Dicarboxylic acid production by *Yarrowia lipolytica* strains

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by

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Few people face with courage all that life gives them and you've shown me that I am one of them. To believe in myself and never quit.

"**Remember after the storm, a rainbow always follows and the sun shines bright**"

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Chapter 1

1. Introduction

1.1 *n*-Alkanes - products of the petrochemical industry

Aliphatic hydrocarbons are insoluble hydrocarbon molecules composed entirely of carbon-carbon and carbon-hydrogen linkages. They may be saturated (alkanes) or unsaturated (alkenes or alkynes). They also range from gases such as methane and ethane, through liquids to long chain molecules of 40 or more carbon atoms that are solid at physiological temperatures. They maybe straight chain, simple branched or highly branched compounds (Watkinson *et al.*, 1990).

Crude oil is a fossil fuel, which consists of a mixture of olefins (alkenes), aromatics, paraffins (saturated alkanes) and napthenes (cycloalkanes) (Freudenrich, 2002). The number of carbons per molecule, range from 2 to 70, with the largest fraction containing less than 20 carbons per molecule. During the oil refining process the crude oil is fractionated to give for example gasoline or motor fuel (alkanes or cycloalkanes of C_4 to C_{12}), diesel (alkanes containing more than 12 carbons) and heavier oil fractions such as lubricating oil (long chain $C_{20} - C_{50}$ alkanes, cycloalkanes and aromatics). New gas-to-liquid (GTL) fuel projects, based on Fischer-Tropsch technology, that are coming online worldwide produce almost pure long chain hydrocarbons without any aromatics. These long-chain hydrocarbons are mixtures of *n*-alkanes, isoalkanes (branched alkanes) and alkenes (Czernichowski *et al.*, 2001). The ranges of carbon chain length vary depending on the GTL process used. Some processes produce solid waxes as by-products (more than 16 carbons per molecule) while others produce only liquid hydrocarbons with between 5 and 20 carbons per molecule.

1.2 Bioconversion of *n*-alkanes to value added products

n-Alkanes represent a wide range of potential substrates for microorganisms (Watkinson *et al.*, 1990). From the mid 1960's until the mid 1970 hydrocarbon biochemistry became a world-wide theme for industrial research when mainly petroleum companies became interested in the use of microorganisms for the production of single-cell protein as an alternative foodstuff derived from alkanes, as well as for biochemical synthesis of amino acids, fatty acids, sterols, vitamins and other substances of commercial interest (Mauersberger *et al.*, 1996). By the mid-1970s Western industrial countries and Japan lost interest in this technology, because petroleum products became too expensive. However, in the old Soviet Union, GDR and in East European countries

this research continued until the early-1990s. Several commercial processes for the production of fodder yeasts using *n*-alkane fractions from their oil-refining processes as feedstocks, were put into operation. Pure *n*-alkanes became attractive substrates for bioconversion to other value added or as carbon source for fermentation processes due to their abundance and availability at very low costs, and free of aromatics.

Microbial alkane degradation has been studied over the years, and a number of bacteria, yeasts and fungi have been shown to possess metabolic pathways for the degradation of a wide variety of hydrocarbons (table 1). Three pathways for the degradation of alkanes have already been established and the enzyme reactions have been elucidated, these include:

I. Monoterminal oxidation

($R\text{-}CH_3 \rightarrow RCH_2OH \rightarrow RCHO \rightarrow RCOOH$

This pathway is common in *Pseudomonas* spp.

II. Diterminal oxidation

($H_3CRCH_3 \rightarrow H_3CRCH_2OH \rightarrow HOCH_2RCH_2OH \rightarrow HOOCRCOOH$)

This pathway occurs in several types of bacteria and fungi including *Yarrowia lipolytica* in which methyl groups at both termini of n-alkanes are sequentially hydroxylated (Rehm and Reiff (1981).

III. Subterminal oxidation

$RCH_2CH_3 \rightarrow RCH(OH)CH_3 \rightarrow RC(O)CH_3$

This pathway has been recognized in *Nocardia* spp (Klug and Markovetz, 1967).

Table1. Some genera of microorganisms that have been shown to metabolise aliphatic hydrocarbons.

Bacteria	**Yeasts**	**Filamentous fungi**
Acetobacter	*Candida*	*Aspergillus*
Actinomyces	*Yarrowia*	*Cladosporium*
Alcaligenes	*Pichia*	*Corollaspora*
Bacillus	*Cryptococcus*	*Dendryphiella*
Corynebacterium	*Debaryomyces*	*Gliocladium*
Flavobacterium	*Hansenula*	*Lulworthia*
Mycobacterium	*Rhodotorula*	*Penicillium*
Nocardia	*Torulopsis*	*Varicospora*
Pseudomonas	*Trichosporon*	

Although initially interest on alkane degradation biochemistry was focused mainly on production of single-cell protein from *n*-alkanes, attention later turned towards production of primary oxidation products and other metabolites directly from these substrates. Potential products listed in table 2 are common to the use of alkanes or carbohydrates as the carbon and energy source. Some of these include carboxylic acids, amino acids, nucleic acids, vitamins, antibiotics and enzymes. In these processes the substrate of choice depends to a large extent on the relative cost of the raw materials. The enhanced level of acetyl-CoA formed during alkane degradation favours some of these products, such as carotenoids, steroids, coenzyme Q and polyhydroxyalkanoates.

Table 2. A summary of products, which were derived or specifically produced or enhanced through the use of alkanes as carbon sources.

PRODUCT	MICROORGANISM
Amino acids L-glutamate, L-lysine, L-alanine and L-tyrosine	*Corynebacterium hydrocarboclastus, Corynebacterium alkanolyticum, Arthrobacter paraffineus, Alcaligenes marshallii, Brevibacterium ketoglutamicum, Nocardia*
Organic acids Citrate, 2-methylisocitrate, fumarate	*Candida lipolytica, Candida zeylanoides, Candida citrica, Candida hydrocarbofumarica, Candida blankii*
Carbohydrates and Lipids Rhamnolipids, mannitol, erythritol, arabitol	*Pseudomonas aeroginosa, C. lipolytica, C. zeylanoides, Candida tropicalis*
Nucleic acids Hypoxanthine, nucleosides, guanilic, inosinic, adenylic acids	*Candida petrophilum, Pseudomonas sp., A. simplex*
Vitamins Biotin, coenzyme A, cytochrome c	*Pseudomonas, Corynebacterium* sp., *Pseudomonas alkanolytica, Candida albicans, C. lipolytica*
Antibiotics Cryomycin, cepharosporins, phenazine derivatives	*Streptomyces griesus, Pacilomyces carneus, Ps. aeroginosa, A. paraffineus*
Enzymes **Protease, catalase, amino acid oxidase, uricase**	*Ps. aeroginosa, Fusarium* sp., *C. lipolytica, C. tropicalis*

On the other hand some of the products were synthesized directly via biotransformation of alkane through oxidation that required specifically alkanes as substrates and the products included were monocarboxylic acids, dicarboxylic acids, and fatty alcohols as listed in table 3. Accumulation of dicarboxylic acids only occurred if β-oxidation is blocked through mutagenesis or genetic engineering (Mauersberger *et al.*, 1996).

Table 3. The biotransformation of alkanes to produce valuable monoterminal and diterminal intermediates.

Product	Microorganism	Reference
Linear alcohols Octanol; Longer chain alkanols	*Pseudomonas;Corynebacterium, Rhodococus*	Mathys *et al.*, 1999, Ludwig *et al.*, 1995
Monocarboxylic acids Octanoate	*Pseudomonas*	Rothen, 1998
Wax esters Cetyl palmitate Dideacyldecane-1,10-diol	*Acinetobacter; Corynebacterium*	Buhler and Schinder, 1988
Dicarboxylic acids Brassylic acid; Dodecanedioic acid	*C. maltosa; C. tropicalis*	Picataggio *et al.*, 1993, Picataggio *et al.*, 1997
Polyhydroxyalkanoates PHB; PHB/HV	*A. eutrophus*, rec *E. coli; A. eutrophus*	Sonnleitner *et al.*, 1979, Kessler and Witholt 1999, Doi *et al.*, 1988

1.3 *Yarrowia lipolytica* – an industrial alkane utilizing yeast

Interest in *Y. lipolytica* previously known as *Candida lipolytica* initially arose from its rather uncommon physiological characteristics (Barth and Gaillardin, 1996). At that time, the specie was classified as *Candida*, since no sexual state had been described. *Y. lipolytica* can utilize alkanes, fatty acids and fats as the sole carbon source (Wang *et al.*, 1999).

Large-scale industrial processes for the production of single-cell protein and citric acid by *Y. lipolytica* grown on *n*-alkanes were developed in the 1960s (Barth and Gaillardin, 1996). A process for the production of γ-decalactone from alkyl ricinoleate (a derivative of castor oil) by

Y. lipolytica was patented in 1993 and a process for α-keto-glutarate production was patented in 1972. *Y. lipolytica* is attractive for industrial applications as it is non-pathogenic (Barth and Gaillardin, 1996).

Yarrowia is a dimorphic yeast which forms both yeast-like cells and true mycelium (Gaillardin *et al.,* 1997). It has a haploid genome and a sexual life cycle as compared to *Candida* yeasts, which are diploid or partly diploid and do not have a sexual state. This makes *Y. lipolytica* advantageous for genetic and molecular manipulation (Iida *et al.*, 2000). A variety of genetic tools have been developed for *Y. lipolytica.* These include vectors for the deletion of selected genes, a transposon-based tagging mutagenesis system (Neuvéglise *et al.*, 1998) as well as transposon-based cloning systems for the heterologous expression of proteins (Nicaud *et al.*, 2002).

1.4 Aim of this study

In *Y. lipolytica* five *POX* genes, labelled *POX1* through *POX5*, that encode five acyl-CoA oxidases (*AOX1* through to *AOX5*) have been identified (*Wang et al.*, 1999). The acyl-CoA oxidases, catalyse the first step of β-oxidation, which is the second rate-limiting step after hydroxylation by monooxygenases in alkane degradation (Hashimoto *et al.,* 2000). In a recent article, Wang *et al.*, (1999) reported the construction of single, double, triple and quadruple *POX* deleted mutants of *Y. lipolytica* derived from the wild type strain W29. A paper by Picataggio *et al.* (1992) and a few recent patents (Picataggio *et al.*, 1993, Picataggio *et al.*, 1997) described the accumulation of dioic acids by genetically engineered strains of *Candida maltosa* and *C. tropicalis* with acyl-CoA oxidase encoding genes disrupted. However there is no information in the literature on production of long chain dicarboxylic acids by genetically modified strains of *Y. lipolytica* with *POX* genes deleted. The potential value of these long chain dicarboxylic acids motivated us to conduct a study, focused on genetically engineered strains of *Y. lipolytica.* We had through our collaboration with Dr J.-M. Nicaud of the INRA-CNRS in France access to the above mentioned series of *Y. lipolytica* strains with the acyl-CoA oxidase encoding genes disrupted.

Therefore the aims of this project were:

- To compile a literature review on dicarboxylic acid production by alkane utilizing yeasts.
- To develop a simple and cost effective turbidimetric method to monitor growth of *Y. lipolytica* strains on different chain length alkanes and their derivatives.
- To investigate the toxicity of the different alkane degradation intermediates to *Y. lipolytica* and the preparation of strains tolerant to a toxic intermediate in the hope that such strains might accumulate dioic acids as a means of detoxification.
- To investigate the bioconversion of alkanes and alkane degradation intermediates to dioic acids by the wild type strain *Y. lipolytica* W29 and its derivatives MTLY21 (*ΔPOX2, POX3*), MTLY35 (*ΔPOX2, POX3, POX5*) and MTLY37 (*ΔPOX2, POX3, POX4, POX5*).

Chapter 2

2. The production of dicarboxylic acids by alkane utilizing yeasts – a literature review

2.1 Dicarboxylic acids as industrial products

Long-chain dicarboxylic acids are versatile chemical intermediates useful as raw materials for the preparation of perfumes, polymers and adhesives (Picataggio *et al.*, 1992). These include such commercially important acids as adipic acid, maleic acid (butanedioic acid, C4), sebacic acid (decanedioic acid, C10), azelic acid (C9) and dodecanedioic acid (C12) etc. Adipic acid (hexanedioic acid, C6) is a feedstock for the synthesis of nylon. Sebacic acid (decanedioic acid, C10) is a component of the engineering nylon 6:10 and its dibutyl ester is one of several plasticisers sanctioned for use in plastics that are likely to come into contact with foods. The lithium and aluminium salts of azelaic acid (nonanedioic acid, C9) are lubricants, while its alkaline and ammonium salts are useful additives to anti-freeze mixtures (Green *et al.*, 2000). Besides the chemical uses mentioned above, azelaic acid has antibiotic properties that are useful in the treatment of acne, while brassylic acid (tridecanedioic, C13) is a synthetic musk. According to patents, hexadecanedioic acid (C16) can also be used for the production of peptides, lipids, oil resistant polyamide based adhesives and powder coatings

Chemical synthesis of dicarboxylic acids with chain-lengths up to C12 is possible, but usually results in numerous by-products, requiring extensive purification. Several tons of azelaic acid (C9) are produced each year by treating oleic acid with ozone and smaller amounts of sebacic acid (C10) are obtained from oxidation of ricinoleic acid. Dodecanedioic (C12) and eicosanedioic acids (C20) are synthesized from petrochemical input (Green *et al.*, 2000).

The synthesis of long-chain dicarboxylic acids (chain lengths more than C12) is very difficult. Therefore, bioconversion of *n*-alkanes or fatty acids to these products becomes an attractive option. Brassylic acid (C13), for instance, is the product of the microbial oxidation of tridecane.

2.2 Alkane assimilation in yeasts

About 20% of all yeasts species have been reported to have the ability to utilize hydrophobic compounds like *n*-alkanes, fatty acids and triglycerides, as carbon substrates (Mauersberger *et al.*,

1996). Alkane-assimilating yeasts include *C. tropicalis*, *C. maltosa*, *Pichia guilliermondii* and *Y. lipolytica*. Alkane assimilation by yeasts was found to occur mainly via the monoterminal and diterminal oxidation pathways (Fig. 1). However, monoterminal oxidation was considered to be the main pathway of alkane utilization by yeasts (Fukui and Tanaka, 1981).

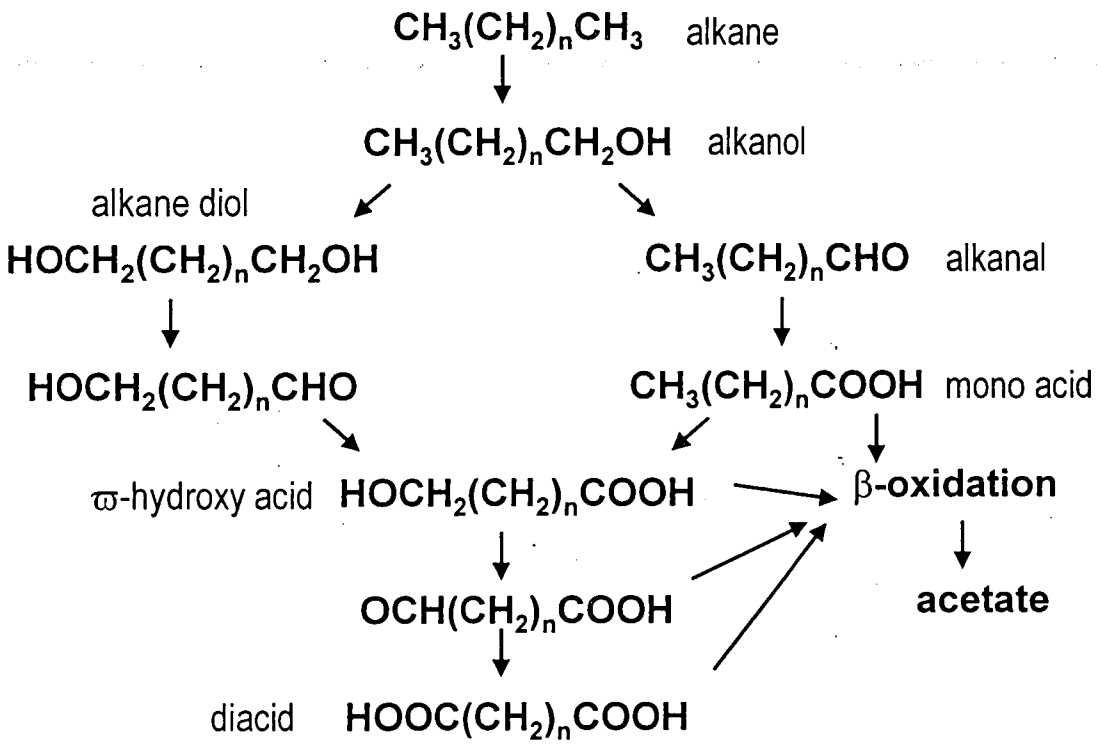

Figure 1 The diterminal alkane degradation pathway in yeasts.

The first enzymatic step of hydrocarbon assimilation by yeasts is the terminal hydroxylation of alkane to alcohol, catalysed by a membrane-bound enzyme complex consisting of a cytochrome P450 monooxygenase and a NADPH cytochrome reductase. This complex is located in the endoplasmic reticulum. Both these enzymes were first obtained in a highly purified state from alkane grown *C. maltosa* (Rehm and Reiff 1981). The hydroxylase complex is responsible for the primary oxidation of the terminal methyl group in alkanes and fatty acids (Gilewicz *et al.*, 1978). The genes, which encode the cytochrome P450 monooxygenase and NADPH reductase, have been cloned and sequenced from a number of alkane degrading yeasts (Sanglard and Loper, 1989).

The second step involves formation of fatty acids from the alcohol. This oxidation step is catalysed by two enzymes namely the fatty alcohol oxidase and the fatty aldehyde dehydrogenases. These enzymes have been purified from several alkane degrading yeasts (Kemp *et al.*, 1990). The fatty acids can then be oxidized through the same pathway to the corresponding dicarboxylic acids (Sanglard and Loper, 1989).

