Oxidation of long-chain n-alkanes by mutants of a thermophilic alkane-degrading bacterium: *Thermus sp.* ATN1

Dem Promotionsausschuss der

Technischen Universität Hamburg-Harburg

zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften (Dr. rer. nat.)

genehmigte Dissertation

von

José Luis García Ojeda

aus

Parral, Chihuahua, Mexiko

2013

Gutachter:

1. Prof. Dr. rer. nat. Rudolf Müller

2. Prof. Dr.-Ing. Wolfgang Calmano

Tag der mündlichen Prüfung: 5. April 2013

Abstract

In this work, mutants of the thermophilic bacterium, *Thermus sp.* ATN1, capable to degrade long-chain n-alkanes at 70°C, have been constructed by classical mutagenesis. Long-chain n-alkanes were converted constitutively by these mutants to long-chain aldehyde intermediates, mono- and dicarboxylic acids.

Additionally, the gene encoding an alcohol dehydrogenase, suspected to be involved in the metabolism of long-chain n-alkanes in this strain, was disrupted by homologous recombination in an attempt to create mutants producing terminally di-hydroxylated compounds. Only fatty acids were obtained. Over-oxidation of the alcohol products by the alkane monooxygenase complex in this strain or the presence of another alcohol dehydrogenase are proposed as explanation for these results.

Nevertheless, the constructed mutants were capable to produce long-chain α,ω-dicarboxylic acids with up to 28 carbon atoms by bioconversion of the corresponding n-alkanes and it is expected that saturated substrates with up to 32 carbon atoms can be converted by these mutants. This is the first known report on bioconversion of aliphatic hydrocarbons of chain lengths larger than 18 carbon atoms to mono and dicarboxylic acids with a thermophilic bacterium.

The broad range of long-chain diacid products obtained with these mutants could be produced for new types of polymers, adhesives, lubricant additives, pharmaceuticals and other novel applications. In addition, *Thermus sp.* ATN1 offers several advantages over other microorganisms for the production of long-chain α,ω-dicarboxylic acids. The strain is non-pathogenic and its thermophilic nature provides unique characteristics for process control to avoid culture contamination.

Finally, the production of a biosurfactant by this thermophilic strain and its characterization are described. This biosurfactant showed advantages over commercial surfactants in the characterization tests, especially at high temperatures. The use of this biosurfactant as hydrocarbon bioavailability enhancer is demonstrated in the utilization of n-hexadecane by a mesophilic strain.

Zusammenfassung

In dieser Arbeit wurden Mutanten des thermophilen, Alkan-abbauenden Bakteriums *Thermus sp.* ATN1 durch klassische Mutagenese hergestellt. Langkettige n-Alkane wurden durch diese Mutanten zu langkettigen Aldehydezwischenprodukten, Mono- und Dicarbonsäuren konstitutiv bei 70 °C umgesetzt. Zusätzlich wurde das Gen, das für eine Alkoholdehydrogenase kodiert, durch homolge Rekombination ausgeschaltet. Dieses Enzym ist vermutlich am Abbau von langkettigen n-Alkanen in diesem Bakterienstamm beteiligt. Diese Mutanten sollten eigentlich α,ω-di-hydroxylierte Verbindungen produzieren, es wurden jedoch nur Fettsäuren erhalten. Überoxidation der Alkoholprodukte durch den Alkanmonooxygenasekomplex in diesem Stamm oder das Vorhandensein einer weiteren Alkoholdehydrogenase werden als Erklärungen für diese Ergebnisse vorgeschlagen.

Dennoch waren die konstruierten Mutanten in der Lage, langkettige α,ω-Dicarbonsäuren mit bis 28 Kohlenstoffatomen durch Biokonversion der entsprechenden n-Alkane zu produzieren. Es wird erwartet, dass gesättigte Substrate mit bis 32 Kohlenstoffatomen durch diese Mutanten umgesetzt werden können. Dieses ist der erste bekannte Bericht über eine Biokonversion von aliphatischen Kohlenwasserstoffen mit mehr als 18 Kohlenstoffatomen zu den entsprechenden Mono- und Dicarbonsäuren mit einem thermophilen Bakterium.

Das breite Spektrum von langkettigen α,ω-Dicarbonsäuren welche durch diese Mutanten hergestellt werden können, könnte für neue Typen von Polymeren, von Klebstoffen, von Schmierstoffadditiven, von Pharmazeutika und für andere neue Anwendungen genutzt werden. Außerdem bietet *Thermus sp.* ATN1 einige Vorteile über anderen Mikroorganismen für die Produktion von langkettigen α,ω-Dicarbonsäuren. Der Stamm ist nicht pathogen und seine thermophile Natur bietet einzigartige Vorteile für die Prozesskontrolle, da bei hohen Temperaturen die Kontaminationswahrscheinlichkeit sehr viel geringer ist.

Schließlich wurde die Produktion eines Biotensids durch diesen thermophile Stamm und dessen Charakterisierung beschrieben. Dieses Biotensid zeigte Vorteile gegenüber kommerziellen Tensiden, besonders bei hohen Temperaturen. Die positive Wirkung dieses Biotensids zur Erhöhung der Bioverfügbarkeit und Abbaubarkeit von Kohlenwasserstoffen, wurde am Beispiel des Abbaus von n-Hexadekan durch einen mesophilen Stamm bewiesen.

Acknowledgments

Many have been those that crossed my path and contributed in different but significant ways to the completion of this project. My gratitude goes to all of them.

I would like to express my sincerest gratitude and appreciation to my supervisor Prof. Dr. Rudolf Müller for his invaluable support and guidance over these years, especially for trusting me the opportunity to work in his research group despite the particular circumstances under which this research was completed. I would also like to thank Prof. Dr. Wolfgang Calmano and Prof. Dr. An-Ping Zeng for evaluating this thesis.

Special thanks are also extended to Prof. Dr. Andreas Liese for supporting this work at the Institute of Technical Biocatalysis. I am also very grateful to all the colleagues I met at the Institute and in particular to those that directly contributed to this project: Maren Breuer, Pamela Vazquez and Ranganathan B. Venkatesan.

Finally I would like to thank my family for their always unconditional support and in special to Dona, for her patience and encouragement during my pursuit of this Ph.D.

Oxidation of long-chain n-alkanes by mutants of a thermophilic alkane-degrading bacterium: *Thermus sp.* ATN1

Contents

List of Figures

List of Tables

Chapter 1

1. Introduction

Throughout history, there have been many examples of processes designed to convert a given raw material into numerous "refined" products while making efficient use of the resource (Lynd *et al.,* 2005). Petroleum refining and extraction of natural oils are regarded as essential processes for modern society; the first being the most important source of primary energy and producing the major percentage of today's chemical industry feedstocks[1], the latter being a fundamental part of the food sector but also producing the most important renewable raw materials for the oleo-chemical industry[2] (Metzger *et al.*, 2006, 2009).

The chemical industry provides innumerable useful and valuable products. However, because of its reliance on fossil resources, its environmentally unfriendly production processes, and the production of toxic by-products, waste and products that are not readily recyclable or degradable after their useful life, the industry has come under increasing pressure to make chemical production more eco-friendly (Hatti-Kaul *et al.*, 2007).

[1] World's annual fossil resources consumption was about 9,800 million metric tons oil equivalent in 2009, with about 37% accounting for North America and the European Union demand, proven oil reserves would meet 45 years of demand at current consumption rates, while known supplies of natural gas would last for more than 60 years and coal for up to 120 years (BP, 2010). In the U.S. the chemical industry consumes about 8% of the fossil energy demand being half of that percentage used directly as feedstock (U.S. Department of Energy, 2007) whereas in Europe consumption by the industry represents 11% of the fossil energy demand (Metzger et al. 2006); both regions add about 330 million metric tons oil equivalent annual consumption just for the chemical industry.

[2] Between 15 and 17 million metric tons of natural oils and fats are consumed annually by the chemical industry, representing about 14% of the global natural oils production which is about 120 million metric tons (Metzger et al. 2006).

For the past two decades governments have increased efforts to promote the development of chemical technologies that improve industry's sustainability performance guided by the concept of "green chemistry" based on principles like the use renewable feed stocks, selective catalysts and alternative, non-toxic solvents; minimizing risks, waste generation, energy consumption and design of safer and biodegradable chemicals (Anastas, P. and Warner, J., 1998).

Although the industry continues to explore and implement the use of renewable feed stocks to address long-term sustainability, extensive capitals assets and decades of technological development and manufacturing experience suggest that to completely supplant current processes will be very difficult (Thomas *et al.*, 2002).

Thus there is great interest not only in shifting the resource base for chemical production from fossil to renewable derived raw materials, but also into continue upgrading and developing processes that can contribute to improve the eco-efficiency of the chemical industry (as defined by WBCSD, 2000).

Major improvements in productivity and energy efficiency have been made to chemical processes during the past few decades and, to continue improving, it is probable that a biotechnological approach will be adopted with several existing feed stocks (including still vast fossil resources) and processes to extract higher values from feed stocks, process by-products and waste streams (Thomas *et al.*, 2002).

1.1 Chemicals through biotransformation

Industrial or white biotechnology provides tools for adapting and modifying the biological machinery of microorganisms and the utilization of substrates, processes and systems found in nature to develop processes that are more eco-efficient and products that are more environment-friendly, beyond performance that could normally be achieved using conventional chemical technologies (OECD, 2001).

Biocatalysis or biotransformation encompasses the use of biological systems to catalyze the conversion of one compound to another. The catalyst part of the biological system can thereby consist of whole cells, cellular extracts, or isolated enzyme(s). Biocatalytic processes are also denominated bioconversions when the catalytic activity involves living organisms. Different from fermentation processes, where there are several catalytic steps between the substrate and the product, biotransformations involve only one or two steps and the chemical structures of the substrate and the product resemble one another which is not necessarily the case for fermentations (Liese *et al.*, 2006).

The application of biological systems has been significantly developed for the production of high-value products like pharmaceuticals, food additives, commodity chemicals and fine chemicals while meeting several green chemistry principles (Hatti-Kaul *et al.*, 2007). Because of its demonstrated potential, biotechnology increasingly attracts attention also for the production of industrial chemicals. Table 1 shows some examples of biotransformation products in the metric ton scale, mainly fine chemicals (Ghisalba *et al.*, 2010).

Table 1.1 - Some examples of biotransformation products used in the metric ton scale (Ghisalba *et al.*, 2010).

Product	Metric tons/Year
Acrylamide	~250 000
Aspartame	10 000
Nicotinamide	15 000
L-Carnitine	>1000
L-DOPA	>150
7-aminocephalosporanic acid (7-ACA)	4000
(+)-6-aminopenicillanic acid (6-APA)	10 000
(*S*) Naproxen	>1000
Lysine	>1000 000
Glucose–Fructose syrup	12 000 000
Vitamin C	>100 000
Citric acid	1000 000

The main technical parameters influencing the costs of a biocatalytic process (metric ton scale), and therefore dictating its success, are productivity (as higher productivity is associated with lower capital costs), product concentration (which influences ease of product recovery and purification), yield (which dictates the cost of raw materials and the amount of byproduct which needs to be dealt with) and biocatalyst consumption. Successful product developments in the chemical

industry with biocatalysis involve on average a yield of 78%, a volumetric productivity of 15.5 g/L·h and a final product concentration of 108 g/L, figures that account for fine chemicals, whereas in contrast, pharmaceuticals production are focused on time-to-market (Straathof *et al.*, 2002).

Table 1.2 - Efficiencies of biocatalytic processes for the production of fine chemicals* (Straathof *et al.*, 2002).

Target compound class	Biocatalysts/enzymes used	Volumetric productivity (g/(L·h))				Final product concentration (g/L)				% Yield
		Average	Min	Max	Total number of processes	Average	Min	Max	Total number of processes	
Amino acids	Decarboxylase, aspartase, oxidoreductases, amidases, lyase; (enzyme and cell)†	54.6	27	130	5	102	11	330	14	82‡
Alcohols	Lipase, oxidoreductase, fumarase, kinase; (enzyme and cell)	4.2	0.1	7.5	7	107	0.2	800	14	88
Carbohydrates	Transferase, amylases, aldolases; (enzyme)	3.0	3	3	1	237	50	350	4	90
β-Lactams	Amidases, acylases, oxidase, lipase, peptidases; (enzyme)	18.5	18.5	18.5	1	87	7	200	9	94
Nucleotides	Lactamase, deaminase; (cell)	–	–	–	ng§	65	30	100	2	47‡
Acids	Lipases, esterases, amidases, hydroxylases, oxygenase; (enzyme and cell)	1.7	1	2.4	4	108	2	228	7	81‡
Epoxides	Oxygenase; (cell)	1#	1	1	1	7#	7	7	1	90
Hydroxy aromatics	Hydroxylases; (cell)	1.4	0.3	2.4	3	59	10	190	5	72
Amines	Lipase, oxidoreductase; (enzyme and cell)	12.8	12.8	12.8	1	80	20	200	4	43.5‡
Amides	Hydratase, oxidoreductases; (cell)	42.0	4	80	2	225	30	500	4	96 (44‡)
Total		15.5	0.1	130	25	108	0.2	800	64	78‡

*Annual production is over 1 metric ton. †The bioprocess is either based on whole cells (cell) or an isolated enzyme (enzyme) as biocatalyst. ‡The average % yield is limited by the application of classical kinetic resolutions in (part of) the processes. §ng, not given #Average for the production of a group of epoxide derivatives.

As well, it is expected that bulk chemicals including polymers may involve biotransformations such as conversion of methane to methanol (Chevron Research & Technology and Maxygen) or conversion of sugars to 3-hydroxypropionic acid (Cargill Inc. USA) or dehalogenation step in Dow□s alkene oxide process. Thus, the next generation of biocatalysis based processes will target large volume chemicals and polymers and will compete directly with petroleum-based products (Liese *et al.*, 2006) and/or will continue to incorporate biological systems into industrial existing processes or develop new ones with fossil based feed stocks; this is the case for the relatively recent production of long-chain dicarboxylic acids (DCA's) from aliphatic hydrocarbons (Cathay Industrial Biotech Ltd. China) which can also utilize naturally derived fatty acids for DCA's production (Schörken & Kempers, 2009).

1.2 Two important industrial chemicals - Long-chain alcohols and long-chain dicarboxylic acids

Long-chain terminal oxidized compounds like fatty alcohols and long-chain dicarboxylic acids (LCDAs) are important industrial chemicals mainly used as intermediates for additives used in valuable products like cosmetics, washing detergents, paints, coatings, lubricants and polymers.

These chemicals are industrially produced either from petro-chemical or renewable feed-stocks by chemical processing. Biocatalysis based production of these chemicals from fossil feed-stocks has typically remained at a research/exploratory stage. On the other hand, biotransformation of natural oils has been successfully developed up to an industrial scale in recent years. However, biotechnology is still a niche technology in the oleo-chemical industry and production of fatty alcohols and LCDAs still relies on chemical transformation processes (Schörken *et al.*, 2009).

1.2.1 Long-chain alcohols

1.2.1.1 Applications for long-chain alcohols

Long-chain or fatty alcohols (C12 and higher) are indispensable intermediates for the production of surfactants, but are also employed as free alcohols in cosmetics and some other applications (intermediates to amines and other chemicals). Surfactants account for 70 – 75 % of fatty alcohol production (Brackmann & Hager, 2004). The hydroxyl group may undergo a large number of chemical reactions making fatty alcohols versatile intermediates. Their amphiphilic character, which results from the combination of a non-polar, lipophilic carbon chain with a polar, hydrophilic hydroxyl group, confers surface activity upon these compounds (Presents *et al.,* 2000).

Surfactants are used in a wide range of fields. By far, the most important field of application is the washing and cleansing sector as well as textile treatment and cosmetics. These use more than 50 % of the total amount of surfactants. Surfactants are also used in the food sector, in crop protection, mining, and the production of paints, coatings, inks, and adhesives (Hill, 2007).

Global fatty alcohol production was predicted to be more than 2 million metric tons in 2010 with a 3.8% increase in annual demand until 2020 (Colin A. Houston & Associates, 2006). Market value was estimated to be about 3 billion $US, with an approximate price of 1,500 $US/metric ton (Steen *et al.,* 2010). Fatty alcohols manufacture is dominated by tropical-oil-based production (oleochemical), but around 35 % (~700 k metric tons) are being still produced from petrochemical feed stocks (Figure 1).

Oleochemical- and petrochemical-based surfactants have traded market dominance over the years based on factors such as consumer preference, capacity availability, and especially increasing crude oil price (McCoy, 2005).

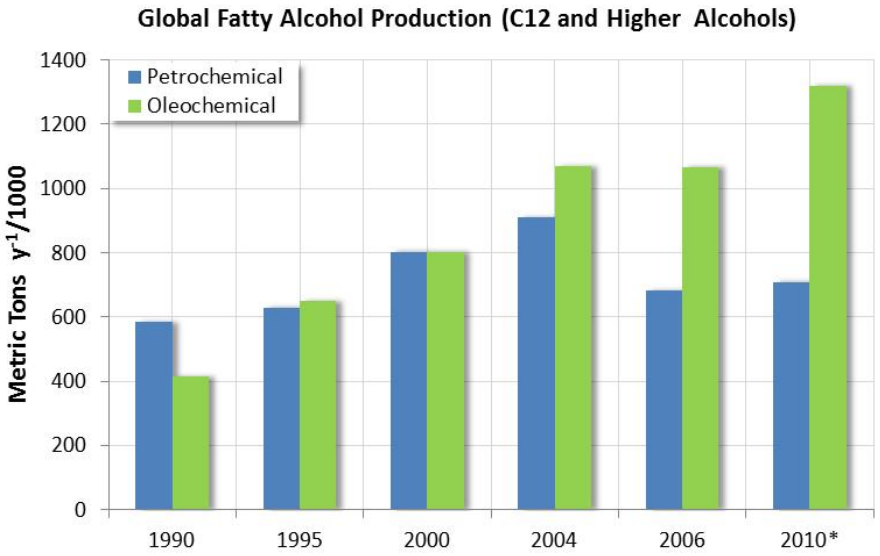


Figure 1.1 - Global fatty alcohol production. Sources: CESIO, 2001 (1990-2000); Colin A. Houston & Associates, 2006 (2004-2010*). * Predicted.

1.2.1.2 Industrial production processes

Fatty alcohols derived from natural fats and oils are normally produced by the hydrogenation of the corresponding fatty acid methyl-esters. Most of the methanol is recovered in this process and is recycled for use in the ester exchange step. Another route of manufacturing is the hydrolyzation of fats and oils to the corresponding fatty acids followed by a subsequent catalytical reduction to the alcohols.

A number of synthetic routes have been developed for producing detergent-range alcohols from petroleum-derived raw materials. Ethylene, olefins, or n-paraffins are the basic chemical starting materials and the Ziegler chemistry (ELPAL®, ALFOL®) and OXO process are the most important routes (Figure 1.2).

In the Ziegler process ethylene is added to triethyl aluminum to build a mixture of high-molecular-weight trialkyl aluminums known as the ethylene growth product. After the oxidation with air the corresponding aluminum alkoxides are formed. The subsequent hydrolysis of these alkoxides leads to a mixture of linear primary alcohols having the same number of carbon atoms as the alkyl groups in the trialkyl aluminum growth product. Ziegler alcohols have even-numbered carbon chain lengths just like natural oil-based alcohols.

The OXO reaction as applied to the synthesis of detergent-range alcohols is currently employed commercially in a variety of modifications. Although each of these processes represents unique technology, they all involve the reaction of olefins with synthesis gas (CO/H_2) in the presence of an OXO catalyst to yield higher alcohols. The major differences among the processes are the type and source of the olefin, catalyst and process conditions. Most of the OXO plants in the world use processes in which first the intermediate aldehydes are isolated, purified and then hydrogenated in a second reactor. The Shell SHOP process with a cobalt type catalyst allows the

hydroformylation and hydrogenation of the intermediate aldehyde in the same reactor (Brackmann & Hager, 2004).

OXO-alcohols, which contain 20-40% branching of the alkyl chain, consist of both even- and odd-numbered carbon chain lengths. These alcohols also compete directly in some markets with natural oil-based alcohols (Brackmann & Hager, 2004). Balanced mixed surfactant systems based on branched and linear alcohols are important for cleaning performance depending on application. Branched alcohols are only industrially available from petrochemical processes.

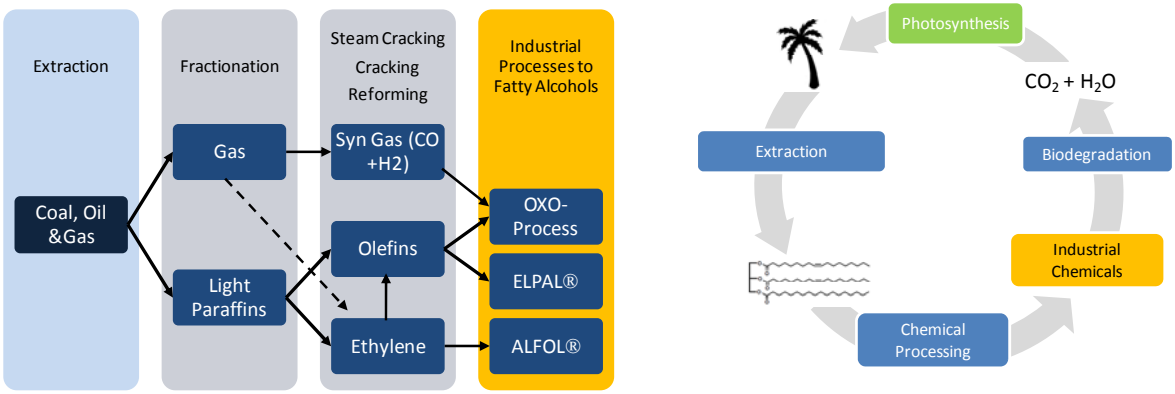


Figure 1.2 - Commercial routes to obtain fatty alcohols from petrochemical and oleochemical feed stocks.

A series of studies in the mid-1990s examined the production of surfactants from a life-cycle inventory (LCI) point of view (Hirsinger *et al.,* 1995a; Hirsinger *et al.,* 1995b; Hirsinger, 1998; Stalmans *et al.,* 1995). The LCI work has resulted in calculations that compare the total energy used and waste generation (and emitted to air, water, and soil) in the processing of surfactants based on oleochemicals and petrochemicals. In general, there are environmental trade-offs for both sources. For example, while oleochemical surfactants are derived from a renewable resource, they typically produce more air emissions and solid wastes. On the other hand, petrochemical surfactants consume more total energy, as the raw materials are produced from energy resources. Whether the feedstock source is animal fat, plant oil, or crude oil, there are energy requirements and environmental wastes that are a part of the feedstock and production stages of turning raw materials into surfactants.

Reducing significantly energy consumption in the production of fatty alcohols from petrochemical feed stocks would make these products more competitive in a market that in the last decade has shifted production to oleo based alcohols. A higher variety of fatty alcohols (even and odd carbon chain lengths, linear and branched, and di-alcohols) could be available for a wider range of applications overcoming inherent limitations of natural base products or those limitations imposed by the use of costly petrochemical based alcohols.

For instance, long-chain linear or branched >C14 paraffins (up to 7% in some crude oil sources, Espada *et al.,* 2010) could be directly oxidized to alcohols by selective catalysis (e.g. biocatalysis) reducing significantly energy consumption used to produce building blocks employed in synthesis of petrochemical long-chain alcohols.

1.2.1.3 Long-chain alcohols through biocatalysis

There are no commercial production processes of fatty alcohols through biocatalysis. However, research efforts have been made to investigate the possibility to produce these alcohols either from natural oils and fats by enzymatic reduction of fatty acids or by the conversion of n-alkanes to the corresponding fatty alcohols through terminal hydroxylation (see 1.3).

1.2.1.3.1 Long-chain alcohols by enzymatic reduction of fatty acids

Fatty acid biosynthesis is the preferred pathway to accumulate energy storage compounds in many organisms. During biosynthesis, fatty acids are activated as thioesters with coenzyme A (i.e. fatty acyl-CoAs) or acyl carrier protein (i.e. fatty acyl-ACPs). Fatty alcohols can be synthesized by enzymes reducing these fatty acyl-thioester substrates. These enzymes are referred to as fatty alcohol-forming fatty acyl-CoA reductases (FAR). Several of these enzymes such as that encoded by acr1 from *Acinetobacter calcoaceticus* BD413 have been described, but the best-studied fatty alcohol-generating enzymes (FARs) are eukaryotic (Schirmer *et al.*, 2009). Naturally, fatty alcohols produced by FARs are often incorporated as esters to waxes, cuticles and other structures, serving as hydrophobic/protection barriers and typically non-esterified fatty alcohols are only found in very limited amounts. Yields and productivities in the range of 1 g/L and 0,05 g/L·h of fatty alcohols by this route have been found with recombinant microorganisms engineered to express heterologous FAR enzymes (McDaniel *et al.*, 2011; Steen *et al.*, 2010; Schirmer *et al.*, 2009).

1.2.2 Long-chain dicarboxylic acids

Aliphatic α,ω-dicarboxylic acids are organic chemicals that contain two carboxylic acid functional groups with the formula of HOOC-R-COOH and where R is an alkyl or alkenyl chain. These compounds are versatile chemical intermediates of different chain length and are used as raw materials for the preparation of polymers (e.g. polyesters, polyamides and polyurethanes), perfumes, adhesives and macrolide antibiotics (Huf *et al.*, 2011).

1.2.2.1 Applications for long-chain dicarboxylic acids (LCDAs)

Long-chain dicarboxylic acids are here defined as diacids with more than 10 carbon atoms. These are used in a wide variety of polymers and chemical applications, most commonly as the primary monomer building block in high performance polyamides (nylons) used in automobiles or industrial applications where exposure to chemicals or moisture is expected. Nylon engineering plastic is the largest downstream application sector of long-chain dicarboxylic acids. Other current downstream applications for LCDAs include polyamides, adhesives, fragrances, corrosion inhibitors, lubricants and powder coatings (Cathay Biotech, 2011; CCR, 2011).

For instance, dodecanedioic acid (DC-12) currently competes with azelaic (DC-9) and sebacic (DC-10) acids used to make aliphatic polyesters in large scale and which, in turn, are used in fibers, films, casting resins, plasticizers, synthetic lubricants, and adhesives (e.g. hot melt adhesives).

Brassylic acid or tridecanedioic acid (DC- 13) is used in the manufacture of synthetic musk as well as of nylon 13,13.

Table 1.3 - Applications for LCDAs (Modified from Cathay Biotech, 2011).

Application Category	Product Application(s)	LCDA Product(s)
Polyamide / Nylon	PA612, PA614, electric cable sheaths, toothbrush fibers	DC-12, DC-14
Adhesives & Performance Coatings	Co-polyamide adhesives, polyester adhesives, paints	DC-11, DC- 12, DC-13, DC-14
Coatings GMA Powder Coat Cross-Linkers	Automobile wheels	DC-12
Anti-Corrosion	Metal working fluids, industrial cooling systems	DC-11, DC-12, PureMix
Synthetic Lubricants (Dibasic Esters)	High performance automobiles	DC-12
Personal Care—Synthetic Musk & Ketone	Household cleaners, high quality fragrances	DC-11, DC-12, DC-13, DC-15, DC-16
Pharmaceuticals	Adjuvant	DC-16

1.2.2.1.1 Application opportunities for LCDAs

Polymers prepared from higher LCDAs (>C10) possess improved properties, especially in the areas of flexibility, chemical resistance and moisture resistance (Cathay Biotech, 2011).

Desired properties are also acquired by incorporation of LCDAs co-monomers into certain engineering thermoplastic resins. These copolymer products retain the mechanical properties of the homopolymer resins, such as high impact strength, while offering a lower melt viscosity than the conventional resin. Lower melt viscosity helps to reduce the processor's cycle time and increase productivity because the molten plastic will flow into existing molds more quickly. Copolymers with LCDAs (high-flow resins; as defined by Mobley, 1999) are also important to designers who use plastics, because it means that thinner-walled and lighter weight parts can be made for applications such as laptop computers (Mobley, 1999).

The growth rate of the high-flow resin market is limited by the availability and cost of suitable diacid co-monomers. The cost of the cheapest suitable LCDA available in bulk quantities is above the average selling price of the conventional resins and high-flow resin must be sold at a premium, which limits its market penetration. LCDAs produced through biotransformation, either as individual chain lengths or as mixtures could potentially yield the same properties as chemically synthesized diacids. Mobley (1999) estimated that, if biotechnology could provide a cheaper source of diacid monomer for the high-flow resin, sales of the high-flow resin could rise dramatically.

Other applications such as the use of octacosanedioic acid (DC-28) for tumor treatment and of other LCDAs having a total number of carbon atoms divisible by four have been reported in the

patent literature (Rubin, 1999). The use of unsaturated LCDAs in cosmetic sun screen preparations (UV blocker) has also been reported in the patent literature (Samorski & Dierker, 2010).

Moreover, LCDAs can be converted by standard industrial processes to the respective diols and diamines, thus offering opportunities for the production of established and also of new polyesters and polyamides (completely from renewable feed stocks if natural fatty acids are used). Polyesters derived from long-chain diacids and diols (> C18) seem to be most interesting, having properties similar to polyethene and being biodegradable (Metzger, 2009).

1.2.2.2 LCDAs at industrial production scale

The LCDA produced in the largest quantity is dodecanedioic acid (DC-12) with about 25,000 metric tons in 2012 and below the almost 59,000 metric tons of high quality sebacic acid produced in the same year (CMAI, 2010). DC-12 is becoming more competitive against DC-10 in some applications. China is the major producer of LCDAs. Up to the fourth quarter of 2010, the total capacity to make long-chain dicarboxylic acids in China reached 53,500 metric tons/year and will further expand (CCR, 2011).

DC-12 and DC-13 hold a majority proportion in LCDAs produced in China (via Biotransformation). They are mainly exported to the international market. China's total output of LCDAs was around 10,000 tons in 2006, and with the startup of some production units, the output reached around 17,000 tons in 2009. The demand for LCDAs in the sectors such as engineering plastic, perfume and high-grade hot melt adhesive has increased constantly in recent years. The rapid development of downstream sectors in China and abroad will promote further development of the LCDAs sector. China's output of long-chain dicarboxylic acids was expected to be about 19,000 tons in 2010 (~40 % of installed capacity), and will increase to 50,000 tons in 2015 (CCR, 2011).

- **Dodecanedioic acid (DC-12)**

Currently, the largest market for DC-12 is polyamide 6,12 where it is 66% of the polymer. Polyamide 6,12 is made using DC-12 and hexamethylene diamine. The use of DC-12 in the polymer improves the moisture and chemical resistance of the polymer and provides greater flexibility when compared to polyamide 6,6 resins. It is used in a variety of automobile applications and in the production of monofilaments for toothbrushes, paint brushes and cosmetic brushes. Companies like DuPont, Evonik Industries AG, IFF, Arkema and Novo are important consumers.

Worldwide demand for high performance DC-12 is estimated to grow approximately 4.6% per year from 2010 to 2015 to 32,213 metric tons. In 2010, the China market accounted for 27% of the global market. The demand for DC-12 in the China market is estimated to grow at a compound annual growth rate of 8.4% from 6,681 metric tons in 2010 to 10,004 metric tons in 2015 (Figure 1.3).

The global production base for DC-12 is limited to four principal producers: Invista, Cathay Biotech, Evonik Industries AG and UBE. Invista, Evonik Industries AG and UBE primarily produce LCDAs (DC-12) through chemical processes. In 2010 DC-12 installed capacity by these companies

was about 35,000 metric tons/year, with 12,000 metric tons capacity (34%) from Cathay Biotech via biotransformation of n-dodecane (Cathay Biotech, 2011).

