When oleic acid was used as inducer activity was induced much sooner and more strongly, but it was maintained for a relatively short period (period of maximum activity 24 – 36h). It is not clear why energy from oleic acid and stearic acid was not available to drive hydroxylation? Further work should be done to determine the best co-substrates to drive hydroxylation by native and cloned

hydroxylases. If ethanol is a good substrate to drive such hydroxylations, then it will be better to clone hydroxylases under the ***ICL1*** promoter, rather than under the ***POX2*** promoter.

CHAPTER 6
CONCLUSION

Two points of control exist within the hydrocarbon degradation pathway of ***Y. lipolytica***. The first point of control is the P450 monooxygenase system. The overexpresion of ***P450*** genes such as these from the CYP52 family coding for the alkane hydroxylases may lead to an increase in activity and increased formation of possible useful products from hydrocarbon metabolism. The second point of control exists within the β-oxidation system and is the second enzymatic step in the pathway catalyzed by acyl-CoA oxidase. By deleting the genes coding for this enzyme, the pathway can be disrupted. This leads to the accumulation of products, such as dioic acids, that would normally be broken down.

The experiments done investigated these two points of control. Strains tested were either disrupted in β-oxidation or overexpressing a ***CPR*** and ***CYP*** gene. Strains where β-oxidation was disrupted as well as overexpressing a ***CPR*** and ***CYP52F1*** gene coding for alkane hydroxylase from ***Y. lipolytica*** were also investigated. The overexpression of ***CYP557*** in ***Y. lipolytica*** coding for a putative fatty acid hydroxylase originally from ***Rhodotorula retinophilla*** was also investigated.

The biotransformation of undecane, dodecane, undecene and hexylbenzene was first investigated using the β-oxidation disrupted strain, ***Y. lipolytica*** MTLY37, which has four out of six ***POX*** genes, coding for acyl CoA oxidases, deleted. Dodecanedioic and undecanedioic acid were accumulated from the corresponding alkanes. The alkane substrates in many cases disappeared from the media even when dioic acid yields were only between 18 and 32%, suggesting a β-oxidation system that may still be functional. When undecene, which could not be transformed to a dioic acid, because it has a double bond at the one end, was used at substrate, no product was detected, but all the substrate disappeared. Only a small amount of phenylacetic acid (0.4mM) was accumulated when hexylbenzene was used as substrate. A small amount of another product

(4 mM) possibly the mono acid, phenylhexanoic acid, was accumulated. In experiments using strains derived from ***Y. lipolytica*** E150, with intact β-oxidation a 100% conversion of hexylbenzene to phenyl acetic acid was achieved with strains containing additional ***CPR*** and ***CYP52*** genes. ***Y. lipolytica*** displayed a 100% conversion when ethanol was added as the co-substrate. Strains with disrupted β-oxidation containing additional copies of ***YlCPR*** and ***CYP52F1*** showed little to no formation of phenylacetic acid from hexylbenzene. No phenylhexanoic or alkanoic acids could be picked up by GC. When decylbenzene was used as substrate these strains achieved 84% conversion to phenylacetic acid but once again no mono acids could be picked up by GC. Taken together these results indicated that ***Y. lipolytica*** shows a strong resistance to accumulate monocarboxylic acids.

The accumulation of the intact 5-methylundecanedioic acid from 5-methyl undecane could be observed in all strains tested. ***Y. lipolytica*** MTLY37 (disrupted β-oxidation, without additional ***CPR*** or ***CYP*** genes) and MTLY76 (disrupted β-oxidation, additional ***CPR*** and ***CYP52F1*** gene) accumulated maximum concentrations of 6.8 and 5.7mM, respectively. GC-MS confirmed the formation of the 5-methylundecanedioic acid as well as the degradation products. MTLY76 with ethanol as co-substrate was the only strain that did not degrade the initial dioic acid. The results indicated that it is possible for ***Y. lipolytica*** to hydroxylate branched alkanes such as 5-methyl undecane.

The effect that additional ***CPR*** and ***CYP52*** genes had on the hydroxylation of hydrocarbons was also investigated using hexylbenzene. TVN493 (additional ***CPR*** and ***CYP52F1*** genes, functional β-oxidation) accumulated the highest concentration of phenyl acetic acid (52mM) when ethanol was used as inducer or co-substrate. The control strain TVN91 (expressing additional ***CPR*** and ***CYP53*** genes, functional β-oxidation) accumulated the highest concentration of phenyl acetic acid (39mM) when dodecane was used as inducer. In contrast the rate of phenyl acetic acid production in TVN493 was higher ($19 \mu mol^{-1} min^{-1} l^{-1}$) when dodecane and oleic acid were

used as inducers than when ethanol (13µmol.min^{-1}l^{-1}) was used as inducer. The rate achieved with TVN91 (14 -15µmol.min^{-1}l^{-1}) when dodecane and oleic acid were used as inducers was only slightly less than the rates achieved with TVN493 with the same inducers. From these results it is clear that the expression of additional ***YlCPR*** and ***CYP52*** genes only slightly improved the hydroxylation rates achieved with the native enzymes.

It appeared that ***CYP557A1*** was functionally expressed in ***Y. lipolytica***. A comparison of the maximum rates of dioic production between strains expressing ***CYP52*** and strains expressing ***CYP557A1*** when decylbenzene was used as substrate and ethanol as inducer and co-substrate, showed that TVN356 and TVN348 (functional β-oxidation, expressing ***CPR*** and ***CYP557A1***) achieved a slightly higher rate (14µmol.min^{-1}l^{-1}) compared to strains expressing ***CYP52s*** (12µmol.min^{-1}l^{-1}), pointing towards the active expression of ***CYP557A1***. In strains with disrupted β-oxidation it was not possible to unequivocally demonstrate functional expression of CYP557A1, although these strains accumulated dioic acids from stearic acid when strains only expressing the ***CPR*** did not.