The omega-oxidation of fatty acids proceeds via the omega-hydroxy fatty acid and it's aldehyde derivative to form dicarboxylic acids without the requirement for CoA activation. However, both fatty acids and dicarboxylic acids can be transmitted into microbodies and then degraded by the β-oxidation pathway in the peroxisomes, leading to chain shortening (Sanglard and Loper, 1989).

2.3 Alkane uptake

Alkane uptake in bacteria and fungi, including yeasts, is generally assumed to be a passive process, which is facilitated by special hydrophobic structures associated with the cell walls. Alkane assimilating yeasts also secrete surfactants and proteins that act as emulsifying agents. During emulsification the surfactants form micelles that encapsulate the hydrocarbon molecules resulting in small droplets that can be easily taken up by the yeast cells.

Significant differences in the chemical composition of the cell wall between glucose and alkane grown cells of *C. maltosa* have been reported. The cell wall of alkane grown cells were more hydrophobic and the lipid content increased two-fold as compared to glucose grown cells (Mauersberger *et al.*, 1996). Watkinson and Morgan, (1990) also reported that extensive changes in membrane lipid composition have been found to occur during growth on alkanes.

Microscopic studies of alkane-degrading yeasts gave evidence that there were pores in the cell wall that permitted the penetration of hydrocarbons to the surface of the cell membrane and this meant that the hydrocarbon transport to the cell might be possible only by direct contact between the yeast cell and the hydrocarbon droplet (Watkinson and Morgan, 1990, Shennan and Levi, 1974).

2.3.1 Cytochrome P450 monooxygenase enzyme system

Enzymes belonging to the cytochrome P450 superfamily have been found in many microorganisms including bacteria, archaea, and fungi. The cytochromes P450 constitute a superfamily of heme-containing enzymes that exhibit a spectrophotometric absorption peak at or near 450 nm when carbon monoxide is bound to the reduced forms of the enzyme. This enzyme catalyses the transformation of hydrophobic compounds to hydrophillic ones, by introducing an oxygen atom derived from molecular oxygen (Iida *et al.*, 2000). The alkane inducible cytochrome P450 (P450 ALKs) that are classified into the CYP52 family have been found in several alkane assimilating yeasts, such as *C. maltosa, C. tropicalis* and *Y. lipolytica* (Iida *et al.*, 2000). The P450ALKs catalyse the terminal monoxygenation of alkanes and convert them to long-chain fatty alcohols. These are further oxidized to fatty acids. They also hydroxylate alkane metabolites, such as fatty acids to form long-chain dicarboxylic acids (Picataggio *et al.*, 1992). Alkane utilizing yeasts contain multiple genes that encode different P450ALK species which form a P450 multigene family. It has been shown that in the genomes of *C. maltosa, C. tropicalis* and *Y. lipolytica* there are in each case eight P450ALK genes present and their products differ in their substrate specificity and inducing substances (Iida *et al.*, 1998, 2000).

2.3.2 Fatty alcohol oxidases and fatty aldehyde dehydrogenases

These two enzymes play an important role in alkane assimilation. The oxidation of fatty acids from alkanes is catalysed by fatty alcohol oxidases and fatty aldehyde dehydrogenases.
In the 1980's it was shown that molecular oxygen is the essential electron acceptor of fatty alcohol oxidation and simultaneously H_2O_2 is formed. Consequently the enzyme responsible for fatty alcohol oxidation was identified as a H_2O_2 forming oxidase (FAOD). The alkane induced fatty alcohol oxidases of alkane assimilating yeasts were characterized and found to have broad substrate specificity. Genes coding for what was classified as fatty acid alcohol oxidases were identified in *C. maltosa* and *C. tropicalis* (Mauersberger *et al.*, 1996).

In several yeasts the enzymes responsible for oxidation of fatty aldehydes into fatty acids have been demonstrated to be membrane-bound NAD-dependent dehydrogenases, called fatty aldehyde dehydrogenases (FALDH) (Mauersberger *et al.*, 1996). The expression of these enzymes have been shown to be repressed by glucose.

2.4 Peroxisomes

Peroxisomes are subcellular organelles present in most eukaryotic cells. These organelles are involved in various metabolic functions including fatty acid β-oxidation (Picataggio *et al.*, 1991). In mammalian cells both mitochondria and peroxisomes oxidize fatty acids via β-oxidation, however, in contrast to mammalian cells, yeasts such as *C. tropicalis* and *Y. lipolytica* possess only the peroxisomal β-oxidation system. These peroxisomes are the sole sites of oxidation of fatty acids (Tanaka and Ueda, 1993). In most cases these peroxisomes proliferate in response to cultivation on fatty acids or *n*-alkanes Tanaka and Ueda, (1993).

2.4.1 Peroxisomal β-oxidation

Peroxisomal β-oxidation is the cyclic degradation of a fatty acid with each cycle yielding a fatty acid, two carbon atoms shorter, and an acetyl-CoA molecule (Picataggio *et al.*, 1991). Human peroxisomal membranes contains at least two acyl-CoA synthetases: a long chain acyl-CoA synthetase, which activates long chain fatty acids and a very long chain fatty acyl-CoA synthetase, which activates very long chain fatty acids (Hashimoto *et al.*, 2000).

This peroxisomal fatty acid β-oxidation consists of four steps:

(i) An oxidation reaction in which the acyl-CoA is desaturated to a 2-trans-enoyl-CoA;

(ii) a hydration reaction, which converts the enoyl-CoA to a 3-hydroxyl acyl-CoA;

(iii) a second oxidation step, which dehydrogenates the hydroxy intermediate to a 3-ketoacyl-CoA; and

(iv) thiolytic cleavage, which releases acetyl-CoA and an acyl-CoA, two carbon atoms shorter than the original molecule. Acyl-CoA can re-enter the cycle for the next round of β-oxidation.

The first oxidation step is catalyzed by a H_2O_2 generating fatty acyl-CoA oxidase, the second and third steps by a bifunctional protein displaying enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity. The fourth step (thiolytic cleavage) is catalysed by a thiolase (Hashimoto *et al.*, 2000).

Peroxisomal β-oxidation is not linked to the production of metabolic energy (Ratledge, 1984). The acetyl-CoA produced during β-oxidation goes into the tricarboxylic acid (TCA) cycle for energy generation and into the glyoxylate cycle for the production of gluconeogenic intermediates and TCA cycle intermediates.

2.4.2 Acyl-CoA oxidases

In the yeast *Candida tropicalis* peroxisomal acyl-CoA oxidases are octomeric flavoproteins with molecular weight of 600 kD (Picataggio *et al.*, 1991), while in *Y. lipolytica* they present as heteropentameric, co-factor-containing complexes (Titorenko *et al.*, 2002). The peroxisomal acyl-CoA oxidases, which are the first and rate limiting enzymes of the *β*-oxidation pathways in peroxisomes, catalyse the first reaction in the β-oxidation pathway by the stoichiometric conversion of acyl-CoA to enoyl-CoA for substrates with chain lengths from 4 to 20 carbons (Picataggio *et al.*, 1991). The oxidation of the long chain acyl-CoA thioester yields the corresponding trans-2-enoyl-CoA. A number of these acyl-CoA oxidase encoding genes have been cloned from plants, animals, and some microorganisms. Several of these acyl-CoA oxidase genes are found to occur in one organism often with different substrate specificities (Wang *et al.*, 1999). In yeast cells one gene has been identified in *Saccharomyces cerevisiae*, two and three genes have been identified in *C. maltosa* and *C. tropicalis* respectively and five genes have been identified in *Y. lipolytica.* It has been demonstrated in *C. maltosa* and in *C. tropicalis* that disruption of β-oxidation specifically at the level of the fatty acyl-CoA oxidases leads to the accumulation of dicarboxylic acids (Picataggio *et al.*, 1993, 1997).

2.5 Dicarboxylic acid accumulation by yeast mutants deficient in β-oxidation

2.5.1 Mutants derived from chemical mutagenesis

Over the years many different mutants of alkane degrading yeasts were isolated after physical (ultraviolet or gamma irradiation) or chemical (MNNG) mutagenesis. Of special interest were the alkane non-utilizing mutants. Mauersberger *et al.*, (1996) classified the alkane non-utilizing mutants into five phenotypes designated *alk*A to *alk*E, based on substrate utilization tests on agar plates with different alkane oxidation intermediates as carbon sources. Utilization of alkanes and alkane oxidation intermediates by these different phenotypes is listed in table 4 (Mauersberger *et al.*, 1996). The *alk*D mutants (i.e. Alk^-, FA^-, Ac^+) were found to accumulate in some instances very high concentrations of dicarboxylic acids.

Table 4: Differentiation of *alk* mutants from the auxotophic mutants with *n*-alkanes (C_{10}, C_{12}, C_{16}) or glucose as carbon sources by testing their growth on minimal medium (Mauersberger *et al.*, 1996).

Phenotype	Growth on carbon source (chain lengths)					
	Alkanes (C_8, C_{10}) (C_{12}, C_{16})	Alcohols (C_{12}-ol)	Aldehydes (C_{12}-al)	Fatty acids (C_{12}OOH) (C_{16}OOH)	Acetate or ethanol	Glucose
Alk^+	+	+	+	+	+	+
*alk*A	-	+	+	+	+	+
*alk*B	-	-	+	+	+	+
*alk*C	-	-	-	+	+	+
*alk*D	-	-	-	-	+	+
*alk*E	-	-	-	-	-	+

Casey *et al.*, (1990) reported a nystatin enrichment procedure, which yielded 288 Alk^- mutants after MNNG mutagenesis. Of the 288 Alk^- mutants 84 were *alk*D mutants. The bulk (75) of the *alk*D mutants accumulated hexadecanedioic acid (up to 7 g/l in shake flask experiments) from hexadecane and palmitic acid.

Industrial production of brassylic acid by the Nippon Mining Company, which started in Japan in 1987, is based on a mutant strain of *C. tropicalis* labelled M2030 (Mauersberger *et al.,* 1996). Biochemical analysis of this mutant revealed the absence of two acyl-CoA oxidases and 3-ketoacyl-CoA thiolase activity. Activity of the bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase) was also drastically decreased.

Also in Japan the Ajinomoto Company developed already in the early 1970s a mutant of *C. maltosa,* labelled M-12, which produced under optimised conditions in a fermenter up to 100 g/l hexadecanedioic acid from hexadecane using acetate as carbon and energy source. In shake flasks it produced up to 61 g/l hexadecanedioic acid. This strain was obtained from a wild type strain (with a tendency to accumulate dioic acids while growing on alkanes, after two rounds of mutagenesis. There is nothing in the recent literature on the production of dicarboxylic acids by mutants of *Y. lipolytica* although there are some old patents describing the use of *C. lipolytica* mutants for the production of dicarboxylic acids. (Picataggio *et al.*, 1993, 1997)

2.5.2 Mutants derived through genetic engineering

Picataggio and co-workers patented their work using genetically modified strains of *C. maltosa* and *C. tropicalis,* which were constructed through genetic engineering of auxotrophic mutants (Picataggio *et al.*, 1993, 1997). Deletion of all the *POX* genes from *C. maltosa* ATCC 90677 and *C. tropicalis* ATCC 20913 yielded strains with the ability to convert alkane substrates to form long chain dicarboxylic acids. The substrates utilized were dodecane and methyl myristate, which resulted in high concentrations of dodecane dioic acid being produced.

A strain of *C. maltosa* ATCC 90677 that lacked the Ura^3 gene marker was derived from the wild type strain ATCC 28140 through mutagenesis (Fig 2). This auxotrophic mutant strain ATCC 28140 was not able to accumulate any dicarboxylic acids. By disrupting the *POX* genes a mutant strain designated 11/11 was derived. Two strains were derived from strain 11/11. Strain ATCC 74431 was derived from 11/11 by restoring the adenine and histidine auxotrophic markers. Strain ATCC 74430 was derived by overexpressing the P450alk and P450 reductase genes. These genetically modified strains were able to accumulate high concentrations of dodecane dioic acid from dodecane. The mutant strains ATCC 74430 accumulated 21.6g/l of dodecanedioic acid in

51 h, whereas, ATCC 74431 accumulated 28.8g/l in 69 h. The additional P450alk and P450 reductase genes thus did not improve dicarboxylic acid production.

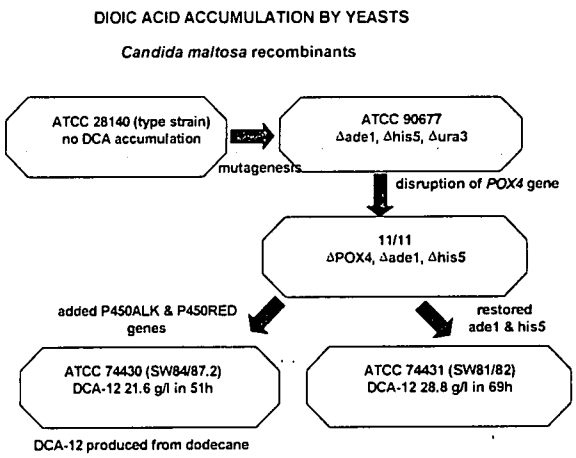

Figure 2: Construction of recombinant *C. maltosa* strain for the production of dicarboxylic acids

A similar route was followed to obtain *C. tropicalis* strains with the POX genes disrupted (ATCC 20962) and with the P450alk and P450 reductase genes amplified (ATCC 20987). Strain ATCC 20987 accumulated up to 150 g/l tetradecanedioic from methyl myristate after 95 h in a fermenter.

2.6 Process conditions influencing dicarboxylic acid production

During production of dioic acids, the conversion process is usually carried out by batch fermentation. This batch process consists of two phases namely, the growth and the conversion (transformation) phases (Mobley and Shank, 2000). The growth phase is initiated when the yeast biocatalyst is used to inoculate the batch fermenter, containing nutrient medium. The cell biomass increases depending on the factors influencing growth such as, the cell type and the nutrient contents of the media. When substrates such as, alkanes or fatty acids, are added to the media it marks the beginning of the conversion phase and this results in the formation of the desired products. The most recent comprehensive report on factors affecting dicarboxylic acid production is a patent by Mobley and Shank (2000). They used for genetically engineered *C. tropicalis* strain ATCC 20987 with all *POX* genes disrupted, and P450alk and P450 reductase genes (*CPR*)

overexpressed. They conducted their experiments in a stirred, aerated fermenter (5 L) charged with 2.27 L of a chemically defined medium. They found that the following factors were crucial for obtaining high specific dicarboxylic acid productivity:

- The conversion phase should be started at or soon after the maximum growth rate is attained;
- no carbon limitation, best growth during continuous addition of glucose throughout the growth and conversion phase;
- no carbon accumulation;
- optimum temperatures between 27 and 33 °C; and
- pH of 6.5 during the growth phase and 7.0 to 8.5 at beginning of the conversion phase.

Under optimal conditions Mobley and Shank (2000) obtained average specific productivities of 1.0 g/l/h and final dicarboxylic acid concentrations of 75 – 80 g/l. In one example they accumulated 94 g/l dicarboxylic acid after 97 h cultivation on oleic acid.

Green *et al.*, (2000) recently investigated the accumulation of dioic acids from dodecane and lauric acid by a mutant of C. *maltosa* (*cloacae*) FERM-P736. This strain has a block in the β-oxidation pathway, but can still grow on *n*-alkanes and fatty acids. They found that the toxicity of fatty acids and to a lesser extent dodecane inhibited growth on these substrates, but that the addition of 5 % (v/v) pristane alleviated the problem. The pH of the media influenced distribution of lauric acid between the aqueous and the pristane phases, with the acid preferring the organic phase at low pH (< 5). Dioic acid production was favoured between pH 4 and 5, but the cells still retained viability at pH 3 (Green *et al.*, 2000). Eventually a pH switch was implemented, pH 6.5 during the growth phase and pH 5.0 during the conversion phase (Green *et al.*, 2000). Under optimal conditions 5 g/l dodecanedioic acid was accumulated from 10 g/l lauric acid.

Much of the early research on production of dioic acids revealed that oxygen supply played an important role in aerobic fermentations (Watkinson and Morgan, 1990). According to Jiao *et al.*, 2001, insufficient oxygen supply lead to losses in biomass yields as well as products of low quality. Jiao *et al.*, (2001) showed that during cultivation of *C. tropicalis* CT1-12, it was capable of converting H_2O_2 to oxygen and water by the enzyme catalase and this resulted in an improved oxygen transfer that enhanced the product yield. Jiao and co-workers also discovered that enhancement of dicarboxylic acid production by H_2O_2 is not only due to improved oxygen

supply, but also to increased cytochrome P450 activity. In shake flasks they increased the brassylic acid concentration after 96h from 18 g/l to 23 g/l by adding 1 ml of a 2 mM H_2O_2 solution to 50 ml bioconversion medium every 3 h. In a 22 L bioreactor they improved the dioic acid yield by 14.7% by regular feeding of H_2O_2, maintaining the H_2O_2 concentration at 2mM.

When β-oxidation was completely blocked, alkanes or fatty acids could not serve as carbon or energy source. It was then essential that an alternative carbon and energy source be added during the growth phase and an alternative energy source during the conversion phase (Mobley and Shank, 2000). Even when β-oxidation was only partially blocked the addition of a secondary carbon and energy source improved biomass production during the growth phase and dioic acid accumulation during the conversion phase (Green *et al.*, 2000). Although glucose at relatively high concentrations (i.e. 1.5%) inhibits production of the enzymes involved in alkane oxidation (i.e. P450 monooxygenases, FAOD and FADH) (Mauersberger *et al.*, 1996), most of the recent publications described the use of glucose or sucrose as carbon and energy source during the growth and bioconversion phases (Mobley and Shank, 2000; Green *et al.*, 2000 and Jiao *et al.*, 2001). It was however essential that during the conversion phase the glucose concentration be kept very low (Mobley and Shank, 2000).

There are only a few publications describing the use of other carbon and energy sources. Casey *et al.*, (1990) used sorbitol as carbon and energy source when screening Alk^-, FA^-, Ac^+ mutants of *C. maltosa* for dioic acid production. According to Mauersberger *et al.*, (1996) the β-oxidation blocked *C. maltosa* M-12 strain produced high concentrations of dioic acids after growth on acetate.