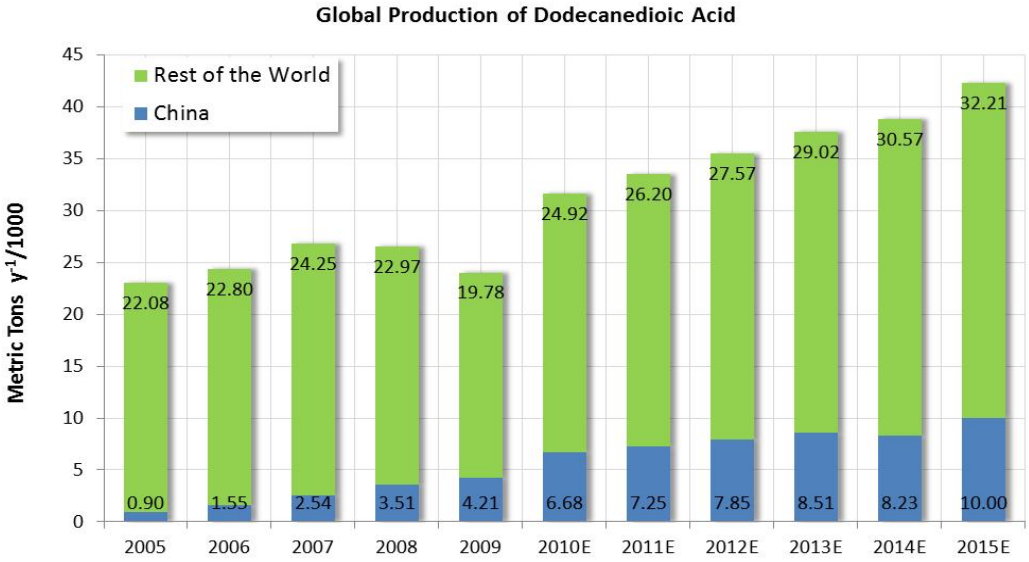


Figure 1.3 - Global and Chinese DC-12 market development (Cathay Biotech & CMAI, 2010). E = Estimated.

- **Decanedioic acid (DC-10)**

Decanedioic acid (DC-10) is also known as sebacic acid is used in several low volume applications such as polyamides, plasticizers, hot melt adhesives, polyesters, lubricants and cosmetics. More than 70% of global sebacic acid demand is for nylons 10,10 and 6,10 according to CMAI (2010).

In 2010, the global demand for high performance DC-10 was about 58,700 metric tons with more than 90% produced in China, where the installed capacity reaches 125,000 metric tons/year.

Sebacic acid prices are highly dependent on castor oil prices (DC-10 is produced from chemical treatment of ricinoleic acid obtained from castor oil). According to CMAI, castor oil market price has risen almost 80% from 2006 to 2010 resulting in increased prices for sebacic acid. An increase of the cost of polyamide 6,10 and 10,10 production makes other polyamides more cost competitive. Polyamide 6,12, made with DC-12, has greater moisture and chemical resistant characteristics and is typically preferred over polyamide 6,10, made with DC-10, at a comparable price. As a result, bioprocess-based DC-12 is expected to become a preferred alternative to sebacic acid. In the future higher LCDAs could also result attractive for high performance polyamide or polyester applications (Cathay Biotech, 2011).

1.2.2.3 Industrial production processes of LCDAs

Most α,ω-dicarboxylic acids are exclusively produced by chemical conversion processes that suffer a number of disadvantages, including limitations in the range of products, use of multi-step conversion processes, and generation of unwanted and hazardous by-products. While short-chain diacids can be synthesized in high yields, costs of long-chain diacids production rise significantly

due to the generation of various by-products and are connected mostly to a costly purification (Mobley, 1999; Metzger, 2009). Currently short and long-chain diacids are produced industrially from non-renewable and renewable feed stocks. There are three process categories for producing long-chain diacids: (1) cracking of vegetable oils, (2) organic synthesis and (3) biotransformation of aliphatic hydrocarbons and fatty acids by certain yeast species (CCR, 2011).

1.2.2.3.1 Cracking of vegetable oils

Because of the limited raw materials choice, LCDAs producers can only produce tridecanedioic acid (DC-13) and pentadecanedioic acid (DC-15) by cracking processes under harsh conditions and the product purity is rather low. This approach is also used to produce shorter large volume diacids.

Brassylic acid can be produced by ozonolysis of erucic acid (22:1 Δ13). Azealic acid (nonanedioic acid) is produced industrially from oleic acid (C18:1Δ9) in the same way (about 20,000 metric tons/year of oleic acid are reacted to azelaic acid). Both reactions generate nonanoic acid as by-product.

Sebacic acid (DC-10) is produced by splitting of ricinoleic acid with caustic soda using a ratio of 2:1 at 250–275 °C.

Because ozone is very expensive and the industrial ozonolysis presents some difficulties, an alternative process is required. The direct catalytic cleavage with H_2O_2 as oxidant was investigated extensively. A catalytic process using peracetic acid and ruthenium catalysts or catalysts based on H_2O_2 and Mo, W, or Re was reported, yielding only 50–60% diacids. An efficient catalytic process using oxygen from the air has not yet been developed (Metzger, 2009).

1.2.2.3.2 Organic Synthesis

The large-scale industrial production of LCDAs by organic synthesis process is affected because of the limitations of complex production processes; currently it is limited to dodecanedioic acid, which is the longest straight-chain diacid available from organic synthesis using butadiene as starting material (Mobley, 1992; Cathay Biotech, 2011).

Cole-Hamilton reported quite recently on the methoxycarbonylation of unsaturated fatty esters and acids to α,ω-diesters with very high selectivity using palladium catalysts with bulky bis-(ditertiarybutylphosphinomethyl) benzene (DTBPMB) as ligand. The double bond is isomerized to the ω-position, which is methoxycarbonylated. Most importantly, dimethyl nonadecanedioate was obtained not only from oleic acid but also from linoleic (C18:2n-6) and linolenic acid (C18:3n-6). Thus, all unsaturated C18 fatty acids in the fatty acid mixture were reacted to the same saturated diacid (reviewed by Metzger, 2009).

Chemical production of long-chain DCA is also possible through olefin metathesis (scission and regeneration of carbon - carbon double bonds, that allow olefin fragment redistribution/conjugation), but depending on the substrate used in the process it is also connected with

the occurrence of by-products. In principle any unsaturated fatty acids or unsaturated derivatives thereof can be used for the metathesis reaction. Terminal single double bonds are preferred for this reaction since otherwise product mixtures difficult to separate are obtained. Gaseous ethene is obtained as by-product, which can be easily removed. Several linear diacids have been synthesized by metathesis reaction of unsaturated fatty acids. For instance, dimethyl octadecanedioate and dimethyl hexacosanedioate can be obtained by self-metathesis of methyl oleate and methyl erucate after hydrogenation, respectively. Hydrogenation gives quantitatively the respective saturated acids (Rybak & Meier, 2007).

The spectrum of diacids obtainable from unsaturated fatty acids was enlarged by combination of the metathesis reaction with ω-methoxycarbonylation in a one-pot reaction. For example, methyl oleate was cross-metathesized with 2-butene using second-generation Hoveyda–Grubbs catalyst giving methyl 9-undecenoate and 2-undecene. The unreacted 2-butene was then evaporated and, without workup, the ω-methoxycarbonylation was performed, giving very high conversion of methyl 9-undecenoate and 2-undecene to dimethyl dodecanedioate and methyl dodecanoate, respectively, after hydrogenation (Zhu *et al.*, 2006).

A comprehensive summary of routes to obtain linear fatty α,ω-dicarboxylic acids including the above described has been presented by Metzger (2009).

It is important to remark that chemical conversion has not achieved selective ω-oxidation of natural (e.g. unsaturated) fatty acids. Thus the synthesis of long-chain DCA via microbial production and biotechnological transformation can provide a cost-efficient and greener process alternative (Metzger, 2009).

1.2.2.3.3 Biotransformation of paraffins and fatty acids

Biotechnology offers an innovative way to overcome the limitations and disadvantages of the chemical processes to produce LCDAs. Yeast biocatalysts are able to convert aliphatic hydrocarbons (long-chain alkanes) as well as long-chain fatty acids (from renewable agricultural products) directly to long-chain diacids via ω-oxidation. The biocatalyst can produce a variety of diacid products and produces no hazardous by-products (Mobley, 1999).

A number of companies (in China) produce LCDAs via fermentation with genetically optimized *Candida tropicalis* strains. The biotransformation process can currently produce a series of long-chain dicarboxylic acids with 11 to 18 carbon atoms (DC11-DC18), this is carried out under mild process conditions and can realize large-scale industrial production (CCR, 2011).
Nippon Mining Co. Laboratories in Japan first produced brassylic acid (DC-13) with the fermentation process in 1987. The capacity of the production unit was 200 metric tons/year and the products were used to produce musk-T perfume. The process was based on n-tridecane biotransformation by *Candida sp.* strains (Huf *et al.,* 2011). Since 2001, the production unit has stopped production. In the 1990s, Chinese producers also reached 600 metric tons/year capacity for DC-13 with the same approach, but it was also stopped (CCR, 2011) since brassylic acid can be produced chemically from oxidative cleavage (by ozonolysis) of erucic acid, that can be obtained from rapeseed oil (Metzger, 2009).

Cognis Inc. (Henkel Research Corporation, Sta. Rosa, California, USA) engineered *C. tropicalis* strains and developed fermentation processes utilizing paraffins and long-chain fatty acids as substrates for the production of LCDAs since the 1990s (Schörken & Kempers, 2009).

The Institute of Microbiology, Chinese Academy of Sciences developed a fermentation technology to produce long-chain dicarboxylic acids and successfully realized the industrial production of LCDAs with this technology in 2005 (CCR, 2011).

Cathay Industrial Biotech Co., Ltd. located in China is known as the world's biggest microbial LCDA manufacturer. Cathay was founded 1997 and established their pilot plant for microbial LCDAs production from long-chain alkanes in Shanghai in 2001. In 2003 the production facility for LCDAs in Shandong was completed and in 2007 the capacity was expanded to 15,000 metric tons/year. Cathay can produce LCDAs based on both paraffins and renewable fatty acid feed stocks. Odd numbered LCDA products by this route are petroleum based (Huf *et al.,* 2011).

Interestingly, most producing companies are located in China. It is known that different companies as well as academia in Japan, USA and Germany have done a lot of research on strain and process development for *C. tropicalis* for a high-yield production but did not achieve commercialization (see table 1.4 for some relevant studies). One reason for missing plants outside China, e.g. Germany, might be the classification of *C. tropicalis* as pathogenic microorganism in Europe which requires a high security standard for an industrial process. For the use of non-pathogenic microorganisms also strains like *Y. lipolytica* and *S. cerevisiae* have been studied and developed. However the high-yield LCDA concentrations and productivities obtained with *C. tropicalis* have not been reached yet so that more research has to be done in this direction (Huf *et al.,* 2011).

1.2.2.4 Competing production processes

The dodecanedioic acid process is a good example of competing production processes. In the organic synthesis route, the basic starting material is butadiene. DC-12 is manufactured via Ti/Al catalyzed cyclic trimerization of butadiene, followed by hydrogenation to cyclododecane, air oxidation to a mixture of cyclododecanone and cyclododecanol, finally nitric acid oxidation renders dodecanedioic acid (Figure 1.4). This process is based on petrochemical feed stocks. The multistep conversion process produces unwanted byproducts such as cyclooctadiene and vinyl cyclohexene, which result in yield losses. The nitric acid oxidation step yields NOx, which is either released to the atmosphere or must be destroyed in a reduction furnace (Mobley, 1999).

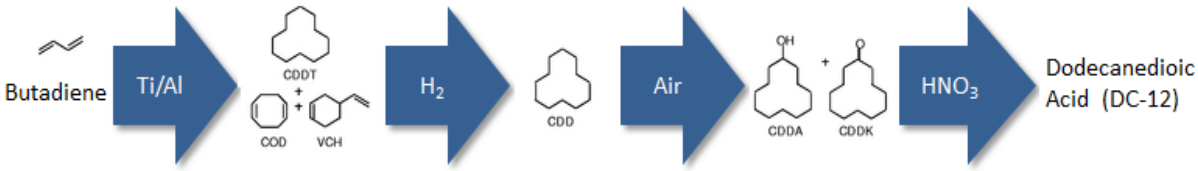


Figure 1.4 - Production of dodecanedioic acid from butadiene (Cathay Biotech, 2011).

With a market price calculated approximately at $6/kg, dodecanedioic acid has a high price for a bulk monomer. Butadiene based dodecanedioic acid has a production cost of about $4.4/kg (Cathay Biotech, 2011).

The conversion of long-chain aliphatic hydrocarbons and long-chain fatty acids directly to long-chain dicarboxylic acids by optimized *C. tropicalis* strains is performed at industrial scale. Currently about 35% of the global DC-12 production is done by this route (in China). Dodecanedioic acid based on α,ω-oxidation of dodecane by *C. tropicalis* has a production cost of about $4.3/kg (as calculated from the initial public offering filing for the NASDAQ stock exchange by Cathay Industrial Biotech , 2011).

Diacids with carbon numbers greater than 12 are chemically difficult to synthesize. Long-chain fatty acids are readily available from renewable agricultural and forest products such as soybean oil, tallow, corn oil, or tall oil whereas long-chain hydrocarbons can be easily obtained from crude refining opening a broad spectrum of substrates for the biotransformation route that would result in various LCDA products.

1.2.2.5 LCDAs through biotransformation - Process development

Several classes of microbial monooxygenases are able to convert alkanes into different types of oxidized compounds. The terminal oxidation of carboxylic acids and linear alkanes into dicarboxylic acids by yeast species possessing these enzymes has been widely examined (table 1.5) and also gained industrial relevance. The best production organism for dicarboxylic acids of different chain length is *Candida tropicalis*.

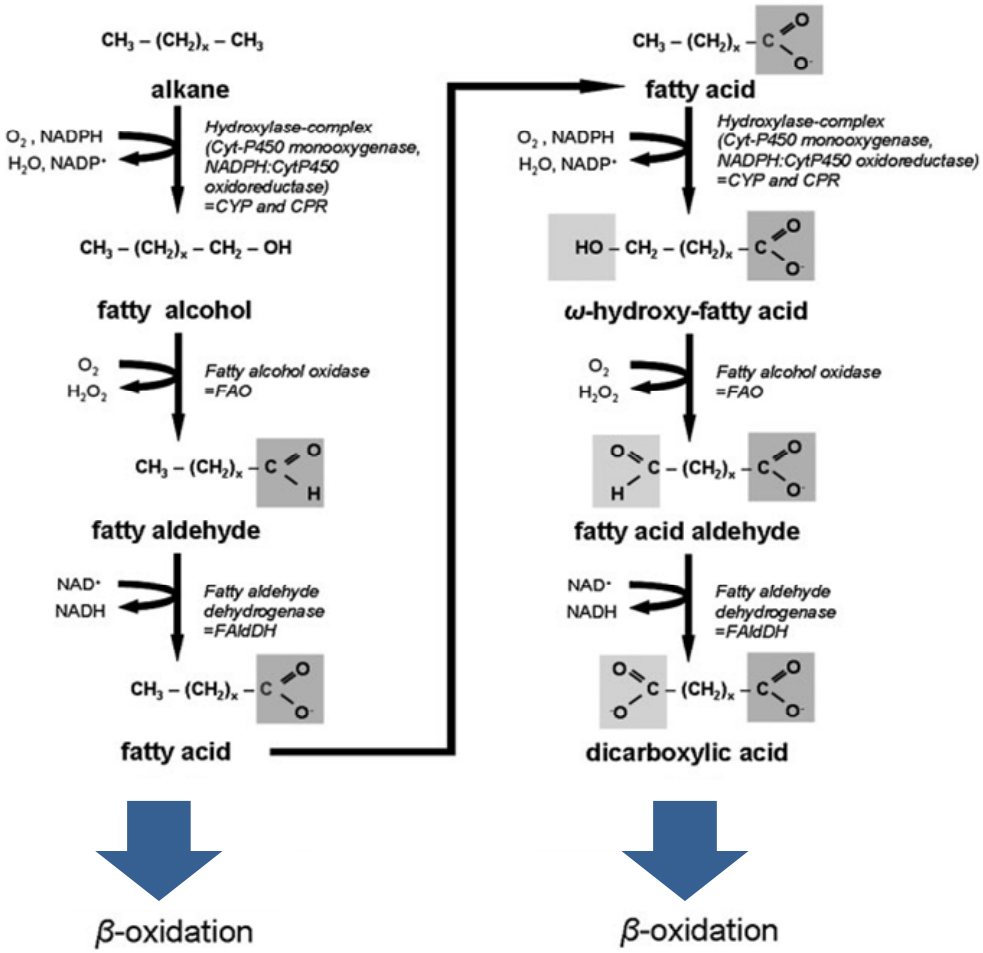


Figure 1.5 - Terminal oxidation of alkanes and fatty acids in peroxisomes of some yeast (Modified from Huf *et al.,* 2011).

Monooxygenases of the P450 (CYP52) family catalyze the terminal hydroxylation of aliphatic chains, and in cooperation with alcohol oxidases and aldehyde dehydrogenases the acid is formed under consumption of molecular oxygen, ω-oxidation of fatty acids leads to diacids via ω-hydroxy fatty acids (figure 1.5). The first step in this oxidation pathway is generally accepted to be rate-limiting. This step is mediated by the ω-hydroxylase complex consisting of a cytochrome P450 monooxygenase and an associated NADPH-cytochrome P450 reductase.

Wild type *C. tropicalis* also efficiently metabolizes fatty acids and the dicarboxylic acid intermediates through the β-oxidation pathway, enabling the organism to grow on fatty acids or alkanes as the sole carbon source.

Classical strain improvement techniques (e.g. chemical mutagenesis) have been used to develop strains that are partially deficient in their ability to grow on diacids, fatty acids, or alkanes (table 1.5). While these strains show enhanced production of diacids, they also produce diacids that are shorter by one or more pairs of carbon atoms than the alkane or fatty acid substrate and form unsaturated or 3-hydroxy - dicarboxylic acid by-products due to residual activity of the β-oxidation pathway. Partial metabolism of the substrate also results in costly yield losses (Mobley, 1999).

To overcome yield losses, β-oxidation blocked mutants of *C. tropicalis* have been generated and several genes of the oxidation pathway have been cloned to improve LCDAs production. The organism possesses at least ten different CYP52 genes with different substrate affinities. Overexpression of the oxidation-relevant genes in *C. tropicalis* was shown to be a method for optimizing the organisms' productivity (Picataggio *et al.,* 1992). Alternatively the genetic manipulation of the acetyl-CoA transportation pathway was proposed (Schörken & Kempers, 2009).

C. tropicalis accepts a broad range of substrates of different chain length, making it a versatile tool for the production of the industrially relevant dicarboxylic acid including brassylic and dodecanedioic, sebacic and azelaic acid and for the production of new dibasic acids, which cannot be accessed chemically in a cost-efficient manner today. The organism is able to transform alkanes, free fatty acids and esters with similar productivities and recently the bio-oxidation of residual waste fatty acids from oil distillates was also described (Schörken & Kempers, 2009).

1.2.2.5.1 Biocatalyst improvement

Cognis Inc. (Henkel Corporation) used a unique approach to inactivate genes in *C. tropicalis* which encode for acyl-CoA oxidase, enzyme involved in the first reaction in the β-oxidation pathway (Figure 1.6). Because of the diploid nature of the organism and the existence of two isozymes, four acyl-CoA oxidase genes were sequentially disrupted. With the acyl-CoA oxidase activity eliminated, the transformed strain showed nearly 100% efficiency of conversion of substrate to products with 100% retention of chain length. This strain was also shown to accept a variety of fatty acid substrates, both saturated and unsaturated. While the β-oxidation pathway blocked strain showed good productivity, the productivity was further enhanced by amplifying the cytochrome P450 monooxygenase and the NADPH-cytochrome reductase genes encoding the rate-limiting ω-hydroxylase complex to enhance the ω-oxidation pathway (Picataggio *et al.,* 1992).

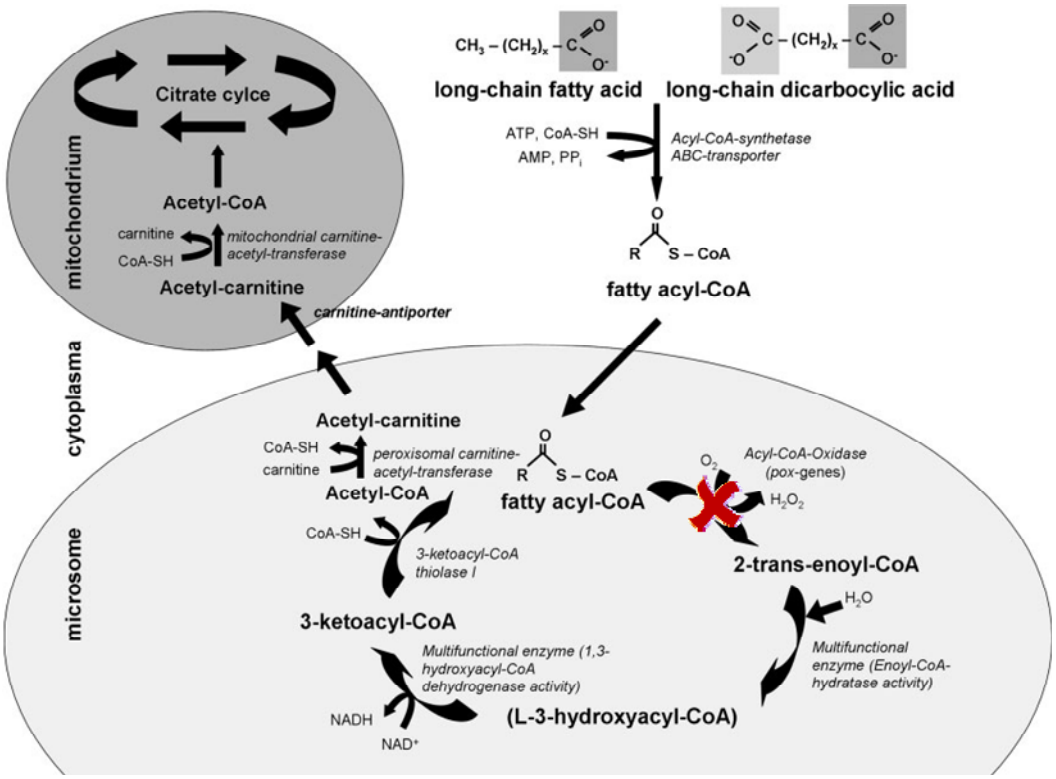


Figure 1.6 - β-oxidation pathway in peroxisomes (microsomes) of yeasts. Blockage of the Acyl-CoA-Oxidase(s) has been performed to in *C. tropicalis* to improve diacid strain productivity (Modified from Huf *et al.,* 2011).

1.2.2.5.2 Bioprocess economics and optimization for successful development

With the batch bioprocess technology demonstrated in the 1990s and assuming the use of the genetically modified *C. tropicalis*, estimations on process economics were made.

Raw materials costs are a large contributor to the overall cost. Their impact could be minimized by use of low-cost fermentation medium components for growth and maintenance of the yeast biocatalyst. Further opportunities for reduction of raw materials costs were identified in the use of low-cost fatty acid substrates and in the reduction of the rates of use of antifoam and of the base used for pH control.

The cost of fatty acid substrates is dependent on their source and composition. Pure fatty acid substrates are generally priced higher than mixed fatty acids derived directly from plant or animal sources, which are significantly cheaper.

Assuming that raw materials costs could be minimized, the preliminary economic analysis indicated that capital-related costs and utilities costs were the next most significant cost components. This analysis also suggested that the most effective way to reduce conversion costs (figure 1.7) was to increase the overall bioreactor productivity (diacid productivity in g/L·h).

Increases in bioreactor productivity reduce capital costs (since smaller bioreactors will do the same job) and utility costs (since less bioreactor volume needs to be stirred and aerated for less time).

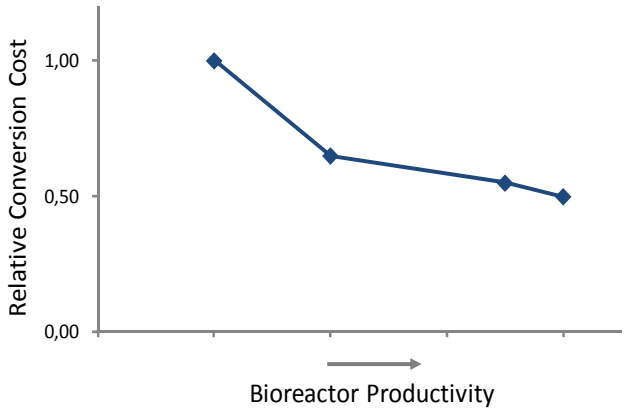


Figure 1.7 - After reducing raw materials costs, additional and significant cost reductions can be realized by increasing bioreactor productivity (Mobley, 1999).

Bioreactor productivity can be increased by improving the specific productivity of the biocatalyst and by optimizing the fermentation process conditions. Economically successful biological processes for bulk chemicals share two characteristics: high product concentrations and high bioreactor productivities. The diacid bioprocess with yeast already demonstrates high product concentrations. Further development must focus on increasing productivity.

In batch fermentations, the genetically modified *C. tropicalis* yielded high concentrations of diacid (80–100 g/L, in the final broth) and peak bioreactor productivities of over 1 g/L·h with some substrates for part of the conversion cycle (Mobley, 1999; Picataggio *et al.,* 1992).

Cathay Biotech reported improved productivities, relative to their productivity in 2001 of 110% in 2004, 130% in 2009 and 230% in 2010 by means of development of high yield and temperature tolerant strains and optimized proprietary fermentation techniques (medium, pH temperature, aeration and agitation) allowing reduction of raw materials and energy costs (Cathay Biotech, 2011).

Improvements in cell disruption and purification technology to recover polymer-grade LCDAs greatly contribute to improve bioprocess economics. Simultaneous demulsification and removal of residual raw materials and cell mass from the LCDA production process dramatically shorten the purification process. Solvent extraction is also required for the purification, improved solvents allowing the use of lower extracting volumes is expected to significantly reduce energy use and costs.

Table 1.4 - Some relevant studies on microbial production of long-chain dicarboxylic acids by terminal oxidation of long-chain n-alkanes.

Reference	Journal	Year	Author(s)	Title
1	European Journal of Applied Microbiology & Biotechnology (1982) 14:254-	1982	Zu-Hua Yi and Hans-Jiirgen Rehm	Metabolic formation of dodecanedioic acid from n-dodecane by a mutant of *Candida tropicalis*
2	Applied Microbiology and Biotechnolgy, 24:168-- 174	1986	F. F. Hill, I. Venn, and K. L. Lukas	Studies on the formation of long-chain dicarboxylic acids from pure n-alkanes by a mutant of *Candida tropicalis*
3	US Patent Office	1989	Stephen Picataggio, Kristine Deanda, L. Dudley Eirich / Henkel Research Corporation	Patent US5254466 - Site-specific modification of the *Candida tropicalis* genome
4	Annals of the New York Academy of Sciences Volume 613, Enzyme Engineering 10 pages 697–701	1990	M. Takagi, N. Uemura, K. Furuhashi	Microbial Transformation Processes of Aliphatic Hydrocarbons
5	Nature Biotechnology 10, 894 - 898	1992	Stephen Picataggio, Tracy Rohrer, Kristine Deanda, Dawn Lanning, Robert Reynolds, Jonathan Mielenz & L. Dudley Eirich (Microbial Technology Department, Cognis)	Metabolic Engineering of *Candida Tropicalis* for the Production of Long–Chain Dicarboxylic Acids
6	Department of Microbial, Biochemical & Food Biotechnology, Faculty of Natural and Agricultural Sciences, University of the State Bloemfontein, South Africa	2005	Newlande Van Rooyen	Thesis : Biotransformation of alkanes, alkylbenzenes and their derivatives by genetically engineered *Yarrowia Lipolytica* strains
7	Biotechnology Journal, 1, 68–74	2006	Zhuan Cao, Hong Gao, Ming Liu and Peng Jiao	Engineering the acetyl-CoA transportation system of *Candida tropicalis* enhances the production of dicarboxylic acid

Reference	Strain	Mutation	Substrates Tested	Substrate (Best)	Product α,ω-diacid	Productivity g/L·h	Yield (g/L medium)	Conversion	Time (h)	Cultivation Type	Cell density (g/l)	Added substrate (g)	Working Volume (L)	Concentration in Broth (g/L)
1	*C. tropicalis* 1230	Wild Type	C12	Dodecane	Dodecanedioic acid	0.007	0.36	1.42%	48	Batch	-	0.374	0.02	18.7
	C. tropicalis Mutant S76	Classical Mutagenesis - Not growing in DCA's or Alkanes	C12	Dodecane	Dodecanedioic acid	0.059	2.13	16.85%	36	Batch	-	0.187	0.02	9.4
			C12	Dodecane	Dodecanedioic acid	0.065	3.13	12.38%	48	Batch	-	0.374	0.02	18.7
2	*C. tropicalis* Mutant 7/34	Classical Mutagenesis	C11-C16	Dodecane	Dodecanedioic acid	0.30	36	20.35%	120	Fed-Batch	19	775	6.5	7.15
				Tetradecane	Tetradecane dioic acid	0.47	56	17.10%	120	Fed-Batch	19	773	6.5	7.14
				Hexadecane	Hexadecane dioic acid	0.33	39	3.18%	120	Fed-Batch	38	770	6.5	7.11
3	*C. tropicalis* Mutant H5343	β Oxidation Pathway Completely Blocked	C12-C14	Dodecane	Dodecanedioic acid	0.54	125	80.00%	232	Fed-Batch	-	116	5	4-60
				Tridecane	Brasylic acid	0.88	100	92.00%	114	Fed-Batch	-	82	5	4-60
				Tetradecane	Tetradecane dioic acid	0.63	101	96.00%	160	Fed-Batch	-	81	5	4-60
4	*C. tropicalis* Mutant M2030	Classical Mutagenesis (4th generation)	C13	Tridecane	Brasylic acid	1.17	140	>95%	120	Fed-Batch	-	-	20,000	-
5	*C. tropicalis Mutant* H43	β Oxidation Pathway Partly Blocked	C12-C16 saturated C18-C22 unsat.	Dodecane	Dodecanedioic acid	0.51	119	29.75%	234	Fed-Batch	-	296	5	5-30
	C. tropicalis Mutant H5343	β Oxidation Pathway Completely Blocked		Dodecane	Dodecanedioic acid	0.90	140	80.00%	156	Fed-Batch	-	129	5	5-30
	C. tropicalis Mutant AR40	β Oxidation Pathway Completely Blocked / Multiple Cytochrome-P450 monooxygenase (P450alk1) and NADPH oxydoreductase (CPR) genes, encode both components of the ω-hydroxylase complex		Tridecane	Brasylic acid	0.79	135	100.00%	170	Fed-Batch	-	102	5	5-30
6	*Y. Lipolitica* MTLY37	β Oxidation Pathway Partly Blocked	C11-C12	Dodecane	Dodecanedioic acid	0.10	13.8	34.07%	144	Fed-Batch	20	90	3	30.0
7	*C. tropicalis* Mutant W10-1	Classical Mutagenesis	C13	Tridecane	Brasylic acid	0.67	80	32.34%	120	Batch	-	560	3	186.7
	C. tropicalis Mutant CZ-15	Inhibition of acetyl-CoA transportation system in W10-1	C13	Tridecane	Brasylic acid	0.82	98	39.62%	120	Batch	-	560	3	186.7

1.3 Bacterial long-chain alkane metabolism - Biocatalytic application for the production of chemicals

Microbial biodegradation of pollutants has been a field of growing importance because of its potential use in bioremediation but also in biocatalysis. The study of the fate of persistent organic chemicals in the environment has revealed a large reservoir of enzymatic reactions with a large potential in preparative organic synthesis, which has already been exploited on pilot and even on industrial scale for some oxygenases (Meyer & Panke, 2008). Moreover, there is an increasing interest of technology development for the production of commercially valuable chemicals from less soluble hydrophobic substrates such as polyaromatics, fats, aliphatic hydrocarbons as well as polymeric compounds by means of biocatalysis since existing but also new-valuable products for different applications could be synthesized (Otto, 2001).

1.3.1 Alkanes

Alkanes or paraffins are saturated hydrocarbons, formed exclusively by carbon and hydrogen atoms bonded exclusively by single bonds. They can be linear (n-alkanes) or branched (iso-alkanes) and are virtually insoluble in water. Those having between one and four carbon atoms (methane to butane) are gaseous at ambient temperature whereas larger molecules are liquid or solid. Saturated hydrocarbons (including cycloalkanes) can constitute about 20-50% of crude oil, depending on the source of the oil. In addition, alkanes (predominantly long-chain compounds) are produced throughout the biosphere by living organisms (plants, algae and bacteria) as a waste product, a structural element, a defense mechanism, or as a chemo attractant (van Beilen *et al.,* 2003). This probably explains why alkanes are present at low concentrations in most soil and water environments (Rojo, 2009).

Alkanes are high-energy and inflammable compounds and as main components in fuels and oils they are of outstanding value for modern life (Wentzel *et al.,* 2007). Alkanes are non-polar molecules that are chemically very inert (Labinger & Bercaw, 2002). Their metabolism by microorganisms poses challenges related to their extremely low water solubility, their tendency to accumulate in cell membranes, and the energy needed to activate the molecule. For these reasons, their release to the environment results in ecological problems. Despite these challenges, several microorganisms, both aerobic and anaerobic, can use diverse alkanes as carbon and energy source (Wentzel *et al.,* 2007).