The importance of time of substrate or inducer addition was first investigated using ***Y. lipolytica*** MTLY37 and dodecane as substrate. A higher rate (14µmol.min^{-1}l^{-1}) of dioic acid formation was achieved when the substrate was added after 24h with 4% w/v glucose in the medium. These results suggest that the time of substrate addition may play a very important role in the optimization of dioic acid production.

During the biotransformation of dodecane by ***Y. lipolytica*** MTLY37, the best results were obtained when 4% (44% yield) glucose was added to the culture instead of 2% glucose (44% yield vs 7% yield). With ***Y. lipolytica*** TVN91 and TVN493, phenylacetic acid production from hexylbenzene was better when 1% glucose was added after 24h compared to cultures where nothing was added. This suggests that the addition of a co-subtrate is necessary. It remains unclear why cloned alkane hydroxylases maintained

high activity for the longest time when ethanol was added as inducer, although the induction of the cloned and native alkane hydroxylase was delayed by ethanol. With oleic acid and stearic acid as inducers high activity was induced quickly but not maintained.

Alkylbenzenes proofed useful substrates to investigate many of the questions we asked. In future the same system can be used to investigate expression of the ***CYP*** genes under the ***ICL1*** promoter. Indications are that use of the ***ICL1*** promoter might make it easier to distinguish between native and cloned genes, since ethanol apparently represses the native alkane and fatty acid hydroxylases. It might even allow accumulation of dioic acids by strains with intact or partially disrupted β-oxidation, since it also seems to repress all or some of the acyl CoA oxidases.

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SUMMARY

A variety of microorganisms, including yeasts, are capable of utilizing ***n***-alkanes as carbon source (Schmitz ***et al***., 2000; Watkinson & Morgan, 1990). The over expression of P450 genes such as the CYP52 family coding for the alkane hydroxylases may lead to an increase in activity and increased formation of possible useful products from hydrocarbon metabolism (Iida ***et*** al., 2000). Disruption of the β-oxidation pathway by deleting the genes coding for acyl CoA-oxidases, also leads to the accumulation of products that would normally be broken down (Picataggio ***et al***., 1991). The genetic engineering of these two points of control opens up many possibilities for the accumulation of different products from hydrocarbons. Although some work was done concerning these systems in ***Candida tropicalis*** very little work has been done in ***Yarrowia lipolytica***.

It was the aim of the project to investigate the biotransformation of alkanes, alkylbenzenes and their derivatives by different groups of genetically engineered ***Y. lipolytica*** strains in order to investigate a number of questions. The possible accumulation of monocarboxylic acids in ***Yarrowia lipolytica*** was inestigated by using substrates such as undecene and hexylbenzene. ***Y. lipolytica*** MTLY37 a β-oxidation disrupted strain with ***POX2, POX3, POX4*** and ***POX5*** genes deleted could not accumulate any monocarboxylic acid from undecene. The undecene was however fully utilized indicating that this strain still had some β-oxidation activity. Little phenylacetic acid was formed (0.4 mM) from hexylbenzene. Another product that could not be positively identified at the time, but which might have been phenylhexanoic acid accumulated (4mM). No monocarboxylic acids other than phenylacetic acid could also be accumulated from alkylbenzenes in strains with blocked β-oxidation expressing ***CPR*** and ***CYP*** genes, leading to the conclusion that ***Y. lipolytica*** can not accumulate monocarboxylic acids.

Y. lipolytica strains with disrupted β-oxidation as well as a strain with functional β-oxidation expressing additional ***YlCPR*** and ***CYP52F1*** genes accumulated the full-length dioic acid from 5-methylundecane. All these strains also sequentially broke down the 5-methylundecanedioic acid to 5-methylnonanedioic acid, 3-methylheptanedioic acid and 3-methylpentanedioic acid. ***Y. lipolytica*** MTLY76 was the only strain that did not degrade the 5-methylundecanedioic acid completely.

Using hexylbenzene as substrate it was possible to establish that ethanol delayed the induction of both the native ***ALK*** genes as well as the inserted ***CYP*** genes. However, the cloned genes were later induced quite strongly (probably by the phenylalkanoic acids formed from hexylbenzene) for an extended period, while the native genes were only weekly induced. The maximum activity of ***Y. lipolytica*** was slightly lower when ethanol was used as inducer (13µmol.min^{-1}l^{-1}) than when oleic acid was used as inducer (19µmol.min^{-1}l^{-1}). The alkane hydroxylase activity was however maintained for a longer time when ethanol was used as inducer. When dodecane was used as inducer native genes were strongly induced for a relatively long period, but not as long as the cloned genes after ethanol.

Alkylbenzenes as substrate was also useful to distinguish between alkane hydroxylase activity of native and cloned monooxygenases. A significant difference in the activity of ***Y. lipolytica*** TVN356 expressing ***CPR*** together with ***CYP557A1*** (putative fatty acid hydroxylase from ***Rhodotorula retinophila***) and ***Y. lipolytica*** TVN91 expressing CPR together with ***CYP53*** (benzoate ***para***-hydroxylase from ***R. minuta***) could be observed (14µmol.min^{-1}l^{-1} and 8µmol.min^{-1}l^{-1} respectively) when decylbenzene was used as substrate. To better study the hydroxylase activity of inserted P450s, it may be better to use the ***ICL1*** promoter to drive the expression of the inserted ***CYP*** genes and use ethanol as inducer.

Keywords: ***Yarrowia lipolytica***, ***n***-alkanes, P450 monooxygenase, β-oxidation, monocarboxylic acid, dioic acid, alkylbenzenes, ethanol, hydroxylase activity, promoter.