2.7 Conclusions

Primary oxidation products such as fatty alcohols, fatty acid, hydroxy fatty acid and especially dicarboxylic acids of different chain lengths of alkanes are of industrial importance in the production of surfactants, detergents, lubricants and cosmetics (Mauersberger *et al.*, 1996). Some alkane utilising yeasts can accumulate dicarboxylic acids from alkanes or fatty acids. The degradation of *n*-alkanes by alkane utilising yeasts consists of several steps involving quite a number of enzymes (Barth and Gaillardin 1996, Mauersberger *et al.*, 1996). According to

literature wild type and genetically modified strains of mostly *C. maltosa* and *C. tropicalis* with β-oxidation blocked, in most cases at the level of the acyl-CoA oxidases, were exploited in order to accumulate these valuable intermediates on industrial scale (Picataggio *et al.*, 1993, 1997). A number of process conditions such as time of alkane addition, pH, temperature, glucose concentration and aeration are crucial for obtaining high dioic acid concentrations (Mobley and Shank 2000). Under optimal conditions dicarboxylic acid concentrations as high as 150 g/l can be obtained.

References (chapter 1 and 2)

Barth G, Gaillardin C (1996) *Yarrowia lipolytica*, p. 313-388. In K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Germany.

Buhler M, Schindler J (1984) Aliphatic hydrocarbons. In Rehm H, Reed G (ed), Biotechnology. Verlag, Chemie, Weinheim.

Casey J, Dobb R, Mycock G (1990) An effective technique for enrichment and isolation of *Candida cloacae* mutants defective in alkane catabolism. *J. Gen. Microbiol.* **136**: 1197-1202

Czernichowski A (2001) GlidArc assisted preparation of the synthesis gas from natural and waste hydrocarbons gases. *Oil and gas Sci. Tech.* **56**: 181-198

Doi Y, Tamaki A, Kunioka M, Soga K (1988) Production of copolyesters of 3-hydroxybutyrate and 3-hydroxyvalreate by *Alcaligenes eutrophus* from butyric and pentanoic acids. *Appl. Microbiol. Biotechnol.* **28**: 330-334

Freudenrich C (2002) How oil refining works. http://science.howstuffworks.com/oil-refining.htm

Fukui S, Tanaka A (1981) Metabolism of alkanes by yeast. *Adv. Biochem. Eng.* **19**: 216-237

Gaillardin C, Casaregola S, Feynerol C, Diez M, Fournier P (1997) Genomic organization of the yeast *Yarrowia lipolytica. Chromosoma* **106**: 380-390

Gilewicz M, Zacek J, Azoulay E (1978) Hydroxylase regulation in *Candida tropicalis* grown on alkanes. *Can. J. Microbiol.* **25**: 201-206

Green KD, Michael KT, Woodley JM (2000) *Candida cloacae* oxidation of long-chain fatty acids to dioic acids. *Enzyme Microb. Technol.* **27**: 205-211.

Hara A, Ueda M, Matsui T, Arie M, Saeki H, Matsuda H, Furuhashi K, Kanai T, Tanaka A (2001) Repression of fatty acyl-CoA oxidase-encoding gene expression is not necessarily a determinant of high-level production of dicarboxylic acids in industrial dicarboxylic acid producing *Candida tropicalis. Appl. Microbiol. Biotechnol.* **56**: 478-485

Hashimoto T, Reddy J, Cook W, Yeldandi A, Rao M (2000) Less extrahepatic induction of fatty acid β-oxidation enzymes by PPARα. *Biochem. Biophys. Res. Comm.* **278**: 250-257

Iida T, Sumita T, Ohta A, Takagi M (2000) The cytochrome P450ALK multigene family of an *n*-alkane-assimilating yeast, *Yarrowia lipolytica*: cloning and characterization of genes coding for new CYP52 family members. *Yeast.* **16**: 1077-1087

Jiao P, Huang Y, Li S, Hua Y, Cao Z (2001) Effects and mechanisms of H_2O_2 on production of dicarboxylic acid. *Biotechnol and Bioeng.* **75**: 456-462

Kemp G, Dickinson F, Ratledge C (1990) Light sensitivity of the *n*-alkane-induced fatty alcohol oxidase from *Candida tropicalis* and *Yarrowia* lipolytica. *Appl. Microbiol. Biotechnol.* **32**: 461-464

Kessler B, Witholt B (1999) Poly(3-hydroxyalkanoates). In Flickinger M, Drew S (ed), Encyclopedia of Bioprocess Technology: Fermentation Biocatalysis and Bioseparation. John Wiley and Sons.

Klug M, Markovetz A (1967) Degradation of hydrocarbons by members of the genus *Candida*. *J. Bacteriol.* **93**: 1847-1852

Ludwig B, Akundi A, Kendall K (1995) A long-chain secondary alcohol dehydrogenase from *Rhodococcus erythropolis* ATCC 4277. *Appl. Environ. Microbiol.* **61**: 3729-3733

Maeng J, Yasuyoshi S, Yoshiki T, Kato N (1996) Isolation and characterization of a novel oxygenase that catalyzes the first step of n-alkane oxidation in *Acinetobacter* sp. Strain M-1. *J. Bacteriol.* **178**: 3695-3700

Mathys R, Schmid A, Witholt B (1999) Integrated two-liquid phase bioconversion and product-recovery for the oxidation of alkanes: process design and economic evaluation. *Biotechnol and Bioeng.* **64**: 456-462

Mauersberger S, Ohkuma, M., Schunck, W.-H., Takagi, M (1996) *Candida maltosa*, p. 411-580. In K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Germany.

Mobley, D.P. and Shank, G.K. (2000) Method for high specific bioproductivity of alpha, omega-alkane dicarboxylic acids using *Candida tropicalis*. World IPO patent 0017380A1

Neuveglise C, Nicaud J, Macdonald P, Gaillardin C (1998) A shuttle mutagenesis system for tagging genes in the yeast *Yarrowia lipolytica*. *Gene* **213**: 37-46

Nicaud J, Madzak C, van den Broek P, Gysler C, Duboc P, Niederberger P, Gaillardin C (2002) Protein expression and secretion in the yeast *Yarrowia lipolytica*. *Fems. Microbiol.* Org. **2**: 371-379

Picataggio S, Deanda K, Mielenz J (1991) Detemination of *Candida tropicalis* Acyl Coenzyme A oxidase Isozyme function by sequential gene disruption *Mol. Cell. Biol.* **11**: 4333-4339

Picataggio S, Rohrer T, Deanda K, Lanning D, Reynolds R, Mielenz J, Eirich D (1992) Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. *Biotechnol.* **10**: 894-898

Picataggio, S., Deanda, K and Eirich, L.D. (1993) Site-specific modification of the *Candida tropicalis* genome. US Patent 5254466

Picataggio, S., Rohrer T. and Eirich, L.D. (1997) Method for increasing omega-hydroxylase activity in *Candida tropicalis*. US Patent 5648247

Ratledge C (1984) Microbial conversions of alkanes and fatty acids. *J. Am. Oil. Chem. Soc.* **61**: 447-453

Rehm H, Reiff I (1981) Mechanisms and occurrence of microbial oxidation of long-chain alkanes. *Adv. Biochem. Eng.* **19**: 175-215

Rothen S, Sauer M, Sonnleitner B, Witholt B (1998) Biotransformation of octane by *E. coli* HB101[pGEc47] on defined medium: octanoate production and product inhibition. *Biotechnol and Bioeng.* **58**: 356-365

Sangalard D, Loper J (1989) Characterization of the alkane-inducible cytochrome P450 (P450*alk*) gene from the yeast *Candida tropicalis*: identification of a new P450 gene family. *Gene* **76**: 121-136

Shennan J, Levi J (1974) The growth of yeast on hydrocarbons, p. 1-57. In Hockenhull, D. J. D (ed.), Progress in Industrial Microbiology. Churchill Livingstone, Edinburgh

Sonnleitner B, Heinzle E, Braunegg G, Lafferty R (1979) Formal kinetics of poly-β-hydroxybutyric acid (PHB) production in *Alcaligenes eutrophus*H16 and *Mycoplana rubra*R14 with respect to dissolved oxygen tension in ammonium-limited batch cultures. *Eur. J. Appl. Environ. Microbiol. Biotechnol.* **7**: 1-10

Tanaka A, Ueda M (1993) Assimilation of alkanes by yeasts: Functions and biogenesis of peroxisomes. *Mycol. Res.* **97**: 1025-1044

Titorenko V, Nicaud J, Wang H, Chan H, Rachubinski R (2002) Acyl-CoA oxidase is imported as a heteropentameric, cofactor-containing complex into peroxisomes of *Yarrowia lipolytica*. *J. cell. Biol.* **156**: 481-494

Wang H, Ledall M, Wache Y, Laroche C, Belin J, Gaillardin C, Nicaud J (1999) Evaluation of Acyl Coenzyme A oxidase (AOX) Isozyme function in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*. *J.Bacteriol.* **181**: 5140-5148

Watkinson R, Morgan P (1990) Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegr.* **1**: 79-92

Chapter 3

A turbidimetric method for measuring growth of *Yarrowia lipolytica* on hydrocarbons

A turbidimetric method for measuring growth of *Yarrowia lipolytica* on hydrocarbons

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Abstract

A simple, cost effective turbidimetric method to monitor growth of *Yarrowia lipolytica* on or in the presence of hydrophobic substrates was developed and evaluated. Cyclohexane and NaOH were added to samples prior to separation of the biomass from the culture medium by centrifugation. Increasing the pH of samples to 14 abolished to a large extent the hydrophobicity of *Y. lipolytica* cells that otherwise prevented proper pelleting of cells in the presence of an organic solvent. This method gives accurate, repeatable turbidity measurements with no interference from the hydrophobic substrates using small samples of 500 µl.

Introduction

Very few researchers use turbidimetric methods to monitor the growth of microorganisms on or in the presence of hydrophobic substrates such as *n*-alkanes (Marino *et al.* 1998). Dry weight determinations (Green *et al.* 2000), enumeration of colony forming units (CFUs) on agar (Kim *et al.* 1999), oxygen uptake (Bouchez-Naïtali *et al.* 2001) and titration of acids with NaOH (Mauersberger *et al.* 2001) are methods used in recent

articles that described the growth of different microorganisms on *n*-alkanes. However, these methods are either time consuming, require relatively large volumes of sample (i.e. 3 to 10 ml) or require specialised equipment. The reasons why turbidimetric methods, which are generally regarded as rapid simple procedures for the indirect estimation of biomass, are not used when hydrocarbons are present are (i) the possible interference of the water insoluble oils or solids with the measurements and (ii) the tendency of cells to adhere to hydrophobic solids.

Yarrowia lipolytica is an alkane-utilising yeast for which extensive genetic techniques have been developed (Barth and Gaillardin 1996), allowing the construction of genetically engineered strains with disruption or over-expression of selected genes (Wang *et al.* 1999, Mauersberger *et al.* 2001). To investigate the growth of different *Yarrowia lipolytica* strains on or in the presence of a range of liquid and solid *n*-alkanes or alkane derivatives, we required a rapid method using small samples to estimate biomass production. When centrifuged in the presence of a hydrophobic organic solvent, a large percentage of *Y. lipolytica* cells cling to the water/solvent interface (Kim *et al.*, 1999). This is due to the strong hydrophobicity of the yeast cells that is characteristic of many alkane-utilising microorganisms (Marino *et al.*, 1998). *Y. lipolytica* cells display this hydrophobicity even when grown in the absence of hydrophobic substrates (Kim *et al.*, 2000). It is, therefore, not possible to efficiently harvest cells of *Y. lipolytica* in the presence of an organic solvent by centrifugation. However, to monitor growth in the presence of such a water insoluble substrate, it is necessary to remove this substrate by washing or extracting culture samples with an organic solvent such as cyclohexane, which is a good solvent for dissolving a wide range of alkanes and alkane derivatives.

The procedure described in this paper for screening a large number of strains on or in the presence of several substrates used samples of only 500 µl, 1.5 ml microcentrifuge tubes and a microtitre plate reader to produce optical density (OD) measurements that correlated well with dry weight determinations.

Materials and methods

Microorganism and growth conditions

Twelve *Yarrowia lipolytica* strains were used in this study as indicated in Table 1. The French and German strains were obtained from the Laboratoire Microbiologie et Génétique Moléculaire, Institute National Agronomique Paris-Grignon, France. All strains were maintained and stored under liquid nitrogen in the MIRCEN yeast culture collection of the University of the Free State, South Africa.

Cultivation in liquid media was performed with 50 ml YNB medium or YP broth in 1000 ml Erlenmeyer flasks (Table 1, experiment 1) or with 12.5 ml YP broth in 250 ml Erlenmeyer flasks (Table 1, experiments 2 to 5) on a rotary shaker at 180 r min^{-1} at 25°C. These shake flasks were inoculated with 24 h YP cultures that had been incubated as above.

YP broth contained (per litre distilled water): 10 g yeast extract (Merck), 10 g peptone (Merck) and either 40 g glucose or 20 ml hexadecane. Where indicated (Table 1), dodecanol (Fluka) dodecanal, (Fluka) dodecanoic acid (Sigma) or dodecanedioic acid (Fluka) were added at concentrations of 0.5% or 1% (w/v) to YP broth supplemented with glucose. YNB medium contained (per litre) 0.05 M phosphate buffer at pH 6.8:. 1.7 g YNB (Yeast nitrogen base without amino acids and ammonium sulphate, Difco), 4 g NH_4Cl, (Merck) 0.1 g Uracil, (Sigma) 1 g Yeast extract, 20 ml pristane, (Fluka) 1 ml Tween 80 (Sigma) and 10 g eicosane, (Fluka).

Table 1 *Yarrowia lipolytica* strains and culture media used in different experiments.

Experiment	Strain	Genotype	Medium
1	H222[a]	*MATA*, wild type	YP broth supplemented with 4% (w/v) glucose and YNB broth supplemented with pristane (2%, v/v), Tween 80 (0.1%, v/v) and eicosane (1%, w/v).
2	UOFS Y-0097[b] UOFS Y-1701[b]	Wild-type Wild type	YP broth supplemented with 4% (w/v) glucose, or 4% (w/v glucose and dodecanol (0.5% or 1%, v/v) or 2% (v/v) hexadecane
3	H222[a]	*MATA*, wild type	YP broth supplemented with 4% (w/v) glucose or 4% (w/v) glucose and dodecanol 1% (v/v), dodecanal 1% (w/v), dodecanoic acid 1% (w/v) or dodecanedioic acid 1% (w/v).
4	W29[c] MTLY 21[d] MTLY 35[d] MTLY 35A[d,e] MTLY 37[4]	*Mat*A, wild type *pox*2Δ, *pox*3Δ *pox*5Δ, *pox*2Δ, *pox*3::*URA*3 *pox*5Δ, *pox*2Δ, *pox*3::*URA*3 *pox*5Δ,*pox*2Δ,*pox*3Δ,*pox*4::*Ura*3	YP broth supplemented with 4% (w/v) glucose.
5	H222-41[f] P01d[g] E129[h] E150[h]	*MATA ura*3-41 *MATA leu*2-270 *ura*3-302 *xpr*2-322 *SUC*2 *MATA leu*2-270 *lys* 11-23 *ura*3-302 *xpr*2-322 *SUC*2 *MATB leu*2-270 *his ura*3-302 *xpr*2-322 SUC 2	YP broth supplemented with glucose (4%, w/v) or glucose (4%, w/v) and dodecanol (0.5% or 1% v/v).

[a] German wild-type strain (Barth & Gaillardin 1996).
[b] South African wild-type strains from the UFS yeast culture collection.
[c] French wild-type strain (Barth & Gaillardin 1996).
[d] Acyl coenzyme A oxidase deleted mutants derived from W29 via P01d (Wang et al. 1999).
[e] A dodecanol tolerant strain derived from MTLY 35 (unpublished results).
[f] A URA3 disrupted mutant of H222 (Mauersberger et al. 2001).
[g] A derivative of W29 (Barth & Gaillardin 1996).
[h] Inbred French strains derived from W29 (Barth & Gaillardin 1996).

Turbidimetric measurements and cell counts

Sample preparation

Method 1

Samples of 500 µl each were added to 200 µl cyclohexane plus 100 µl 5 M NaOH in 1.5 ml microcentrifuge tubes, vortexed for 5 min and centrifuged at 12 000 r min^{-1} for 10 min. The supernatants were discarded and the pellets were resuspended in 500 µl of physiological saline solution consisting of 0.9 % (w/v) NaCl.

Method 2

Samples of 500 µl each were added to 200 µl cyclohexane in 1.5 ml microcentrifuge tubes, vortexed for 5 min. and centrifuged at 12 000 r min^{-1} for 10 min. Subsequently, the cell mass was harvested and resuspended as above.

Method 3

Samples of 500 µl were transferred to 1.5 ml microcentrifuge tubes and centrifuged at 12 000 r min^{-1} for 10 min. Subsequently, the cell mass was harvested and resuspended as above.

Method 4

Samples of 500 µl each were taken from flasks and used without any pre-treatment or centrifugation.

The turbidity of 200 µl samples, suitably diluted before transfer to a microtitre plate, were measured at 620 nm using a Labsystems iEMS reader MF (Thermo BioAnalysis company, Helsinki Finland). Cell counts were also performed on suitably diluted samples using a haemacytometer (Boeco, Germany).

Dry weight measurements

Method 1

Cyclohexane (2 ml) and 5 M NaOH (400 µl) were added to 4 ml samples of broth in test tubes, vortexed for 5 min and then filtered under vacuum through dried and pre-weighed glass fibre filters (GF52 47MM BX200; Schleicher & Schuell). The biomass on the filter

was washed with a mixture of distilled water (4 ml), cyclohexane (2 ml) and 5 M NaOH (400 µl) followed by washing with

26 ml of distilled water.

Method 2

As above, but with the omission of the NaOH.

Method 3

Samples (4 ml) of broth without pre-treatment (NaOH and cyclohexane omitted) were filtered as above and washed with 26 ml of distilled water only.

The biomass was gravimetrically determined after drying the filters overnight at 105°C to constant mass.