1.3.2 Degradation of long-chain n-alkanes

Biodegradation of hydrocarbons by microorganisms receives considerable attention due to the possibilities such processes can offer to remove pollutants from the environment and also to upgrade of oil refinery products (Van Hamme *et al.,* 2003).

Aerobic n-alkane degradation is a widespread phenomenon in nature, and several microbial strains and enzymes involved in n-alkane degradation have been isolated and studied in detail (Wentzel *et al.,* 2007).

Degradation of higher paraffins by *Aspergillus versicolor* was first reported by Hopkins and Chibnall in 1932. This fungal strain is able to grow on odd and even-numbered n-alkanes up to a carbon chain length of C35 (Britton, 1984). Numerous mesophilic and extremophilic strains (thermophiles and psychrophiles) capable of degrading hydrocarbons have been described and novel strains continue to be discovered. A large number of microorganisms belonging to the phyla of eubacteria, yeast, and fungi and also some algae capable of using long-chain n-alkanes as carbon and energy source are known (reviewed in van Beilen *et al.,* 2003).

Significant amounts of hydrocarbon-degrading microorganisms can be found in typical soil, sand or ocean sediment, and their numbers increase considerably in oil-polluted sites. Various alkane degrading bacteria have a very versatile metabolism and most frequently alkanes are not the preferred substrates for growth, these bacteria will rather utilize other compounds before turning to alkanes (Harayama *et al.*, 2004). On the other hand hydrocarbonoclastic bacterial species are highly specialized in degrading hydrocarbons. They play a key role in the removal of hydrocarbons from polluted environments (Yakimov *et al.*, 1998; 2007). Particular attention has been paid to *Alcanivorax borkumensis*, a marine bacterium that can assimilate linear and branched alkanes, but which is unable to metabolize aromatic hydrocarbons, sugars, amino acids, fatty acids and most other common carbon sources. *Alcanivorax sp.* are present in non-polluted sea waters in low numbers, probably living at the expense of the alkanes that are continuously produced by algae and other sea organisms and that are present at low but constant concentrations. *Alcanivorax sp.* strains become predominant after a spill of crude oil and are believed to play an important role in natural bioremediation of oil spills worldwide (Rojo, 2009).

Hydrocarbon degraders are also of particular interest for future biotechnological applications. For instance, thermophilic long-chain n-alkane-degrading strains may provide the basis for genetic engineering of strains for biocatalytic processes under extreme conditions. Since the first description of a thermophilic n-alkane degrading bacterium by Mateles *et al.* in 1967, alkane-degrading bacterial strains have been isolated from several high-temperature habitats like oil reservoirs and volcanic environments. Several of these are also capable of using long-chain n-alkanes of C18 and longer like some *Geobacillus* and *Thermus sp.* (Wentzel *et al.,* 2007).

1.3.2.1 Microbial uptake of long-chain n-alkanes as a carbon and energy source

Most substrates promoting microbial growth need to undergo cellular uptake or attachment to become accessible by the cell's catabolic machinery (Wentzel *et al.,* 2007). The extreme low solubility of long-chain n-alkanes in water hampers their uptake by microorganisms (Rojo, 2009). The solubility of alkanes decreases dramatically as the molecular weight increases (Bell, 1972; Eastcott *et al.,* 1988) reducing their bioavailability (Otto, 2001), see table 1.5.

The hydrophobic nature of the bacterial cell surface is important for cell contact with hydrophobic substrates because the initial step in aliphatic and aromatic hydrocarbon degradation is often

mediated by oxidation reactions catalyzed by cell-membrane-associated oxygenases (Wentzel *et al.,* 2007).

The uptake mechanism may differ depending on the bacterial species considered, the molecular weight of the alkane and the physico-chemical characteristics of the environment (Rojo, 2009). Microorganisms may gain access to medium- and long-chain-length n-alkanes by either adhering to hydrocarbon droplets or by a surfactant-facilitated process. Most alkane-degrading bacteria secrete diverse surfactants that facilitate emulsification of the hydrocarbons (Ron & Rosenberg, 2002).

Table 1.5 - Solubilities of normal medium and long-chain alkanes corresponding aliphatic acids and alcohols in water (Bell, 1972; *Eastcott et al., 1988).

No. of carbon atoms in chain	Solubility of alkane [mg/L]	Solubility of undissociated fatty acid [mol/L]	Solubility of alcohol [mol/L]
5	38.5	2.40E-01	2.50E-01
6	9.5	6.80E-02	6.10E-02
7	2.93	1.30E-02	1.55E-02
8	0.66	2.20E-03	4.50E-03
10	0.052		
12	0.0084	1.15E-05	2.30E-05
14	0.007	8.00E-07	1.46E-06
15			4.50E-07
16	0.000052*	1.20E-07	1.70E-07
18		1.10E-07	

1.3.2.2 Bacterial aerobic n-alkane degradation pathways

Microorganisms have established effective strategies involving specialized enzyme systems and metabolic pathways to access n-alkanes. In-depth characterization of genes and enzyme systems involved in the utilization of long-chain n-alkanes has led to an improved understanding of microbial long-chain n-alkane metabolism (Wentzel *et al.,* 2007). Understanding aerobic degradation of long-chain alkanes by bacteria is of utmost relevance for the present work.

The first step of alkane degradation by alkane-assimilating microorganisms involves selective hydroxylation of the alkane at the terminal or sub-terminal carbon (van Beilen *et al.*, 2003).

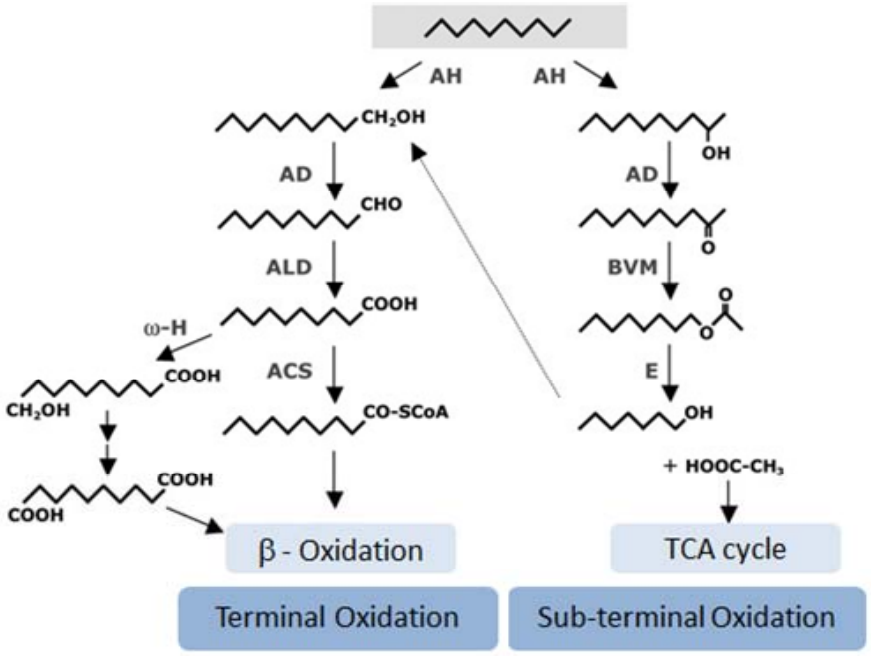


Figure 1.8 - Aerobic pathways for the degradation of long n-alkanes by terminal and subterminal oxidation; AH, alkane hydroxylase; AD, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; ACS, acyl-CoA synthetase; w-H, w-hydroxylase; BVM, Baeyer–Villiger monooxygenase; E, esterase; TCA, tricarboxylic acid cycle (Rojo, 2009).

The oxidation of a terminal methyl group yields a primary alcohol, which is further oxidized to the corresponding aldehyde, and finally to a fatty acid (figure 1.8). In the microbial cell, the fatty acid is activated by long fatty acyl CoA synthetase to form Acyl-CoA and further processed by β-oxidation to generate acetyl-CoA yielding ATP during the process. In some cases, both ends of the alkane molecule are oxidized through ω-hydroxylation of fatty acids at the terminal methyl group, rendering an ω-hydroxy fatty acid that is then converted into a dicarboxylic acid and further processed (Rojo, 2009).

Subterminal oxidation of n-alkanes has also been reported. The product generated, a secondary alcohol, is converted to the corresponding ketone (figure 1.8), and then oxidized by a Baeyer–Villiger monooxygenase to produce an ester. The ester is hydrolyzed by an esterase, generating an alcohol and a fatty acid. Both terminal and subterminal oxidation can coexist in some microorganisms (Rojo, 2009).

Strains degrading medium-chain alkanes (C5–C11), or long-chain alkanes (> C12), frequently contain integral membrane non-heme iron dependent monooxygenases related to the well-characterized *Pseudomonas putida* GPo1 *alk*B alkane hydroxylase. *alk*B-type enzymes function in complex with two electron transfer proteins, a binuclear iron rubredoxin, and a mononuclear iron rubredoxin reductase channeling electrons from NADH to the active site of the alkane hydroxylase (van Beilen *et al.,* 2003). However, some bacteria contain enzymes that belong to a family of soluble cytochrome P450 that hydroxylate C5–C11 alkanes. Finally, some strains assimilating alkanes of more than 18 carbon atoms contain alkane hydroxylases unrelated to the former ones

(reviewed in van Beilen *et al.*, 2003; van Beilen & Funhoff, 2007). Multiple alkane hydroxylases, exhibiting overlapping substrate ranges (including both *alkB*-type and cytochrome P450 enzymes), have been reported for many n-alkane degraders (Wentzel *et al.*, 2007).

1.3.2.3 Regulation of bacterial n-alkane catabolic pathways

Inducible n-alkane utilization has been demonstrated for many n-alkane-oxidizing bacterial species. The nature of the inducers and regulation mechanisms seem to be species-specific (Wentzel *et al.,* 2007).

In general, expression of the genes involved in the initial oxidation of alkanes is tightly controlled. A specific regulator gene assures that the pathway genes are expressed only in the presence of the appropriate alkanes. In addition, superimposed to this specific regulation there are several mechanisms that modulate the induction of the pathway genes according to cell needs (Rojo, 2009).

Regulation has been studied in depth for *Pseudomonas putida* GPo1 described to utilize C5-C14 n-alkanes (van Beilen *et al.*, 1994). The strain harbors the OCT plasmid carrying the *alk*BFGHJKL operon containing the genes for n-alkane utilization and a regulatory locus (alkR) containing the alkS gene responsible for activation of the *alkB*FGHJKL operon expression (figure 1.9). The expression of the AlkS regulator is controlled by two promoters, which allow the protein to regulate its own expression, positively and negatively. AlkS expression occurs from the σ^S-dependent P*alkS1* promoter in absence of alkanes and mostly during the stationary phase. In the presence of alkanes the P*alkS2* promoter becomes active whilst P*alkS1* is repressed. Transcription from P*alkS2* appears to be subject of catabolite repression by a mechanism that should allow both rapid induction of the n-alkane utilization pathway, and a fast down regulation thereof when the n-alkanes are consumed (reviewed in Wentzel *et al.*, 2007).

For some of the known specific regulators there is evidence supporting that n-alkanes or n-alkanols act as effectors. As alkanes are non-polar molecules that most likely accumulate in the cytoplasmic membrane, while transcriptional regulators are normally cytoplasmic proteins, it may be that these regulators have affinity for the inner side of the cytoplasmic membrane, where they have easy access to the alkanes acting as effectors. After binding the alkane, the regulator should move and find its binding site on the DNA to activate the degradation pathway (reviewed in Rojo, 2009).

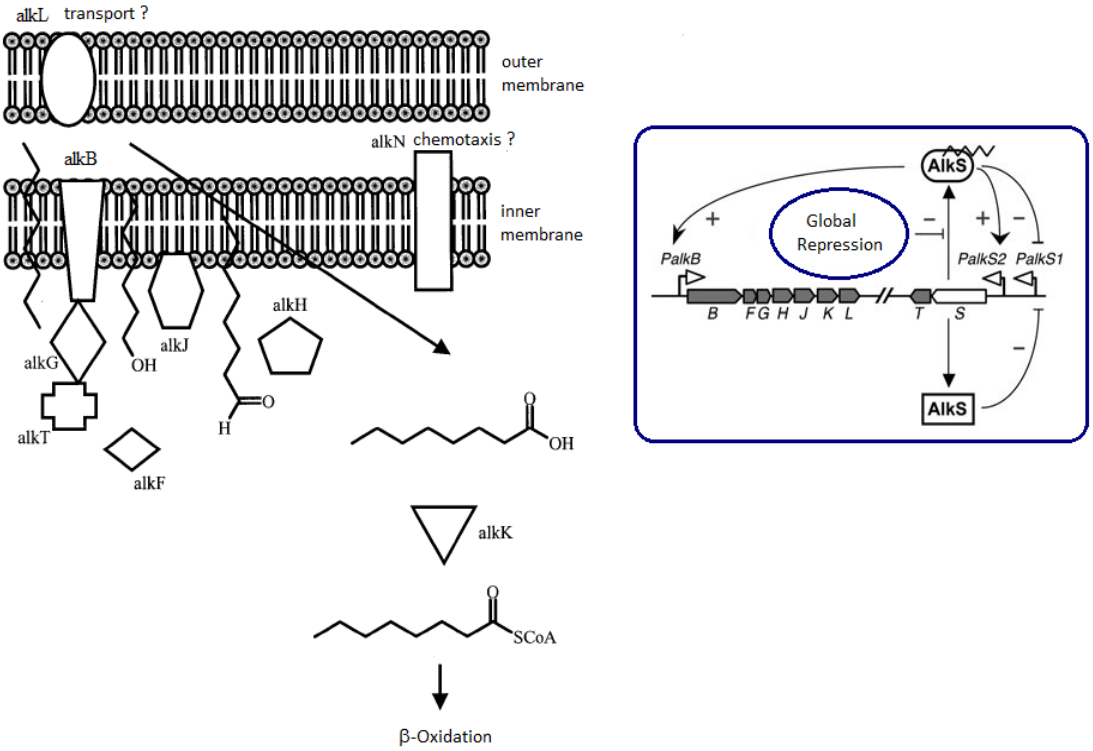


Figure 1.9 - Scheme of alkane degradation in *Pseudomonas putida* GPo1 (gram-negative bacteria), showing the locations and functions of the *alk* operon gene products: *alk*B (alkane hydroxylase), *alk*F and *alk*G (rubredoxins), *alk*H (aldehyde dehydrogenase), *alk*J (alcohol dehydrogenase), *alk*K (acyl-CoA synthetase), *alk*L (outer membrane protein that may be involved in uptake), *alk*N (a methyl-accepting transducer protein that may be involved in chemotaxis), *alk*T (rubredoxin reductase). *alk*S is a regulator of the *alk*BFGHIJKL operon that controls its own expression and (*alk*ST genes) by means of the P*alkS* promoters (van Hamme *et al.*, 2003; van Beilen *et al.*, 1994, 2001).

The induction of the alkane-degradation genes can be modulated in several ways to accommodate it to the existing environmental or physiological conditions. Three examples are considered by Rojo in his 2009 review on bacterial alkane degradation: differential regulation, product repression and catabolite repression.

1.3.2.3.1 Differential regulation of multiple alkane hydroxylases

Some bacterial strains contain only one alkane hydroxylase, as it is the case for the well-characterized alkane degrader *P. putida* GPo1. However, many other strains have several alkane-degradation systems, each one being active on alkanes of a certain chain-length or being expressed under specific physiological conditions. Differential regulation is directed by regulators that respond to a particular range of effectors and/or physiological signals.

For example, *Acinetobacter sp.* strain M-1 contains two *alk*B related alkane hydroxylases, named AlkMa and AlkMb, which are differentially regulated depending on the alkane present in the medium. Expression of AlkMa, which is controlled by the AlkRa regulator, is induced by alkanes having a very long-chain length (> C22), while that of AlkMb is induced by AlkRb in the presence of

C16-C22 alkanes (Tani *et al.*, 2001). These hydroxylases share the constitutively expressed auxiliary proteins rubredoxin and rubredoxin reductases, which are components of the AlkM hydroxylase complex. Both of these proteins, in addition to alkane utilization, may be involved in other redox reactions important for normal cell functioning (reviewed in Wentzel *et al*., 2007).

Hydrocarbonoclastic bacteria and some species having a versatile metabolism possess three or more alkane oxidation systems. For example, *A. borkumensis* has two *alk*B like alkane hydroxylases and three genes coding for cytochromes P450 believed to be involved in alkane oxidation (van Beilen *et al.*, 2004; Schneiker *et al.*, 2006). Expression of all these alkane oxidation genes should be differentially induced according to the substrate present under each circumstance, however the regulators and/or the signals involved have not been thoroughly characterized (Rojo, 2009).

1.3.2.3.2 Product repression

In some cases the activation of the pathway genes is inhibited when the concentration of the alkane degradation products increases over a certain threshold, a phenomenon called product repression (Rojo, 2009). This regulatory mechanism may be a way to coordinate the generation of fatty alcohols and fatty acids from alkanes with their further metabolism, because these compounds tend to accumulate in the cell membrane, modifying its composition with deleterious consequences to cell physiology (Chen *et al.*, 1995; 1996).

1.3.2.3.3 Catabolite repression

Bacterial species that have a versatile metabolism usually possess global regulation systems that assure assimilation of preferred carbon sources over other non-preferred substrates. Hydrocarbons are typically non-preferred growth substrates. The expression of alkane-degradation pathways is inhibited by the presence of many other carbon sources in several strains. The preferred compounds and the mechanisms used to modulate the expression of catabolic pathways vary depending on the bacterial species considered. Catabolite repression probably arises from a number of global regulatory mechanisms directed to optimize carbon metabolism and energy generation in response to different signals. It is not clear what these signals are, but they may be related to the concentration of key metabolites or molecules in the cell that in turn depend on the efficiency of the different catabolic pathways in terms of energy gain (Rojo, 2009).

One good example is the case of *P. putida* GPo1, where activation of the promoters P*alk*B and P*alkS2* by *alk*S is negatively regulated when cells grow in a complete medium containing alkanes, or in a minimal salts medium containing alkanes and other alternative carbon sources such as amino acids, succinate or lactate (see figure 1.10). Compounds such as citrate, pyruvate or glycerol, which are also metabolized, do not exert this inhibitory effect. Repression is particularly strong during exponential growth in a complete medium, where amino acids are the carbon source used, and rapidly fades away when cells enter into stationary phase (reviewed in Rojo, 2009).

1.3.3 Biotransformation of hydrocarbons: Long-chain alcohols by terminal hydroxylation of long-chain n-alkanes

Efficient routes for region and stereo-selective oxidation/hydroxylation of non-activated carbon atoms are sought for production of higher value chemicals from hydrophobic substrates such as petroleum hydrocarbons (Schmid *et al.,* 2001). Synthetic catalysts are plagued by low selectivity leading to over-oxidation and often require harsh reaction conditions (Meinhold *et al.,* 2006).

The chemical functionalization of non-activated methyl groups of organic compounds is considered a crucial problem in organic synthesis. Even hydroxylation, one of the simpler functionalization reactions, remains a difficult chemical task. Major problems arise from the activation of the carbon atom, because of the use of strong oxidants not always compatible with the substrate, and from the occurrence of side reactions that produce other oxidation products, such as aldehydes or carboxylic acids (Bosetti *et al.,* 1992).

As previously introduced, the first step of alkane degradation by most alkane-assimilating microorganisms involves selective hydroxylation of the alkane at the terminal carbon. Biochemical and genetic studies have focused on a limited number of enzymes that catalyze this reaction (van Beilen *et al.,* 2003). The best known is the alkane hydroxylase from *Pseudomonas putida* GPo1 (Baptist *et al*., 1963), which has been studied in recombinant strains like *Pseudomonas putida* PpS8141 (Bosetti *et al.,* 1992) and *E. coli* GEC137 pGEc47ΔJ for the production of alkanols (Grant *et al.,* 2011).

Typically only formation of mono alcohols no longer than 16 carbon atoms has been studied. But formation of α,ω-diols (di-alcohols) has been also reported (Fujii *et al.,* 2006).

In the work by Bosetti *et al.* (1992), the alkane hydroxylation system of *Pseudomonas putida* GPo1 was introduced into *Pseudomonas putida* PpS81 (alcA81). The resulting recombinant strain PpS8141 was able to oxidize n-alkanes to the corresponding 1-alkanols but unable to utilize alkanols, which were accumulated in the medium. PpS8141 was grown in two-liquid-phase bioreactors, an upper alkane bulk organic phase (20% of the total volume) over the aqueous phase containing the growth substrate citrate, octanoate, or pyruvate. The strain was able to grow well in the presence of a bulk apolar phase (C7-C11) only when pyruvate was used as the growth substrate. 1-alkanols production rates were found superior compared to the ones obtained with *Pseudomonas putida* GPo1. The highest production rates were obtained with n-octane and n-nonane. More recently Feng *et al.,* 2007 and Dong *et al.*, 2012 reported the formation of 1-hexadecanol from n-hexadecane with the purified long-chain alkane monooxygenase LadA from *Geobacillus thermodenitrificants* NG80-2 expressed in *E. coli* but without figures about whole-cell biotransformation.

Mathys *et al.* (1999) presented a process design and economic evaluation for the production of 1-octanol from n-octane utilizing the recombinant *Pseudomonas putida* strain studied by Bosetti (1992). Production costs were estimated between 6,000 and 10,000 $US/metric ton assuming the use of a strain/process with doubled biocatalytic activity compared to the figured used for the

study (Table 1.6, reference 2). These figures are far above current market prices for bulk fatty alcohols, making the realization of such a production process unfeasible.

However, the scale of the higher alcohols market suggests that similar compounds, like longer chain alcohols (>C16) or long-chain α,ω-diols (which are not yet industrially available) could find a place in the already vast variety of applications for fatty alcohols because new and interesting properties could be introduced in products like additives for cosmetics, building blocks for new polymers, dispersants in paints and lubricants, flow modifiers in coatings, chemical reducing agents, etc. Research interest in this regard is demonstrated by recent publications.

Table 1.6 summarizes relevant publications for the microbial production of long-chain alkanols by terminal hydroxylation of long-chain n-alkanes.

Table 1.6 - Some relevant studies on microbial production of long-chain alkanols by terminal hydroxylation of long-chain n-alkanes.

Reference	Journal	Year	Author(s)	Title
1	Enzyme and Microbial Technology, 14, 702-708	1992	Aldo Bosetti, Jan B. van Beilen, Hans Preusting, Roland G. Lageveen and Bernard Witholt	Production of primary aliphatic alcohols with a recombinant *Pseudomonas* strain, encoding the alkane hydroxylase enzyme system
2	Biotechnology and Bioengineering, 64(4), 459-477	1999	Renata G. Mathys, Andrew Schmid, Bernard Witholt	Integrated Two-Liquid Phase Bioconversion and Product-Recovery Processes for the Oxidation of Alkanes: Process Design and Economic Evaluation
3	Bioscience Biotechnology & Biochemistry, 70(6),1379-1385	2006	Fujii, T., Narikawa, T., Sumisa, F., Arisawa, A., Takeda, K. and Kato, J.	Production of α,ω-alkanediols using *Escherichia coli* expressing a Cytochrome P450 from *Acinetobacter sp.* OC4
4	Enzyme and Microbial Technology, 48, 480–486	2011	Chris Grant, John M. Woodleyb, Frank Baganza	Whole-cell bio-oxidation of n-dodecane using the alkane hydroxylase system of *P. putida* GPo1 expressed in *E. coli*

Reference	Strain	Substrates Tested	Substrate (Best)	Product	Productivity g/L·h	Yield (g/L) medium volume only	Conversion	Time (h)	Cultivation Type	Cell density (g/L)	Added substrate (g)	Working Volume (L)	Substrate in Broth (g/L)
1	*Pseudomonas putida* PpS8141	C6-C12	Octane	1-Octanol	0.052	1.25	0.62%	24	Batch	2	70.30	0.40	175.8
2	*Pseudomonas oleovorans-Pseudomonas putida*	C6-C12	Octane	1-Octanol	0.742	17.80	72.00%	24	Fed-Batch	20	Only Calculated Data		
			Octane	1-Octanol	1.483	35.60	30.00%	24	Continuous	10			
3	*E.Coli* BL21 Harboring pDo1ABC	C5-C14	Octane	1-Octanol	0.094	2.25	0.84%	24	Resting Cells	200	0.35	1.50E-03	234.3
			Octane	α,ω-alkanediol	0.011	0.26	0.09%	24	Resting Cells	200	0.35	1.50E-03	234.3
			Dodecane	1-Dodecanol	0.002	0.05	0.02%	24	Resting Cells	200	0.38	1.50E-03	250.0
			Dodecane	α,ω-alkanediol	0.000	0.00	0.00%	24	Resting Cells	200	0.38	1.50E-03	250.0
4	*E. coli* GEC137 pGEc47ΔJ	C6-C12	Dodecane	1-Dodecanol	0.008	0.40	0.24%	48	Batch	10.7	15.00	0.10	150.0
			Dodecane	Dodecanoic Acid	0.085	4.08	2.31%	48	Batch	10.7	15.00	0.10	150.0
			Octane	1-Octanol	0.034	0.82	0.51%	24	Batch	2.4	14.06	0.10	140.6
			Octane	Octanoic Acid	0.022	0.62	0.35%	28	Batch	2.4	14.06	0.10	140.6

1.3.4 Biotransformation of hydrocarbons: LCDAs by α,ω-oxidation of long-chain n-alkanes

Research efforts to investigate the possibility to produce long-chain dicarboxylic acids by terminal oxidation of n-alkanes with bacterial cells or enzymes have been limited. Most probably because of the much higher yields, productivities and spectrum of LCDAs that can be obtained with some yeast strains.

Formation of aliphatic dicarboxylic acids from hydrocarbons by bacteria was first described by Kester & Foster in 1963 for the production of dicarboxylic acids from the C10–C14 alkanes by a *Corynebacterium* sp. Strain 7E1C. With this strain, Broadway *et al.* (1993) reported n-dodecane giving the highest yield of the corresponding dicarboxylic acid DC-12 (300 mg/L) and less DC-14 (45 mg/L) produced from n-tetradecane adding substrates at 2% v/v in the cultures during 120 h of incubation at 30 °C. No dicarboxylic acids were detected from hexadecane with this strain. Dahlstrom & Jaehning (1973) reported mutants derived from this *Corynebacterium sp.* strain (*Corynebacterium sp.* ATCC 21744 to 21747) which produce dicarboxylic acids (DC-12) in increased amounts (up to 23 g/L with molar conversions above 85%). The same range of dicarboxylic acids was produced with these mutants compared to the wild-type strain (Broadway *et al*., 1993).

As well, conversion of n-pentadecane to DC-15 is stimulated by organic solvent- and detergent-treated *Cryptococcus neoformans* and *Pseudomona aeruginosa* and inhibited by elevated levels of DC-15 (Chan *et al.*, 1990). To avoid product inhibition the use of a continuous process with immobilized *Cryptococcus* cells led to a significant increase in yield compared with the batch type production of DC-15. Diacid production was continued once accumulated DC-15 was removed from the conversion medium, and the yield of DC-15 reached approximately 7.2 g/L. The capability of DC-15 production of *C. neoformans* was maintained at least 250 h (at 30 °C) by removing DC-15 every 50-h period. The average productivity was estimated to around 0.028 g/L·h (Chan *et al.*, 1997).

1.4 Thermophilic bacteria and degradation of hydrocarbons

Extremophiles are organisms, which are able to survive extreme environmental conditions. These microorganisms produce biocatalysts working under extreme conditions, comparable to those prevailing in various industrial processes. Thermophiles (growing optimally at 45-80°C) and hyperthermophiles (growing optimally above 80°C) are extremophiles of particular interest because they produce enzymes working at elevated temperatures (Madigan *et al.*, 2004).

The knowledge about thermophilic degradation pathways is still limited and properties of many of the involved enzymes are not known (Müller *et al.*, 1998). Considerable research is necessary to exploit this interesting area for novel enzymes, which can lead to novel biotechnological processes. Running biotechnological processes at elevated temperatures has many advantages, such

as decreased viscosity of the substrates and elevated diffusion coefficients, meaning higher reaction rates (Otto, 2001).

In addition to their high thermostability, these microorganisms also promise to have greater tolerance to organic solvents and a longer useful life. The possibility of recovering volatile products directly from a culture provides the opportunity to develop simplified bioprocesses (Sonnleitner & Fiechter, 1983). In the same way non-volatile liquid products that solidify at lower temperatures could be easily separated from the culture broths. Increased insensitivity of processes to contaminations compared to those involving mesophiles is also attractive for process development.

It is recognized that enzymes from thermophiles are more resistant to physical and chemical denaturation. Compared to similar mesophilic enzymes, the stability of thermophilic enzymes is achieved by the difference of amino acids sequence. It appears that critical amino acid substitutions at only a few locations in the enzyme allow it to fold in a way that is consistent with heat stability. Heat stability is also enhanced by increasing the number of ionic bonds between alkaline and acidic amino acids (Sonnleitner & Fiechter, 1983).

Oil pollution resulting from the spillage or leakage of crude oil and fuels in the environment can be addressed with bioremediation. Temperature plays an important role in controlling the nature and efficiency of microbial hydrocarbon degradation, which is of major significance for in situ bioremediation (Leahy & Colwell 1990). Faster growth rates would be an advantage of using thermophiles in bioremediation processes (Meintanis *et al.*, 2006).

Thermophilic hydrocarbon degraders of *Bacillus*, *Thermus*, *Thermococcus* and *Thermotoga* species occurring in natural high-temperature or sulfur-rich environments are of special significance as they could be efficiently used for bioremediation of oil-polluted environments were relative high temperatures can be encountered (Feitkenhauer *et al.,* 2003; Shimura *et al.*, 1999; Meintanis *et al.*, 2006).

Thermophiles growing on medium chain and long-chain alkanes constitute a great biotechnology perspective and the number of characterized thermophiles with biodegradation potential has increased during the last years (Feitkenhauer *et al.,* 2003; Hao *et al.*, 2004). Although there is still much to explore about the main alkane hydroxylase systems of the thermophilic degradation pathways, homologues of the *Pseudomonas* genes that allow hydrocarbon degradation found in thermophilic bacteria have been related with high biodegradation rates at high temperatures (Meintanis *et al.*, 2006).

1.5 Biosurfactants

Surfactants are organic amphiphilic molecules that contain both hydrophobic and hydrophilic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. This characteristic

allows them to form stable micellar structures and dissolve in aqueous solutions and localize at surfaces. Surfactants are capable of reducing surface and interfacial tension and forming micro-emulsions, e.g. hydrocarbons in water. Because of their excellent detergency, emulsifying, foaming, and dispersing traits, surfactants are among the most versatile process chemicals (Desai & Banat, 1997).

The growth of microorganisms on hydrocarbons is often accompanied by the emulsification of the insoluble carbon source in the culture medium. In most cases, this has been attributed to the production of extracellular emulsifying agents during the hydrocarbon fermentation (Rosenberg *et al.*, 1978), which are designated as biosurfactants. Biosurfactants are biological molecules, which manifest properties similar to the well-known synthetic surfactant family (Sim *et al.*, 1997).

Both chemically and biologically derived surfactants have the potential to increase the bioavailability of hydrophobic substrates (e.g. organic pollutants) by several mechanisms like emulsification of non-aqueous phase liquids or enhancement of the apparent solubility of the hydrophobic substances (Parales *et al.*, 2008).

Surfactants have been reported to increase the uptake and assimilation of alkanes such as n-hexadecane in liquid cultures, but their usefulness in soils and other situations is less evident. In addition biosurfactants probably have other roles as well, such as facilitating cell motility on solid surfaces, or the adhesion/detachment to surfaces or biofilms (Rojo, 2009).

Biosurfactants have a wide range of potential applications in cosmetic, food and beverage and pharmaceutical industries or for treating oil spills or enhanced oil recovery. Advantages of biosurfactants over conventional synthetic surfactants include biodegradability, low toxicity and ease of production by fermentation as well as high selectivity and specific activity at extreme temperatures, pH, and the ability to be synthesized from renewable feed stocks. Unfortunately, low yields and productivities have limited their commercial realization (Desai & Banat, 1997; Gartshore *et al.*, 2000).