Results and discussion

Kim *et al.*, (2000) reported that pronase treatment destroys the cell hydrophobicity of *Y. lipolytica*. We discovered that 5 M NaOH had a similar effect when used in a sample treatment procedure where cyclohexane and 5 M NaOH were added to samples, followed by vortexing for 5 min and then centrifuging for 10 min.

The efficacy of the above procedure was evaluated by comparing turbidimetric measurements, haemacytometer counts and dry biomass measurements from samples treated with cyclohexane and NaOH, samples treated with only cyclohexane and samples harvested without any treatment. Two different media were used for this evaluation. YP broth supplemented with glucose was used as a control while YNB medium supplemented with eicosane, a solid water insoluble substrate was used as the alkane test medium. The latter medium was also supplemented with tween80 and pristane. Alkane degrading yeasts only grow on solid alkanes in the presence of a co-solvent such as pristane (Green *et al.*, 2000). The pristane was not degraded. Tween 80 serves as an emulsifier (Barth and Gaillardin 1996). Samples for turbidimetric measurements were taken at regular intervals during a 48 h period, whereas samples for haemacytometer counts and dry biomass determinations were taken after 48 h of incubation. For turbidimetric measurements and haemacytometer counts, cells were harvested by

centrifugation, whereas dry weight determinations were done on cells harvested by filtration. Table 2 shows that treatment with cyclohexane and NaOH had no significant effect on the dry biomass determinations, indicating that the solvent did not extract a significant amount of intracellular material. In the case of the cultures grown on eicosane, however, we had expected the hydrocarbon residues to contribute to the dry weight of the untreated samples. The fact that there was no significant difference in the dry weights suggested that all the eicosane had been consumed after 48 h. Table 2 and Figure 1 show that samples centrifuged after treatment with only cyclohexane gave, as expected, the lowest optical density (OD) values and cell counts, because a significant percentage of cells adhere to the hydrophobic solvent

Table 2. Turbidimetric measurements of 200 µl samples in a microtitre plate, cell counts and dry weight measurements of 48 h cultures of *Yarrowia lipolytica* H222 grown in YP medium supplemented with 4% (w/v) glucose or in YNB medium supplemented with 1 % (w/v) eicosane, 2 % (v/v) pristane and 0.1 % (v/v) Tween 80. Mean values and standard deviations of the mean for measurements done in triplicate on duplicate flasks are shown.

Culture medium	Sample treatment	OD (620 nm)	Cell counts (cells ml^{-1})	Dry biomass (g l^{-1})
YP with glucose	None	6.3 ± 0.3	1.33(± 0.04) x 10^9	6.3 ± 0.5
	Cyclohexane	3.3 ± 0.5	0.33(± 0.03) x 10^9	6.3 ± 0.2
	Cyclohexane and NaOH	7.4 ± 0.5	1.63(± 0.07) x 10^9	6.4 ± 0.2
YNB with pristane, Tween 80 and eicosane	None	4.4 ± 0.2	2.5(± 0.1) x 10^7	3.7 ± 0.3
	Cyclohexane	1.8 ± 0.2	1.5(± 0.1) x 10^7	3.7 ± 0.3
	Cyclohexane and NaOH	4.6 ± 0.1	3.4(± 0.1) x 10^7	3.83 ± 0.3

The pre-treatment procedure with cyclohexane and NaOH yielded the highest cell counts and OD values, since the NaOH treatment apparently destroys the cell hydrophobicity. The samples centrifuged without pre-treatment gave OD measurements and cell counts that were slightly lower than that of the samples treated with cyclohexane and NaOH. In the case of the alkane grown cultures this is probably due to the cells adhering to the

hydrophobic pristane. However, the finding that a small percentage of glucose grown cells did not pellet even in the absence of any hydrophobic solvent, was unexpected and is difficult to explain. Figure 1 suggests that between approximately 10 and 24 h of incubation the cells exhibited a strong hydrophobicity. Even in the case of the glucose-grown cultures a significant percentage of cells did not pellet when samples were centrifuged without treatment with cyclohexane and NaOH.

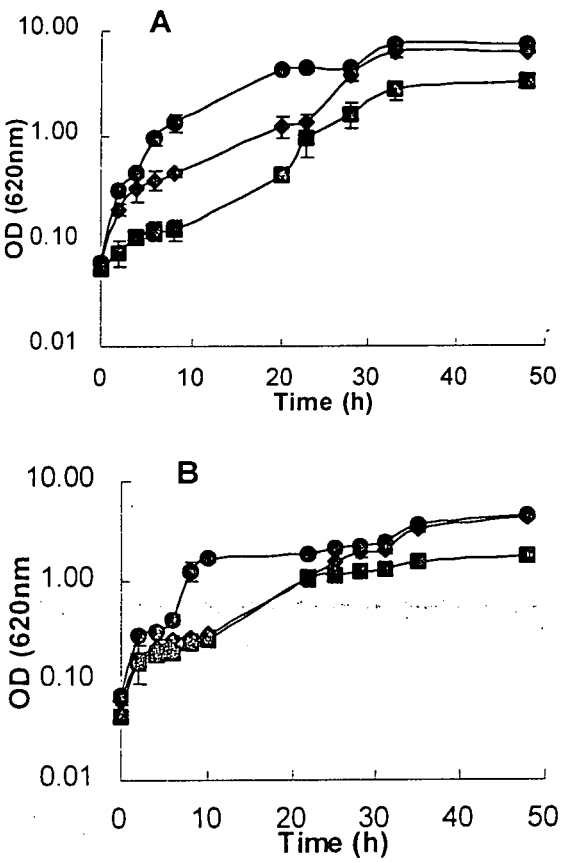

Figure 1. Effect of sample treatment on turbidimetric measurements taken during the growth of *Y. lipolytica* in (A) YP medium supplemented with 4 % w/v glucose and (B) YNB medium supplemented with 2% v/v pristane, 0.1% v/v tween 80 and 1% w/v eicosane. Mean and standard deviations for samples taken in triplicate from two flasks. Samples were centrifuged without any treatment (◆◇), centrifuged after treatment with cyclohexane (■□) and centrifuged after treatment with cyclohexane and NaOH (●○).

We subsequently proceeded to use the cyclohexane-NaOH treatment procedure to monitor growth of different *Y. lipolytica* strains on or in the presence of different hydrocarbons. The uneven growth curves as seen in fig. 1, however, remained a concern. As a further test of the cyclohexane-NaOH treatment procedure we compared in one experiment turbidimetric measurements of samples vortexed and centrifuged after addition of cyclohexane plus NaOH, with assays performed directly on diluted culture broth (i.e. without prior cell harvesting). Haemacytometer counts were done on both cyclohexane-NaOH treated samples and untreated samples directly after inoculation and after 48h (in triplicate). After 48h dry biomass determinations were done in triplicate on samples washed with cyclohexane and NaOH. In this experiment strains UOFS Y-0097 and UOFS Y-1701 were grown in YP medium supplemented with glucose, hexadecane and glucose plus dodecanol (0.5% and 1%, v/v). Growth was inhibited in the cultures containing dodecanol.

The growth curves of strains UOFS Y-0097 and UOFS Y-1701 on glucose and hexadecane (fig. 2) show that both methods gave uneven growth curves following generally the same trends. The most notable differences in OD values obtained with the two different procedures were observed directly after inoculation and between 28 and 48h for strain UOFS Y-1701 grown on hexadecane, when the OD values of the untreated samples were significantly higher than the OD values of the cyclohexane-NaOH treated samples. Comparison of cell counts done directly after inoculation revealed that a significant percentage of cells did not pellet directly after inoculation even with cyclohexane-NaOH treatment. When cells were counted directly without harvesting the average initial cell count for the eight cultures was 51 (±9) million cells ml^{-1}, while it was 5.8 (±0.8) million cells ml^{-1} after harvesting.

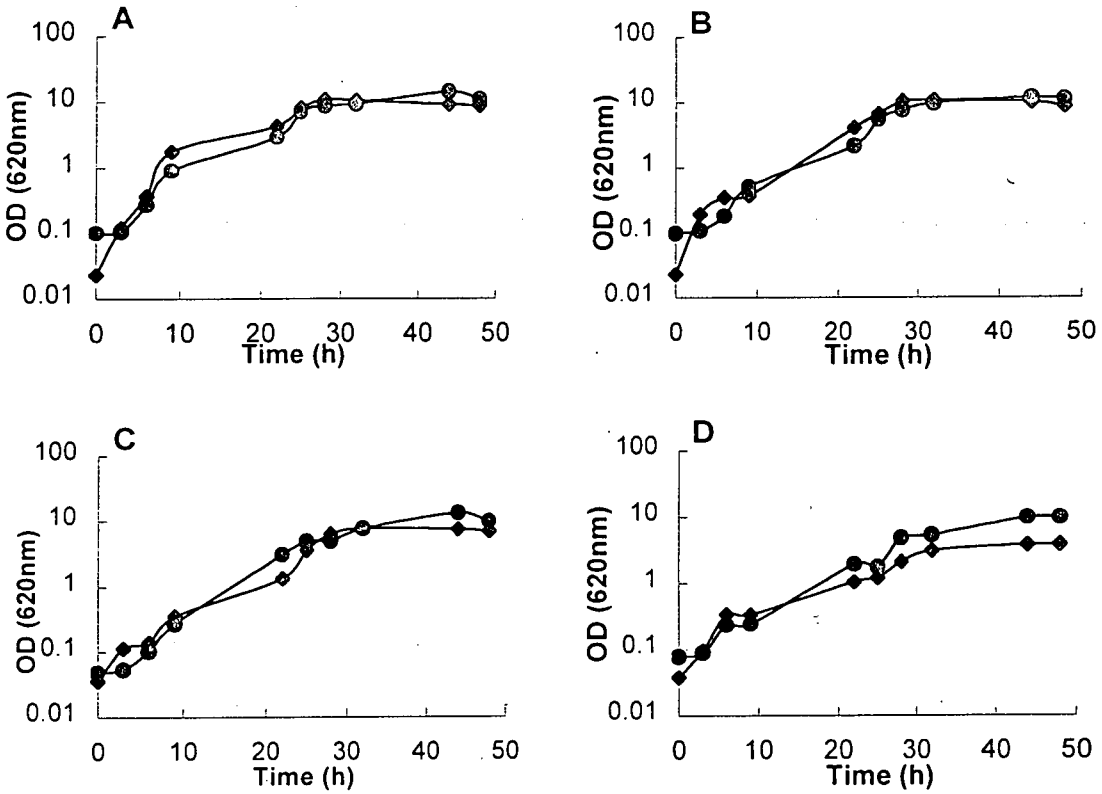

Figure 2. Effect of sample treatment on turbidimetric measurements taken during the growth of *Y. lipolytica* strains UOFS Y-0097 (A and C) and UOFS Y-1701 (B and D) in (A and B) YP medium supplemented with 4 % w/v glucose and (C and D) YP medium supplemented with hexadecane (2 % v/v). Samples were used directly without harvesting biomass (●) or centrifuged after treatment with cyclohexane and NaOH (◆).

Comparison of OD values with cell counts (fig 3A) and dry weights (fig. 3B) and of cell counts and dry weights (fig. 3C) taken after 48h for the eight cultures (including the cultures grown in the presence of dodecanol) revealed, however, that OD values and cell counts for strain UOFS Y-1701 grown on hexadecane apparently overestimated growth when the cells were not harvested (circled data points).

Comparison of OD values (corrected by multiplying with the dilution factor) with haemacytometer counts (fig 3a) further revealed that in this case sample treatment had little effect on the regression line equations (slopes of 1.3 vs 1.2), but the correlation

coefficient (R^2 value) was lower in the case of the untreated samples (0.8927 vs 0.8057). Figures 3B and 3C, correlating OD values and haemacytometer counts, respectively, with dry biomass concentration, indicate that some biomass was lost during centrifugation after treatment with cyclohexane and NaOH. By extrapolation of the regression lines to the x-axis it was determined that these turbidimetric measurements underestimated the biomass concentration by 1.68 g l^{-1}, whereas in the case of haemacytometer counts the biomass concentration was underestimated by 3 g l^{-1} when using the cyclohexane plus NaOH pre-treatment. Turbidity determinations and haemacytometer counts directly performed on the culture broth without any pre-treatment gave a slight over-estimation of biomass, namely 0.61 g l^{-1} and 0.38 g l^{-1}, respectively. The correlation coefficients were, however, again significantly lower (Figure 3). The observation that a significant percentage of cells failed to pellet during centrifugation, even after treatment with cyclohexane and NaOH, might partially explain the uneven growth curves shown in Figures 1 and 2.

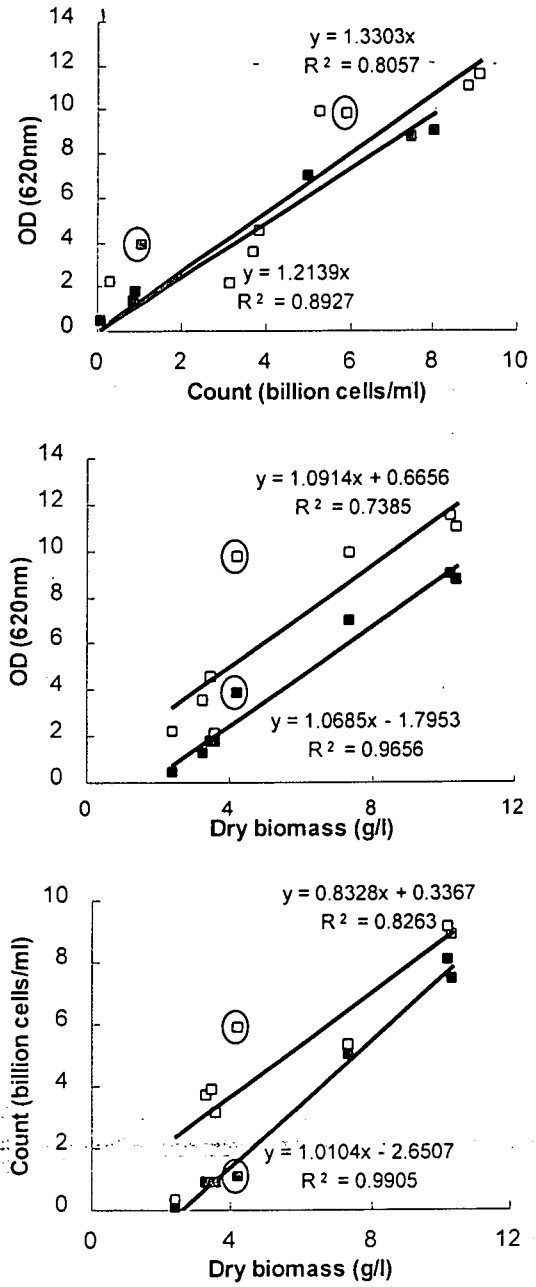

Figure 3. Correlation between (A) turbidimetric measurements and haemacytometer counts, (B) optical density and dry biomass concentration and (C) haemacytometer counts and dry biomass concentration of samples from 48 h cultures of two wild type strains of *Y. lipolytica* (UOFS Y-0097 and UOFS Y-1701) grown in YP broth supplemented with glucose (4%, w/v) and dodecanol (0%, 0.5% and 1%, v/v) or hexadecane (2%, v/v). Samples for turbidimetric measurements and haemacytometer counts were centrifuged after treatment with cyclohexane and 5 M NaOH (method 1) (■) or used directly without any treatment or centrifugation (method 4) (□). Samples for dry biomass were washed with cyclohexane and 5 M NaOH.

In order to determine whether OD values and/or cell counts can be used to compare growth of different *Y. lipolytica* strains under different conditions, data from experiments 1 and 2 as well as from another three experiments, in total representing 12 different strains (see Table 1 for details), were combined to evaluate the correlation between turbidity, haemacytometer counts and dry biomass concentration (figure 4). All samples were from 48 h cultures and all were treated with cyclohexane and NaOH prior to centrifugation or filtration. Even with data from such a diversity of strains and growth conditions, the correlation between turbidity and dry biomass (Figure 3A) was still acceptable (R^2 = 0.8984), although the regression equation differed from that of Figure 3B, so that an OD of 4 would translate to a dry biomass concentration of 5.4 g l^{-1} when using the equation from Figure 2B and 5.0 g l^{-1} when using the equation from Figure 4A. With the larger data set the correlation between haemacytometer counts and dry biomass was very poor, however (R^2 = 0.3634). This might be due to differences in the cell size of different strains grown under different conditions.

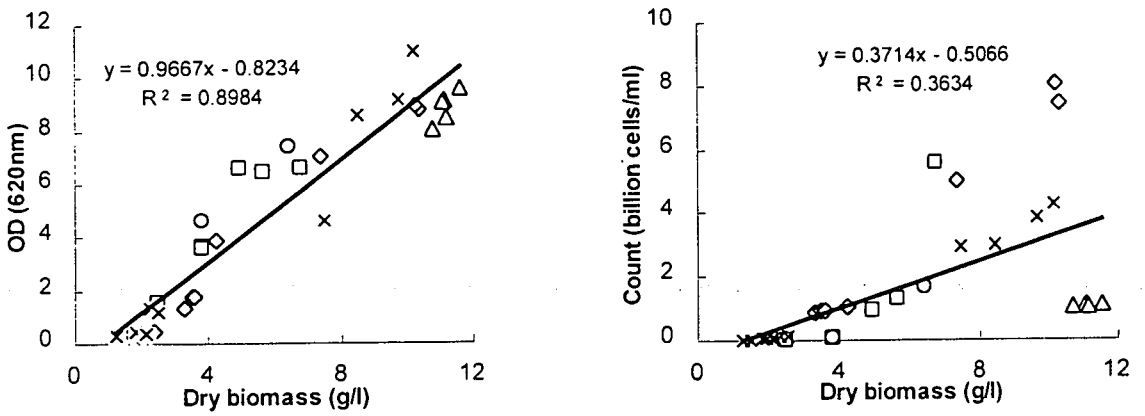

Figure 4. Correlation between (A) optical density and dry biomass concentration and (B) haemacytometer counts and dry biomass concentration of samples from 48 h cultures from five different experiments conducted with 12 different strains of *Y. lipolytica*. Experiment 1 (O): Strain H222 grown in YP broth supplemented with glucose (4%, w/v) and YNB broth supplemented with eicosane (1%, w/v). Experiment 2 (◇): Strains UOFS Y-0097 and UOFS Y-1701 grown in YP broth supplemented with glucose (4%, w/v) and dodecanol (0%, 0.5% and 1%; v/v) or hexadecane (2%, v/v). Experiment 3 (□): Strain H222 grown in YP broth supplemented with glucose (4%, w/v) and dodecanol (1%, v/v), dodecanal (1%, w/v), dodecanoic acid (1%, w/v) or dodecanedioic acid (1%, w/v). Experiment 4 (△): Strains W29, MTLY37, MTLY35, MTLY35A and MTLY21 grown in YP broth supplemented with glucose (4%, w/v). Experiment 5 (×): strains H222-41, PO1d, E129 and E150 in YP broth supplemented with glucose (4%, w/v) and dodecanol (0%, 0.5% and 1%, v/v). Samples for turbidimetric measurements and haemacytometer counts were centrifuged after treatment with cyclohexane and 5 M NaOH (method 1). Samples for dry biomass were washed with cyclohexane and 5 M NaOH.