1.5.1 Types of bacterial biosurfactants

Unlike chemically synthesized surfactants, which are classified according to the nature of their polar moiety (e.g. anionic, cationic, amphoteric), biosurfactants are categorized mainly by their chemical composition and microbial origin. The biosurfactant-producing microbes are distributed among a wide variety of genera and various microorganisms are known to produce specific kind of biosurfactants (Desai & Banat, 1997; Rahman & Gakpe, 2008).

In general, biosurfactant structure includes a hydrophilic moiety consisting of amino acids or peptides anions or cations, mono-, di-, or polysaccharides and a hydrophobic moiety consisting of unsaturated or saturated fatty acids. The major groups of biologically produced surfactants include glycolipids, phospholipids, lipopeptides, lipoproteins and biopolymers (Desai & Banat, 1997).

Bacterial biosurfactants have also been classified according to molecular size (Rosenberg & Ron, 1999). Low molecular weight molecules that efficiently lower surface and interfacial tensions and high molecular weight polymers that bind tightly to surfaces. The low molecular weight biosurfactants are generally glycolipids in which carbohydrates are attached to a long-chain aliphatic acid or lipopeptides. Glycolipid bioemulsifiers, such as rhamnolipids, trehalose lipids and sophorolipids, are disaccharides that are acylated with long-chain fatty acids or hydroxy fatty acids (Ron & Rosenberg, 2002).

One of the best-studied classes of glycolipids are rhamnolipids, produced by several species of P*seudomonas*, which can consist of two molecules of rhamnose and two molecules of β-hydroxydecanoic acid (Itoh *et al.*, 1971; Para *et al.*, 1989; Tahzibi *et al.*, 2004). Glucose lipids produced by *Alcanivorax borkumensis* have also been described. These consist of an anionic glucose lipid with a tetrameric oxyacyl side-chain (reviewed in Ron & Rosenberg, 2002).

The high molecular weight bacterial surfactants are produced by a large number of bacterial species from different genera and are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers. The high molecular weight surfactants are less effective in reducing interfacial tension, but are efficient at coating the oil droplets and preventing their coalescence. They are highly efficient emulsifiers that work at low concentrations (0.01%–0.001%), representing emulsifier-to-hydrocarbon ratios of 1:100–1:1000. These high molecular weight bioemulsifiers exhibit considerable substrate specificity. For example, some emulsify efficiently mixtures of aliphatic and aromatic (or cyclic alkane) hydrocarbons, but will not emulsify pure aliphatic, aromatic or cyclic hydrocarbons. Others can also emulsify pure hydrocarbons but only of a high molecular weight. Among the best-studied biosurfactants are the bioemulsans produced by different species of *Acinetobacter*. Bioemulsans are complexes of anionic (hetero) polysaccharides and proteins whose surface activity results from the presence of fatty acids that are attached to the polysaccharide backbone via O-ester and N-acyl linkages (Ron & Rosenberg, 2002).

1.5.2 Physiology and production of bacterial biosurfactants

1.5.2.1 Physiological role of biosurfactants

Biosurfactants are produced by a variety of microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on hydrophobic substrates (Desai & Banat, 1997).

The functioning mechanism of biosurfactants in microbial cells is not fully understood. There has been speculation about their involvement in emulsification of water-insoluble substrates and other mechanisms of bacterial hydrophobic substrate uptake. For instance, non-producing biosurfactant mutants of *P. aeruginosa KY-4025* and *P. aeruginosa PG-201* showed poor growth compared to the parent strains on n-paraffins and hexadecane, respectively, and addition of rhamnolipids to the medium restored growth on these hydrocarbons (Itoh *et al.*, 1972; Koch *et al.*, 1991). Direct contact of bacterial cells (*Pseudomonas* sp.) with hydrocarbon emulsified droplets and their interaction have been recently described (Cameotra & Singh, 2009). In addition,

biosurfactants have been shown to be involved in cell adherence which imparts greater stability under hostile environmental conditions and virulence, in cell desorption to find new habitats for survival and in antagonistic effects towards other microbes in the environment (reviewed in Desai & Banat, 1997).

Bacteria attached and growing on an oil droplet become nutrient-starved once the group of hydrocarbons they can utilize is depleted. Cell-bound biosurfactants can cause the microbial cell surface to become more or less hydrophobic, depending on its orientation suggesting that microorganisms can use their biosurfactants to regulate their cell-surface properties to attach or detach from surfaces according to need. The detachment of bacteria from the depleted oil drop enables them to move to other drops where they metabolize the specific group of utilizable hydrocarbons. Therefore, detachment of bacteria from oil drops results in more efficient oil degradation since the hydrocarbons left would be available for other bacteria capable of utilizing those (Ron & Rosenberg, 2002).

1.5.2.2 Cell growth and biosurfactant production

Biosurfactant production exhibits many variations among various systems and only a few generalizations can be made. Production can be growth-associated and growth-limiting conditions related (stationary phase), see figure 1.10.

For growth-associated biosurfactant production, parallel relationships exist between growth, substrate utilization, and biosurfactant production. The production of rhamnolipids by some *Pseudomonas spp.*, glycoprotein AP-6 by *P. fluorescens*, surface-active agent by *B. cereus* IAF 346, and biodispersan by *Bacillus sp.* strain IAF-343 are all examples of growth-associated biosurfactant production. Production of cell-free emulsan by *A. calcoaceticus* RAG-1 has been reported to be a mixed growth-associated and non-growth-associated type. Emulsan-like substance accumulates on the cell surfaces during the exponential phase of growth and is released into the medium when protein synthesis decreases (reviewed by Desai & Banat, 1997).

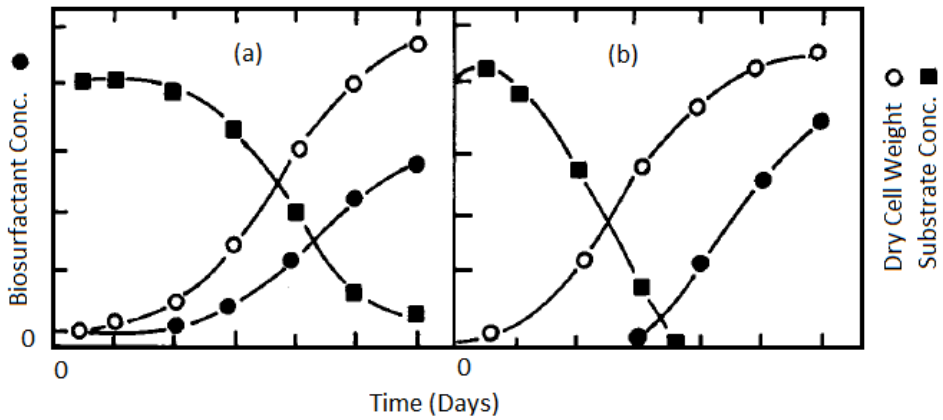


Figure 1.10 - Fermentation kinetics for biosurfactant production. (a) Growth-associated production observed in *Acinetobacter calcoaceticus*. (B) Production under growth-limiting condition (exhaustion of nitrogen) in the case of *Pseudomonas spp*. Modified from Desai & Banat, 1997.

Production under growth-limiting conditions is characterized by a sharp increase in the biosurfactant level as a result of limitation of one or more medium components. A number of investigators have demonstrated an overproduction of biosurfactants by *Pseudomonas spp*. when the culture reaches the stationary phase of growth due to the limitation of nitrogen and iron. Production of biosurfactants by several microorganisms has also been reported to follow this pattern (Desai & Banat, 1997).

In several cases it was shown that emulsifier production is induced by molecular signals involved in quorum sensing. This regulatory feature appears to be general, and probably applies to the production of both low and high molecular weight emulsifiers, as in all cases emulsifier production is concurrent with the increase in cell density and the onset of the stationary phase of growth (Ron & Rosenberg, 2002).

In general, three mechanisms, namely, induction, repression, and nitrogen and multivalent ions, operate in the regulation of biosurfactant production. The induction of biosurfactant synthesis by addition of long-chain fatty acids, hydrocarbons or glycerides to the growth medium has been described for various microorganisms (Desai & Banat, 1997).

Repression of biosurfactant production by *Acinetobacter calcoaceticus* and *Arthrobacter paraffineus* on hydrocarbon substrates has been observed with organic acids and D-glucose, respectively. Similarly, drastic reduction in the synthesis of rhamnolipids by *P. aeruginosa* and of liposan by *C. lipolytica* upon the addition of D-glucose, acetate and tricarboxylic acids has been noted.

Nitrogen- or metal ion-dependent regulation also played a prominent role in the synthesis of biosurfactants. The synthesis of rhamnolipids in *P. aeruginosa* upon exhaustion of nitrogen and commencement of the stationary phase of growth has been observed by several investigators. Moreover, the addition of a nitrogen source caused an inhibition of rhamnolipid synthesis in resting cells of *Pseudomonas sp*. strain DSM-2874. The limitation of multivalent cations also causes overproduction of biosurfactants. For instance, iron limitation stimulates biosurfactant production in *P. fluorescens* and *P. aeruginosa* (reviewed in Desai & Banat, 1997).

1.5.3 Involvement of biosurfactants in oil bioremediation

There are at least two ways in which biosurfactants are involved in bioremediation: increasing the surface area of hydrophobic water-insoluble substrates and increasing the bioavailability of hydrophobic compounds (Ron & Rosenberg 2002).

1.5.3.1 Increasing the surface area of hydrophobic water insoluble substrates

For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil. When the surface area becomes limiting, biomass increases arithmetically rather than exponentially. The evidence that emulsification is a natural process brought about by extracellular agents is indirect, and there are certain conceptual difficulties in under-

standing how emulsification can provide an (evolutionary) advantage for the microorganism producing the emulsifier. Emulsification is a cell-density-dependent phenomenon, the greater the number of cells, the higher the concentration of extracellular product. The concentration of cells in an open system, such as an oil-polluted body of water, never reaches a high enough value to effectively emulsify oil. Furthermore, any emulsified oil would disperse in the water body and would not be more available to the emulsifier-producing strain than to competing microorganisms. One way to reconcile the existing data with these theoretical considerations is to suggest that the emulsifying agents do play a natural role in oil degradation, but not in producing macroscopic emulsions in the bulk liquid. If emulsion occurs at, or very close to, the cell surface and no mixing occurs at the microscopic level, then each cluster of cells creates its own microenvironment and no overall cell-density dependence would be expected (Ron & Rosenberg 2002).

1.5.3.2 Increasing the bioavailability of hydrophobic water-insoluble substrates

The low water solubility of many hydrocarbons, especially the polycyclic aromatic hydrocarbons (PAHs), is believed to limit their availability to microorganisms, which is a potential problem for bioremediation of contaminated sites resulting in prolonged persistence of these compounds in the environment. It has been assumed that surfactants would enhance the bioavailability of hydrophobic compounds. Several non-biological surfactants have been studied, and both negative and positive effects of the surfactants on biodegradation were observed. For example, the addition of Tween 80 to two *Sphingomonas* strains increased the dissolution rate of solid-phase phenanthrene and resulted in an overall increase in growth. By contrast, the same surfactant inhibited the rate of fluoranthene mineralization by two strains of *Mycobacterium*, and no stimulation was observed in other studies using several surfactants (reviewed in Ron & Rosenberg 2002). Biosurfactants are more effective than chemical surfactants in increasing the bioavailability of hydrophobic compounds. The high molecular weight bioemulsifier Alasan was recently shown to significantly increase the rate of biodegradation of several PAHs (Barkay *et al.*, 1999).

Lower water solubility and high molecular weight of hydrophobic substrates also increase their sorption to surfaces limiting bioavailability. When organic molecules are bound irreversibly to surfaces, biodegradation is inhibited. Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility. Surfactants that lower interfacial tension dramatically are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. Low molecular weight biosurfactants that have low critical micelle concentrations (CMCs) increase the apparent solubility of hydrocarbons by micellization (reviewed in Ron & Rosenberg 2002).

Much less is known on how polymeric biosurfactants increase apparent solubilities of hydrophobic compounds. Recently, it has been demonstrated that Alasan increases the apparent solubilities of PAHs 5–20-fold and significantly increases their rate of biodegradation (Barkay *et al.*, 1999).

1.5.4 Utilizing biosurfactants and oil-degrading bacteria for bioremediation

Bioremediation involves the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g. nutrients and oxygen), conditions (e.g. pH and moisture content), and prevailing microorganisms. Thus, bioremediation usually consists of the application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content (soils), if necessary, supplying air and often adding bacteria. The addition of emulsifiers (including surfactants) is advantageous when bacterial growth is slow (e.g. at cold temperatures or in the presence of high concentrations of pollutants) or when the pollutants consist of compounds that are difficult to degrade, such as PAHs (Ron & Rosenberg 2002).

Bioemulsifiers can be applied as an additive to stimulate the bioremediation process (Barkay *et al.*, 1999; McKew *et al.*, 2007; Aparna *et al.*, 2011). However, with advanced genetic technologies it is expected that the increase in bioemulsifier concentration during bioremediation will be achieved by the addition of bacteria that overproduce bioemulsifiers. These bioemulsifiers producing bacteria can also participate in oil degradation even if they cannot degrade hydrocarbons. They can function in a bacterial consortium, supplying the emulsifier for other bacteria that carry out the degradation process (Ron & Rosenberg, 2002).

Bacteria that cannot grow on particular hydrocarbons have previously been shown to impart an important synergistic effect in the degradation of petroleum (Rambeloarisoa *et al.*, 1984). *Alcanivorax borkumensis* cannot degrade PAHs but produces a powerful extracellular glucose lipid biosurfactant (Yakimov *et al.*, 1998). PAHs degradation by *Cycloclasticus sp*. was enhanced by bioaugmentation with *A. borkumensis* that may have increased bioavailability of PAHs by means of its biosurfactant (McKew *et al.*, 2007).

The bioemulsifier can also diffuse in the soil or can even be transferred to the other bacteria on close contact, such as in biofilms. Recently, horizontal transfer of capsule polysaccharide has been demonstrated in bacteria, resulting in bacteria coated with emulsifying polysaccharide capsule produced by bacteria of another species. The effect of these phenomena on oil bioremediation remains to be further investigated. Optimization of the bioremediation process would involve selecting the best oil-degrading microorganisms, the most suitable biosurfactant, the best bioemulsifier producers and the most effective combination of these (Ron & Rosenberg 2002).

1.6 The scope of this study

The search for chemicals produced from hydrocarbon substrates has been objective of several studies and much research work has been done with several mesophilic yeast and bacterial strains (and their enzymes) to obtain chemicals from long-chain n-alkanes. Although in some cases, e.g. C*andida spp.*, challenges in regard to product yields and productivities have been overcome, the limited range of products and pathogenicity of the involved strains continue to limit process development.

In this context, this project aims to take advantage of the ability of a thermophilic strain to degrade hydrocarbons by producing intermediate metabolites from long-chain n-alkanes that could have potential application in the industrial and/or fine chemicals sector. So far no studies with thermophilic microorganisms have been reported for this purpose.

This work includes the construction of mutants of the thermophilic alkane degrading *Thermus sp.* ATN1. These mutants are capable to oxidize one or both terminal methyl groups of long-chain n-alkanes, producing the corresponding terminal α,ω-oxidized products. These products had to be identified and isolated.

Highlighting the possibility of producing existing and novel chemicals applying this biotechnological approach to upgrade refined mineral oil products was the main objective of this investigation.

In addition, it was important to describe factors influencing the biotransformation of long-chain alkane by the constructed mutants, in order to understand possible optimization routes. For this purpose it was necessary to develop product formation monitoring and quantification techniques, including an approach to product separation.

Finally, it was an objective of this research to provide deeper insights into the alkane uptake and utilization mechanisms by *Thermus sp.* ATN1, since the alkane oxidizing system in the wild-type strain has been described only partly (Otto, 2001). This included the isolation and characterization of a biosurfactant produced by this strain suspected to be involved in the alkane uptake process. Additionally, possible application of this biosurfactant to enhance hydrocarbon bioavailability for other microorganisms is also described.

1.6.1 *Thermus sp.* ATN1, a thermophile capable to degrade long-chain n-alkanes

Thermus sp. ATN1, strain subject of this study, is capable to utilize long-chain n-alkanes as carbon and energy source. Similarly to *Pseudomonas putida* Gpo1, terminal oxidation has been proposed for the degradation of long-chain n-alkanes by this strain and its ability to oxidize alkanes of various chain lengths was described (Otto, 2001). *Thermus sp.* ATN1 is a thermophilic strain obtained from a compost heap in Hamburg, Germany. It was isolated and cultured at the Hamburg University of Technology, TUHH (Feitkenhauer, 1998). The thermostable alkane oxidizing system of this strain was partly described: an alcohol dehydrogenase (TADH) encoding gene (AlkDH) suspected to be involved in the long-chain alkane catabolic pathway in this strain was identified, cloned and expressed in *E. coli* and the enzyme was purified and characterized (Otto, 2001, Hollrigl *et al.,* 2008).

1.6.2 Obtaining α,ω-oxidized aliphatic products with the hydrocarbon degradation machinery from *Thermus sp.* ATN1

With basis on existing knowledge about the alkane oxidizing system in this strain, it was thought that the construction of mutants ideally blocked in the second step of the proposed n-alkane degradation pathway (figure 1.11) would result in long-chain α,ω-oxidized compounds, as those with alcohol groups at both ends (primary diols).

For this purpose, mutant construction through directed mutagenesis was executed by truncating (gene disruption) the AlkDH gene with a kanamycin resistance gene (Moreno *et al.,* 2003) by homologous recombination.

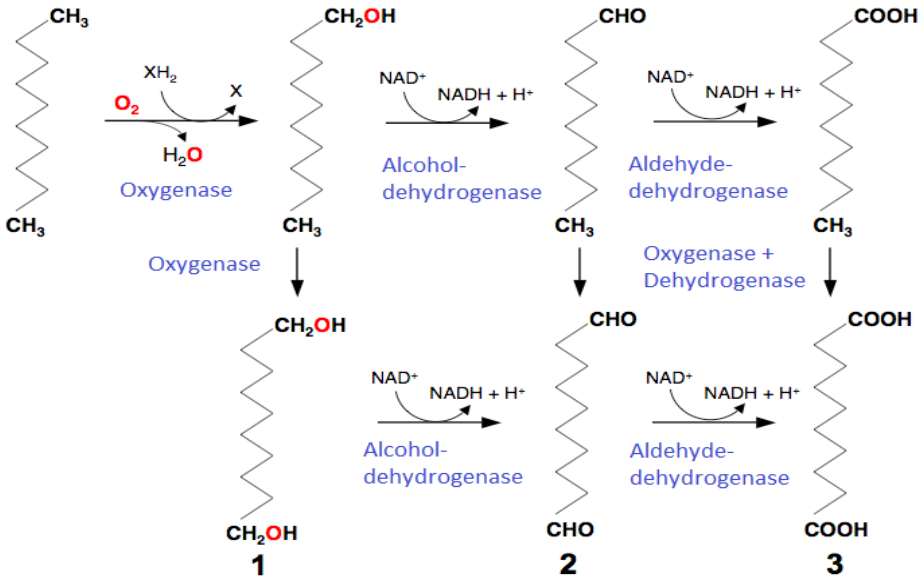


Figure 1.11 - Long-chain alkane oxidation pathways in *Thermus sp.* ATN1.

Constitutive long-chain n-alkane utilization has not been described for this strain and its hydrocarbon metabolic regulation mechanisms are unknown. Furthermore, evidence of involvement of the known alcohol dehydrogenase (TADH) in the long-chain alkane metabolism is only indirect. Because of this, also classical (random) mutagenesis combined with selective phenotype screening was performed on the wild-type strain as strategy to obtain mutants capable to produce long-chain α,ω-oxidized products.

1.6.2.1 Random and directed mutagenesis

Strain improvement through mutagenesis is an essential and important part of process development for a biotechnological process. The overall goal is to reduce costs by developing strains with increased productivity, ability to use cheaper or alternate substrates, or other unique and desirable characteristics (Rowlands, 1984). For the purposed of this work, mutants of *Thermus sp.* ATN1 should be constructed by random and directed mutagenesis. It was assumed that both methods could result in the desired strain phenotype.

Classically, mutants are generated by treating the test organism with chemical or physical agents that modify DNA (mutagens). Random mutagenesis has been used reliably for many years for titre improvement, first in penicillin production and then in a variety of other fermentations. It still plays a central role in many industrial fermentation processes today (Rowlands, 1984; Primrose & Twyman, 2006; Rowlands, 2011).

This method is applied on the basis that is not possible to predict which type of mutation is needed to obtain the desired phenotype in a particular strain. Therefore, it is common to use several types of mutagenic treatments during screening to yield a range of mutants as wide as possible. As any gene in the organism can be mutated and the frequency with which mutants occur in the gene of interest can be very low, selection strategies must be highly effective (Rowlands, 1984; Primrose & Twyman, 2006).

The new phenotype of the desirable mutants must be expressed maximally under the screening conditions used to facilitate effective screening. A number of factors can affect expression, and hence the efficiency of screening. These factors include: *expression delay* e.g. some time is required for initial DNA damage to be fixed into a stable mutation by the appropriate DNA repair pathway; *modified regulatory mechanism* e.g. can cause false positives or regulatory mutants as the mutation occurs in the regulatory mechanism and not in the targeted protein or enzyme activity; and *impure culture* as the effect of the desirable mutation in one cell will be diluted or masked by the other cells.

Sub-culturing before screening will generally solve *expression delay* and *impure culture* issues. This allows mixed cells to segregate to form pure colonies, enhances the viability of the mutants after mutagenesis and provides time for expression pathways to be completed, so the mutant phenotype can be fully expressed at the screening step (Rowlands, 1984).

Even when mutants with the desired phenotype are isolated (e.g. after selective shake flask screens to eliminate false positives), there is no guarantee that the mutation has occurred in the gene of interest. Prior the development of gene-cloning and sequencing techniques it was not possible to determine the gene or where in the gene the mutation had occurred and whether it arose by a single base change, an insertion of DNA, or a deletion. With current molecular biology techniques it is possible to isolate and study single genes and mutagenesis has been also refined. Instead of randomly mutagenizing many cells and then analyzing thousands of offspring to isolate a desired mutant, it is now possible to modify specifically any given (known) gene. This technique is generally referred as site-directed mutagenesis. The mutation can be a single base change (a point mutation), deletion or insertion.

Gene activity can be increased and/or novel activity can be created (gain-of-function mutagenesis), e.g. by a point mutation. Site-directed mutagenesis has become a basic tool for protein engineering. Engineered proteins may have only minor changes but it is not uncommon for entire domains to be deleted or new domains added (Primrose & Twyman, 2006).

In a similar way, gene activity can be reduced or eliminated (loss-of-function mutation), e.g. by replacement of the endogenous gene with engineered DNA containing long homologous regions

of the endogenous gene (known as gene targeting). The normal gene is replaced by homologous recombination with the engineered gene-targeting vector. As effective recombination occurs at a very low frequency, it is important to detect the few cells that have integrated the modified gene. Gene targeting vectors are designed with this in mind. The simplest strategy is to include an antibiotic resistance gene on the vector, which interrupts the sequence homologous to the gene of interest and thus makes the inserted gene non-functional. The introduced "selectable marker" gene makes the cells that possess it resistant to antibiotics. This characteristic can be used to eliminate cells that are not genetically modified (Joyner, 1999).

It must be considered that even when the desired directed mutation can be confirmed in the gene of interest, mutants may not exhibit the desired phenotype due to expression regulation of this gene under the screening conditions.

1.6.2.2 Enzyme vs. whole cell biocatalysis

Oxy-functionalization of hydrophobic substrates is difficult. Biologically it generally involves enzyme systems which often consist of multiple and membrane-bound components. This complicates the handling of isolated enzymes and often results in unstable activities under process conditions besides requiring expensive cofactors in stoichiometric amounts. Because of these facts, the use of many isolated monooxygenases for industrial purposes on large scale is not feasible (Julsing *et al.*, 2008; Urlacher & Girhard, 2012).

Thus, whole-cell systems are typically used to guarantee continuous cofactor regeneration and enzyme synthesis. Whole-cell biocatalysis can also be carried out in two-phase systems (e.g. aqueous–organic solvent), which is advantageous because substrates and products are often hydrocarbons and thus not water-soluble. However, other factors such as substrate uptake, product transport, toxicity of substrates and products, insufficient cofactor supply, host physiology (in the case recombinant strains) and strain stability have to be taken into account (Urlacher & Girhard, 2012).

Finally, all necessary components have to be available at the right time, concentration, and location in the cell for optimal reaction conditions. The biocatalyst can be a wild-type microorganism, but in most cases optimization of the biocatalyst is necessary to improve activity and/or stability (Julsing *et al.*, 2008).

The need of cofactors, their regeneration and the sensitivity of multi-component systems favour the selection of whole cell biotransformation as the method for this project.

Chapter 2

2. Materials and Methods

2.1 Abbreviations

MTBE	tert-Butyl methyl ether
C #	Alkane of # carbon atoms
FAME	Fatty Acid Methyl Ester
DCA	Dicarboxylic Acid
DNA	Deoxyribonucleic acid
dNTPs	Desoxyribonucleotides
GC-MS	Gas Chromatography coupled to Mass Spectrometry
E.	Escherichia
Min	Minutes
T	Temperature

Fwd	Forward Primer
Rev	Reverse Primer
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
bp	base pairs
TADH	Alcohol dehydrogenase from *Thermus sp.* ATN1
ADH	Alcohol dehydrogenase
AlkDH	Alcohol dehydrogenase encoding gene from *Thermus sp.* ATN1
AlkDH2	356 bp 5' terminal flanking region of AlkDH
AlkDH3	481 bp 3' terminal flanking region of AlkDH
KAT	Kanamycin resistance encoding gene + PslpA promoter
AlkDH2-KAT	AlkDH2 (3') ligated to KAT (5')
KAT-AlkDH3	KAT (3') ligated to AlkDH3 (5')
AlkDH2-KAT-AlkDH3 / AlkDHtKAT	Alcohol dehydrogenase KAT truncated gene
PCR	Polymerase chain reaction
LDH	Lactate Dehydrogenase
NADH/NAD	Reduced/Oxidized Nicotinamide-adenine dinucleotide
BSA	N,O-bis(trimethylsilyl)acetamide
SDS	Sodium Dodecyl Sulphate

DNSA	Dinitrosalicylic acid
FTIR	Fourier transform infrared spectroscopy
ATR	Attenuated total reflectance
ICP	Inductively coupled plasma mass spectroscopy
LCDA	Long-chain dicarboxylic acid

2.2 Materials

2.2.1 Chemicals

Chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany), Merck (Darmstadt, Germany) and Carl Roth GmbH (Karlsruhe, Germany).

2.2.2 Strains

All strains used in this study are listed in table 2.1.

Table 2.1 - Strains used in this study.

Strain	Characteristics	Source
Thermus sp. ATN1	*Thermus sp*. wild type able to grow on (degrade) long-chain alkanes as sole carbon source.	Isolated and cultured from compost heap in Hamburg (Feitkenhauer, 1998)
Thermus sp. ATN1 random mutants: RMS2, RMS5, RMS17 & RMS26	Mutants obtained by random (NTG) mutagenesis of *Thermus sp*. ATN1followed by selective phenotype enrichment.	This study
Thermus sp. ATN1 AlkDHtKAT-RMS5 mutants (DG)	Mutants obtained by disruption of an alcohol dehydrogenase gene, AlkDH (TADH) gene (GenBank: EU681191.1; Otto, 2001, Hollrigl *et al.*, 2008) in *Thermus sp*. ATN1-RMS5 mutant.	This study
E. coli DH5α	*F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoAsupE44 λ-thi-1 gyrA96 relA1*	Gibco®, Invitrogen™ (Hanahan, D., 1985)
Pseudomonas sp. Strain 273	A gram-negative, aerobic bacterium capable to grow on C5 to C12 α,ω-dichloroalkanes in the presence of oxygen as the sole source of carbon and energy as well as in non-halogenated aliphatic compounds	Isolated and cultured from soil in Stuttgart area (Wischnak, 1998)

2.2.3 Culture media

All glassware, culture media and buffer solutions were autoclaved at 121°C for 20 min and the pH was adjusted according to the value needed using sterile NaOH 2M, unless otherwise specified. Medium containing Gelrite was autoclaved for 30 min and poured at T>65°C. Media composition is listed in tables 2.2 and 2.3.

Table 2.2 - Media used for culture and enrichment of *Thermus sp.* ATN1 and selected mutants.

Complex medium for *Thermus sp*. (DSMZ, No. 74)	
Polypeptone	8.0 g
Yeast Extract	4.0 g
NaCl	1.0 g
Add 1000 mL Millipore water and adjust pH to 6.5	
For solid media the following were added:	
Gelrite	8.0 g
$MgCl_2$	1.0 g
Mineral salts medium for *Thermus sp.* (Feitkenhauer, 1998)	
Media prepared from solutions autoclaved separately and mixed after cooling down:	
$MgCl_2$ $6H_2O$	0.1 g
$CaCl_2$ $2H_2O$	0.1 g
KCl	0.04 g
in 250 mL Millipore Water	
$FeSO_4$ $7H_2O$ in 50 mL Millipore Water	0.1 mg
Na_2HPO_4	0.42 g
NaH_2PO_4 in 680 mL Millipore Water	0.20 g
Trace Elements Solution (as described in DSMZ 141)	10 mL
Yeast Extract (in 10 mL Millipore water and sterilized through 0,45 µm filter)	0.1 g
Total volume 1000 mL, pH 6.5	
For solid media the following were added:	
Gelrite	8.0 g
$MgCl_2$	1.0 g
Trace element solution composition (in DMSZ 141)	
Nitrilotriacetic acid	1.50 g
$MgSO_4$ $7H_2O$	3.00 g
$MnSO_4$ H_2O	0.50 g
NaCl	1.00 g
$FeSO_4$ $7H_2O$	0.10 g
$CoSO_4$ $7H_2O$	0.18 g
$CaCl_2$ $2H_2O$	0.10 g
$ZnSO_4$ $7H_2O$	0.18 g
$CuSO_4$ $5H_2O$	0.01 g
$KAl(SO_4)_2$ $12H_2O$	0.02 g
H_3BO_3	0.01 g
Na_2MoO_4 $2H_2O$	0.01 g
$NiCl_2$ $6H_2O$	0.03 g
Na_2SeO_3 $5H_2O$	0.30 mg
Millipore Water	1000 mL
First dissolve nitrilotriacetic acid and adjust pH to 6.5 with KOH, then add minerals. Final pH 7.0 (with KOH). Store in the dark at 4 °C	

Table 2.3 - Media for transformation and mutant enrichment in molecular cloning experiments.

TM medium (Hashimoto, 2001) – Transformation and enrichment of *Thermus sp.* ATN1	
Tryptone	4.0 g
Yeast Extract	2.0 g
NaCl	1.0 g
Add 1000 mL Millipore water and adjust pH to 7.5	

TT medium (Hashimoto, 2001) – Transformation and enrichment of *Thermus sp.* ATN1	
Tryptone	4.0 g
Yeast Extract	2.0 g
NaCl	1.0 g
$CaCl_2$	to 0.4 mM
$MgCl_2$	to 0.4 mM
Add 1000 mL Millipore water and pH adjusted to 7.5	

LB medium (Sambrook *et al.,* 2001) – Transformation and enrichment of *E. coli* DH5α	
Tryptone	10.0 g
Yeast Extract	5.0 g
NaCl	5.0 g
Add 1000 mL Millipore water and pH adjusted to 7.0	

2.2.4 Substrates and co-substrates (Carbon sources)

Alkanes liquid at room temperature (20 °C; C16 and lower chains) as well as co-substrates in solution were sterilized through a 0.45 µm filter and added to the mineral salts medium to desired concentration. Solid substrates (alkanes, alcohols and fatty acids) were either added as MTBE or hexane solutions into sterile culture flaks, then solvent was left to evaporate under a sterile hood or they were added as solids and sterilized by autoclaving.

As substrates, alkanes were added in concentrations ranging from 5 to 10 mM whereas alcohols ranged from 2 to 5 mM. Only straight chain saturated compounds were used in this study. Carbon sources (co-substrates) were added to initial concentrations ranging from 5 to 15 mM for batch cultivation or controlled at a concentration of about 10 mM during fed-batch cultivation. Choice of substrate and co-substrate depended on type of cell culture experiment according to table 2.4.

Table 2.4 - Cell culture experiments and substrate/co-substrate choice.

Cell Culture Experiment	Mutant Screening and Selection	Bioconversion Screening	Bioconversion for DCA production
Substrates	-	Alkanes (C8, C10, C11, C12, C16, C18, C20), Alcohols (C10, C12, C14, C16, C18)	Alkanes (C16, C17, C18, C20, C24 and C28)
Co-substrates (Carbon Source)	Alkane (C16), Alcohols (C12, C16) and Fatty Acid (C16)	Acetate, Citrate, Lactate and Pyruvate Sodium Salts, Glycerol, Glucose and Fatty Acids (C12, C16)	Pyruvate and Acetate Sodium Salts

2.2.5 Buffers and solutions

Buffers were prepared from stock solutions as shown in table 2.5 (Sambrook *et al.,* 2001). All solutions were prepared with deionized water (Millipore, Germany).

Table 2.5 - Buffers and solutions.

Buffers and Solutions	Application
Potassium Phosphate Buffer 10 and 20 mM, pH 7.6	Cell washing and re-suspension
Triethanolamine-HCl buffer - Triethanolamine-HCl 250 mM, pH adjusted to 7.5 with NaOH at 25°C - EDTA-disodium salt 2.5 mM	Pyruvate Determination Assay
NADH buffer - NADH-disodium salt 7 mM - NaHCO3 120 mM	Pyruvate Determination Assay
Tris-Acetate-EDTA buffer (TAE) Stock solution 50X (1L), pH 8.0 - Tris 242 g - Glacial acetic acid 57.1 mL - EDTA 500 mM solution 100 mL	Agarose gels and DNA Electrophoresis
Buffer B1 - Tris-HCl, pH 8.0 50 mM - EDTA, pH 8.0 50 mM - Tween-20 0.5% - Triton X-100 0.5%	Genomic DNA Extraction
Buffer B2 - Guanidine HCl 3 M - Tween-20 20%	Genomic DNA Extraction
Denaturation solution - 1.5 M NaCl, 0.5 M NaOH Neutralization solution - 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), 1 mM EDTA Blotting buffer: 20X SSC, pH 7.0 - 3 M NaCl, 0.3 M sodium citrate 100X Denhardt's solution - 2% (w/v) BSA + 2% (w/v) Ficoll™ - 2% (w/v) PVP (polyvinylpyrrolidone) Pre-hybridization solution - 6X SSC, 5X Denhardt's solution, 50% formamide, 0.5% SDS.	Southern Blotting, Hybridization and Detection
DNSA Reagent - *1% 3,5-dinitrosalicylic acid and 30% Sodium Potassium Tartrate in 0.4 M NaOH*	Carbohydrates determination
Bradford Reagent - 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol + 100 ml 85% (w/v) phosphoric acid. Diluted to 1 L (after dye is dissolved). Filtered with Whatman #1 paper before use.	

2.2.6 Enzymes and molecular biology tools

2.2.6.1 Enzymes

- LDH 150 - L-Lactate Dehydrogenase 150 (evo-1.1.150, >2000 U/mL; >20 U/mg)
  - Source: *E. coli* recombinant, Evocatal, Düsseldorf, Germany
- TADH (Höllrigl *et al.*, 2008), thermostable alcohol dehydrogenase from *Thermus sp.* ATN1 (1 mg/mL stock solution)
  - Source: *E. coli* recombinant, Dr. Katja Bühler (Laboratory of Chemical Biotechnology, Department of Biochemical and Chemical Engineering, TU Dortmund)
- All enzymes used in DNA manipulation were bought from Fermentas (St. Leon-Rot, Germany)

2.2.6.2 Molecular biology tools

Molecular biology tools are summarized in tables 2.6 and 2.7

Table 2.6 - Plasmids and oligonucleotides.

Plasmids	Characteristics	Source
pKT1	pUC-derived plasmid, containing sequences from *Thermus thermophilus*. Shuttle vector with a KAT cassette under the control of a bifunctional promoter	Biotools, (Moreno R., 2003)
pUC19	Cloning vector; lacZα, Amp^r pUC origin of replication	Fermentas (Yanisch-Perron *et al.,* 1985)
pUC19-AlkDHtKAT	pUC19 derived plasmid containing the KAT truncated alcohol dehydrogenase gene from *Thermus sp*. ATN1	This study
Oligos (all were ordered from biomers.net GmbH)		**To amplify** / **Characteristics & Restriction Sites**
5'– GAA TTC ATG CGC GCA GTG GTT TTT G – 3' Fwd 5'– GGA TCC TGG AGA TTG CCA AAC ATG G – 3' Rev		AlkDH2	356 bp 5' terminal homologous region of AlkDH Fwd:*Eco*RI /Rev:*Bam*HI)
5'– TCT AGA CCA GGT ATT GGG TGC GAG – 3' Fwd 5'– AAG CTT TCA CCC CCT GAC AAC CAA A – 3' Rev		AlkDH3	481 bp 3' terminal homologous region of AlkDH Fwd:*Xba*I /Rev:*Hind*III)
5'–GGA TCC CCC CGG GAG TAT AAC AGA AAC–3' Fwd 5'–TCT AGA TTC AAA ATC GTA TGC GTT TTG AC–3' Rev		Promoter +KAT	962 bp PslpA promoter + thermophilic KAT Fwd: *Bam*HI/Rev:*Xba*I

Table 2.7 - Molecular biology kits used for DNA manipulation, purification and detection.

Molecular Biology Tools	Used to	Acquired from
GeneRuler™ 100 bp Plus DNA Ladder	DNA gel electrophoresis	Fermentas
GeneJET™ PCR Purification Kit	Purify the PCR products	Fermentas
GeneJET™ Gel Extraction Kit	Extract target size DNA from agarose gel	Fermentas
Transformation kit	To transform the plasmid to *E. coli*	Fermentas
Nucleo-Spin Plasmid	To isolate the plasmid from *E. coli* strain to use for transformation	Macherey-Nagel
Genomic DNA Purification Kit	To isolate genomic DNA from *Thermus sp.*	Fermentas
Biotin Decalabel DNA Labeling Kit Biotin Chromogenic Detection Kit	To carry out the southern hybridization experiments	Fermentas

2.3 Molecular biology methods (DNA manipulation)

2.3.1 DNA amplification (PCR), restriction digestion and ligation

PCR was performed with *Taq* or *Pfu* DNA polymerases. Ligation reactions were performed with T4 DNA Ligase. Several restriction enzymes were utilized in this study: *Eco*RI, *Bam*HI, *Xba*I, *Hind*III, *Alw*NI, *Sac*I and *Bsa*BI. Unless otherwise indicated, all enzymes were used according manufacturer's recommended protocols. Please refer to details in section 2.4.2.

2.3.2 DNA electrophoresis, extraction and purification

Electrophoresis was performed with 0.8% agarose in TAE buffer gels (Sambrook *et al.*, 2001). The runs were performed at 75-80 Volt and gels were stained with Sybr Green® according to manufacturer's recommendations.

Genomic DNA was extracted from *Thermus sp.* ATN1 and its mutants to be used as a template in PCR screening experiments and to perform DNA hybridization procedures. Extraction was performed either according to instructions provided with the Genomic DNA Purification Kit (Fermentas) or by the below described method. The latter provided the highest genomic DNA yields.

Genomic DNA extraction procedure:

- 5 mL of a *Thermus sp.* ATN1 DSMZ74 overnight culture were used for cells harvesting by centrifugation at 4500 rpm for 20 min (4°C). The supernatant was discarded.
- The pellet was re-suspended in 500 µL buffer B1 (containing 22 µL of 10 mg/mL RNase A).
- 30 µL Lysozyme (100 mg/mL) and 50 µL Proteinase K (20 mg/mL) were added to the bacterial suspension and incubated at 37°C gently shaking for an hour.
- 200 µL of buffer B2 were added and the lysate was shaken in a vortex mixer for few seconds. The lysate was then incubated during 30 min at 50°C in a water bath.
- 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the lysate and shaken in a vortex mixer, followed by centrifugation during 20 min at 4500 rpm (4°C) to separate the phases.
- The upper aqueous phase was transferred to a new falcon tube and 0.1 volume of 3M sodium acetate solution, pH 4.8 and 0.7 volume of isopropanol were added
- DNA was collected by centrifugation for 20 min at 4500 rpm (4°C). The DNA pellet was washed with 400 µL cold 70% ethanol and dried at room temperature.
- The DNA was dissolved in 50 µL sterile distilled water (Practical course on Environmental Microbiology, TUHH, 2008).

PCR products used for ligation during AlkDHtKAT construction were purified with the GeneJET™ PCR purification kit; GeneJET™ Gel Extraction Kit was used for purification of target DNA fragments from agarose gels run in TAE buffer, both available from Fermentas. Plasmid DNA was extracted and purified with a kit according to manufacturer instructions (NucleoSpin® Plasmid, Macherey-Nagel).

2.4 Mutants construction and selection

2.4.1 Random mutagenesis

Thermus sp. ATN1 cells were treated with N-methyl-N´-nitro-nitroso-guanidine (MNNG or NTG) for chemical mutagenesis. Survivors were selectively enriched by cultivation on media containing penicillin and n-hexadecane as sole carbon source.

2.4.1.1 NTG treatment

A modification of the procedure described by Adelberg *et al.* (1965) was used. Cells were grown overnight in 100 ml DSMZ 74 medium at 65 °C. 4 ml of this culture were used for cell harvesting by centrifugation at 9000 rpm for 15 minutes. Cells were re-suspended in 1 ml 20 mM potassium phosphate buffer pH 6.5 containing NTG in concentrations ranging from 0 to 160 µg/ml. These preparations were incubated 40 minutes at 70°C and 350 rpm in a thermomixer. After cooling down, cells were pelleted at 9000 rpm for 2 minutes and were washed 3 times with 1 ml 10 mM potassium phosphate buffer containing 0.85 %w NaCl, then they were re-suspended in 1 ml 20

mM potassium phosphate buffer and finally dilutions were prepared and volumes of 50 µl were spread on DSMZ74 Gelrite plates. Plates were incubated for 72 h at 65 °C. NTG concentrations rendering survivor rates between 1 and 10 % were selected for mutagenesis in combination with mutant selective enrichment.

2.4.1.2 Penicillin enrichment

On the basis that penicillin can kill only growing cells by inhibiting the cross-linking of peptidoglycan polymers essential for the structural integrity of the cell wall, a mutant enrichment step with Penicillin V followed the NTG treatment (Fitzgerald *et al.*, 1975).

Conditions for the mutagenic treatment prior enrichment were similar to those reported for other type of mutations caused by NTG treatment in *Thermus thermophilus*, studied by Kobashi *et al.* (1999). Cells treated with 20 and 40 µg/mL NTG were enriched over one, two or three penicillin enrichment cycles, plated and selected for a preliminary phenotype screening procedure.

Mutants were incubated in minimal medium with n-hexadecane as sole carbon source and penicillin concentrations of 250 U/mL and 2500 U/mL were tested (values in literature ranged from 100 up to 4000 U/mL for this type of treatment). Time of treatment for one enrichment cycle corresponded to 12 h (1 generation time of the wild type strain in n-eicosane, as reported by Otto, 2001). Optimally penicillin was added at a concentration of 250 U/mL for the treatment, but only after a pre-enrichment starvation time of 6 to 8 h (to permit cessation of growth of the desired mutants, Herdman *et al.*, 1980). Enrichment was stopped by ice chilling followed by centrifugation, cell washing and re-suspension in complex medium (DSMZ74). After 3 h of incubation, dilutions were prepared and spread on DSMZ74 Gelrite plates. Repeated penicillin enrichment steps before plating were executed to improve mutant yield.

2.4.1.3 Random mutants screening

The approach to screen for disruption of the long-chain alkane metabolic pathway in the random mutants included 2 stages:

2.4.1.3.1 Plate screening

Transferring complex-media-growing colonies to mineral medium plates supplemented with long-chain n-alkanes and intermediate expected metabolites (fatty alcohol and fatty acids) as single carbon sources.

Mutants on DSMZ74 Gelrite plates were replicated on mineral medium plates with 1.13 g/L n-hexadecane or n-octadecane as sole carbon source (5 mM equivalent). Replicas on 0.37 g/L 1-dodecanol (2mM) and 1.28 g/L hexadecanoic acid (5 mM) were also tested. Incubation was at 65 °C for 6 to 8 days. As *Thermus sp.* ATN1 colonies grow flat and almost colorless on mineral medium, a colony staining method using 0.1% Coomassie Brilliant Blue R stain was used.

The staining solution was poured on the plate and left for 45 seconds, then the solution was poured off followed by a rinsing step with (4:5:1) water/methanol/acetic acid during 1 minute. After pouring the rinsing solution off the plates were inverted on a paper towel to dry them. After colony staining, it was possible to differentiate mutants still growing on the n-alkanes from those showing no apparent growth, the later were further studied.

It was assumed that cells growing on the long-chain n-alkane had no mutations that could lead to terminal oxidized products. The same assumption was made for colonies not growing in the alkane but able to grow on fatty alcohols, since for this case mutation(s) would have occurred at the first step of the metabolic pathway (possibly a damaged monooxygenase system). In contrast cells able to grow in the fatty acid (fatty acid auxotrophs) but not able to grow on the alkane nor on the alcohol were considered as positive mutants and with potential to produce interesting intermediates (table 2.8).

Table 2.8 - Possible disruptions of the long-chain n-alkanes metabolic pathway of *Thermus sp.* ATN1 that could be detected with the proposed phenotype screening approach. Only mutant types 3 and 4 (not selectable with the screening strategy) are interesting for further study within the scope of this thesis.

	Growth in Substrate as Sole Carbon Source			Possible Pathway Disruption					Possible Products from Long-chain n-Alkanes Bioconversion (Growing on a different Carbon Source)			
Mutant Type	Long-chain n-Alkane	Fatty Alcohol	Fatty Acid	None	Monooxygenase	Alcohol Dehydrogenase	Aldehyde Dehydrogenase	β-Oxidation	Fatty Alcohol	α,ω - Long-chain Diols	Fatty Acids	α,ω - Long-chain Dioic Acids
1	✓	✓	✓	✓	×	×	×	×	×	×	×	×
2	×	✓	✓	×	✓	×	×	×	×	×	×	×
3	×	×	✓	×	✓	✓	✓	×	✓	✓	✓	✓
4	×	×	×	×	✓	✓	✓	✓	✓	✓	✓	✓

2.4.1.3.2 Liquid culture screening

A subsequent screening procedure for mutants selected from plate screening was performed in liquid mineral medium containing the same substrates and concentrations as used in solid media plates. Experiments were carried out in 15 mL Falcon tubes containing 5 mL medium incubated at 70°C and 120 rpm. Incubation for each set of cultures ran for several days. Mutants showing growth in liquid mineral media with n-hexadecane as sole carbon source were discarded as possible candidates for n-alkane bioconversion.

2.4.2 Directed mutagenesis - Gene disruption by homologous recombination

Targeted gene disruption was performed similarly as described by Hashimoto *et al.* (2001) and Fujiwara *et al.* (2006). Homologous recombination with a thermostable kanamycin-resistant marker (Moreno *et al.*, 2003) flanked by homologous regions of the gene AlkDH encoding the known *Thermus sp.* ATN1 alcohol dehydrogenase TADH (Hollrigl *et al.*, 2008) was used for gene knock out. TADH is suspected to be involved in the long-chain n-alkanes metabolism. Directed mutagenesis was performed in the wild-type and with selected mutant strains obtained by random mutagenesis.

2.4.2.1 Construction of truncated AlkDH gene

Figure 2.1 presents the scheme of the three types of DNA constructs that were used for the transformation of the wild type strain and selected mutants in order to obtain AlkDH disrupted mutants.

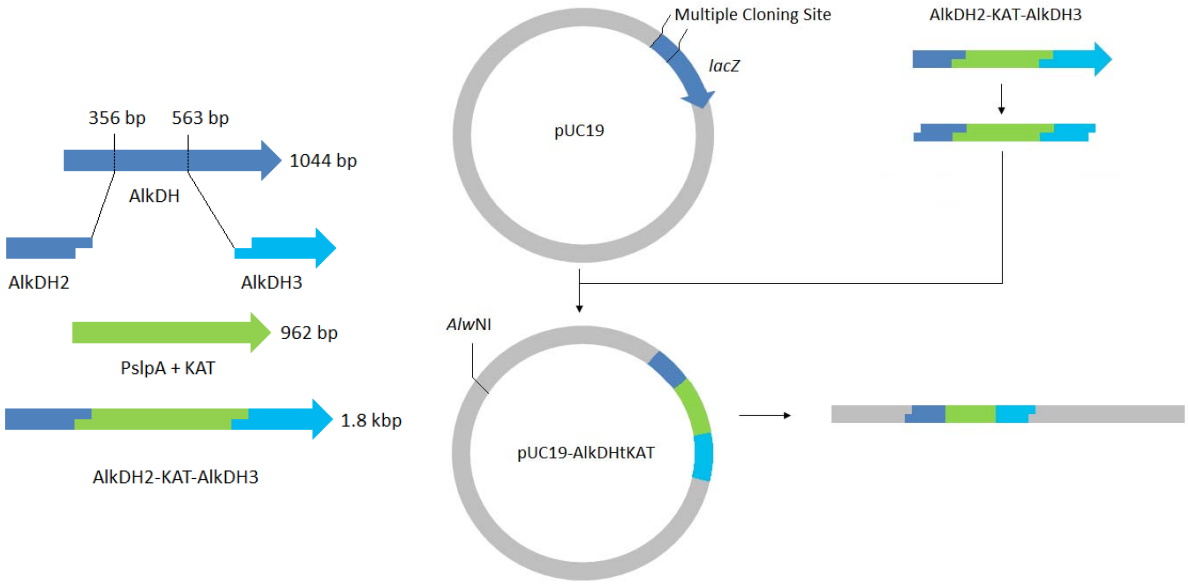


Figure 2.1 - Preparation of recombinant DNA constructs for disruption of the TADH encoding gene (AlkDH) in *Thermus sp.* ATN1: (a) Amplification of AlkDH2, AlkDH3 and KAT followed by sticky end ligation to obtain AlkDH2-KAT-AlkDH3; (b) Sticky end ligation of ligation of AlkDH2-KAT-AlkDH3 into the multiple cloning site of the plasmid cloning vector pUC19 to obtain plasmid pUC19-AlkDHtKAT; (c) Digestion of plasmid pUC19-AlkDHtKAT for linearized plasmid transformation experiments (Hashimoto, 2001).

Construction of the truncated alcohol dehydrogenase gene was the first step in this process. Truncated AlkDH (AlkDH2-KAT-AlkDH3 or AlkDHtKAT) was prepared by sticky end ligation of a kanamycin resistance encoding gene + promoter (KAT cassette) with flanking regions of the known *Thermus sp.* ATN1 alcohol dehydrogenase (TADH) encoding gene (AlkDH).
Amplification of AlkDH flanking regions and KAT was performed with *Taq* polymerase and primers listed in table 2.5 at concentrations of 50 fM, 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. Table 2.9 presents data for the PCR mix and thermocycler program. Higher primer concentrations resulted in unspecific PCR products.

Table 2.9 - PCR mix and thermocycler program for amplification of AlkDH flanking regions and KAT.

PCR Mix			Thermocycler Program		
Component	Volume [µL]	Concentration	Time [min]	Tempera-ture [°C]	Cycles
10X *Taq* polymerase Buffer	5	1X	3	95	1
25 mM MgCl2	2.5	1.25 mM	1	95	5
2 mM dNTPs	5	0.2 mM	1	54	
Taq Polymerase (5 U/µL)	0.25	0.025 U	2	72	
Nuclease free water	34.25	-	1	95	15
Forward primer (2.5 pmol/µL)	1	50 fM	1	55	
Reverse primer (2.5 pmol/µL)	1	50 fM	2	72	
Template (~1 nmol/µL)	1	20 pM	1	95	20
Total Volume	50		1	57	
			2	72	
			15	72	1
			∞	8	1

AlkDH2, AlkDH3 and KAT PCR products were purified and dephosphorylated with FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas) prior to restriction digestion (sticky-ends preparation). Dephosphorylation was performed to avoid self-ligation due to compatible sticky ends or blunt ligation.

The digestion reactions for both AlkDH flanking regions and KAT PCR products were performed overnight at 37°C. Reaction mixtures were prepared according to table 2.10. Restriction enzymes were thermally inactivated at 80°C for 20 min to stop the digestion.

Table 2.10 - Digestion mixtures preparation to produce sticky-end AlkDH flanking regions and KAT DNA fragments.

	AlkDH2	AlkDH3	KAT
DNA	30 µL(0.028 µg/µL)	15 µL(0.063 µg/µL)	15 µL(0.063 µg/µL)
Buffer	5 µL	5 µL	5 µL
	10X *Bam*HI buffer	10X Tango buffer	10X Tango buffer
Enzyme	1.5 µL *Bam*HI	1.5 µL *Xba*I	1.5 µL *Bam*HI
			1.5 µL *Xba*I
Nuclease free water	13.5 µL	28.5 µL	27 µL

The two homologous regions of alcohol dehydrogenase gene, AlkDH2 and AlkDH3, were ligated to the KAT gene with T4 DNA ligase according to manufacturer's protocol. The double ligation reaction was performed overnight at 22°C. T4 DNA ligase enzyme was inactivated at 65°C for 10 min to stop the reaction. Reaction mix is described in table 2.11.

Table 2.11 - Double ligation reaction mix to produce truncated AlkDH.

Component	Volume [µL]
AlkDH2 (0.017 µg/µL)	10
AlkDH3 (0.019 µg/µL)	10
KAT (0.019 µg/µL)	10
10X T4 DNA ligase buffer	4
T4 DNA ligase (5 U/µL)	0.5
Nuclease free water	5.5

In order to check for the correct ligation product a PCR reaction was performed using the ligated sample as template with three different sets of primers: (a) AlkDH2 forward and AlkDH3 reverse, (b) AlkDH2 forward and KAT reverse and (c) KAT forward, AlkDH3 reverse.

2.4.2.2 Polymerase incomplete complementary sequence extension

As double ligation after double digestion of the KAT gene (at once or stepwise) presented several challenges and many DNA manipulation steps, it was found easier to hybridize single strand sequences of AlkDH2-KAT and KAT-AlkDH3 at the KAT region and to complete the other sequence which is left unfilled at regions of AlkDH2 and AlkDH3 using dNTPs by *Pfu* polymerase. No primers were used in this reaction. The KAT complementary templates used for this reaction were AlkDH2-KAT and KAT-AlkDH3 amplified, extracted and purified from gel from previous step. Reaction mixture and thermocycler program for this reaction is presented in table 2.12. The product from this polymerase reaction was used as template for the amplification of whole AlkDHtKAT construct.

Table 2.12 - Polymerase incomplete complementary sequence extension mix and thermocycler program for construction of AlkDH2-KAT-AlkDH3.

PCR Mix			Thermocycler Program		
Component	Volume [µL]	Concentration	Time [min]	Tempera-ture [°C]	Cycles
10X Pfu polymerase Buffer	5	1X	3	95	1
25 mM MgSO4	2.5	1.25 mM	1	95	45
2 mM dNTPs	5	0.2 mM	1	60	
Pfu DNA Polymerase (2.5 U/µL)	0.5	0.025 U	2	72	
Nuclease free water	7	-	15	72	1
AlkDH2-KAT (10 ng/µL)	15	3 ng/µL	∞	8	1
KAT-AlkDH3 (10 ng/µL)	15	3 ng/µL			
Total Volume	50				

2.4.2.3 Construction of plasmid pUC19-AlkDHtKAT

The 1.8 kb construct containing the kanamycin resistance gene was subcloned into the plasmid cloning vector pUC19. Plasmid and construct were digested with the restriction enzymes *Eco*RI and *Hind*III. To prevent recircularization, a dephosphorylation step on the linearized pUC19 vector was performed. The construct was then ligated (sticky-end) in an overnight incubation at 22°C with T4 DNA ligase.

Self-ligation will occur if the ends of the prepared vector are compatible or blunt and 5'-phosphorylated, as the enzyme used to ligate the DNA molecules requires a 5´-phosphate group on one of the DNA substrates to work. Dephosphorylation of the linearized vector DNA, prior to ligation to the insert fragment, decreases the background of the recircularized vector, whereas a DNA insert containing phosphorylated 5´ termini can be ligated into the vector. Alkaline phosphatases are commonly used in cloning experiments to dephosphorylate the 5´ ends of vector DNA.

The product(s) of the ligation reaction was transformed into *E. coli* DH5α competent cells with a transformation kit (Fermentas). Positive transformants were selectively picked up by blue white screening.

White colonies were transferred to liquid LB medium containing 30 µg/mL kanamycin. Transformants growing under this condition have incorporated the plasmid pUC19-AlkDHtKAT encoding for kanamycin resistance. Positive transformants were further tested using PCR to confirm the presence of truncated gene construct (in pUC19). The strains containing a KAT truncated AlkDH gene were sub-cultured overnight in LB medium. Cells were then harvested by centrifugation. The plasmid was isolated and quantified from agarose gel electrophoresis. This plasmid was used for transformation experiments to *Thermus sp*. ATN1 and its selected mutants.

2.4.2.4 Transformation of *Thermus sp.* ATN1 wild-type and mutant strains

An overnight culture of each strain grown in TM medium was diluted 1:20 with TT medium and shaken at 70°C for 2h (~$1x10^8$ cells/mL). This subculture (400 µL) was mixed with a 50 µL DNA solution (see figure 2.1). The mixture was shaken at 70°C for 2h and then spread on plates containing 100, 200, 300 µg/mL of kanamycin. The plates were incubated at 70°C for 48h (Hashimoto, 2001).

The colonies, which were grown on kanamycin, were selected and were further grown on liquid complex medium containing 100 µg/mL kanamycin. Control colony PCR for AlkDH and KAT amplification was done with these strains in combination with phenotype screening (see 2.4.2.6).

2.4.2.5 Transformation efficiency into *Thermus sp.* ATN1

Transformation efficiency with the three types of DNA constructs built for gene disruption (figure 2.1) was studied by transforming *Thermus sp.* ATN1-RMS5 mutant strain (obtained from chemical mutagenesis). The pUC19-AlkDHtKAT plasmid was linearized by digesting it with the restriction enzyme *Alw*NI. Various amounts of the AlkDHtKAT construct (0.384, 0.768, 1.152, 1.536 and 1.92 µg of DNA) were used for transformation. For experiments with the circular and linear forms of the pUC19-AlktKAT plasmid, concentrations of 12.5, 25, 50, 75 and 100 ng of DNA were used. 50 µL of transformation mixtures were spread on DSMZ74 Gelrite plates containing 200 µg/mL kanamycin. Colonies, which appeared to be isolated and well grown, were considered as positive transformants.

2.4.2.6 Phenotype screening of kanamycin resistant mutants

2.4.2.6.1 Pararosaniline test – Detection of aldehydes

It was expected that mutant strains with a truncated alcohol dehydrogenase gene (in this case AlkDHtKAT) will not produce a fatty aldehyde from a fatty alcohol substrate. This was the basis for the phenotypic screening of mutants. The procedure was slightly modified from the work of Conway *et al.* (1987). Mixtures of pararosaniline and bisulfite are often referred to as Schiff reagent and have been widely used to detect aldehydes. 2 mg of pararosaniline and 20 µL of a sodium bisulfite solution (~40 %) were dissolved in 500 µL 95% ethanol, 50-100 µL of this solution were spread on grown mineral media plates containing a long-chain n-alkane or a fatty alcohol and 20 mM sodium pyruvate (as carbon source) after colonies had been grown for 72h at 65 °C. Plates were incubated for another 24-36 h at 55°C. Strains still containing an alcohol dehydrogenase appeared intensely red. Medium or slight coloration was regarded as background coloration.

2.4.2.6.2 TADH activity assay

Another approach to test AlkDH disruption was to test TADH activity (see 2.7.1) in crude extract prepared from cultures of kanamycin resistant mutants.

2.4.2.7 Southern blotting, hybridizing and detection

Incorporation of the kanamycin resistance encoding gene into the *Thermus sp.* ATN1 genome at the position of the AlkDH gene was tested by southern blotting. This technique combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization. For the Southern blot, alkaline transfer from agarose gel to a positive charged nylon membrane (SensiBlot™ Plus Nylon Membrane) was performed as described by Sambrook *et al.*, 2001. Non-radioactive biotinylated probes were used for hybridization and detection (Leary *et al.*, 1983), this was performed with Biotin Decalabel™ DNA labeling kit and Biotin chromogenic detection kit available from Fermentas (See appendix for detailed procedure).

Overnight cultures of wild type and mutant strains were used for genomic DNA extraction. Extracted DNA was digested overnight with *Sac*I or with *Bsa*BI according to protocol recommendations. The agarose gels used for blotting were prepared with 0.8% agarose in TAE-buffer.

2.5 Bioconversion screening experiments - Cell cultures

Thermus sp. ATN1 and selected mutants were cultured in mineral medium containing long-chain n-alkanes and a co-substrate as carbon and energy source to screen for alkane bioconversion products. Experiments were performed varying the substrate and co-substrate (see 2.2.4). Experiments were carried out under limited oxygen conditions in (1) sealed serum vials and (2) screw cap shaking flasks.

2.5.1 Cell cultures in sealed serum vials

Mineral medium supplemented with a co-substrate (5-10 mM) was inoculated with 5% v/v of an overnight complex medium culture of the corresponding strain (either wild-type or selected mutant strains). 5 mL of this culture were then transferred under clean bench conditions to 30 mL sterile serum vials containing 10-25 µmol of substrate each, depending on the experiment (2 to 5 mM equivalent in 5 mL volume as described in section 2.2.4). Vials were sealed and incubated at 70°C and 150 rpm.

To monitor cell growth and substrate and co-substrate (only for sodium pyruvate) consumption, vials were taken out of incubation at 12 or 24 hours intervals. Cell density was determined by counting cells under the microscope. Substrate concentration and product formation was determined from solvent extraction of each vial followed by GC-MS detection (see 2.8.). Co-substrate concentration was determined enzymatically (see 2.9).

2.5.2 Cell cultures in screw cap shaking flasks

Experiments were conducted in 1 L screw cap Erlenmeyer flasks containing 500 mL of mineral medium. Incubation ran at 70°C and 150 rpm for at least 60 hours. The medium was supplemented with a co-substrate to initial concentrations ranging from 5 to 15 mM for batch cultivation. Fed-batch cultivation was carried out with sodium pyruvate as carbon and energy source at a controlled concentration of about 10 mM, adjusted each 12 or 24 hours. Alkanes were added to initial concentrations from 5 to 10 mM (see 2.2.4).

5 mL samples were taken from the culture broth every 12-24 hours and analyzed for cell density, pyruvate concentration and alkane conversion. Alkane concentration was determined from solvent extraction of samples taken immediately after vigorous shaking of flasks followed by GC-MS analysis. 3 samples were taken per sampling point, except for the initial sample. Initial sample was prepared from 5 mL fresh mineral medium (containing a co-substrate) and the exact amount of alkane to the desired concentration was added just before solvent extraction.

2.5.2.1 Measuring cell density

Cell density in the broth culture was analyzed by cell counting under the microscope in a Neubauer chamber. A droplet of culture sample taken with an inoculation loop was placed in the chamber for counting. Samples from complex media were counted directly from the broth culture whereas samples from mineral media (containing a hydrophobic substrate) required 5 minutes in an ultrasonic bath before counting.

Neubauer chamber dimensions: 0.02 mm depth, 1/400 mm^2 small square area, 16 small squares/big square. Volume per big square is 0.0008 mm^3. Cell counts were performed from 2 to 3 big squares. Average counts were multiplied by 1.25×10E6 to obtain number of cells present in 1 mL culture.

2.5.2.2 Calculation of generation time

During exponential growth by binary fission, the generation time is the time interval required for the cells (or population) to divide. It can be calculated from:

$$G = \frac{t}{3.3 \log \frac{b}{B}}$$

where,

G = the generation time [h]
t = the time interval [h]
B = number of bacteria at the beginning of a time interval
b = number of bacteria at the end of the time interval

2.6 Bioconversion experiments - Cell cultures

Experiments were carried under limited oxygen conditions (2.5.2) and under aerobic conditions in loose cap shaking flasks.

2.6.1 Cell cultures in loose cap shaking flasks

These experiments were conducted in 2 L loose cap Erlenmeyer flasks containing 1000 mL of mineral medium. Loose cap flasks shall provide better gas exchange and avoid oxygen starvation in the broth culture. Cells were incubated at 70°C and 120 rpm for at least 36 hours. Fed-batch cultivation was carried out with sodium pyruvate as carbon and energy source at a controlled concentration of about 10 mM, adjusted each 12 or 24 hours. Alkanes were added to initial concentrations from 5 to 10 mM (see 2.2.4).

Like in screw cap shaking flask cultivation, 5 mL samples were taken from the broth culture every 12-24 hours and analyzed for cell density, pyruvate concentration and alkane conversion.