Further work demonstrated that the use of the above cyclohexane plus NaOH pre-treatment on *Y. lipolytica* cultures grown on olive oil or sunflower oil failed to give satisfactory pellet formation upon centrifugation (results not shown). Efforts to use this method of sample preparation for turbidity measurements on cultures of *Candida tropicalis* and *Rhodotorula* spp. grown on or in the presence of hydrophobic substrates such as dodecane and hexadecane were also unsuccessful because NaOH addition (high pH) did not abolish cell hydrophobicity of these yeasts (results not shown).

Conclusions

The purpose of this study was to develop and evaluate a rapid, cost-effective method for monitoring and comparing growth of different *Yarrowia lipolytica* strains in the presence of different hydrophobic substrates. It was important to limit sample size because some substrates are expensive and incubator space may be limited. We established that washing with cyclohexane plus NaOH did not result in any significant loss in biomass when filtration was used to harvest biomass from the culture broth. To estimate biomass production in the presence of hydrophobic substrates, one has to consider the accuracy and the repeatability of the method. In our hands turbidity measurements gave a more accurate and repeatable reflection of growth than did cell counts and was much faster and easier to use. Pre-treatment of samples for turbidimetric analysis with cyclohexane and NaOH gave reproducible values, using only single 500 µl samples, but could result in an underestimation of biomass concentration if a suitable standard curve was not used. Increasing the pH of samples to 14 abolished to a large extent the hydrophobicity of *Yarrowia lipolytica* cells that otherwise prevented proper pelleting of cells in the presence of an organic solvent. The fact that hydrophobicity can vary during different stages of growth might lead to slight inaccuracies, but is very difficult to compensate for, requiring standard curves representing different growth phases and different growth conditions.

When liquid hydrophobic compounds are present, turbidimetric measurements can also be performed directly on untreated culture broth. In this case, it would be preferable to use multiple samples (i.e. 3 x 500 µl) to compensate for the greater experimental variation. In the presence of solid substrates it is, however, essential to separate the biomass from the growth medium.

References

Barth G, Gaillardin C (1996) *Yarrowia lipolytica*, p. 313-388. In K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Germany

Bouchez-Naïtali M, Blanchet D, Bardin V, Vandecasteele J (2001) Evidence for interfacial uptake in hexadecane degradation by *Rhodococcus equi*: the importance of cell flocculation. *Microbiology* **147**: 2537-2543.

Green KD, Michael KT, Woodley JM (2000) *Candida cloacae* oxidation of long-chain fatty acids to dioic acids. *Enzyme Microb. Technol.* **27:** 205-211.

Kim T, Lee J, Oh Y, Bae K, Kim S (1999) Identification and characterization of an oil-degrading yeast *Yarrowia lipolytica* 180. *J. Microbiol.* **37:** 128-135.

Kim T, Oh Y, Kim S (2000) The possible involvement of the cell surface in aliphatic hydrocarbon utilization by an oil-degrading yeast *Yarrowia lipolytica* 180. *J. Microbiol.Biotechnol.* **10**: 333-337.

Marino F, Karp J, Cooper D (1998) Biomass measurements in hydrocarbon fermentations. *Biotechnol. Techniques* **12:** 385-388.

Mauersberger S, Wang H, Gaillardin C, Barth G, Nicaud J (2001) Insertional mutagenesis in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*: generation of tagged mutations in genes involved in hydrophobic substrate utilization. *J. Bacteriol.* **183**: 5102-5109.

Wang H, Ledall M, Wache Y, Laroche C, Belin J, Gaillardin C, Nicaud J (1999) Evaluation of Acyl Coenzyme A oxidase (Aox) Isozyme function in the *n*-alkane-assimilating yeast *Yarrowia lipolytica. J. Bacteriol.* **181**: 5140-5148

Chapter 4

Toxicity of fatty alcohols and fatty acids to *Yarrowia lipolytica* and the preparation of dodecanol-tolerant strains

Toxicity of fatty alcohols and fatty acids to *Yarrowia lipolytica* and the preparation of dodecanol-tolerant strains

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Key words: *Yarrowia lipolytica*, hydrophobic substrate, dodecanol-tolerant strains, toxicity

Abstract

The toxicity of dodecane and its terminal and diterminal oxidation products on *Yarrowia lipolytica* wild type strain H222 was investigated. Dodecanol severely inhibited growth of *Y. lipolytica* strain H222 in YP media with glucose whereas in the defined media YNB without additional carbon source dodecanal and dodecanoic acid were the most toxic. The results presented in the first round of experiments indicated to us the possibility of preparing cells that can tolerate C12 alcohol. As a result dodecanol tolerant strains were prepared from the wild type strain H222 and the acyl-CoA oxidase deficient triple POX deleted mutant MTLY35. We succeeded in preparing two dodecanol tolerant strains. The first H222A was prepared by step-wise increasing dodecanol concentrations in YP broth supplemented with glucose to 7.5% (v/v). The second strain MTLY35A was prepared on YP agar plates without glucose by step-wise increasing dodecanol concentrations to 8.5% (v/v). No significant amounts of dodecanedioic acid was accumulated by the dodecanol-tolerant strains H222A and MTLY35A when grown on glucose in the presence of dodecanol. Two dodecanol concentration were tested 3 % and 10 % (v/v).

Introduction

Most research on *n*-alkane assimilation by yeasts was done in the 1960s and 1970s (Mauersberger *et al.*, 1996). These references are today not always readily available and yeast classification underwent several revisions since then. In a review on alkane assimilation by *Candida maltosa* Mauersberger *et al.*, (1996) stated that yeast strains such as *Candida maltosa*, *Candida, tropicalis* and *Candida sake* could assimilate shorter chain volatile hydrocarbons when the substrate concentrations were reduced to non-toxic levels. In this case shorter chain volatile alkanes are defined as *n*-alkanes with less than nine carbons. Mauersberger *et al.*, (1996) also stated that *C. maltosa* can grow well on the terminal and diterminal oxidation products of alkanes, i.e. 1-alkanols, fatty aldehydes, fatty acids, ω-hydroxy fatty acids and dicarboxylic acids, but that the shorter chain lengths ($< C_{10}$) of these substrates are found to be toxic for yeast cells or inhibit growth on alkanes. In a relatively recent article Green *et al.*, (2000) showed that decane and dodecane as well as fatty acids of chain lengths C10, C12, C14 and C16 were toxic to a dicarboxylic acid accumulating strain of *C. cloacae*, but that this toxicity could be alleviated by the addition of 5% (v/v) pristane. *C. cloacae* is a synonym of *C. maltosa* (Mauersberger *et al.*, 1996).

We are interested in alkane assimilation and dicarboxylic acid accumulation by *Y. lipolytica*. Since it appeared from the above mentioned references as if alkane and fatty acid toxicity might be strain dependent, we investigated the toxicity of dodecane and its terminal and diterminal oxidation products towards *Y. lipolytica*. Growth experiments were conducted with *Y. lipolytica* H222, the strain used by Mauersberger *et al.*, (2001) to construct tagged mutants deficient in alkane degradation. After establishing that dodecanol and dodecanal are the most toxic oxidation products, we also investigated the preparation of dodecanol-tolerant strains of *Y. lipolytica*. Both *Y. lipolyica* H222 as well as an acyl coenzyme A oxidase triple deleted mutant *Y. lipolytica* MTLY35 (Δ*POX2*, *POX3*, *POX5*) (Wang *et al.*, 1999) were used for the preparation of dodecanol-tolerant

strains. The possibility that dodecanol-tolerant strains accumulate dodecane dicarboxylic acid as a means of detoxification, was also investigated.

Materials and methods

Microorganisms

Yarrowia lipolytica wild type strain H222 (Wang *et al.*, 1999) and the acyl Coenzyme A oxidase triple deleted mutant MTLY35 (*ΔPOX2*, *POX3*, *POX5*) (Wang *et al.*, 1999) were used in this study. These strains were obtained from the Laboratoire Microbiologie et Génétique Moléculaire, Institute National Agronomique Paris-Grignon, France. *Y. lipolytica* strains H222A and MTLY35A were derived from the above mentioned *Y. lipolytica* strains by adaptation to increasing concentrations of dodecanol. All strains were stored under liquid nitrogen in the MIRCEN yeast culture collection of the University of the Free State, South Africa.

Growth conditions

Cultivation in liquid media was, unless stated otherwise, performed with 25 ml YNB medium or YP broth in 500 ml Erlenmeyer flasks on a rotary shaker at 180 r min^{-1} at 25°C. These shake flasks were inoculated with 24 h YPD cultures for toxicity and bioconversion experiments or with 24 h cultures grown in YP medium supplemented with 1 % v/v hexadecane.

YP broth contained (per litre distilled water): 10 g yeast extract (Merck) and 10 g peptone (Merck). YNB medium contained (per litre) 0.05 M phosphate buffer at pH 6.8: 1.7 g YNB (Yeast nitrogen base without amino acids and ammonium sulphate, Difco), 4 g NH_4Cl, (Merck), 0.1 g Uracil, (Sigma), 1 g Yeast extract. In toxicity and bioconversion experiments test substrates were added to YP broth supplemented with 2% or 4% (w/v) glucose. In carbon source utilization experiments the test substrates were added to YP or YNB medium. In some cases pristane (Fluka) was added as a co-solvent or internal standard and in some cases Tween 80 (Sigma) was added as an emulsifier. The substrates tested were

dodecane (Fluka), dodecanol (Fluka), dodecanal (Fluka), dodecanoic acid (Sigma), dodecanedioic acid (Fluka), hexadecane (Fluka), hexadecanol (Sigma), hexadecanoic acid (Fluka), hexadecanedioic acid (Fluka). Dodecane, dodecanol and dodecanal were added to autoclaved media, while the other substrates were added before media were autoclaved. Final concentrations of glucose, the test substrates, pristane and Tween 80 are given in the legends of figures.

Turbidimetric measurements

Samples of 500 µl each were added to 200 µl cyclohexane plus 100 µl 5 M NaOH in 1.5 ml microcentrifuge tubes, vortexed for 5 min and centrifuged at 12 000 r min^{-1} for 10 min. The supernatants were discarded and the pellets were resuspended in 500 µl of physiological saline solution containing of 0.9 % (w/v) NaCl. The turbidity of 200 µl samples, suitably diluted before transfer to a microtitre plate, were measured at 620 nm using a Labsystems iEMS reader MF (Thermo BioAnalysis company, Helsinki Finland).

Extraction and analysis

Samples (500µl) were taken at 24h intervals and acidified first to a pH of 3 by addition of 1M HCl (50µl). The samples were extracted twice with 300µl of tert-butylmethyl ether (Aldrich) containing undecanol (0.5% v/v) as internal standard and the phases separated by centrifugation. The extracts were combined. Samples of the extracts (50µl) were transferred to new 1.5 ml microcentrifuge tubes and methylated with 50µl trimethylsulfonium hydroxide (TMSH) (Butte, 1983). The samples were analysed by gas chromatography. Concentrations were determined from pre-determined standard curves for different substrates and products.

GC analysis was carried out using a Hewlett Packard Hp 6890 series GC system equipped with a CP wax 52CB polar column measuring 30 x 0.53 mm x 1µm. The GC conditions were as follows: Nitrogen was used as a carrier gas at a rate of 38.4 ml/min and a split ratio of 5:1. Injector temperature: initial oven temperature was at 120°C for 5

min, then increasing at 10°C/min to a final temperature of 260°C for 7min. Flow through the column was at 6.1 ml/min. The temperature of the detector was at 350°C

Adaptation of Y. lipolytica strain H222 to increased concentrations of dodecanol in YP broth

Y. lipolytica wild type strain H222 grown in YP broth containing 4 % w/v glucose was sequentially transferred to YPD broth containing increasing concentrations of dodecanol, as set out in figure 1. Transfer to media containing higher concentrations of dodecanol was continued until a final concentration of 7.5 % v/v dodecanol was reached. After 24h the culture adapted to 7.5 % v/v dodecanol was streaked onto YP agar plates containing only 4 % w/v glucose. From the plates frozen stocks were prepared of this adapted strain labelled H222A.

Adaptation of Y. lipolytica strain MTLY35 to increased concentrations of dodecanol on YP agar plates

MTLY35 was streaked out on plates containing 1% (w/v) yeast extract, 2% (w/v) peptone 2% (w/v) agar supplemented with dodecanol. Initially the medium was supplemented with 0.1% (v/v) dodecanol. Plates were incubated at 30°C until growth was observed. Adaptation was achieved through sequential transfer of culture to plates containing 0.2%, 0.5%, 1%, 2,5%, 7.5% and finally 8.5% (v/v) dodecanol. From the plate containing 8.5% v/v dodecanol a culture was again streaked onto YP agar plates containing only 4 % w/v glucose. Frozen stocks were prepared of this adapted strain labelled MTLY35A.

Results and Discussion

YP broth containing 4% (w/v) glucose was used to determine toxicity of alkanes and alkane oxidation intermediates when glucose is present as a carbon source. YNB medium without glucose was used to evaluate toxicity in a poor medium without an additional carbon source. In the first round of experiments, the toxicity of dodecane and its derivatives dodecanol, dodecanal, dodecanoic acid and dodecane dioic acid was evaluated in the presence of glucose (Figure 2). To compare growth, turbidimetric measurements were taken at regular intervals over a period of 50h following the

procedure previously described (chapter 1). From this experiment it was evident that *Y. lipolytica* H222 was able to grow very well in the presence of dodecane and dodecane dioic acid giving OD measurements of 6.6 and 6.5. These ODs were comparable to that of the control flask containing only glucose in which the final OD was 6.6 after 50h (figure 2). OD measurements of ca. 6.5 translate to a dry biomass concentration of approximately 7.7 g/l (chapter 1). Dodecanal and dodecanoic acid were toxic giving OD measurements of 3.5 and 3.6 respectively after 50h, but not as toxic as dodecanol, which severely inhibited growth so that the OD measurement after 50h was only 1.5. The sudden increase in OD from 0.29 after 24h to 1.35 after 48h instilled the idea that there might be a population of cells that are relatively tolerant to the toxicity of the dodecanol.

In the next experiment we evaluated the ability of *Y. lipolytica* H222 to use the C12 derivatives as carbon sources. . Pristane (2% v/v) was added as a co-solvent and tween80 (0.1 % v/v) as an emulsifier (Barth and Gaillardin 1996). According to Green *et al.*, (2000), the branched alkane tends to reduce the toxicity of fatty acids when it is added to the media at a concentration of 5 % (v/v). Since dodecane and dodecane dioic acid did not show any toxicity in the presence of glucose, they were in this case considered to be the positive controls giving OD measurements of 4.31 and 4.17 respectively after 55h (figure 3). In this experiment the fatty aldehyde and the fatty acid inhibited growth the most giving final OD measurements of only 1.7 and 1.3 respectively after 55h of cultivation. Growth on dodecanol was initially delayed but recovered after 24h to finally reach a maximum OD of 3.7.

Growth of *Y. lipolytica* H222 in YP media containing glucose (4% w/v) was not affected by hexadecane or its derivatives hexadecanol, hexadecanoic acid or hexadecane dioic acid when added to a final concentration of 1% (v/v or w/v) (figure 4). When *Y. lipolytica* H222 was cultivated in YNB media containing the C16 derivatives as carbon sources growth on hexadecanol and hexadecanoic acid was severely inhibited when compared to growth on hexadecane and hexadecane dioic acid. (Figure 5). The OD measurements after 50h cultivation on the C16 alcohol and C16 acid were 1.5, while final OD measurements after 50h growth on hexadecane and hexadecane dioic acid reached 6.4.

Thus growth on hexadecanol as carbon source was worse than growth on dodecanol as carbon source, even though hexadecanol was less toxic than dodecanol when glucose was the carbon source. Growth on hexadecane as carbon source was also better than growth on dodecane as carbon source.