2.6.2 Isolation and enrichment of products (Dicarboxylic acids)

Remaining substrate (alkanes) and products were isolated by processing the culture broth as follows:

1. Centrifugation of warm culture at 6000 rpm during 20 minutes while cooling down to 4 °C to separate cells and insoluble remaining substrates and products.
2. Floating material was removed by filtration with Whatman filter paper at 4°C or decantation.
3. Acidification of free-cell broth with concentrated HCl down to pH 2, followed by 24 h incubation at room temperature to further separate de-saponified carboxylic acids, dispersed alkanes and biosurfactant.
4. Solvent extraction 1:1 with MTBE in a separating funnel followed by 2 washing steps with half volume MTBE.
5. Mono and dicarboxylic acids were isolated free from solvent extracts or as methyl esters from hydrolysis and esterification of removed floating solids:
    a. Free acids were isolated from the organic phase by solvent evaporation followed by chromatographic separation in a preparative column packed with silica gel 60A (pre- treated 2X with 1:1 Ethanol-HCl 0.05 M overnight at 60 °C, washed and dried overnight at 80°C). Elution done with 5x 10 mL fractions of n-heptane and 10x 10 mL fractions of MTBE
    b. Esterified acids (in biosurfactant and/or insoluble material isolated from the cell-free broth) were isolated as methyl esters by treating isolated solids with 2% methanolic sulphuric acid followed by solvent extraction, concentration and chromatographic separation.

2.7 Biochemical methods

2.7.1 Alcohol dehydrogenase activity test

Alcohol dehydrogenase activity was determined by measuring fatty alcohol conversion through GC-MS analysis in crude extract samples with 1-alkanols and NAD. The method was based on a spectrophotometric method previously reported by Otto (2001) and Höllrigl *et al.* (2008) in which consumption or formation of NADH was monitored at 340 nm and 60°C

400 µL crude extract (prepared from a 20 % weight wet cell suspension, 2.7.1.1) were mixed with 100 µL of NAD 50 mM in an 2 mL Eppendorf tube containing 2 µmol of substrate (C14 and C16 alkanols). Samples were incubated at 65 °C in a thermomixer. After incubation samples were extracted with 500 µL MTBE. The solvent extract was derivatized by silylation with BSA and analyzed by GC-MS (see 2.8). Substrate consumption (and product formation) was monitored at different incubation times.

Similar experiments were conducted with 20% w/v resting cell suspensions including alkanes as substrates.

2.7.1.1 Preparation of crude extract

Cells were harvested from broth cultures by centrifugation at 6000 rpm (~15 °C) and washed 3X by re-suspension/centrifugation in 20 mM potassium phosphate buffer (20% w/v cell paste). Cell paste was stored at -80°C.

For crude extract preparation cells were thawed 20% w/v in 20 mM phosphate buffer supplemented with 1 mM DTT and $FeSO_4$. The cells were disrupted by ultrasonication keeping temperature below 6°C, kept in an ice –NaCl bath. Sonication parameters were High Gain, 70% Power, Cycle 5x10%, 20 x 1.5 min with a Bandelin SONOPLUS HD (70-200W) equipment. The extract was centrifuged at 9000 rpm for 20 minutes at 4°C. The supernatant was used immediately for alcohol dehydrogenase activity experiments.

2.7.2 Pyruvate determination assay

Unlike the wild type strain, selected mutant strains were not able to grow on alkanes as sole carbon source. Sodium pyruvate was the co-substrate of choice (see 3.8 results). Pyruvate acts as the carbon and energy source for growth of the mutants of *Thermus sp*. ATN1. Determination of pyruvate concentration in the culture broth was performed not only to monitor consumption but also to adjust its concentration (fed-batch cultivation) during alkane bioconversion experiments. It is expected that if the concentration of pyruvate is exhausted or if it is in excess, the bioconversion of alkanes would be reduced.

Pyruvate concentration was determined enzymatically by the decrease in absorbance at 340 nm due to the oxidation of NADH by a lactate dehydrogenase (LDH). LDH in the presence of NADH converts pyruvic acid (pyruvate) into D-lactic acid and NAD^+.

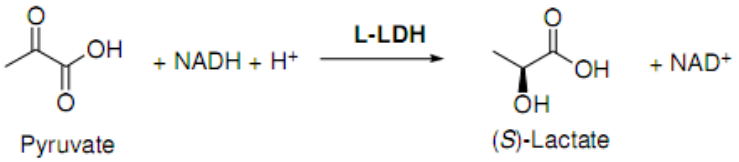


Figure 2.2 - Pyruvate reduction by LDH. The amount of NAD^+ formed in the above reaction is stoichiometric to the amount of pyruvic acid (Bucher *et al.,* 1963).

Culture samples were analysed in reaction mixtures prepared according table 2.13.

Table 2.13 - Reaction mixture for pyruvate determination assay.

Component	Volume [µL]
Triethanolamine-HCl buffer	750
NADH buffer	25
Sample to be measured	25
Lactate dehydrogenase	5

Absorbance at 340 nm was measured at 0 min and after 8 min. The difference in absorbance of the blank was subtracted from the difference in absorbance of each sample. Pyruvate concentration was calculated from a standard calibration curve obtained for a pyruvate concentrations showing linearity up to a concentration of 5 mM. Samples with higher pyruvate concentrations were diluted with water. The blank was prepared using 25 µL of distilled water instead of sample.

2.7.3 Aldehyde detection assay

According to table 2.8, it could have been possible to obtain mutants that possess a damaged aldehyde dehydrogenase in the long-chain n-alkanes metabolic pathway. To elucidate possible aldehyde bioconversion products from n-alkanes a qualitative and semi-quantitative assay was performed with the Purpald® reagent on samples taken during cultivation of selected mutants in bioconversion screening experiments (qualitative) and with crude extract and resting cell suspensions (semi-quantitative) using C14 and C16 alkanols as test substrates.

The assay was a modification of the method described by Small *et al.* (1990) and is based on the reaction of 4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole; 4-Amino-5-hydrazino-4H-1,2,4-triazole-3-thiol (Purpald®) with aldehydes that progresses (after oxidation) to the formation of purple colored adducts (Figure 2.3).

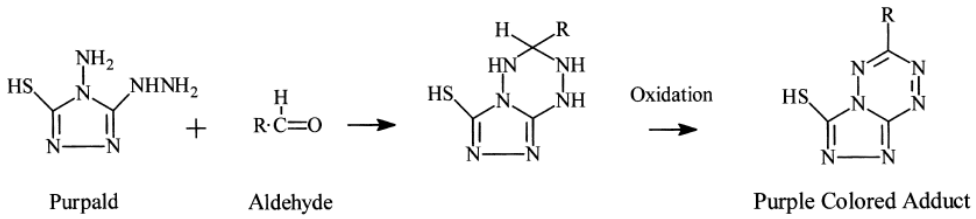


Figure 2.3 - Reaction of Purpald® with aldehydes to give a purple colored adduct.

A 2g/L stock solution of Purpald® in aqueous or methanolic 0.1 N KOH was used for the assay (depending on sample nature). Blank was prepared with 200 µL the Purpald solution and 800 µL of water or isopropyl alcohol in 2 mL Eppendorf tubes. Samples were measured as follows:

For crude extract samples, 200 µL MTBE extracts from TADH activity tests were mixed with 200 µL of the methanolic Purpald® solution and 600 µL of isopropyl alcohol. Eppendorf tubes were left open (to facilitate oxidation by air) at room temperature for 2 hours and absorbance was measured at 540 nm.

For broth culture samples and resting cell preparations (400 µl of 20% w/v cells in 20 mM phosphate buffer with 2 µmol substrate), 200 µL aliquots taken after incubation were mixed with 200 µL of the aqueous Purpald® solution and 600 µL of water. Eppendorf tubes were left open (to facilitate oxidation by air) at room temperature for 2 hours and absorbance was measured at 540 nm.

2.7.4 Protein concentration (Bradford)

Protein concentration was determined by the method of Bradford (Bradford, 1976). Solution was prepared according to table 2.5. Protein standards for the calibration curve were prepared with bovine serum albumin (BSA).

For the test, 20 µL of each sample were mixed with 50 µL of NaOH 1 M and 1 mL of the Bradford reagent in a 2 mL Eppendorf tube. 20 mM phosphate buffer was used in the control sample. After vortexing, preparations were incubated at room temperature for 25 to 30 minutes. Absorbance was measured at 590 nm.

2.8 GC-MS Analysis

2.8.1 Sample preparation

Alkane consumption/conversion and product formation was monitored from 5 mL of samples taken from broth cultures at 12 or 24 h intervals. Samples were acidified to pH <2 with concentrated HCl (10 µl for 5 ml samples) and extracted with 2.5 mL solvent (MTBE), 40 µL (120 g/L) of 1,

12-dichloro-dodecane were added as an internal standard. In order to extract the alkane completely, the possible amphiphilic products and the internal standard from the culture samples and eventually trapped in/behind the cell membrane, partial cell disruption of cells by ultrasonication in a cold water bath was carried out for 30 min. Ice was added to keep the temperature below 6 °C. The equipment used was Bandelin SONOREX RK106S.

After ultrasonication, separation of solvent and aqueous phases was induced by centrifugation at 6000 rpm for 20 min. Solvent extracts were used for GC-MS analysis.

2.8.2 Sample derivatization for GC-MS analysis

GC-MS analysis required derivatization of alkane bioconversion products and reference substances as they were expected to have acidic protons. Two methods were used depending on sample nature and purpose of the analysis.

2.8.2.1 Trimethylsilylester preparation

Trimethylsilyl derivatives are common in gas chromatography to increase volatility and stability of organic compounds containing active hydrogen atoms as is the case of products expected from the terminal oxidation from long-chain n-alkanes. Derivatization products from fatty alcohols and carboxylic acids were detected as trimethyl silyl ethers, esters or as silylation artifacts thereof. Trimethyl silyl derivatives were prepared employing N,O-bis(trimethylsilyl) acetamide (BSA) as silylating agent (Little *et al.,* 1999) under the following conditions:

- Solvent extracts were derivatized with BSA; pyridine was used to catalyze the reaction. Alkanols, fatty acids (up to C20) and glycerol esters were detected after this derivatization procedure. The sample was prepared by adding 85 µL of pyridine and 15 µL of BSA to 200 µL of solvent phase from sample extraction and incubated at 65°C for 30 min. 250 µL of the silylated sample were transferred to micro-vials for GC-MS analysis. The method was applied with reference substances up to 5 µmol in 200 µl (25 mM).

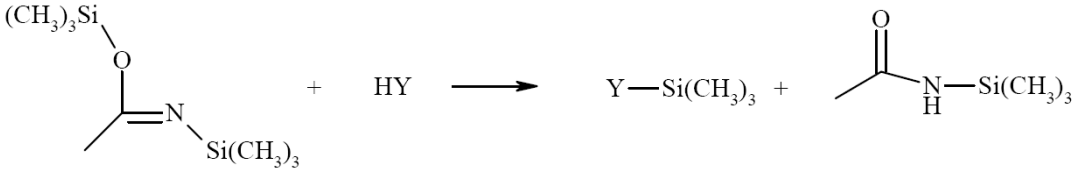


Figure 2.4 - N,O-bis(trimethylsilyl) acetamide (BSA) derivatization reaction.

2.8.2.2 Methylester preparation

Fatty acid and di-acid methyl esters were obtained either by methanolic boron trifluoride or methanolic sulphuric acid derivatization (as described by Christine *et al.*, 1993) according to the following procedures:

- Solid substances isolated from the broth cultures were analyzed as methyl esters prepared with methanolic boron trifluoride (BF3 14% in Methanol) or 2% v/v methanolic sulphuric acid. 10 mg of solid products were re-suspended/dissolved in 800 µl of the derivatizing reagent and incubated for at least 1 h at 80 °C. After ice cooling samples were extracted with 800 µl of n-heptane. 250 µl of the upper phase were transferred to micro-vials for GC-MS analysis. For quantitative purposes, reference substances (e.g. octadecanoic acid) were first dissolved in 1:1 methanol: acetone, methyl esters were obtained mixing 200 µl of the corresponding dilution and 600 µl of methanolic boron trifluoride, followed by incubation and extraction. The method was applied up to 4 µmol of octadecanoic acid in 200 µl solvent (20 mM).

- Solvent extracts were treated to obtain methyl esters fatty acids and dicarboxylic acids, which were not detected by silylation because of the high molecular weight. Fatty acids and DCA's were detected after this derivatization procedure. 100 µL of solvent extract were mixed with 700 µl of the derivatizing reagent and incubated for at least 1 h at 80 °C. After cooling, samples were extracted with 800 µl of n-heptane, followed by GC-MS analysis.

2.8.3 GC-MS methods

GC-MS equipment and operation parameters are listed in table 2.14. It was necessary to adjust oven programs and solvent delay times depending on nature and molecular weight of analytes.

Table 2.14 - GC-MS Equipment and methods.

Equipment:	Agilent 5975C Series (7890A GC/5975VL MSD)
Column:	Capillary column HP-5MS 5% Phenyl Methyl Siloxane (325 °C: 30 m x 250 µm x 0.25 µm) - Agilent Technologies. Helium was used as carrier gas; flow was set at 0.6 mL/min. Back pressure varied with starting oven temperature (20.5 kPa /70 °C and 34.9 /120 °C).
Injector:	Heater was on at 290 °C, sample injection volume was 1 µL on split mode with a 50:1 ratio. Solvent delay was set depending on sample nature, 2.5 min for methyl esters and 4.0 min for BSA derivatives.
Detector:	EM Voltage was set at 1306 eV.
Oven programs:	For compounds <C12 - 70°C to 290°C with a 15°C/min heating up rate. 290 °C for 20 min. For compounds >C12 - 120 °C to 305 °C with a 15°C/min heating up rate. 305 °C for 15 min.

2.9 Biosurfactant from *Thermus sp.* ATN1: Production, isolation and characterization

2.9.1 Monitoring of biosurfactant production

Production of surfactants in the culture medium during cultivation of the wild type and selected mutants in mineral medium for n-alkane bioconversion was monitored by the MBAS method (modified from Longwell *et al.,* 1955) but it was also possible to detect the effect of biosurfactant concentration increase due to n-alkane emulsification observed by infrared analysis.

2.9.1.1 Methylene blue method for active substance (MBAS)

The purpose of the MBAS assay is to determine the presence of surfactants in the culture medium. 1 mL samples from the broth culture were vigorously shaken 30 seconds with 5 µL methylene blue 0.5% in alkaline solution, 1 mL of chloroform was added to the sample. The mixture was left for 20 min to extract the methylene blue anionic surfactant ion pair into the chloroform layer. The tube was centrifuged at 5000 rpm for 2 min. After the extraction with chloroform, the ab-

sorbance of the chloroform phase was measured at 625 nm against a reference of pure grade chloroform.

2.9.1.2 Monitoring of n-alkanes emulsification in *Thermus sp.* ATN1-RMS5 cultures by infra-red technology

Broth culture samples taken at 12 h intervals during bioconversion of n-octadecane (1,3 g/L) by mutant strain RMS5 in screw cap shaking flaks were measured off-line with a ReactIR™ 45m equipment (Mettler Toledo). The equipment is capable to measure with FTIR technology in aqueous matrixes. Cells were removed by centrifugation at 6000 rpm for 20 minutes prior measurement. Cell free supernatant samples were warmed to approximately 50 °C by incubation in a water bath, then, they were vigorously shaken in a vortex mixer for 1 minute and measured.

The evaluation of spectra was done with Spectrum Express (Perkin Elmer) as follows: spectra of samples from a control culture in mineral medium with 10 mM sodium pyruvate as carbon source (without n-alkane) were subtracted from spectra taken from the test culture.

2.9.2 Isolation of biosurfactant

The biosurfactant was isolated from broth cultures of selected mutant strains. The surfactant was part of the insoluble material floating between the aqueous and organic phases in the separating funnel during product recovery processing by solvent extraction (with MTBE) of acidified broth cultures. This insoluble material was drained after the aqueous phase, washed 2X with MTBE and used for some characterization experiments of “crude” biosurfactant (Cirigliano & Carman, 1984).

Alternatively, biosurfactant was also obtained by concentrating 400 mL of a cell-free broth in rotary evaporator from which n-alkanes and floating solids were removed by filtration with Whatman filter paper at 4°C. The concentrate (approximately 30 mL) was lyophilized and the brownish yellow left over material was dissolved in 10 mL warm ethanol and stored at 4°C. A white material precipitated after 24-36h. The tube was centrifuged at 4°C, supernatant was removed and the precipitate was washed 2X with isopropyl alcohol. Finally the material was lyophilized to remove traces of liquid and the dry powder was weighed and stored at 4°C. This material was used for characterization experiments of the “pure” biosurfactant.

2.9.3 Characterization: Emulsification activity and stability

The emulsification activity and emulsion stability of the biosurfactant was measured by a modified method reported by Cirigliano & Carman (1984, 1985). Commercial surfactants SDS and Triton X-100 were measured for comparison. 1 mL broth culture samples or 1 mL of 1g/L surfactant preparations were diluted with distilled water to a final volume of 4 mL, and the solution was mixed with 1 mL of n-hexadecane. The mixture was vigorously shaken in a vortex mixer for 2 min and then allowed to settle for 10 min before measuring absorbance at 540 nm (turbidity). The initial absorbance corresponded to the emulsification activity.

The emulsification stability was measured based on the emulsification activity. The emulsified solutions were allowed to stand for 10 min at room temperature. Absorbance readings were taken every 5 min during 60 min. The log of the absorbance was plotted versus time, and the slope was calculated to express the emulsion stability.

2.9.4 Apparent n-alkane solubility (due to biosurfactant)

Thermus sp. ATN1-RMS5 was cultured in mineral medium supplemented with 1.3 g/L n-octadecane and 10 mM sodium pyruvate. 400 mL of broth culture that reached the stationary phase, from which cells were removed by centrifugation, were filtrated through Whatman filter paper at 4°C and subsequently evaporated to a 50 mL volume. The concentrated permeate was extracted with equal volume of MTBE and was analyzed for octadecane by GC-MS analysis. It was assumed that the alkane concentration in the clear aqueous phase was due to biosurfactant in a micro-emulsion form.

2.9.5 Increasing hydrocarbon bioavailability with *Thermus sp.* ATN1 biosurfactant

The potential of the biosurfactant produced by *Thermus sp.* ATN1 to increase hydrocarbon bioavailability was tested with a mesophilic strain previously reported of being capable to degrade n-alkanes. *Pseudomonas sp.* Strain 273 (Wischnak *et al.,* 1998) was cultured in mineral media containing 1.13 g/L n-hexadecane (5 mM equivalent) as sole carbon source (control) and was compared to cultures containing 1.13 g/L n-hexadecane + 1 g/L biosurfactant and to cultures with 1.13 g/L n-hexadecane + 1g/L SDS.

Mineral medium (with and without surfactants) was inoculated with 5% volume of an overnight complex medium culture of *Pseudomonas sp.* Strain 273. 5 mL of this culture were then transferred under clean bench conditions to 30 mL sterile serum vials containing 25 µmol of n-hexadecane. Vials were sealed and incubated at 37°C and 150 rpm.

To monitor cell growth and n-hexadecane consumption, vials were taken out of incubation at 24 hours intervals. Cells density was determined by cell counting under the microscope. Alkane consumption was determined from solvent extraction of each vial followed by GC-MS detection (see 2.8.).

2.9.6 Biosurfactant composition analysis

The biosurfactant was analysed for the nature of its hydrophilic and hydrophobic moieties. A few milligrams of the purified material where used for the analysis. 1 mg/mL solutions were used to test the nature of the hydrophilic moiety by Bradford (2.7.4) and the DNSA assay. The hydrophobic moiety was analysed by methyl esterification and GC-MS analysis (2.8.2.2).

FTIR-ATR analysis was performed with Spectrum 100 equipment (Perkin Elmer) and additionally elemental analysis by inductively coupled plasma mass spectroscopy (ICP) was ordered for the solid biosurfactant.

2.9.6.1 Dinitrosalicylic acid assay (DNSA)

Reducing sugars were measured to determine the nature of the hydrophilic moiety of the biosurfactant. For this purpose the dinitrosalicylic acid (DNSA) assay was used (Miller, 1959). Equal volumes of sample preparations (~1 mg/mL) and DNSA reagent (500 µL) were mixed and incubated for 10 minutes in boiling water. Samples were cooled to room temperature and diluted 1:4 with water and the absorbance was measured at 570 nm. Rhamnose was used as reference for the calibration curve (0.05-1 mg/mL).

Chapter 3

3. Results

Thermus sp. ATN1 was subject to chemical and directed mutagenesis in order to obtain mutant strain(s) capable to convert long-chain n-alkanes to terminal oxidized products. Mutagenesis results, growth characteristics of the constructed mutants and alkane bioconversion capabilities of the constructed mutants are presented in this chapter.

3.1 Growth characteristics of *Thermus sp.* ATN1

A previous study reported some problems of contamination in cultures of *Thermus sp.* ATN1 with *Bacillus* species (Otto, 2001). Once the purity of the wild-type strain was confirmed (colonies kept color, morphology and growth profile for several generations), *Thermus sp.* ATN1 was cultured on complex and mineral media to observe its growth characteristics.

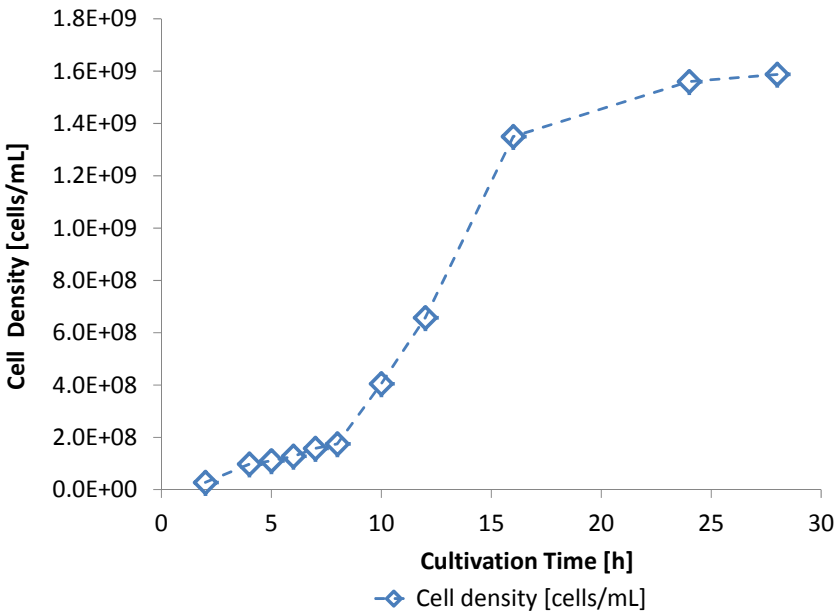


Figure 3.1 - Growth of *Thermus sp.* ATN1 in DSMZ complex medium No. 74; experiment in 250 mL shaking flask w/screw cap at 70°C and 150 rpm.

Growth of the wild-type strain on complex media (figure 3.1) was similar to that observed previously by Otto (2001) with a generation time of about 2.7 h during the exponential phase.

Biotransformation of long-chain n-alkanes with *Thermus sp.* ATN1 mutants would require an additional carbon source (co-substrate) for respiration and growth, as alkane substrates shall be only partly metabolized. Therefore, growth in mineral media was studied to evaluate whether n-alkane metabolism was possible in the presence of other carbon sources.

For co-substrate selection, growth characteristics were observed for wild-type and selected random mutant strains of *Thermus sp.* ATN1 on mineral media containing 0.01% yeast extract and several carbon sources; qualitative results are listed in table 3.1.

The tested sodium salts were the most suitable carbon sources for growth on mineral medium together with the fatty acids. Further bioconversion screening experiments were performed with selected mutants growing on these co-substrates and in the presence of n-alkanes (See 3.5).

Table 3.1 - Preliminary co-substrate screening results for long-chain n-alkanes biotransformation with selected mutants of *Thermus sp.* ATN1.

Sodium Salts	Growth	Alcohols and Fatty Acids	Growth	Others	Growth
Acetate	++	Dodecanol	+	Glucose	+
Citrate	+++	Glycerol	+		
Lactate	++	Dodecanoic Acid	++		
Pyruvate	+++	Hexadecanoic Acid	+++		
+ Limited Growth		++ Medium Growth		+++ Good Growth	

Figure 3.2 shows growth of the wild-type strain on mineral medium with sodium citrate and n-hexadecane. Alkane consumption is limited as long as other carbon source is available. This indicates an inducible and regulated n-alkane metabolism.

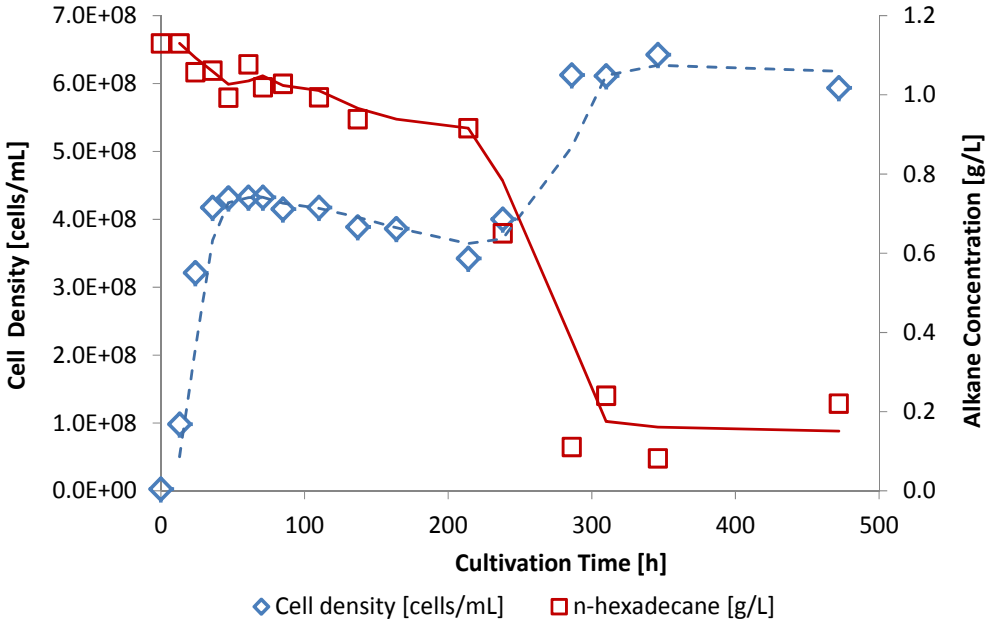


Figure 3.2 - Growth of *Thermus sp.* ATN1 in mineral medium supplemented with 2 carbon sources: 20 mM sodium citrate and 1.13 g/L n-hexadecane. Two exponential phases were observed. Limited alkane depletion before second exponential phase indicates an inducible alkane metabolism. Experiment performed in sealed serum vials.

Two exponential phases are observed in this growth curve, the first corresponding to growth on sodium citrate, followed by a plateau of nearly 200 hours before entering the second exponential phase, during which a considerable decrease in alkane concentration was observed. As previously observed by Otto (2001), growth in sealed serum vials required longer incubation times possibly due to oxygen limitation and/or low mass transfer rates caused by poor gas-liquid mixing in this type of flasks. Generation time was calculated to 3.4 h during the first exponential phase and to about 85 h for the second one.

When the wild-type strain was cultured on n-alkanes only, doubling times were found similar to those previously reported by Otto, as an example a doubling time of 8.3 h was calculated for the growth curve of *Thermus sp.* ATN1 on n-hexadecane presented in figure 3.4. Growth curves similar to figure 3.2 were observed for the wild-type strain growing also in other alkane/co-substrate combinations like n-hexadecane/sodium pyruvate or even in n-dodecane/dodecanoic acid.
This confirmed that the *Thermus sp.* ATN1 grows on different substrates in the presence of long-chain n-alkanes, and starts to metabolize the long-chain n-alkanes only after the other available preferred substrates were consumed. This fact suggested that directed mutagenesis planned by disruption of the known alcohol dehydrogenase gene in the wild-type strain would result in mutants without the ability to produce terminal oxidized products, since the so mutated strain

would not activate the n-alkanes degradation pathway while growing in other substrate and once this pathway is activated there would be no carbon source for growth and NAD regeneration.

Nevertheless, it was possible to generate random mutants that metabolized long-chain n-alkanes while growing on other carbon/energy sources. Probably, the genes involved in the regulation of the n-alkanes metabolic pathway of *Thermus sp.* ATN1 were damaged in these mutants. Under this assumption, directed mutagenesis (alcohol dehydrogenase gene disruption) was chosen as a tool for random mutant improvement looking for the production of long-chain terminal mono and di-oxidized products like diols or dicarboxylic acids.

3.2 Random mutagenesis and mutant screening

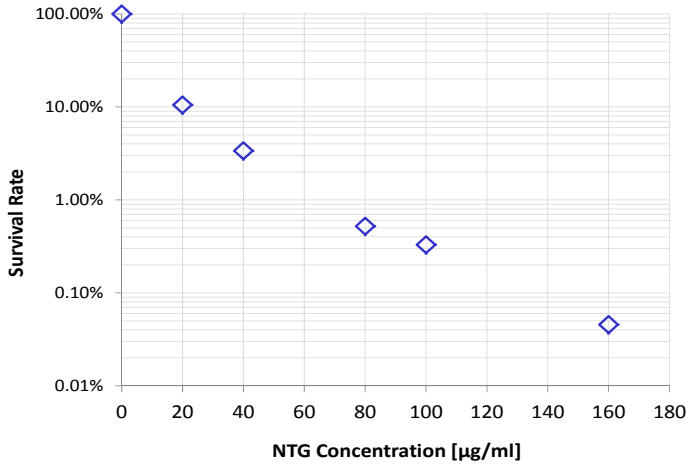
Random mutants were obtained by N-methyl-N´-nitro-nitroso-guanidine (MNNG or NTG) treatment and antibiotic enrichment techniques. A NTG concentration of 40 µg/mL and other conditions for the mutagenic treatment were the same as reported for other type of mutations caused by NTG treatment in *Thermus thermophilus*, studied by Kobashi *et al.* (1999). This NTG concentration resulted in a survival rate of 3.5% when compared to treatment without NTG. In order to maximize the proportion of mutants within survivor cells, survival was targeted for rates ranging around 10%. Survival rates obtained as function of NTG concentration are presented in figure 3.3 (a).

Cells treated with 20 and 40 µg/mL were enriched over one, two or three penicillin enrichment cycles, plated and selected for a preliminary phenotype screening procedure (see 2.4.1.2 Penicillin enrichment).

The criteria to consider a mutant colony as one of those with the desired phenotype were that upon colony transfer to screening plates the mutant strain lost the ability to grow on plates with mineral medium containing n-alkanes and alkanols as sole carbon source, while it could still grow on complex media master plates and on mineral medium supplemented with fatty acids as sole carbon source.

After 5-6 days of incubation at 65 °C, cultivation on mineral medium plates resulted in small, flat and colorless colonies difficult to distinguish from the solid medium surface. Therefore mutant selection could be only done by applying a colony staining procedure. However color contrast and residual growth made it extremely difficult to differentiate between small colonies and non-growing mutants, with a high risk of finding false negative colonies (taking them for non-desired mutants, when they were not really growing), see figure 3.3 (b).

a)



b)

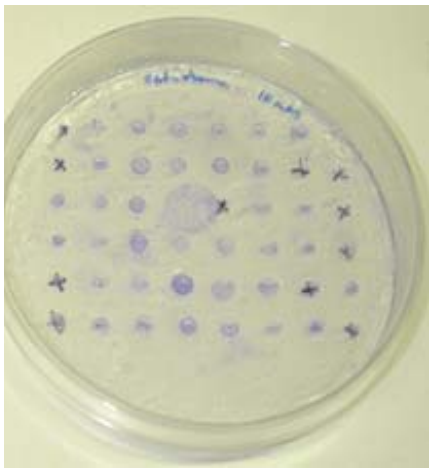


Figure 3.3 - (a) Survival rates of *Thermus sp*. ATN1 vs. NTG concentration during chemical mutagenesis treatment. (b) Selected colonies after 20-40 µg/mL NTG treatment and penicillin enrichment screened on mineral medium plates with n-hexadecane as sole carbon source, growing colonies identified after coomassie blue colony staining were discarded as possible suitable mutants. Spots marked "x" corresponded to colonies on the master plate that did not grow on this plates.

1,300 Colonies were selected for screening, 69% after 1 cycle, 27% after 2 cycles and around 4% after 3 cycles of penicillin enrichment. Colonies showing the desired phenotype represented 0.33% (3 out of 897 mutants) of colonies selected after the 1st penicillin enrichment cycle, 2.85% (10 mutants out of 351) of colonies selected after the 2nd cycle and 27% (14 out of 52 mutants) of those selected after the third enrichment cycle.

A total of 27 colonies were selected based on the phenotype screening on solid media plates and colony staining, representing a total yield of about 2%. All these mutants were fatty acid auxotrophs when growing on mineral media plates with n-alkanes as sole carbon source, but this was not the case during a subsequent screening procedure performed in liquid mineral medium containing the same substrates used in solid media plates.

Finally only 4 colonies were positive to the phenotype screening procedure, resulting in 3.1 mutants per 10^3 survivors (when growing in n-alkanes as sole carbon source). The 4 mutants proved to be stable over at least 5 cultivation cycles and their potential to accumulate metabolites from n-alkanes degradation was studied. The remaining 23 mutants were either revertants and could again grow on n-alkanes as sole carbon source on subsequent cultivation cycles (in liquid media) or simply did not survive multiple cycles due to instability.

A comparison of growth curves of the 4 selected mutants with the wild-type strain during cultivation on 1.13 g/L n-hexadecane as sole carbon source is shown in figure 3.4. Cell counts for the wild-type strain were about 8 times higher than those of the selected mutants which only showed limited growth. This is possibly due to the 0.01 % yeast extract present in the medium.

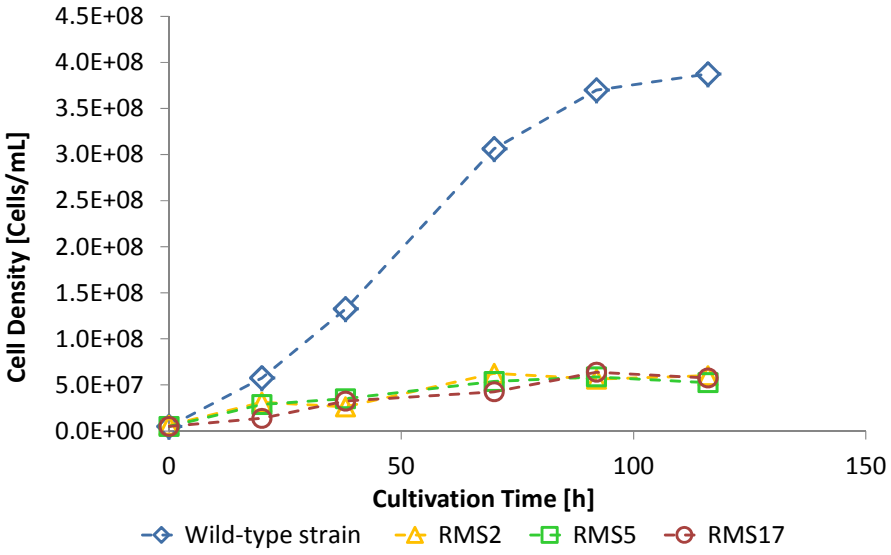


Figure 3.4 - Growth of *Thermus sp.* ATN1 and selected mutants RMS2, RMS5 and RMS17 in liquid mineral medium supplemented with 1.13 g/L n-hexadecane in sealed serum vials. Mutants lost the ability to grow on alkanes under this cultivation conditions (limited oxygen).

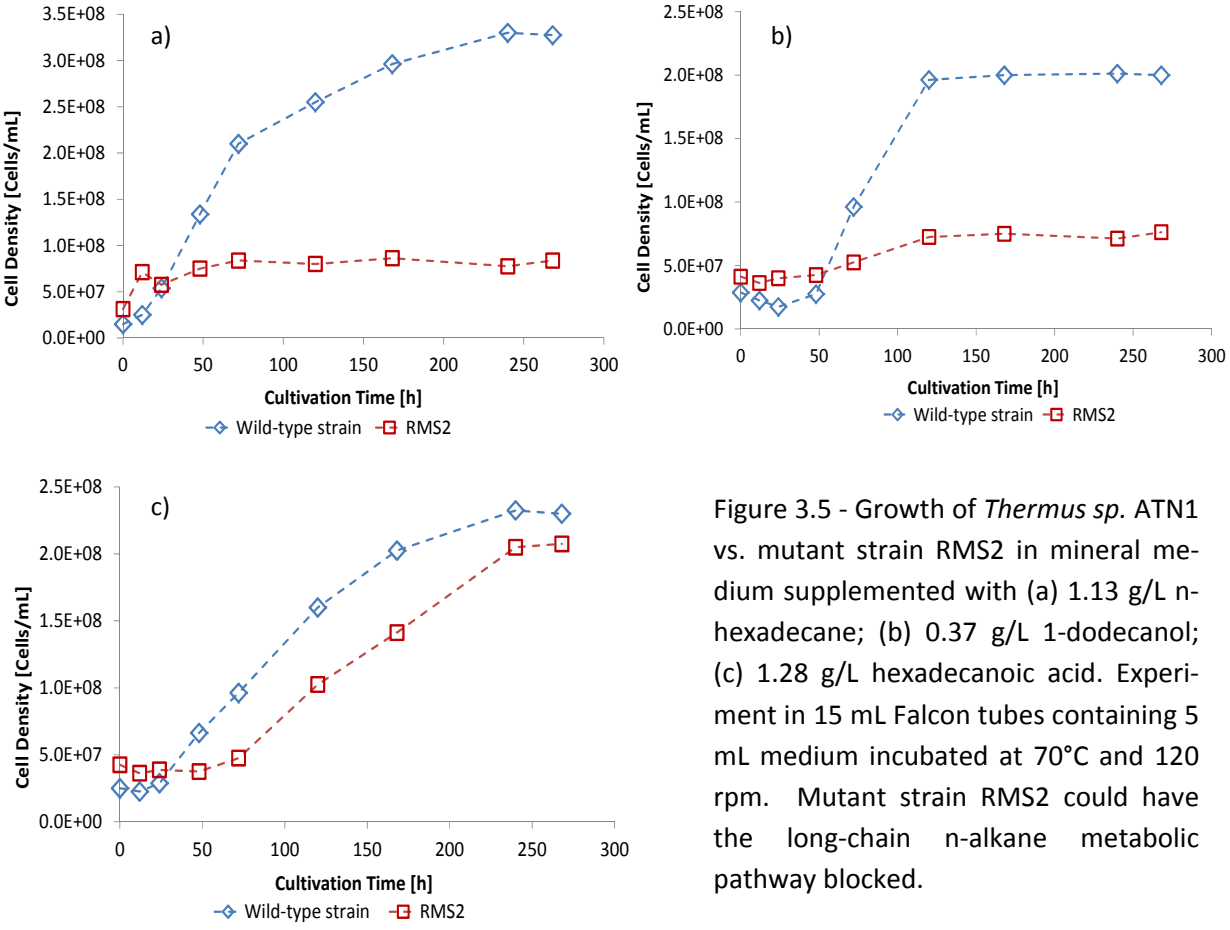


Figure 3.5 - Growth of *Thermus sp.* ATN1 vs. mutant strain RMS2 in mineral medium supplemented with (a) 1.13 g/L n-hexadecane; (b) 0.37 g/L 1-dodecanol; (c) 1.28 g/L hexadecanoic acid. Experiment in 15 mL Falcon tubes containing 5 mL medium incubated at 70°C and 120 rpm. Mutant strain RMS2 could have the long-chain n-alkane metabolic pathway blocked.

Selected mutants showed little or no growth in mineral medium supplemented with either n-hexadecane or 1-dodecanol as sole carbon source when compared to the wild-type strain. However they were able to grow on hexadecanoic acid as can be seen in figure 3.5 (c) for the case of

the mutant strain RMS2. This suggests that mutations preventing oxidation of long-chain n-alkanes or 1-alkanols to the corresponding fatty acids have occurred; making the selected mutant strains suitable candidates for further screening.

3.3 Growth characteristics of selected random mutants and co-metabolism of long-chain n-alkanes

The ability of the selected mutants to co-metabolize long-chain n-alkanes while growing on other carbon sources was studied with co-substrates listed in table 3.1. When growing on sodium citrate and sodium pyruvate observed growth levels reached the highest cell densities.

Figure 3.6 presents growth curves for the 4 selected mutants in comparison to that of the *Thermus sp.* ATN1 growing in sodium citrate in the presence of n-hexadecane in sealed serum vials (limited oxygen). In this experiment, generally longer generation times were observed for all mutants compared to the wild-type strain. In addition the wild-type strain reached cell densities twice as high as those of the mutants while growing in the first carbon source (first exponential phase) and even higher cell densities when starting to metabolize the alkane (as previously shown in figure 3.2).

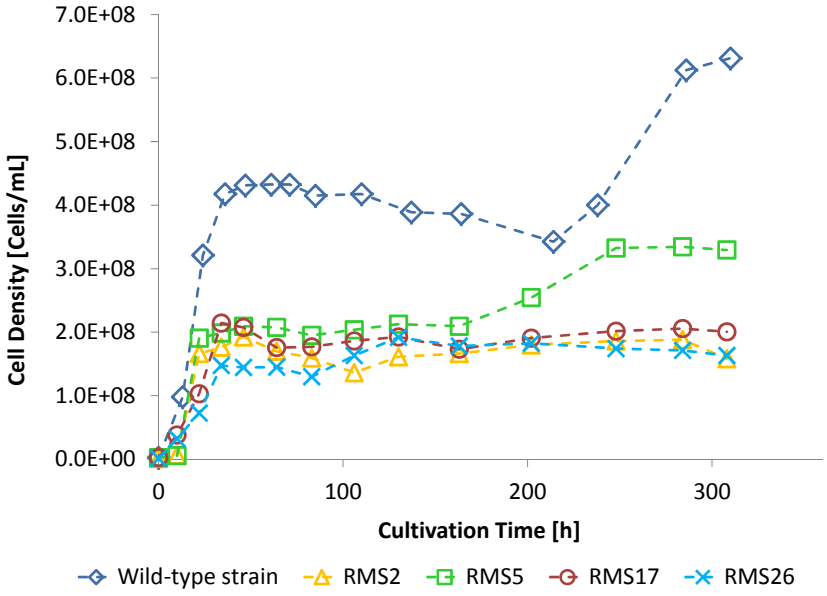


Figure 3.6 - Growth of *Thermus sp.* ATN1 and mutants RMS2, RMS5, RMS17 and RMS26 in mineral medium supplemented with 2 carbon sources: 20 mM sodium citrate and 1.13 g/L n-hexadecane. Experiments carried out in sealed serum vials.

The growth curve from mutant RMS5 resulted most interesting, with a generation time of about 4.7 h during the first exponential phase (compared to 3.4 h of the wild-type strain) and apparently second growing phase starting after 150 h of cultivation with a calculated generation time of 180 h (like the second exponential phase of the wild-type strain with a generation time of about 86 h). This suggests the possibility of mutant RMS5 being able to partly metabolize the alkane present in the medium and to accumulate metabolites during this stage.

Mutants were also cultured on sodium pyruvate in the presence of n-hexadecane. Both, pyruvate concentration and alkane concentration were monitored during these experiments and both were found to decrease at different rates but simultaneously. The highest alkane depletion rates were those observed for mutant RMS5. This lead to the conclusion that in this mutant strain the regulatory mechanism preventing co-metabolism observed with the wild-type strain has been altered. Figure 3.7 shows the growth curve for mutant RMS5 growing in sodium citrate and sodium pyruvate while degrading n-hexadecane.

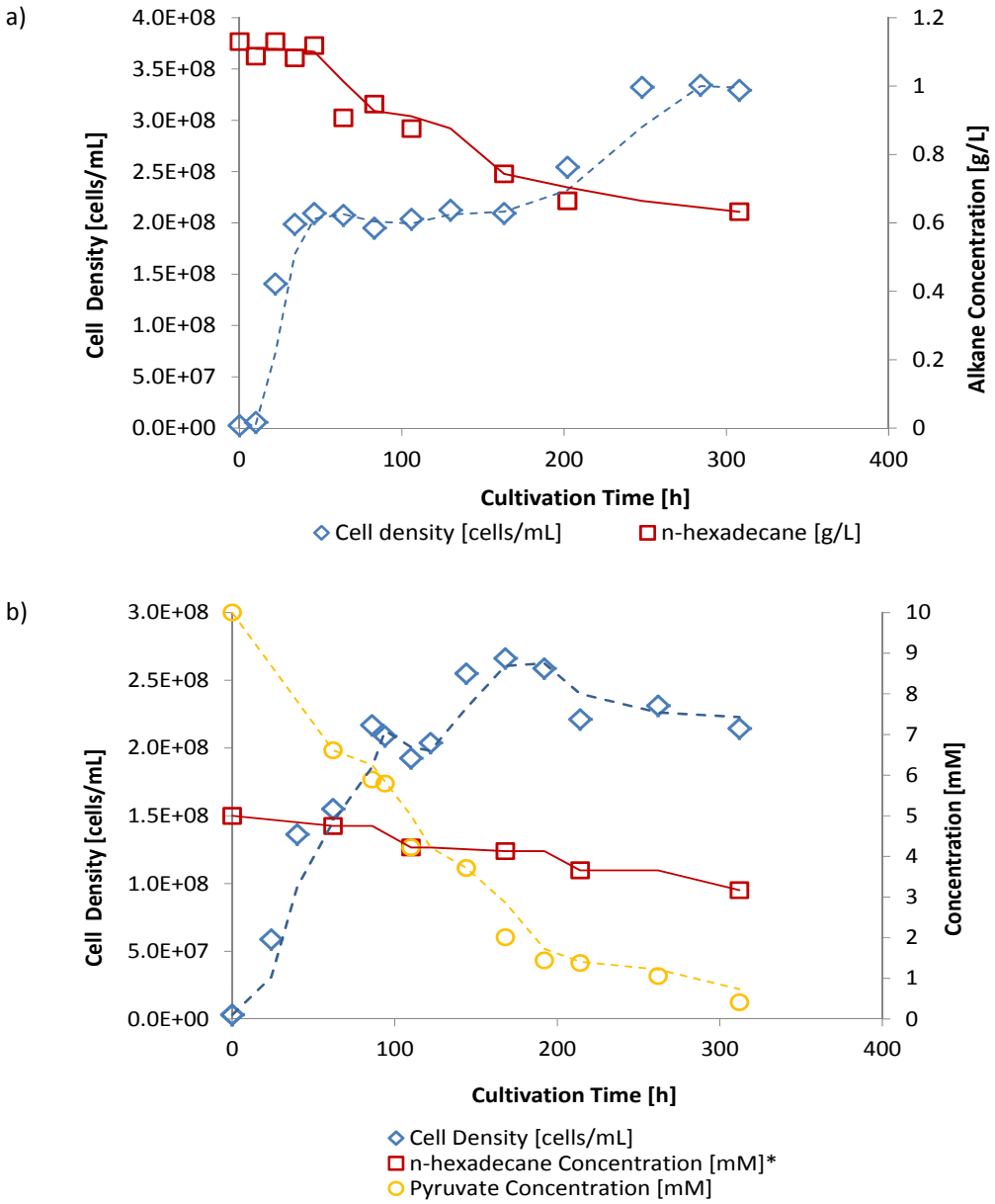


Figure 3.7 - (a) Growth of random mutant strain RMS5 in mineral medium supplemented with 2 carbon sources: 20 mM sodium citrate and 1.13 g/L n-hexadecane. (b) Growth of random mutant strain RMS5 in mineral medium supplemented with 2 carbon sources: 10 mM sodium pyruvate and 1.13 g/L n-hexadecane (*equivalent to 5 mM n-hexadecane in the culture volume). Experiments carried out in sealed serum vials.

As citrate or pyruvate concentration decreased during these experiments, it was assumed that the decreasing alkane concentration was the result of conversion by this mutant. Growth curves and cell densities of cultures containing only the co-substrate were comparable to those containing the co-substrate and the alkane during experiments in shaking flasks. Results of one of these experiments are shown in figure 3.8.

Further work was concentrated in analyzing possible conversion products or accumulated metabolites. These are discussed in section 3.5.

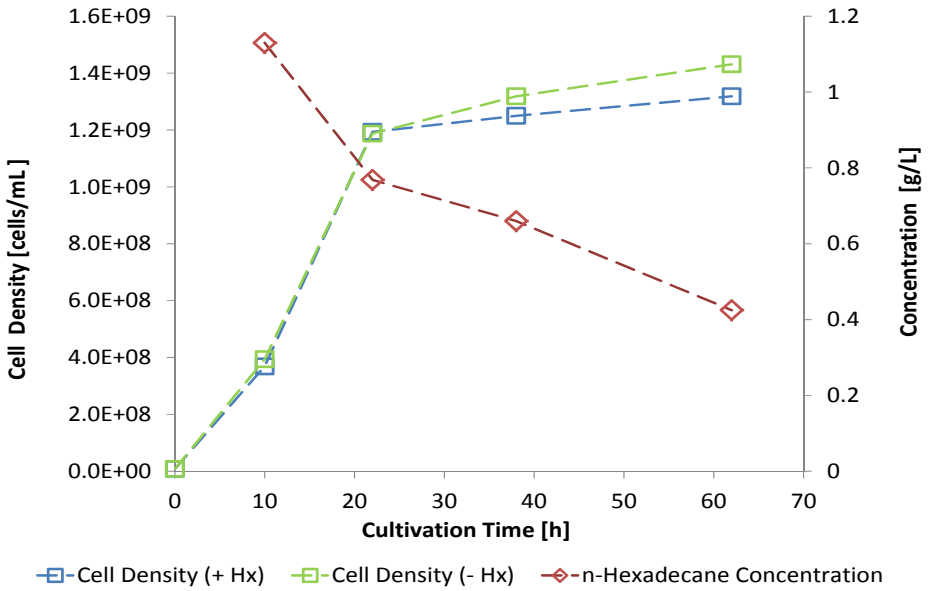


Figure 3.8 - Growth of random mutant strain RMS5 in mineral medium supplemented with 10 mM sodium pyruvate (fed-batch, adjusted to 10 mM each 12h until ~36 h) with (+Hx) and without (-Hx) n-hexadecane (1.13 g/L). Experiments carried out in 1 L screw cap shaking flasks.

3.4 Directed mutagenesis and mutant screening

Directed mutagenesis was performed to improve mutants obtained by chemical mutagenesis, which showed co-metabolism of alkanes while growing on other carbon sources. Co-metabolism would allow the mutant strain(s) to grow in a co-substrate while alkanes could be converted to long-chain terminal mono- and di-oxidized products like diols or dicarboxylic acids if the alkane metabolic pathway could be blocked. Mutagenesis was done by gene disruption of the AlkDH gene by homologous recombination with a KAT truncated AlkDH construct.

3.4.1 Construction of truncated AlkDH gene

Mutants generated by directed mutagenesis were constructed by gene disruption. Preparation of the truncated alcohol dehydrogenase gene was the first step in this process. Truncated AlkDH (AlkDHtKAT) was constructed by sticky end ligation of a kanamycin resistance encoding gene +

promoter (KAT cassette) with flanking regions of the known *Thermus sp.* ATN1 alcohol dehydrogenase (TADH) encoding gene (AlkDH). The gel in figure 3.9 (a) shows genes, flanking regions and truncated AlkDH. All gels presented in this section included a lane with the GeneRule™100 Plus DNA Ladder (SM0321, Fermentas).

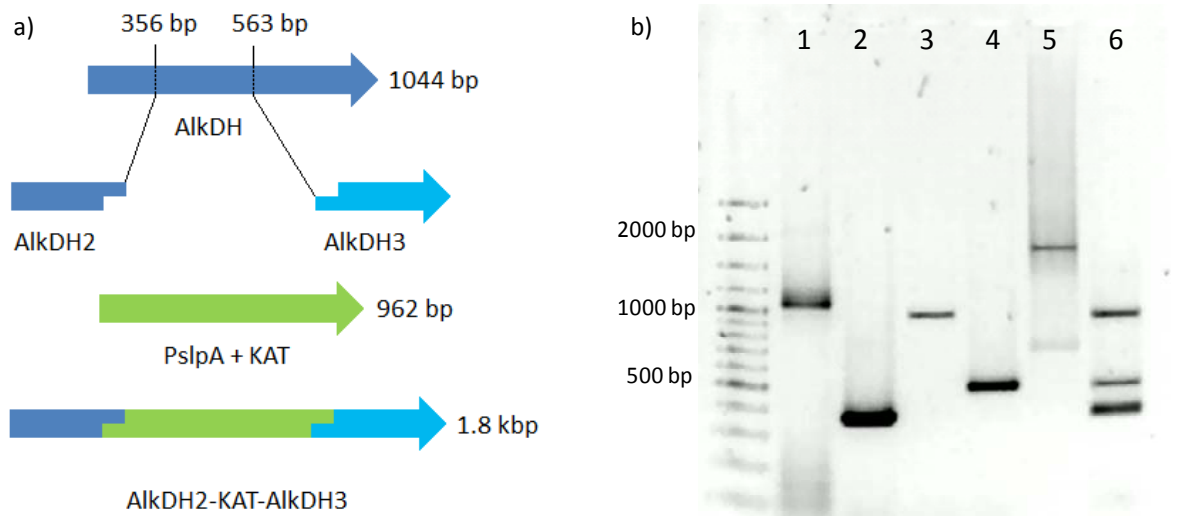


Figure 3.9 - Construction of KAT truncated AlkDH gene. (a) Truncated alcohol dehydrogenase gene construction scheme.

(b) Truncated alcohol dehydrogenase gene (AlkDH2-KAT-AlkDH3, 1.8 kb, lane 5) and its individual regions. TADH encoding gene (AlkDH, 1044 bp, lane 1), AlkDH2 region (356 bp, lane 2), AlkDH3 region (481 bp, lane 4), KAT cassette + PslpA promoter (962 bp, lane 3) and *Bam*HI/*Xba*I digestion of the truncated AlkDH gene (lane 6).

(c) Templates used for incomplete complementary sequence extension and the truncated alcohol dehydrogenase gene: Lane 1- AlkDH2-KAT, 2- KAT-AlkDH3, 3- AlkDHtKAT, 4- digestion of truncated AlkDH.

The construction of truncated AlkDH by double digestion of KAT followed by double ligation of the AlkDH flanking regions resulted only in incomplete ligation products, by this method KAT was either ligated to one or the other flanking regions from AlkDH. As both AlkDH2-KAT and KAT-AlkDH3 were obtained from this step, the whole construct was better obtained by ***incomplete complementary sequence extension*** followed by PCR, avoiding extra DNA manipulation steps needed during a less successful sequential ligation. These DNA constructs are shown in the figure 3.9 (b).

3.4.2 Sub-cloning of truncated AlkDH into pUC19

Truncated AlkDH was purified and prepared for transformation experiments and also ligated into the cloning vector pUC19 to transform it into *Thermus sp.* ATN1 (wild and RSM5) as circular and linearized vector. The pUC19 vector is 2686 bp size, out of which 40 bp were removed by digestion with *Eco*RI and *Hind*III and replaced with the 1761 bp long AlkDHtKAT in this region.

The AlkDH2-KAT-AlkDH3 construct was digested with *Eco*RI and *Hind*III and ligated into the multiple cloning sites region of the *lacZ* operon of pUC19 followed by transformation of the resulting pUC19-AlkDHtKAT plasmid into *E. coli* DH5α competent cells. Positive clones were selected by Blue White screening, followed by 100 µg/mL kanamycin growth screening.

A total of 14 white colonies were selected, 8 of them were able to grow on kanamycin/ampicillin supplemented LB medium. Clones were grown for plasmid isolation and insert PCR check using primers to amplify AlkDH. Out of the 8 clones selected, 2 clones confirmed the presence of an insert large enough as to include the originally chosen AlkDH flanking regions together with the KAT cassette; however the size of this insert was only about 1500 bp (lane 6 in figure 3.10).

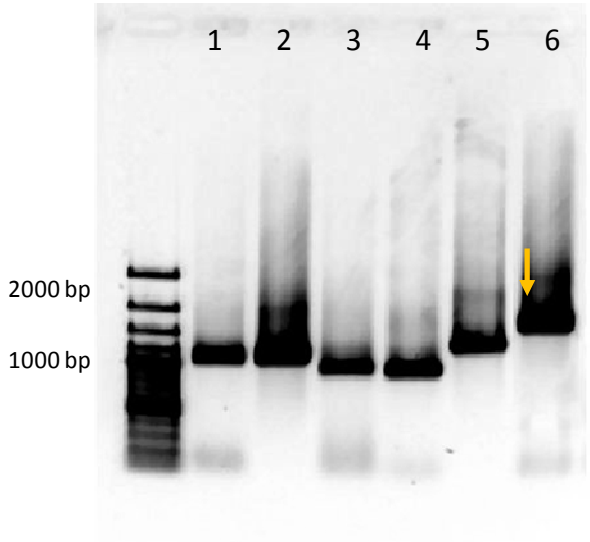


Figure 3.10 - Amplified PCR products (Lanes 1-6) of insert in various *E. coli* DH5α transformants selected by Blue White screening. Lane 6 PCR product size is of about 1500 bp.

The largest insert found in the selected *E. coli* transformants was about 300 bp shorter than the AlkDHtKAT construct; it was assumed that some deletion(s) in the construct sequence had occurred. The *E. coli* transformant in lane 6 of figure 3.10 was selected for further testing and for plasmid isolation for its transformation into *Thermus sp.* ATN1. Different combinations of primers used to amplify AlkDH and KAT regions were used to cross check and to identify the position of missing sequences in the insert of this strain. Figure 3.11 shows PCR results for this experiment.

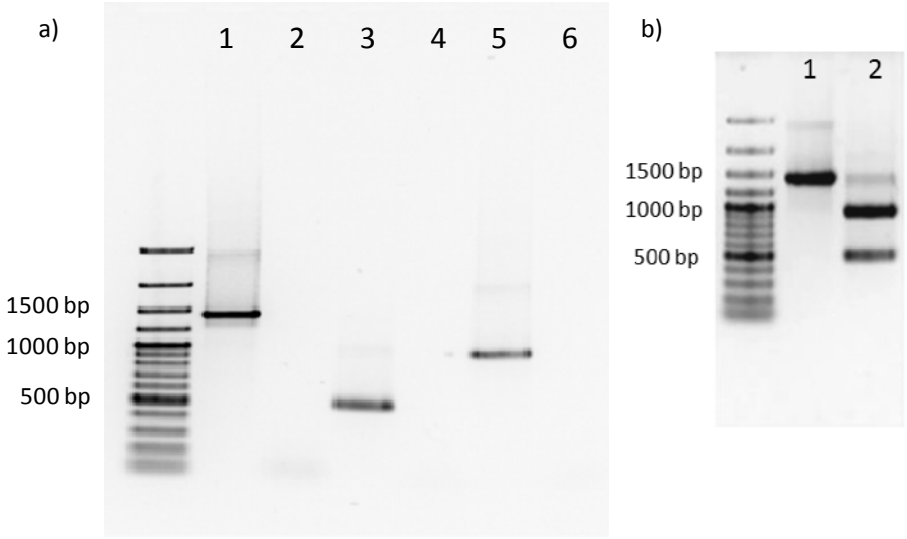


Figure 3.11 - (a) Amplified PCR products of insert in selected *E. coli* transformants with different primers sets. Lane 1 - complete amplified insert, 2 – no product for AlkDH2, 3 – amplified region AlkDH3, 4 – no product for KAT, 5 amplified AlkDH2-KAT and 6 - no product for KAT-AlkDH3; (b) *Bam*HI/*Xba*I double digestion of the 1500 bp insert in selected *E. coli* transformant (Lane 2); Lane 1 - PCR product of whole insert in plasmid isolated from *E. coli* transformant.

The gel in figure 3.11 (a) shows the results of amplification of the insert from the selected *E. coli* strain with various sets of primers. The insert of about 1500 bp (Lane 1) contains an AlkDH3 region close to 500 bp (lane 3), as the original size is 481 bp it was assumed that this region remained intact. It was not possible to amplify either of AlkDH2 region (lane 2) or KAT (lane 4) but the amplification of the construct AlkDH2-KAT (lane 5) gave a product of about 1000 bp, 300 bp shorter than expected.

In addition, the insert amplified from the isolated plasmid from *E. coli* transformant was digested with *Bam*HI and *Xba*I. The restriction enzyme *Xba*I did not work proving that the recognition site for this enzyme is missing probably due to deletion of some nucleotides at the ligation region between AlkDH2 and KAT, figure 3.11 (b). This confirmed a deletion of about 300 bp the ligation region between AlkDH2 and KAT.

Assuming that a homologous region of AlkDH was still present in the AlkDH2 fragment present in the pUC19-AlkDHtKAT plasmid insert, the plasmid was considered suitable for further transformation/gene disruption experiments.

3.4.3 Transformation experiments

Transformation into *Thermus sp.* ATN1 wild-type and mutant RMS5 strains was performed with AlkDHtKAT as well as with the circular and linearized forms of the pUC19-AlkDHtKAT plasmid (as described by Hashimoto *et al.,* 2001). The plasmid isolated from *E. coli* kanamycin resistant clones contained an insert of about 1500 bp.

Transformation efficiencies were compared for the 3 transformation options (Number of kanamycin resistant transformants as a function of amount of DNA used for the transformation). Results are presented in figures 3.12 (a) and (b). The number of kanamycin resistant mutants increased linearly with increasing amounts of DNA used. Transformation with pUC19-AlkDHtKAT gave overall higher transformation efficiencies than with the AlkDH2-KAT-AlkDH3 construct (table 3.2).

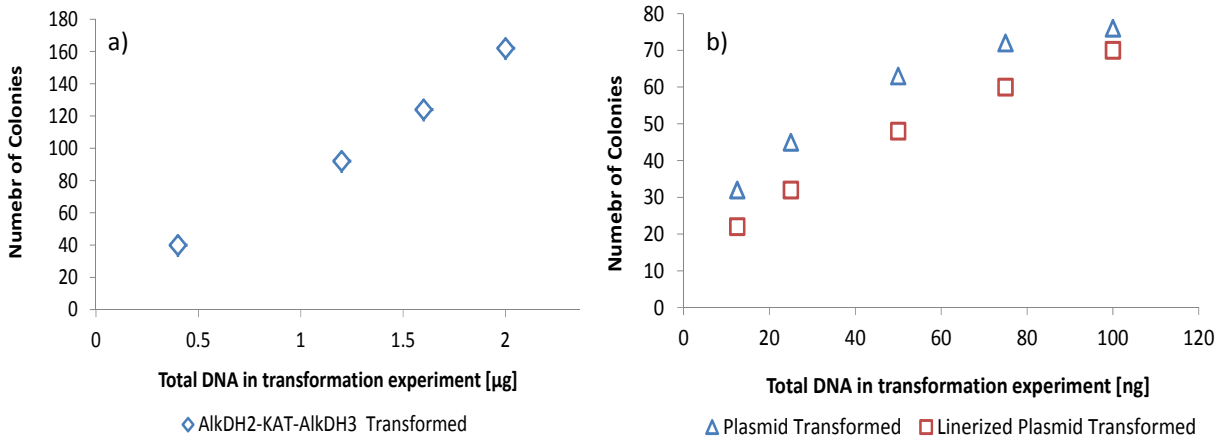


Figure 3.12 - Number of kanamycin resistant colonies formed by transformation into *Thermus sp.* ANT1 mutant strain RMS5 vs. amount of transformed DNA; (a) AlkDH2-KAT-AlkDH3 transformation results, (b) pUC19-AlkDHtKAT transformation results (circular and linearized).

Table 3.2 - Calculated number of kanamycin resistant colonies per µg of DNA in transformation efficiency experiments.

DNA Construct type	Number of colonies per µg of DNA
Alcohol dehydrogenase KAT truncated gene	81
Linearized pUC19-tAlkDH	700
Circular pUC19-tAlkDH	740

Transformation efficiency was studied in the RMS5 mutant strain. It was assumed that altered regulatory mechanisms in this mutant would allow the bioconversion of n-alkanes to 1-alkanols and eventually to α,ω-diols once the AlkDH was disrupted, whilst the strain could grow on an alternative carbon source.

The number of kanamycin resistant colonies was considered sufficient when transforming with the AlkDH2-KAT-AlkDH3 construct. As well, the probability of incorporating the KAT cassette including the PslpA promoter and intact AlkDH flanking regions (no deletions expected in this construct) was assumed higher if this construct was used for transformation. Thus, further screening experiments were predominantly concentrated on mutants obtained from transformation with this construct.

42 kanamycin resistant mutants (DG mutants) were selected from transformation of the RMS5 mutant strain for further screening experiments. 4 kanamycin resistant mutants obtained from the transformation of the wild-type strain were also tested.