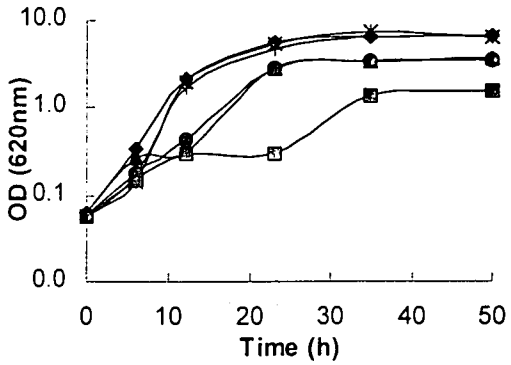


Figure 2. Growth of *Y.lipolytica* strain H222 in YP media with 4% (w/v) glucose supplemented with C12 derivatives, 1% (v/v) dodecane (◆), 1% (v/v) dodecanol (■), 1% (w/v) dodecanal (▲), 1% (w/v) dodecanoic acid (●), 1% (w/v) dodecanedioic acid (∗). A control containing 4% (w/v) glucose (†) was also tested. Samples were treated with cyclohexane and NaOH before centrifugation

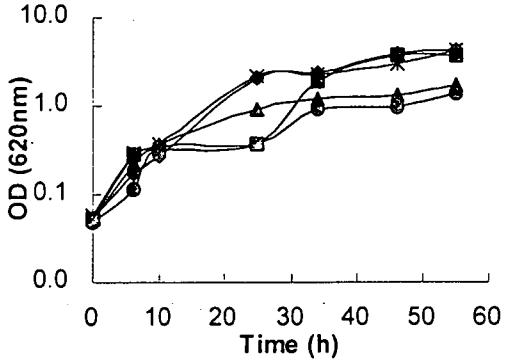


Figure 3. Growth of *Y.lipolytica* strain H222 in YNB media supplemented with the following C12 derivatives as carbon sources: 1% (v/v) dodecane (◆), 1% (v/v) dodecanol (■), 1% (w/v) dodecanal (▲), 1% (w/v) dodecanoic acid (●) and 1% (w/v) dodecanedioic acid (∗). Samples were treated with cyclohexane and NaOH before centrifugation.

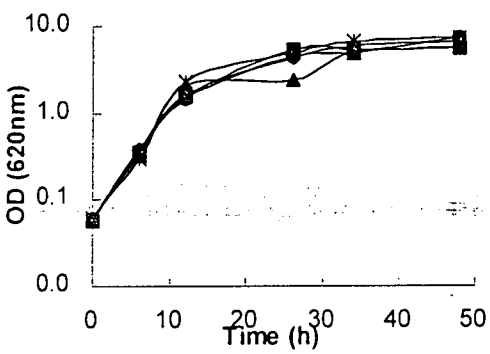


Figure 4. Growth of *Y.lipolytica* strain H222 in YP media with 4% (w/v) glucose supplemented with C16 derivatives 1% (v/v) hexadecane (◆), 1% (w/v) hexadecanol (■), 1% (w/v) hexadecanoic acid (●) and 1% (w/v) hexadecanedioic acid (▲). A control containing 4% (w/v) glucose (∗) was also tested. Samples were treated with cyclohexane and NaOH before centrifugation.

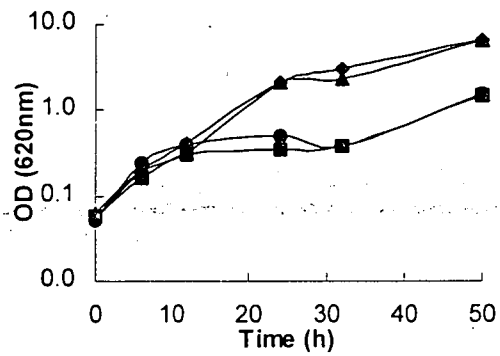
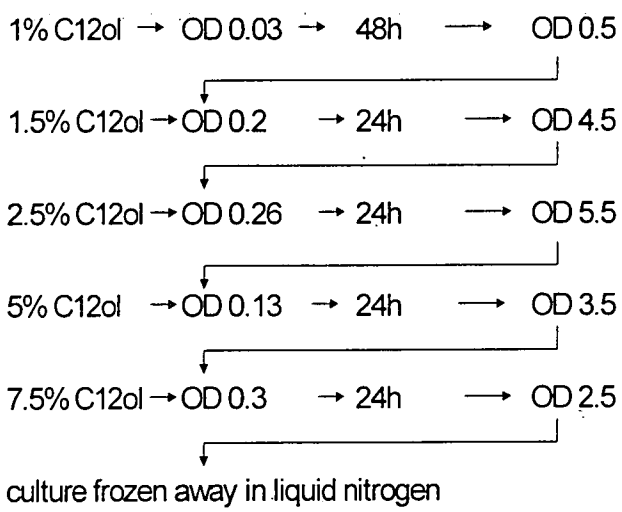


Figure 5. Growth of *Y.lipolytica* strain H222 in YNB media supplemented with C16 derivatives 1% (v/v) hexadecane (◆), 1% (w/v) hexadecanol (■), 1% (w/v) hexadecanoic acid (●) and 1% (w/v) hexadecanedioic acid (▲). Samples were treated with cyclohexane and NaOH before centrifugation.

A dodecanol-tolerant strain of *Y. lipolytica* H222 was prepared by transferring cells every 48 or 24h to fresh YP-glucose broth containing a higher concentration of dodecanol, until a final concentration of 7.5% (v/v) was reached (scheme 1). The dodecanol-tolerant strain labelled H222A was streaked on YP agar containing 4 % glucose



Scheme 1. Transfer procedure followed to adapt *Yarrowia lipolytica* H222 to increased dodecanol concentrations. Cultures were grown in YP broth containing 4% (w/v) glucose and dodecanol at the indicated concentrations (v/v).

Flasks containing YP broth with 2% glucose as well as 3% and 10% (v/v) dodecanol were subsequently inoculated from 24h old pre-cultures of strains H222 and H222A grown in YP broth containing 4% glucose. Strain H222 grew very poorly in the presence of dodecanol (3% and 10% (v/v), whereas the dodecanol-tolerant strain grew almost equally well in the absence and presence of dodecanol (fig. 6 shows results for 10% (v/v) dodecanol, results for 3% dodecanol not shown).

The triple *POX2-POX3-POX5* deleted strain MTLY35 had been derived from the French *Y. lipolytica* strain W29 (Wang *et al.*, 1999). We found that W29, which does not grow as well on n-alkanes as the German strain H222 (Mauersberger *et al.*, 2001), was much more sensitive to dodecanol than H222 (results not shown). We therefore initially thought that it would not be possible to produce dodecanol-tolerant strains from the *POX* deleted

strains derived from W29. However, we eventually succeeded in preparing a dodecanol-tolerant strain of MTLY35, labelled MTLY35A, by growing MTLY35 on YP agar without glucose, containing, initially, only 0.1% (v/v) dodecanol and then transferring to YP agar plates containing increasing concentrations of dodecanol until a concentration of 8.5% (v/v) dodecanol was reached. MTLY35A also grew equally well on glucose in the absence and presence of 10% (v/v) dodecanol (fig. 7). It did not, however, grow on dodecanol as only carbon source when supplied in a liquid YP medium. This was an unexpected result, because during the adaptation glucose was not present as carbon source in the solid YP media.

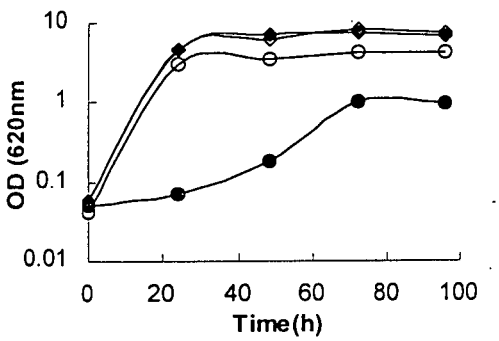


Figure 6. Growth of *Yarrowia lipolytica* wild type strain H222 and the adapted strain with 10% (v/v) dodecanol (●○) and without dodecanol (◆◇). Solid symbols indicate the wild type strain and open symbols the dodecanol adapted strain. The cultures were grown in YP broth supplemented with 2% (w/v) glucose. The experiment was done in duplicate.

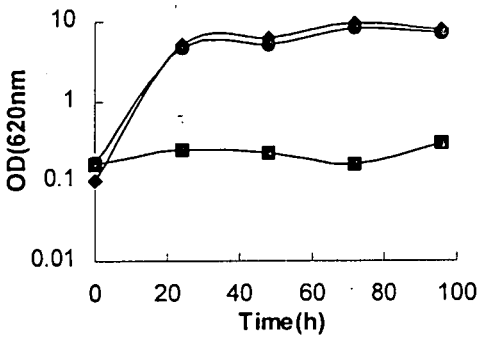


Figure 7. Growth of *Y. lipolytica* MTLY35A in different YP media in the presence of 0.5% (v/v) pristane and 2% (w/v) glucose (◆), 0.5% (v/v) pristane, 2% (w/v) glucose and 10% (v/v) dodecanol (●) and 0.5% (v/v) pristane and 10% (v/v) dodecanol (■). The experiment was done in duplicate.

While growing strains H222, H222A and MTLY35A on glucose containing YP broth supplemented with dodecanol, samples were taken for GC analysis, in order to determine whether these strains accumulated dodecanedioic acid as a means of detoxification. When the dodecanol-tolerant strain H222 was grown in YP media with 2% (w/v) glucose supplemented with 3% (v/v) dodecanol it was possible to observe the disappearance of

the substrate (figure 8A) from samples. When the dodecanol was present at a concentration of 10% (v/v) it formed a second phase, which sometimes solidified at 25°C. Relatively little dodecanol was therefore present in the samples from cultures of strain H222A (figure 8B) despite vigorous mixing during sampling. The situation improved slightly with strain MTLY35A when incubation was done at 30 °C and pristane (0.5% v/v) was also added to the cultures (figure 9). The dodecanol concentration in samples increased after 60h incubation in the case of strain H222A and after 120h incubation in the case of strain MTLY35A (figures 8b and 9). This might be due to increased adherence to the biomass or to the production of a biosurfactant by *Y. lipolytica* (Kim *et al.*, 1999).

Neither strain H222A nor strain MTLY35A accumulated significant concentrations of dodecanedioic acid. In some samples dioic acid concentrations of maximum 1 mg/ml were detected. Slightly higher concentrations of the monocarboylic acid was accumulated. Strain H222A was able to accumulate 1.8 mg/ml of dodecanoic acid after 72h when dodecanol was present at a concentration of 3% (v/v). In the presence of 10% (v/v) dodecanol it accumulated 2 mg/ml dodecanoic acid after 96h. The dodecanoic acid that was accumulated by MTLY35A grown in the presence of 10% (v/v) dodecanol reached a concentration of 9 mg/ml after 168h (figure 9). The triple *POX2-POX3-POX5* deleted strain MTLY35 is deficient in β-oxidation and its growth on oleic acid in solid media was slightly impaired (Wang *et al.*, 1999). Accumulation of mono or dicarboxylic acids from dodecanol by MTLY35A was therefore more likely.

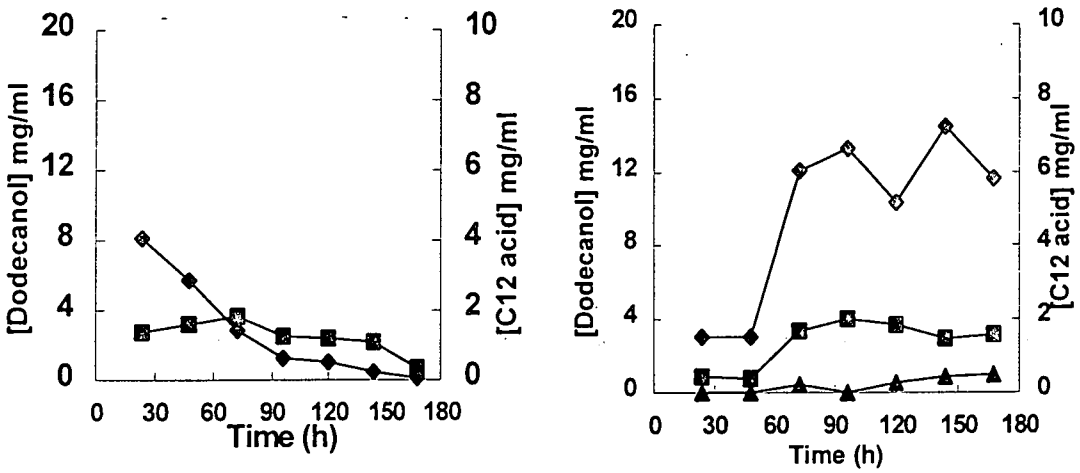


Figure 8. Bioconversion of dodecanol (◆) by the adapted *Yarrowia lipolytica* wild type strain H222 grown in YP media with 2% (w/v) glucose. In A the initial dodecanol concentration was 3% v/v and in B 10% v/v. Graph A shows accumulation of dodecanoic acid (■). Graph B shows accumulation of dodecanoic acid (■) and dodecanedioic acid (▲).

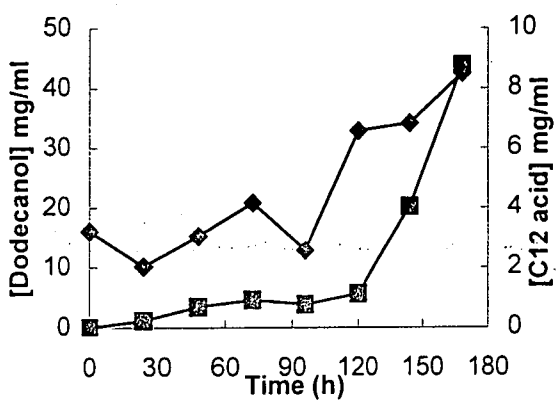


Figure 9. Bioconversion of dodecanol (◆) by the *Y. lipolytica* MTLY35A (ΔPOX2 POX3, POX5) to accumulate dodecanoic acid (■).The strain did not accumulate any dioic acid. The substrate 10% (v/v) was added to glucose grown cultures in the presence of 0.5% (v/v) pristane.

It is evident from the GC analysis results that strains H222A and MTLY35A did not accumulate dioic acid as a means of detoxification. According to literature the tolerance to many toxic solvents were related to the properties of the cell membrane (Isken *et al* 1999, Kawamoto *et al* 2001). Samples of strains H222A and MTLY35A grown in the absence and presence of dodecanol must still be subjected to lipid analysis.

In this study the preparation of dodecanol-tolerant strains from *Y. lipolytica* H222 and MTLY35 did not result in any accumulation of dioic acids. However, this study illustrated that it is possible to prepare dodecanol-tolerant strains of *Y. lipolytica*. It should be possible to use similar procedures to prepare strains tolerant to other solvents. Solvent-tolerant yeasts have great potential for industrial applications as already pointed out by Kawamato *et al* (2001). One example is the use of a two-phase system for asymmetric reduction by baker's yeast of a β-keto acid to a β-hydroxy acid in isooctane (Kawamato *et al.*, 2001). Solvent-tolerant microorganisms can also find application in extractive fermentions (Yabannavar and Wang, 1990) and in bioremediation and removal of pollutants from waste waters and contaminated terrestrial sites (Isken *et al.*, 1999).

Conclusions

The effect of toxic solvents or substrates on different microorganisms can partially or severely inhibit growth, but cells that can adapt to the same solvents can achieve maximum growth in their presence. It is evident that the growth inhibiting effects of these solvents or substrates maybe strain specific. In conclusion we succeeded in preparing dodecanol tolerant strains from *Y. lipolytica* wild type strain H222 and the triple-deleted strain MTLY35. As it has already been mentioned the samples of these strains H222A and MTLY35A are still subjected to lipid analysis, but for this study the adaptation mechanisms that triggered the ability of these *Y.lipolytica* strains to tolerate high concentration of dodecanol are not known. Adaptation of these strains H222 and MTLY35 did not result in any accumulation of dioic acids and although this strains still need to be characterized further it is possible that in the near future the may show potential in industrial applications.

References

Barth G, Gaillardin C (1996) *Yarrowia lipolytica*, p. 313-388. In K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Germany.

Butte W (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for transesterification. *J. Chrom.* **261**: 142-145

Green KD, Michael KT, Woodley JM (2000) *Candida cloacae* oxidation of long-chain fatty acids to dioic acids. *Enzyme Microb. Technol.* **27**: 205-211.

Isken S, Derks A, Wolffs, F, de Bont J (1999) Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. *Appl. Environ. Microbiol.* **65**: 2631-2635

Kawamoto T, Kanda, T, Tanaka, A (2001) Preparation of an organic solvent-tolerant strain from baker's yeast. *Appl. Microbiol. Biotechnol.* **55**: 476-479

Kim T, Lee J, Oh Y, Bae K, Kim S (1999) Idenitification and characterization of an oil-degrading yeast *Yarrowia lipolytica* 180. *J. Microbiol.* **37**: 128-135

Mauersberger S, Ohkuma, M., Schunck, W.-H., Takagi, M (1996) *Candida maltosa*, p. 411-580. In K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Germany.

Mauersberger S, Wang H, Gaillardin C, Barth G, Nicaud J (2001) Insertional mutagenesis in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*: generation of tagged mutations in genes involved in hydrophobic substrate utilization. *J. Bacteriol.* **183**: 5102-5109.

Wang H, Ledall M, Wache Y, Laroche C, Belin J, Gaillardin C, Nicaud J (1999) Evaluation of Acyl Coenzyme A oxidase (AOX) Isozyme function in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*. *J.Bacteriol.* **181**: 5140-5148

Yabannavar V, Wang D (1990) Strategies for reducing solvent toxicity in extractive fermentations. *Biotechnol. Bioeng.* **37**: 716-722

Chapter 5

Dioic acid accumulation by Acyl Coenzyme A Oxidase deficient mutants of *Yarrowia lipolytica*

Dioic acid accumulation by Acyl Coenzyme A Oxidase deficient mutants of *Yarrowia lipolytica*

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Key words: Yarrowia lipolytica, dioic acid; dicarboxylic acid; alkane; acyl coenzyme A oxidase; diterminal oxidation; ω-oxidation; fatty acid

Abstract

Five genes *POX1* through *POX5* encode five acyl coenzyme A oxidase isozymes in *Yarrowia lipolytica*. Dioic acid accumulation from alkanes and alkane degradation intermediates was investigated using *Y lipolytica* wild type strain W29 and POX deleted strains MTLY21 (*ΔPOX2*, *POX3*), MTLY35 (*ΔPOX2*, *POX3*, *POX5*) and MTLY37 (*ΔPOX2*, *POX3*, *POX4*, *POX5*). The quadruple-deleted strain MTLY37 was the only strain that was able to accumulate dioic acids from alkanes, alkanols and monocarboxylic acids. Dodecane was the best alkane substrate for dioic acid accumulation yielding 7 mg/ml dodecane dioic acid after 144h (23% w/v conversion). Lauric acid did not yield any dioic acid (probably due to toxicity), but 5 mg/ml hexadecanedioic acid was accumulated from palmitic acid after 48h. All the strains accumulated dodecanedioic acid from the diterminal functionalised 1,12 dodecane diol and ω-hydroxy dodecanoic acid. The quadruple-deleted strain MTLY37 accumulated a maximum concentration of 20 mg/ml dodecanedioic acid after 48h from 1,12 dodecanediol, while the triple-deleted strain MTLY35 accumulated 18 mg/ml dodecanedioic acid after 48h from 12-hydroxydodecanoic acid.

Introduction

The alkane degradation pathway is well characterized in various yeasts such as *Candida tropicalis, Candida maltosa* and *Yarrowia lipolytica*. This alkane assimilation by yeasts

was found to occur mainly via the monoterminal and diterminal oxidation pathways. Figure 1 shows the first steps of the diterminal alkane degradation pathway.

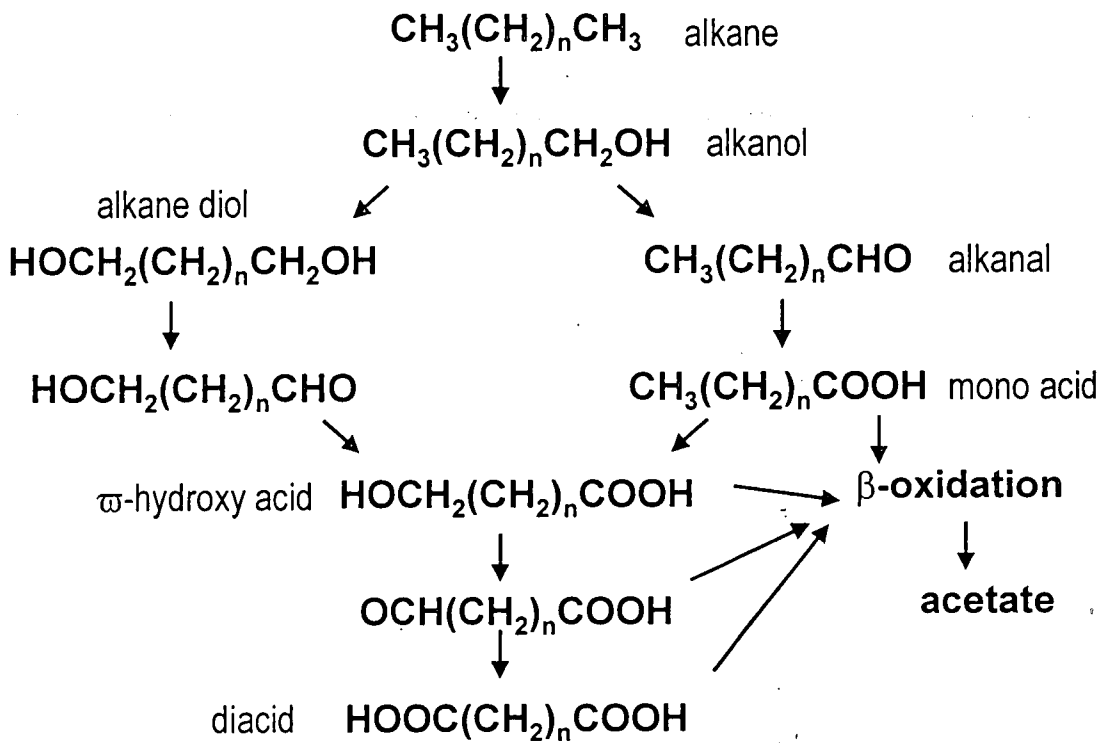

Figure 1. The diterminal alkane degradation pathway in yeasts results in the accumulation of dicarboxylic acids when β-oxidation is blocked. (Casey *et al.*, 1990)

In the mid 1960's, research was focused on production of single cell protein, however with time, attention turned towards production of metabolic products that are of commercial interest, such as dicarboxylic acids (Barth and Gaillardin 1996). Aliphatic dicarboxylic acids are versatile oleochemicals useful as raw materials for the industrial production of surfactants, lubricants, detergents and cosmetics (Picataggio *et al.*, 1992). The shorter chain dioic acids such as adipic acid are readily available but the chemical synthesis of the longer chain dioic acids is more difficult (Green *et al.*, 2000). Over the years several patents have appeared which describe dioic acid production by mutant strains of *C maltosa* and *C tropicalis* defective in their ability to grow on alkanes and fatty acids. Since 1993 Picataggio and others (Picataggio *et al.*, 1993; Picataggio *et al.*,

1997; Fallon *et al.*, 1999; Mobley and Shank, 2000) patented several processes for dioic acid production based on genetically engineered strains of *C. maltosa* and *C. tropicalis* with the acyl coenzyme A oxidase encoding genes (*POX* genes) disrupted. Acyl coenzyme A oxidases catalyse the first step of β-oxidation of fatty acids.

Y. lipolytica is another alkane utilizing yeast for which genetic engineering tools have been developed. It differs from the *Candida* spp. in that it is non-pathogenic and associated with fermented foods such as cheese and sausage (Barth and Gaillardin 1996). Five acyl coenzyme A oxidase isozymes (Aox1 through Aox5) encoded by the *POX1* through *POX5* genes have been identified in *Y. lipolytica* (Wang *et al.*, 1999). Single, double, triple and quadruple disruptants were constructed to evaluate the function of the various isozymes. The quadruple *POX2-POX3-POX4-POX5*-deleted mutant did not grow on fatty acids, while the strain with *POX2*, *POX3* and *POX5* deleted showed only partial growth on fatty acids. All the double disruptants showed normal growth on fatty acids in liquid medium. Growth of the *POX2-POX3*-deleted mutant on plates containing oleic acid as carbon source was slightly inhibited.

In this study we compared the bioconversion of different chain length n-alkanes and alkane degradation intermediates to dioic acids by the wild type strain W29, the double POX2-POX3-deleted mutant, the triple POX2-POX3-POX5-deleted mutant and the quadruple POX2-POX3-POX4-POX5-deleted mutant.

Materials and methods

Microorganisms

Yarrowia lipolytica wild type W29 and POX-deleted strains MTLY21 (Δ*POX2*, *POX3*), MTLY35 (*ΔPOX2*, *POX3*, *POX5*) and MTLY37 *(ΔPOX2*, *POX3*, *POX4*, *POX5*) strains used in this study were obtained from the Laboratoire Microbiologie et Génétique Moléculaire, Institute National Agronomique, Paris-Grignon, France. All the strains were maintained and stored under liquid nitrogen in the MIRCEN yeast culture collection of the University of Free State, South Africa.

Growth and bioconversion conditions

YP broth contained (per litre distilled water): 10 g yeast extract (Merck), 10g peptone (Merck) and 40g glucose. Pre-cultures were grown for 24h in 12.5 ml YP broth in 250

ml Erlenmeyer flasks and 1% (v/v) inoculums of these cultures were used. Bioconversion reactions were performed by adding substrates to 48h old cultures grown in 25 ml YP broth in 500 ml Erlenmeyer flasks. For growth and bioconversion flasks were incubated on a rotary shaker at 180 r min^{-1} at 25°C.

Substrates tested were dodecane (Fluka), tetradecane (Fluka), hexadecane (Fluka), hexadecanoic acid (Fluka), hexadecanol (Sigma), 12-hydroxydodecanoic acid (Fluka), 1,12-dodecanodiol (Fluka) and lauric acid (Sigma). Liquid hydrocarbons were added directly to the culture media to give a final concentration of 3% (v/v). In the case of solid substrates stock solutions/suspensions of 10% (w/v) were prepared and added to the culture media after 48h to give a final concentration of 3% (v/v). After addition of the carbon substrates the pH of the media was adjusted to 8 with 1M NaOH. Samples for GC analysis were taken every 24h.

Extraction and analysis

Samples (500µl) were taken at 24h intervals and acidified first to a pH of 3 by addition of 1M HCl (50µl). The samples were extracted twice with 300µl of tert-butylmethyl ether (Aldrich) containing myristic acid (0.5% w/v) as internal standard and the phases separated by centrifugation. The extracts were combined. Samples of the extracts (50µl) were transferred to new 1.5 ml microcentrifuge tubes and methylated with 50µl trimethylsulfonium hydroxide (TMSH) (Butte, 1983). The samples were analysed by gas chromatography. Concentrations were determined from pre-determined standard curves for dodecanedioic acid (Fluka), hexadecanedioic acid (Fluka) and the different substrates. GC analysis was carried out using a Hewlett Packard Hp 6890 series GC system equipped with a CP wax 52CB polar column measuring 30 x 0.53 mm x 1µm. The GC conditions were as follows: Nitrogen was used as a carrier gas at a rate of 38.4 ml/min and a split ratio of 5:1. Injector temperature: initial oven temperature was at 120°C for 5 min, then increasing at 10°C/min to a final temperature of 260°C for 7min. Flow through the column was at 6.1 ml/min. The temperature of the detector was at 350°C.

Results and Discussion

Production of dioic acids from alkanes

When dodecane was added to a final concentration of 3% (v/v) to 48 h old cultures (15 – 18 g dry biomass/l) of the *Yarrowia lipolytica* wild type strain W29 and the three *POX*-deleted strains MTLY21 *(ΔPOX2, POX3)*, MTLY35 *(ΔPOX2, POX3, POX5)* and MTLY37 *(ΔPOX2, POX3, POX4, POX5)* grown in YP medium containing 4% (w/v) glucose only the quadruple deleted mutant MTLY37 accumulated dodecanedioic acid (results not shown). Figure 2 shows dioic acid accumulation when dodecane, tetradecane and hexadecane were added to *Y lipolytica* quadruple deleted mutant MTLY37. The highest dioic acid concentration reached was 7 mg/ml dodecanedioic acid after 144 h. This mutant strain was only able to accumulate 2.4 mg/ml of tetradecanedioic acid and only 0.7 mg/ml hexadecanedioic acid. It was difficult to follow consumption of the alkanes, because sampling of such hydrophobic substrates is difficult when only small samples are used. However, there was apparently not much consumption of tetradecane and hexadecane, since samples taken after 144h still contained 20 mg/ml tetradecane and 42 mg/ml of hexadecane.

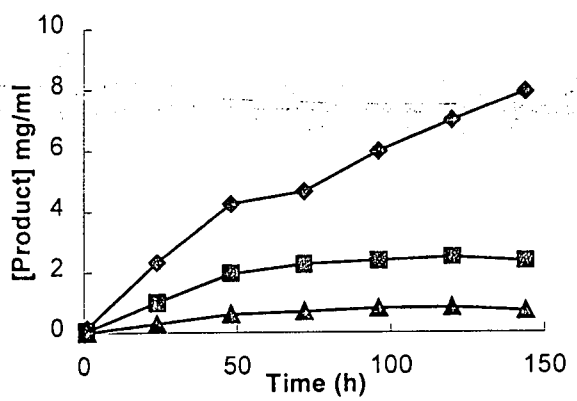

Figure 2. Conversion of dodecane, tetradecane, and hexadecane by *Y.lipolytica* quadruple-deleted strain MTLY 37 *(ΔPOX2, POX3, POX4, POX5)* to form dodecanedioic acid (◆), tetradecanedioic acid (■) and hexadecanedioic acid (▲). The substrates (3 % v/v) were added after 48 h to cultures grown in YP medium containing 4% (w/v) glucose. The pH of the media was every 24h adjusted to 8 with 1M NaOH.

In the case of dodecane there was still 10 mg/ml dodecane present in samples taken after 144h. It therefore appears as if the longer chain alkanes are either not taken up by glucose grown cultures or not hydroxylated. Iida *et al.*, (2000) had shown that the gene product of *YlALK1* was essential for growth on short chain alkanes such as decane and dodecane. It is possible that mainly *YlALK1* was induced under the conditions used for these bioconversion experiments.

Production of dioic acids from monoterminal functionalised alkanes

In a next series of experiment we tested the bioconversion of mono functionalised alkanes in order to better understand the rate limiting steps in dioic acid formation. It was not possible to test for bioconversion of dodecanol or lauric acid, because these substrates are toxic to glucose grown cells of *Y. lipolytica* W29. No dioic acids were accumulated in a bioconversion experiment with lauric acid as substrate using the wild type W29 and *POX*-deleted strains. The acid was not consumed and remained constant at 30 mg/ml in the aqueous media (results not shown).

The longer chain substrates hexadecanoic acid and hexadecanol were not toxic and the hexadecanoic acid concentration in samples was within 1h reduced to ca. 16 mg/ml and after 96h to less than 2 mg/ml in all four cultures of *Y lipolytica* (figure 3A). The initial rapid consumption of hexadecanoic acid in all four cultures might be due to incorporation into the lipid fractions. It was not possible to monitor consumption of hexadecanol because it is a water insoluble solid. The rate at which hexadecanoic acid disappeared from the culture of the quadruple deleted mutant MTLY37 was slightly slower than from the other cultures and this strain was the only one that was able to accumulate hexadecanedioic acid from hexadecanoic acid and hexadecanol. The highest concentration of hexadecanedioic acid that was accumulated from hexadecanoic acid was 5 mg/ml at 48h and from the hexadecanol it was 4 mg/ml at 96h as shown in figure 3B After 96h the dioic acid concentration started to decrease. It is not known whether the remaining *POX1* gene becomes activated in such old cultures.

Production of dioic acids from diterminal functionalised alkanes

Diterminal functionalised alkanes do not require any hydroxylation to be converted to dioic acids. 12-Hydroxydodecanoic acid disappeared at approximately the same rate from cultures of the wild type strain W29 and the double and triple *POX*-deleted strains MTLY21 and MTLY35, while it disappeared at a slightly slower rate from the quadruple deleted mutant MTLY37 (fig 4A). All cultures accumulated dodecanedioic acid, decanedioic acid and octanedioic acid. The wild type strain W29 accumulated 19 mg/ml dodecanedioic acid within 24h, the triple-deleted strain MTLY35 *(ΔPOX2, POX3, POX5)* accumulated 18 mg/ml dodecanedioic acid within 48 h and the double-deleted strain MTLY21 *(ΔPOX2, POX3)* accumulated a maximum of 13 mg/ml within 24h.

Although disappearance of the hydroxy acid was slower from the culture containing quadruple deleted mutant MTLY37 the highest concentration of dodecanedioic acid reached was only 6 mg/ml after 48h (figure 4B) Within 68h dodecanedioic acid had disappeared from all four cultures. The double and triple deleted mutants accumulated relatively high concentrations of 6 and 7 mg/ml decanedioic acid respectively (figure 4C) while the wild type strain W29 accumulated only 2 mg/ml decanedioic acid and the quadruple deleted mutant MTLY37 accumulated almost no detectable decanedioic acid. The rate at which octanedioic acid was accumulated depended on the number of *POX* genes deleted. The wild-type strain W29 and the double deleted mutant accumulated maximum concentrations of 3 and 4 mg/ml octanedioic acid respectively after 48h while the triple and quadruple deleted strains accumulated maximum concentrations of 3 mg/ml after 68h. Octanedioic acid did not completely disappear from the cultures like decanedioic acid, but persisted at a concentration of ca. 1 mg/ml in all cultures up to 120h after substrate addition as shown in figure 4D.

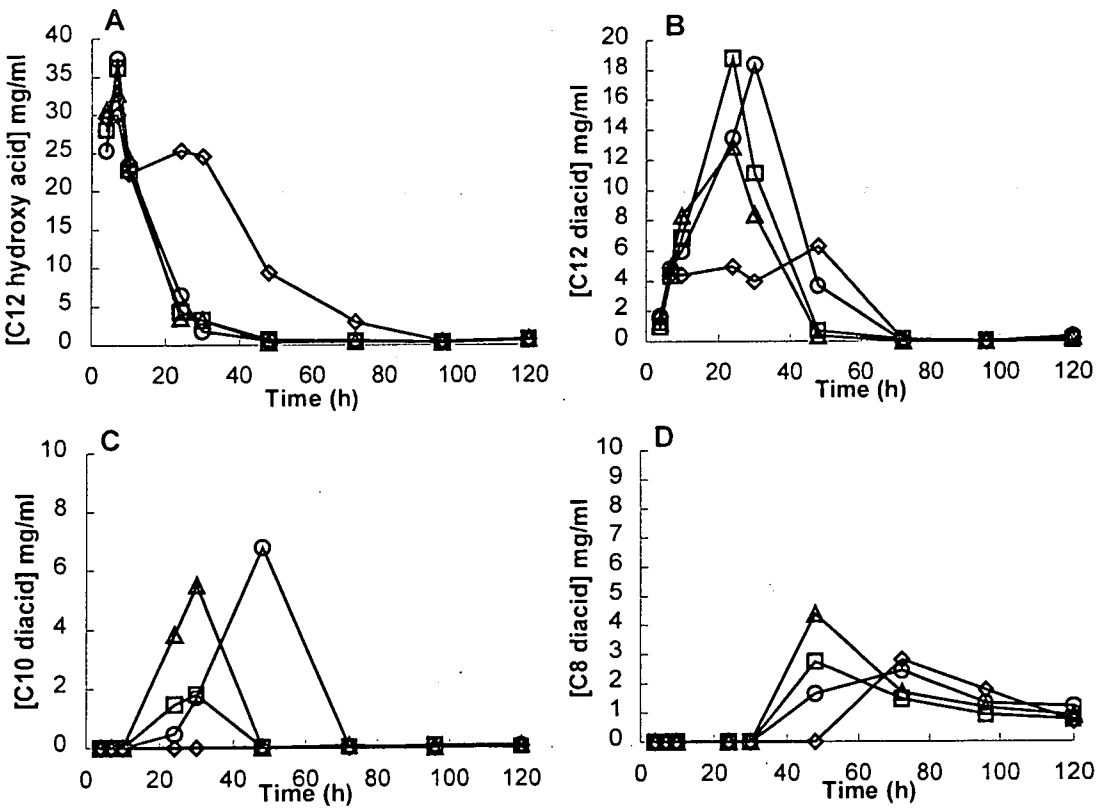

Figure 4. Bioconversion of (A) 12-hydroxy dodecanoic acid by *Yarrowia lipolytica* wild type (□) W29 and *POX*-deleted strains (△) MTLY 21 *(ΔPOX2, POX3)*, (○) MTLY 35 *(ΔPOX2, POX3, POX5)* and (◇) MTLY 37 *(ΔPOX2, POX3, POX4, POX5)* to accumulate (B) dodecanedioic acid, (C) decanedioic acid and (D) octanedioic acid. The substrate (3 % w/v) was added after 48 h to cultures grown in YP medium containing 4% (w/v) glucose. The pH of the media was every 24h adjusted to 8 with 1M NaOH.

The same *Y. lipolytica* strains were used for the bioconversion of 1,12 dodecane diol to form the dioic acids (figure 5A). The substrate 1,12 dodecane diol was oxidized to form the product dodecanedioic acid, that was further degraded to form decanedioic acid and octanedioic acid. In this experiment the disappearance of 1,12 dodecane diol was again not easily monitored, since the substrate is a water insoluble solid and therefore it was not possible to get representative sampling (results not shown). The rate at which dodecanedioic acid was degraded correlated with the numbers of *POX* genes deleted. The

mutant strain MTLY37 took the longest time for the product to disappear and accumulated the highest concentration of dodecanedioic acid (20 mg/ml after 48h) (figure 5A) All these mutant strains were able to accumulate only less than 2 mg/ml of decanedioic acid as shown in figure 4B with the double-deleted strain MTLY 21 accumulating almost 7 mg /ml of octanedioic acid after 48h. The quadruple deleted mutant MTLY37 was the slowest at accumulating octanedioic acid and accumulated the lowest concentration, only 2 mg/ml after 96h (figure 5C).

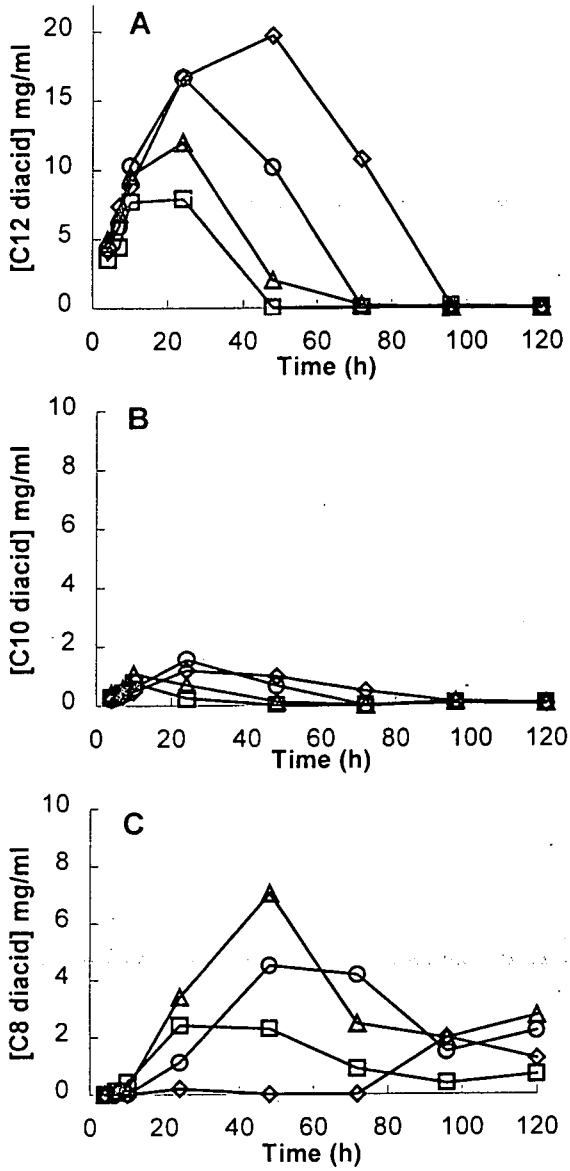

Figure 5. Bioconversion of 1,12 dodecane diol by *Yarrowia lipolytica* wild type (□) W29 and *POX*-deleted strains (△) MTLY 21 *(ΔPOX2, POX3)*, (○) MTLY 35 *(ΔPOX2, POX3, POX5)* and (◇) MTLY 37 *(ΔPOX2, POX3, POX4, POX5)* to accumulate (A) dodecanedioic acid, (B) decanedioic acid and (C) octanedioic acid. The substrate (3 % w/v) was added after 48 h to cultures grown in YP medium containing 4% (w/v) glucose. The pH of the media was every 24h adjusted to 8 with 1M NaOH.

Summary

The rate at which the dioic acids accumulate and the highest concentrations reached is a function of the rate at which they are formed and the rate at which they are degraded (further oxidised). It is evident from this study that the rate of hydroxylation of the alkanes, the alkanols and the monocarboxylic acids are much slower than the rate of dioic acid oxidation. It is only when four of the *POX* genes are deleted that any dioic acids are accumulated from the alkanes or mono functionalised alkanes. It appears that substrate uptake and hydroxylation is complex with regard to chain length specificity, since hexadecane yielded very little dioic acid but hexadecanoic acid yielded a dioic acid concentration that was comparable to the dioic acid concentration reached with dodecane. The fatty alcohols are oxidised to aldehydes and then to carboxylic acids by fatty alcohol oxidases and long chain alcohol and aldehyde dehydrogenases (Mauersberger *et al.*, 1996). It is evident from the experiments with the diterminal functionalised substrates that the rate at which these oxidations occur are slightly faster than the rate at which the fatty acyl CoAs are formed and oxidised. In the case of the C12 substrates both the ω-hydroxy acid as well as the diol are substrates for these oxidative enzymes. It is not clear why the quadruple deleted strain MTLY37 accumulated less dioic acid from the ω-hydroxy acid than the other strains.

This is the first study to report results on production of dioic acids by genetically modified strains of *Y. lipolytica*. In future more research should be directed at optimising growth of *Y. lipolytica* mutants as well as bioconversion conditions in order to increase the production of dioic acids. It is also necessary to study further and understand the genetic regulation of the enzymes involved in the alkane degradation pathway.

References

Barth G, Gaillardin C (1996) *Yarrowia lipolytica*, p. 313-388. In K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Germany.

Butte W (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for transesterification. *J. Chrom.* **261**: 142-145

Casey J, Dobb R, Mycock G (1990) An effective technique for enrichment and isolation of *Candida cloacae* mutants defective in alkane catabolism. *J. Gen. Microbiol.* **136**: 1197-1202

Fallon, R. D., Payne, M. S. and Picataggio, S.K. (1999) Transformed yeast strains and their use for the production of monoterminal and diterminal aliphatic carboxylates World IPO patent 9904014A2.

Green KD, Michael KT, Woodley JM (2000) *Candida cloacae* oxidation of long-chain fatty acids to dioic acids. *Enzyme Microb. Technol.* **27**: 205-211.

Iida T, Sumita T, Ohta A, Takagi M (2000) The cytochrome P450ALK multigene family of an *n*-alkane-assimilating yeast, *Yarrowia lipolytica*: cloning and characterization of genes coding for new CYP52 family members. *Yeast.* **16**: 1077-1087

Jiao P, Huang Y, Li S, Hua Y, Cao Z (2001) Effects and mechanisms of H_2O_2 on production of dicarboxylic acid. *Biotechnol and Bioeng.* **75**: 456-462

Mauersberger S, Ohkuma, M., Schunck, W.-H., Takagi, M (1996) *Candida maltosa*, p. 411-580. In K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag, Berlin, Germany.

Mauersberger S, Wang H, Gaillardin C, Barth G, Nicaud J (2001) Insertional mutagenesis in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*: generation of tagged mutations in genes involved in hydrophobic substrate utilization. *J. Bacteriol.* **183**: 5102-5109.

Mobley, D.P. and Shank, G.K. (2000) Method for high specific bioproductivity of alpha, omega-alkane dicarboxylic acids using *Candida tropicalis*. World IPO patent 0017380A1

Picataggio S, Rohrer T, Deanda K, Lanning D, Reynolds R, Mielenz J, Eirich D (1992) Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. *Biotechnol.* **10**: 894-898

Picataggio, S., Deanda, K and Eirich, L.D. (1993) Site-specific modification of the *Candida tropicalis* genome. US Patent 5254466

Picataggio, S., Rohrer T. and Eirich, L.D. (1997) Method for increasing omega-hydroxylase activity in *Candida tropicalis*. US Patent 5648247

Wang H, Ledall M, Wache Y, Laroche C, Belin J, Gaillardin C, Nicaud J (1999) Evaluation of Acyl Coenzyme A oxidase (AOX) Isozyme function in the *n*-alkane-assimilating yeast *Yarrowia lipolytica*. *J.Bacteriol.* **181**: 5140-5148

Summary

Dicarboxylic acids are value added products, which can be prepared by diterminal oxidation of n-alkanes by bacteria or yeasts with a block in β-oxidation. *Yarrowia lipolytica* is one of the alkane-utilizing yeasts for which genetic tools have been developed. *Y. lipolytica* has a complex set of five acyl Coenzyme A (CoA) oxidase isozymes (encoded by *POX1* through *POX5* genes) with different substrate specificities. Abundant information is available on dioic acid accumulation from n-alkanes by *C. maltosa* and *C. tropicalis* strains deficient in β-oxidation. In many cases β-oxidation had been blocked at the level of the acyl CoA oxidases. In comparison very little information was available on dioic acid accumulation by *Yarrowia lipo*lytica. We had through our collaboration with Dr J-M Nicaud of the INRA-CNRS in France access to the above mentioned series of *Y. lipolytica* strains with the acyl CoA oxidase encoding genes disrupted. It thus became the purpose of my studies to investigate dioic acid accumulation by *Y. lipolytica* strains with impaired β-oxidation.

In order to study the growth of different *Yarrowia lipolytica* strains on or in the presence of a range of liquid and solid n-alkanes or alkane derivatives we required a rapid method using small samples to estimate biomass production. Insoluble hydrophobic substrates interfere with turbidimetric measurements. This problem is more severe if the hydrophobic substrate is a solid. It is not possible to efficiently separate *Y. lipolytica* cells from a hydrophobic substrate by centrifugation in the presence of an organic solvent, because *Y. lipolytica* cells are hydrophic and a large percentage of cells cling to the water/solvent interface. We established that pre-treatment of samples for turbidimetric analysis with 5M NaOH, thus increasing the pH of samples to 14, abolished to a large extent the hydrophobicity of *Y. lipolytica* cells. We also established that washing of the cells with cyclohexane and NaOH when cells were harvested by filtration, did not result in any significant loss in biomass. Based on these observations we developed a simple, cost effective sample preparation procedure for turbidimetric analysis, which gave accurate, repeatable turbidity measurements with no interference from the hydrophobic substrates. This method involved the pre-treatment of small

samples (500 µl) with 5 M NaOH plus cyclohexane, prior to harvesting the biomass by centrifugation. Sample preparation was carried out in microcentrifuge tubes and turbidimetric measurements were done with a microtitre plate reader.

The newly developed procedure was used to investigate the toxicity of dodecane and hexadecane as well as their terminal and diterminal oxidation products to *Y. lipolytica* wild type strain H222. The alkanes and dicarboxylic acids were never toxic to *Y. lipolytica*. Dodecanol severely inhibited growth of *Y. lipolytica* strain H222 in YP media with glucose whereas in a semi-synthetic YNB medium without additional carbon source dodecanal and dodecanoic acid were the most toxic. Hexadecanol and hexadecanoic acid did not inhibit growth of *Y. lipolytica* in YP medium with glucose, but were toxic to Y. lipolytica in a semi-synthetic YNB medium without glucose. The results obtained in the first round of experiments indicated to us the possibility of preparing dodecanol-tolerant strains. Two dodecanol-tolerant strains were subsequently prepared. The first H222A was prepared by step-wise increasing dodecanol concentrations in YP broth supplemented with glucose to 7.5% (v/v). The second strain MTLY35A was prepared on YP agar plates without glucose by step-wise increasing dodecanol concentrations to 8.5% (v/v). Dodecanedioic acid was not accumulated by the dodecanol-tolerant strains H222A or by the triple *POX*-deleted strain MTLY35A, when grown on glucose in the presence of dodecanol. Two dodecanol concentration were tested 3 % and 10 % (v/v).

Dioic acid accumulation from C12 and C16 alkanes and alkane degradation intermediates was investigated using *Y lipolytica* wild type strain W29 as well as the POX deleted strains MTLY21 *(ΔPOX2, POX3)*, MTLY35 *(ΔPOX2, POX3, POX5)* and MTLY37 *(ΔPOX2, POX3, POX4, POX5)*. The quadruple-deleted strain MTLY37 was the only strain that was able to accumulate dioic acids from alkanes, alkanols and monocarboxylic acids. Dodecane was the best alkane substrate for dioic acid accumulation yielding 7 mg/ml dodecanedioic acid after 144h (23% w/v conversion). Lauric acid did not yield any dioic acid (probably due to toxicity), but 5 mg/ml hexadecanedioic acid was accumulated from hexadecanoic acid after 48h. All the strains accumulated dodecanedioic acid from the diterminal functionalised 1,12 dodecane diol and ω-hydroxy

dodecanoic acid. The quadruple-deleted strain MTLY37 accumulated a maximum concentration of 20 mg/ml dodecanedioic acid after 48h from 1,12 dodecanediol, while the triple-deleted strain MTLY35 accumulated 18 mg/ml dodecanedioic acid after 48h from 12-hydroxydodecanoic acid.

Opsomming

Dikarboksielsure is waardevolle produkte wat vanaf alkane berei kan word *via* diterminale oksidasie deur bakterië en giste. *Yarrowia lipolytica* is een van die alkaan-assimilerende giste waarvoor werktuie vir genetiese manipulering reeds ontwikkel is. *Y. lipolytica* het 'n komplekse stel van 5 asiel ko-ensiem A (KoA) oksidase iso-ensieme (gekodeer deur *POX1* tot *POX5* gene) met verskillende substraat spesifisiteite. Baie inligting is beskikbaar rakende dikarboksielsuur produksie vanaf alkane deur *Candida maltosa* en *Candida tropicalis* stamme met onvoldoende β-oksidasie. In baie gevalle was β-oksidasie geblokeer op die vlak van die asiel KoA oksidases. Daar was in verhouding min inligting beskikbaar rakende dikarboksielsuur produksie deur *Y. lipolytica* stamme. Ons het danksy 'n samewerking met Dr. J.-M. Nicaud van die INRA-CNRS in Frankryk toegang gehad tot 'n reeks mutante met verskillende asiel-KoA oksidases uitgelaat. Dit was dus die doel van my studie om ophoping van dikarboksielsure deur hierdie *Y. lipolytica* stamme te ondersoek.

Ons het in die eerste instansie behoefte gehad aan 'n eenvoudige metode om groei te volg. Dit moes verkieslik 'n metode gebaseer op turbiditeitsmeting wees. Onoplosbare hidrofobiese substrate meng normaalweg in met turbiditeitsmetings. Hierdie probleem word vererger wanneer die substraat ook 'n vaste stof is. *Y. lipolytica* selle kan nie d.m.v. sentrifugering geskei word van 'n hidrofobiese substraat omdat die selle hidrofobies is en 'n groot persentasie van die selle klou aan die water/oplosmiddel interfase. Ons het vasgestel dat wanneer die selle met 5M NaOH behandel word (pH 14) dit nie meer hidrofobies is nie. Gebaseer op hierdie waarneming het ons 'n prosedure vir monstervoorbereiding ontwikkel vir biomassa bepaling gebaseer op turbiditeit. In hierdie metode word monsters met 5M NaOH en sikloheksaan behandel, voordat dit d.m.v. sentrifugering geoes word. Monstervoorbereiding kan in mikrosentrifuge buise gedoen word en turbiditeitsmetings met 'n mikrotiterplaatleser.

Die nuwe metode om groei te volg is gebruik om die toksiese effek van dodekaan en heksadekaan asook hulle terminale en diterminale oksidasieprodukte op *Y. lipolytica*

H222 te bepaal. Die alkane en dikarboksielsure was nooit toksies vir *Y. lipolytica* nie. Dodekanol het groei die meeste inhibeer in 'n gisekstrak-peptoon medium met glukose, terwyl dodekanal en dodekanoësuur groei die meeste inhibeer het in 'n semi-sintetiese medium. Heksadekanol en heksadekanoësuur was nie toksies vir *Y. lipolytica* in 'n gisekstrak-peptoon medium met glukose nie, maar was wel toksies in 'n semi-sintetiese medium sonder glukose. Uiteindelik het ons vanaf *Y. lipolytica* H222 en MTLA35, 'n stam met drie asiel-KoA oksidases uitgelaat, stamme berei wat kan groei in die teenwoordigheid van baie hoë konsentrasies dodekanol (10% v/v). Nie een van hierdie stamme het dikarboksielsuur opgehoop, wanneer dit in die teenwoordigheid van hoë konsentrasies dodekanol gegroei is nie (3% en 10% v/v).

Dikarboksielsuurproduksie vanaf C12 en C16 alkane asook hulle geöksideerde derivate is vervolgens ondersoek. 'n *Y. lipolytica* wilde tipe stam W29 en drie stamme met *POX* gene uitgelaat nl. MTLY21 *(ΔPOX2, POX3)*, MTLY35 *(ΔPOX2, POX3, POX5)* en MTLY37 *(ΔPOX2, POX3, POX4, POX5)* is in hierdie ondersoek gebruik. Die stam met vier POX gene uitgelaat, MLTY37, was die enigste wat dikarboksielsure opgehoop het vanaf alkane en geöksideerde derivate. Dodekaan was die beste alkaan substraat vir die ophoping van dikarboksielsuur en het 7 mg/ml gelwer na 144h (23% omskakeling). Dodekanoësuur het geen dikarboksielsuur gelewer nie (waarskynlik omdat dit toksies was), maar heksadekanoësuur het 5 mg/ml dikarboksielsuur gelewer na 48h. Al die stamme het dikarboksielsuur opgehoop vanaf die diterminaal gefunksionaliseerde C12 substrate. MTLY37 het 20mg/ml dikarboksielsuur opgehoop vanaf 1,12-dodekaandiol en MTLY35 het 18 mg/ml opgehoop vanaf 12-hidroksie dodekanoësuur.