3.4.4 Gene disruption tests

Kanamycin resistance could not be used alone as the marker of successful recombination in this case. To confirm that the AlkDH gene was disrupted as targeted, genotype of several kanamycin-resistant clones was analyzed by PCR and Southern hybridization. Additionally phenotype analysis based on TADH activity assays and aldehyde detection techniques were also utilized.

3.4.4.1 PCR amplification of AlkDH and the KAT cassette

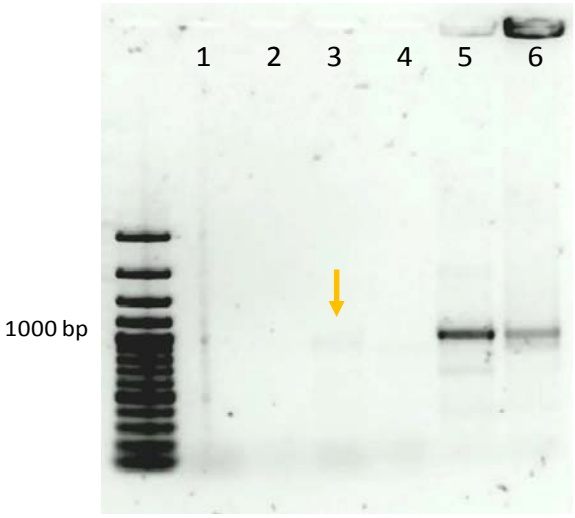


Figure 3.13 - PCR result on AlkDH amplification from genomic DNA extracted from mutants (obtained by gene disruption) capable to grow on complex media containing 100 µg/mL kanamycin. Lanes 1-4 correspond to mutants DG11, DG20, DG27 and DG29 respectively; Lane 5 - *Thermus sp.* ATN1 wild-type strain, Lane 6 - Mutant strain RMS5.

PCR screening was performed using genomic DNA extracted from several kanamycin resistant mutants as a template, as control, PCR amplification was also performed with genomic DNA from the wild and RMS5 strains. No products were obtained from amplification neither with AlkDH primers nor with KAT primers in most cases, though strains acquired kanamycin resistance; except for few of the tested mutants, as mutant DG27, lane 3 in figure 3.13. Most probably recombination resulted in deleted (disruption) or modified DNA sequences not matching any longer with the primers used, controls gave a PCR product in every reaction (figure 3.13, lanes 5 and 6). Several DG mutants giving negative for AlkDH amplification were screened for bioconversion (see 3.5.2).

3.4.4.2 Southern blotting

Figure 3.14 (a) shows hybridization results of the AlkDH biotin labeled probe. Hybridization was positive with PCR amplified AlkDH acting as control in the first lane followed by biotin-AlkDH positive hybridization with digested DNA from the RMS5 mutant. Except for mutant DG27 all DG kanamycin resistant mutants gave a negative result. Mutant strain DG27 gave a positive hybridization result indicating that the strain could have incorporated kanamycin resistance at a different genome position or still preserve an AlkDH fragment long enough to appear as a false positive result. AlkDH PCR with genomic DNA from this strain gave also a positive result (figure 3.13).

a) b)

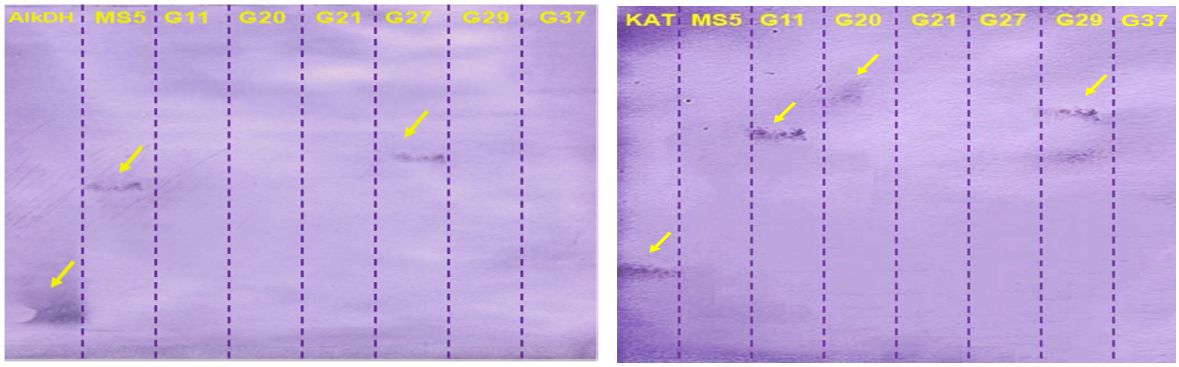


Figure 3.14 - Southern blotting results with *Bsa*BI (*Bse*JI) digested genomic DNA from selected gene disrupted mutants hybridized with biotin labeled probes of (a) AlkDH, mutants positively hybridized RMS5 and DG27; (b) KAT, mutants positively hybridized DG11, DG20 and DG27.

Figure 3.14 (b) shows hybridization results with the KAT biotin labeled probe. PCR amplified KAT acting as a control gave a positive result in the first lane. RMS5 in lane 2 did not hybridize as the strain is not kanamycin resistant. Mutants DG11, DG20 and DG29 were positive to KAT hybridization. It must be notice that although mutants DG21, DG27 and DG37 were negative in this test, these strains were able to grow on 100 µg/mL kanamycin. These negative hybridization results could have originated from low genomic DNA concentration from the extraction step or from insufficient DNA digestion resulting in a DNA concentration not suitable for hybridization. In addition hybridization could not have been detected because of the strong background color observed during this procedure.

The results of this experiment suggested that mutants DG11, DG20 and DG29 acquired the kanamycin resistance gene whilst the alcohol dehydrogenase gene has been disrupted.

3.4.5 Activity assay for alcohol dehydrogenase (TADH) from *Thermus sp.* ATN1

Activity assays for the alcohol dehydrogenase from *Thermus sp.* ATN1 (TADH) had been reported by Otto (2001) and Höllrigl (2008). TADH activity was determined by measuring alcohol conversion through GC-MS analysis in crude extract samples added with 1-alkanols and NAD (previously reported spectrophotometrically by following consumption or formation of NADH at 340 nm and 60°C).

The experiment was designed to demonstrate the effective knock-out of AlkDH in mutants obtained by gene disruption. Crude extract was prepared from a 20 % weight wet cell suspension from mutants RMS5 and DG11 treated by sonication and centrifugation (see 2.4.1.) TADH activity was analyzed according to 2.4.5, substrate consumption and product formation were monitored at different incubation times (figure 3.15).

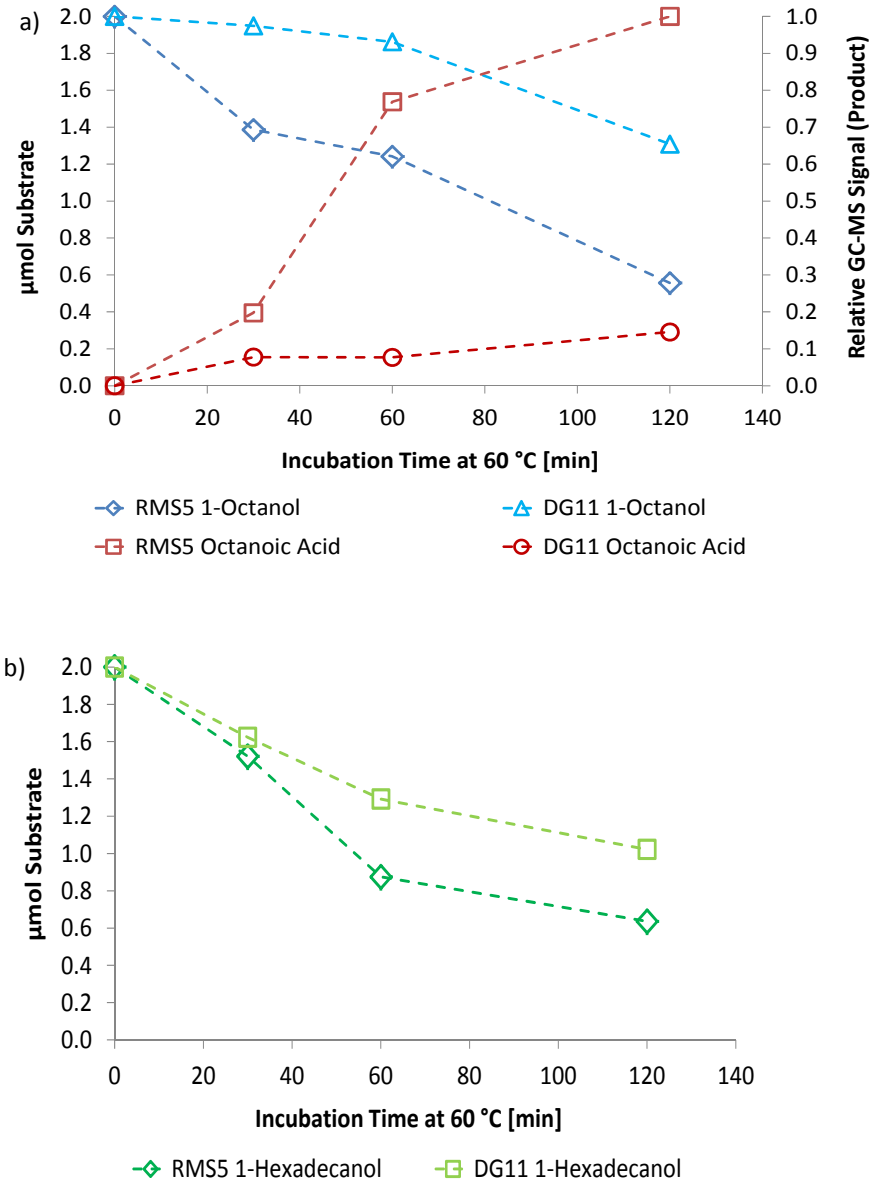


Figure 3.15 - GC-MS monitored alcohol dehydrogenase activity with alkanols in crude extract prepared from mutants RMS5 and DG11: (a) TADH activity with 1-octanol and formation of octanoic acid; (b) Alcohol dehydrogenase activity with 1-hexadecanol, hexadecanoic acid formation was not quantified. Activity was determined measuring substrate conversion during the first 30 min of incubation and is defined as µmol of alcohol oxidized per minute. Specific activity was calculated dividing by the amount of protein (Bradford) in the crude extract. Activity values of crude extracts are presented in table 3.3.

Table 3.3 - Specific TADH activity values of RMS5 and DG11 mutants' crude extracts with 1-alkanols.

Mutant	Protein Concentration [mg/mL]	Crude Extract Volume [mL]	Specific TADH Activity [U/mg]	
			1-octanol	1-hexadecanol
RMS5	3.80	0.4	0.0135	0.0105
DG11	3.36	0.4	0.0013	0.0093

Reaction mix at each incubation time contained 400 µL of crude extract, 2 µmol of the substrate and 100 µL NAD 50 mM (5 µmol). After incubation samples were extracted with 500 µL MTBE; solvent extract was derivatized by silylation with BSA and analyzed by GC-MS.

Results showed a significantly lower specific alcohol dehydrogenase activity with 1-octanol for the mutant DG11 when compared to mutant RMS5. Specific activities with 1-hexadecanol were found comparable. This suggests that, if AlkDH was disrupted in mutant DG11, TADH is not the only dehydrogenase involved in the long-chain n-alkanes metabolism in *Thermus sp.* ATN1. Activity of recombinant TADH has been previously reported with alkanols up to 10 carbon atoms (Höllrigl *et al.,* 2008). TADH activity from crude extract of *Thermus sp.* ATN1 cultures was reported to be low with 1-tetradecanol and was not detected with 1-hexadecanol (Otto, 2001). These results indicate that TADH is most probably not the enzyme involved in dehydrogenation of linear alcohols longer than 14 carbon atoms.

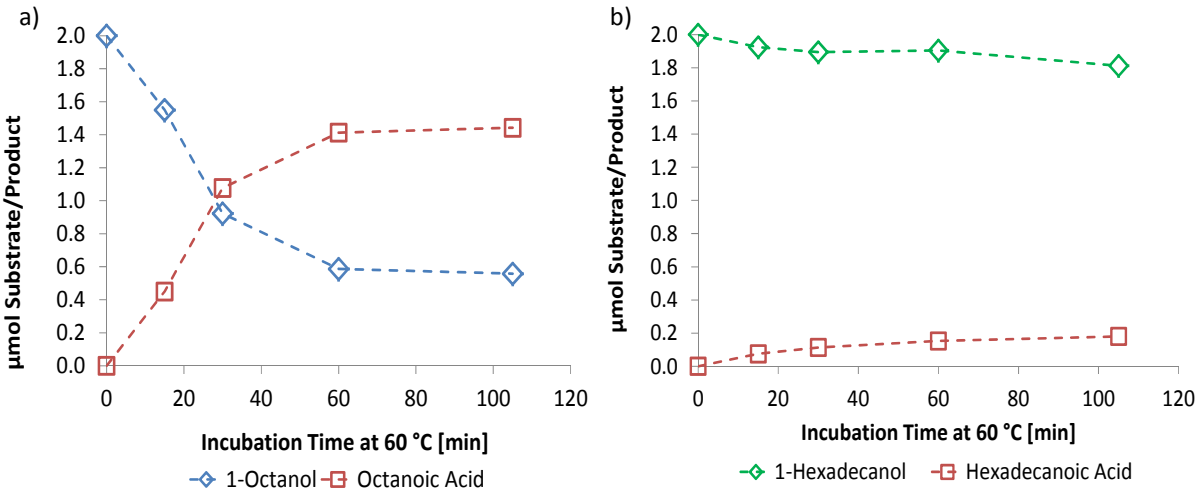


Figure 3.16 - Recombinant TADH activity with 1-octanol and 1-hexadecanol. TADH concentration was adjusted at 10 µg/mL in 20 mM potassium phosphate buffer, reaction mix contained 400 µL TADH solution, 2 µmol substrate and 100 µL NAD 50 mM (5 µmol). After incubation samples were extracted with 500 µL MTBE; MTBE extract was derivatized by silylation with BSA and analyzed by GC-MS.

To corroborate TADH activity characteristics, the purified recombinant enzyme (Höllrigl *et al.,* 2008) was also studied. 1 mg/mL TADH stock solution was kindly provided by Dr. Katja Bühler (Laboratory of Chemical Biotechnology, Department of Biochemical and Chemical Engineering,

TU Dortmund). Activity results obtained under conditions comparable to those for crude extract activity experiments are presented in figure 3.16.

Specific activity (U/mg) of TADH in this test was 8.97 U/mg with 1-octanol being converted to octanoic acid and only 0.87 U/mg with 1-hexadecanol.

Calculated specific activity with 1-octanol was higher than previously reported by Otto but comparable to that reported by Höllrigl in 2008. TADH activity was found considerably lower with 1-hexadecanol.

3.4.6 Aldehyde detection with pararosaniline on mineral medium plates

Thermus sp. ATN1 random mutants (RMS5) were suspected to produce aldehyde intermediates from n-alkane conversion (see 3.5.1.1). It was assumed that blocking AlkDH in the RMS5 strain would result in the production of alcohol intermediates (figure 3.17 b). For screening purposes, gene disrupted mutants were plated on mineral medium supplemented with sodium pyruvate and 1-hexadecanol. After 48 h incubation, pararosaniline solution was added and plates were further incubated (Conway *et al.,* 1987; see 2.4.2.6.1). Colonies appearing red would be producing aldehydes. The test did not discriminate between AlkDH blocked and unblocked mutants. All colonies appeared red (figure 3.17 a) possibly due to high incubation temperatures causing false positives or reaction of pararosaniline with other metabolites.

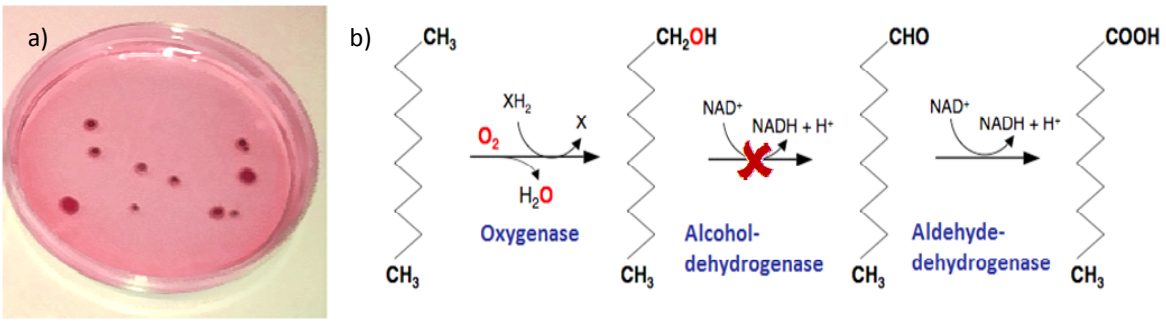


Figure 3.17 - (a) Mutants grown and incubated with pararosaniline; (b) Alkane metabolism with disrupted alcohol dehydrogenase would not produce aldehyde or carboxylic acid metabolites. Gene disrupted mutants were plated on mineral medium containing 20 mm sodium pyruvate and 5 mM equivalent 1-hexadecanol, incubation (staining) with pararosaniline followed after a 72 h incubation period.

3.5 Screening for long-chain n-alkanes bioconversion products

3.5.1 Random mutants

3.5.1.1 Experiments in sealed serum vials (limited oxygen)

Selected random mutants were able to grow on co-substrates whilst n-hexadecane concentration was also decreasing. During the screening phase in sealed serum vials it was possible to monitor alkane consumption by GC-MS analysis. Additionally, after sample derivatization (silylation), it was also possible to observe development of other peaks in the GC-MS chromatograms.

For semi-quantitative analysis a reference substance was added in the same concentration in all samples and peak signals could be normalized based on peak intensity of this internal standard. Thus increase or decrease in alkane and other substances concentration could be monitored. Except for the peak intensity corresponding to the alkane, other peaks showed no clear correlation to incubation time. However co-substrate and its concentration showed some influence on peak developments. Generally speaking, lower molecular weight co-substrates and lower concentrations resulted in these other peaks appearing earlier during the incubation. Figure 3.18 shows normalized signals for n-hexadecane conversion during cultivation of mutant strain RMS17 growing on 10 mM sodium citrate.

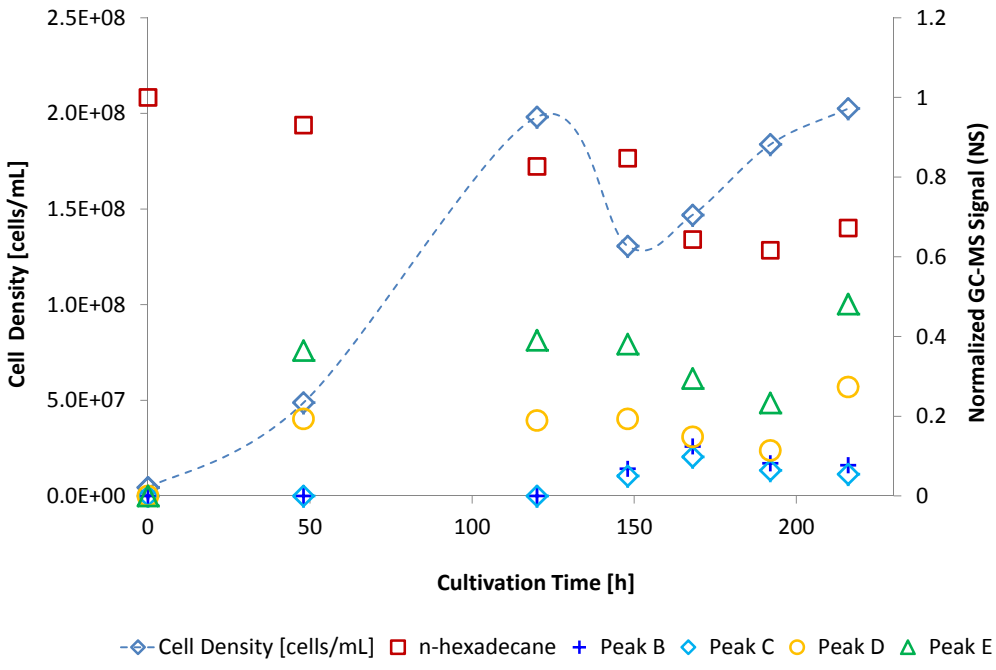
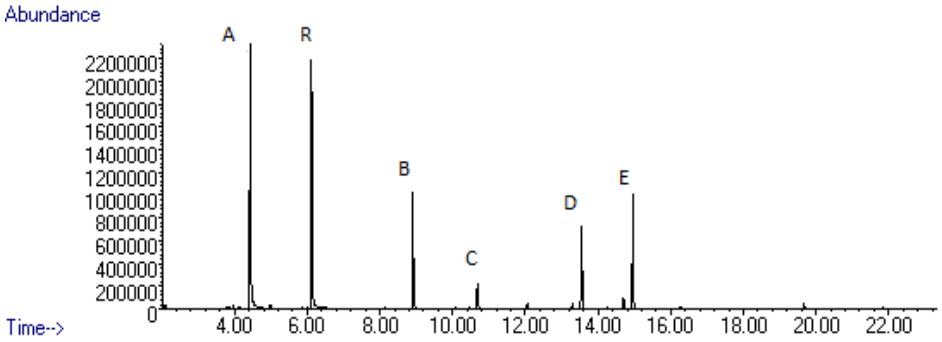


Figure 3.18 - Growth of random mutant strain RMS17 in mineral medium supplemented with 10 mM sodium citrate and 1.13 g/L n-hexadecane. Experiments were carried out in sealed serum vials.



Peak	Retention Time [min]	Substance
A	4.44	n-Octadecane
B	8.91	Hexadecanoic acid, trimethylsilyl ester
C	10.68	Octadecanoic acid, trimehtylsilyl ester
D	13.55	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
E	14.97	Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
R	6.13	1,10 – Dichlorododecane

Figure 3.19 - Peaks detected from the silylated sample extracted from a sealed vial at 168 h incubation during cultivation of mutant strain RMS17 growing on 10 mM sodium citrate and n-hexadecane. 1,10-dichlorodecane was used as internal standard (peak R) for n-hexadecane (peak A) bioconversion screening. Peak identities were determined by library match and later confirmed vs. reference substances.

Peaks for mono or di-alcohols were not detected and it was assumed these metabolites were not accumulated under the cultivation conditions. Neither peak retention times nor mass spectra were comparable to reference substances like 1-hexadecanol or 1,16-hexadecanediol.

Some organic compounds with active hydrogen atoms form additional unexpected derivatives or by-products (artifacts) by the derivatization process used to analyze the samples (as those proposed by Little, 2003). Because of this, it was theorized that silylation artifacts of aldehyde functionalized aliphatic chains or artifacts of fatty acids could have been formed. Although screening procedures and techniques to detect aldehydes gave positive qualitative results (these are discussed in section 3.5.3) no one of the peaks appearing in these experiments could be confirmed as an aldehyde functionalized compound.

A GC-MS chromatogram corresponding to the 168 h sample extracted from a sealed vial from experiment presented in figure 3.18 is shown in figure 3.19. Peak identities were matched to the NITS mass spectral library (2008) and later corroborated by comparison against solutions of reference compounds analyzed by GC-MS.

3.5.1.2 Experiments in screw cap shaking flaks (limited oxygen)

Mutant strain RMS5 showed the highest n-alkane depletion in sealed serum vials. This strain was cultured in shaking flask experiments in order to produce enough cells to conduct resting cells

and crude extract experiments to determine possible n-alkane conversion metabolites. During this phase it was observed that floating solids were formed while the strain grew in mineral medium with n-hexadecane and sodium pyruvate as carbon source. This material was well dispersed in the whole culture volume when flasks were shaken.

The floating solids together with part of the unconsumed alkane were separated from the broth by filtration in the cold (T<4°C). This floating matter was observed in cultures with all long-chain n-alkanes tested and not present when the mutant strain was grown only in sodium pyruvate or other carbon sources tested. Figure 3.20 shows cultures of one of these experiments with three alkanes and a retentate separated from one of these cultures.

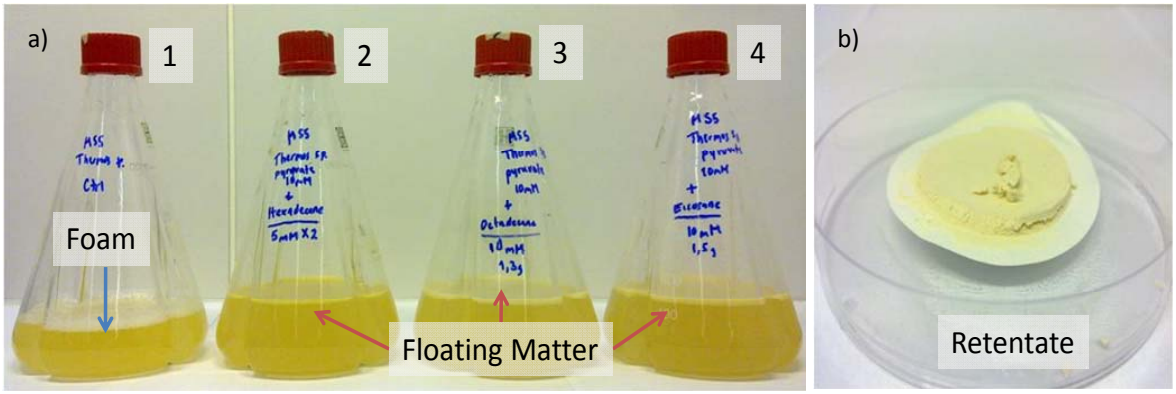


Figure 3.20 - (a) Floating matter observed in cultures of mutant strain RMS5 growing in mineral medium supplemented with sodium pyruvate and long-chain n-alkanes; (1) control culture with sodium pyruvate only (fed-batch, adjusted to around 8 mM each 12h until 48 h), (2) 2,26 g/L n-hexadecane, (3) 2,6 g/L n-octadecane, (4) 3,1 g/L n-eicosane. (b) Retentate obtained after filtration in cold (culture 4). Experiments were conducted in 1 L screw cap shaking flaks (62 hours).

As previously presented in section 3.3, cell densities during the experiment shown in figure 3.20 were very similar or even lower with alkane compared to the control culture without it during sodium pyruvate fed-batch cultivation (Figure 3.21). It was assumed that n-alkanes were not consumed but converted and that conversion products were part in the floating solids. Samples (5 mL) from cultures in screw caps shaking flasks (limited oxygen) were taken immediately after vigorous shaking. Decreasing alkane concentration could be determined from solvent extracts of samples from these cultures taken at indicated incubation times (Figure 3.22).

The floating matter (or part of it) remained insoluble during solvent extraction of the samples. Solvent extracts of these samples were analyzed by GC-MS after derivatization. GC-chromatograms and mass spectra showed a profile very similar to the one observed during sealed serum vials experiments (Figure 3.24 a). Characterization results of the floating solids are presented in section 3.6.

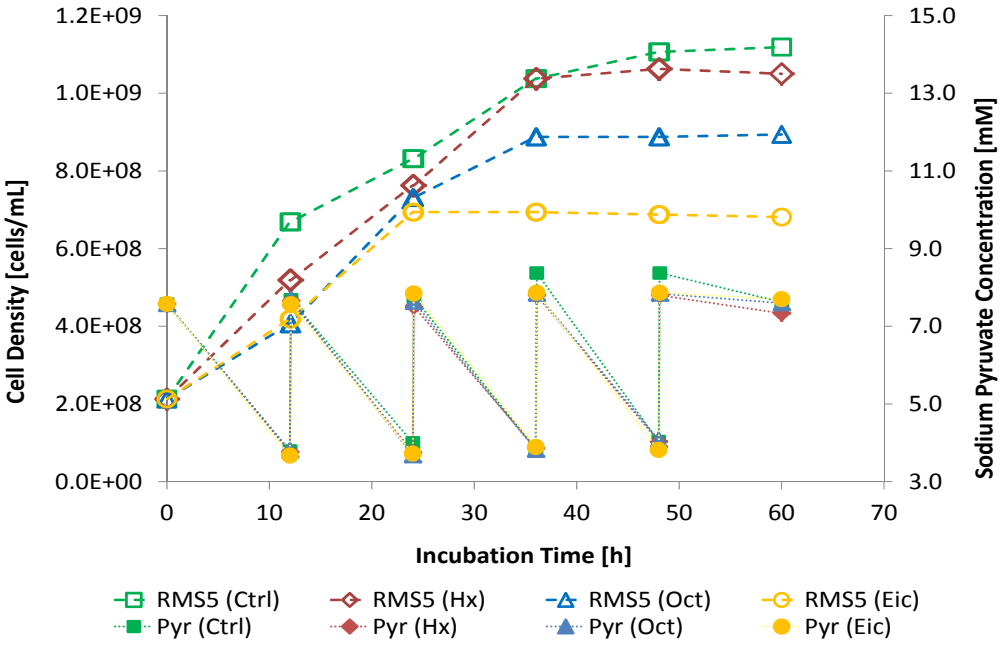


Figure 3.21 - Growth of random mutant strain RMS5 in mineral medium supplemented with 8 mM sodium pyruvate (fed-batch, adjusted to 8 mM each 12h until 48 h) and with 5 mM n-hexadecane (Hx), n-octadecane (Oct), n-eicosane (Eic) and without alkane (Ctrl). Experiments were performed in 1 L screw cap shaking flasks (limited oxygen) during 62 h. Sodium pyruvate concentration was determined enzymatically (see 2.7.2).

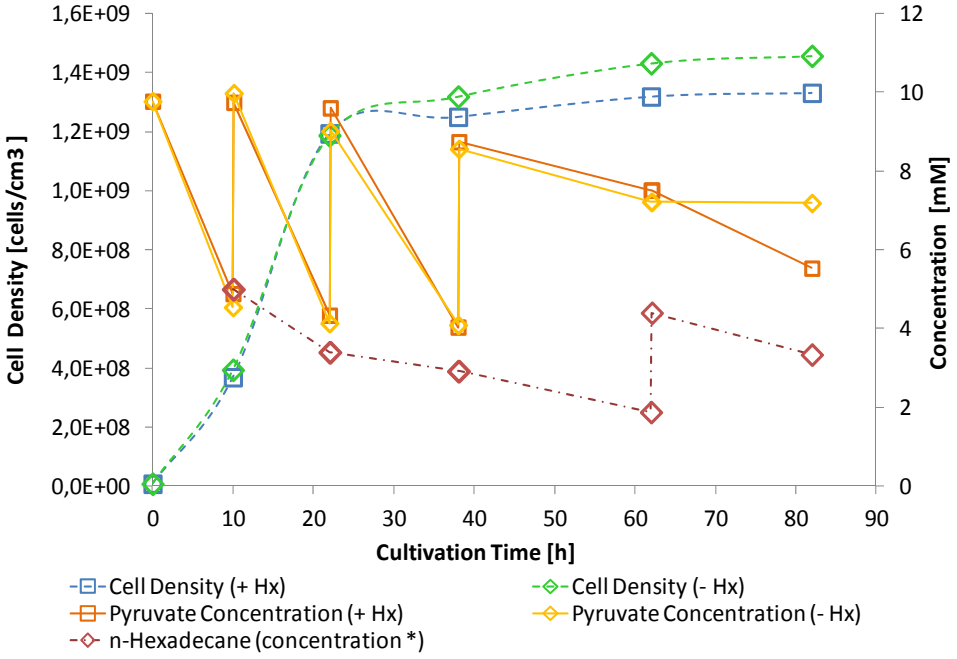


Figure 3.22 - Growth of random mutant strain RMS5 in mineral medium supplemented with 10 mM sodium pyruvate (fed-batch, adjusted to 10 mM each 12h until ~36 h) with (+Hx) and without (-Hx) n-hexadecane (1.13 g/L, 5 mM). Alkane concentration determined from solvent extracts of 5 mL samples taken immediately after vigorous shaking of flasks; n-hexadecane was added at times 10 h and again at 62 h of incubation. Experiments were conducted in 1 L screw cap shaking flasks.

3.5.1.3 Experiments in loose cap shaking flasks

Shaking flasks with loose caps were used for larger culture volumes (1 L). As with the screw cap flasks, objective was to produce larger cell quantities for resting cells and crude extract experiments but especially larger quantities of the floating solids for characterization purposes. Similar to previous shaking flask experiments a fed-batch approach for the co-substrate was applied.

In these experiments, the solids previously observed were also present but as the incubation progressed (generally after 48 h), a complete separation from the liquid phase was no longer visible minutes after stopping agitation. Furthermore, small floating beads appeared after 24-36 h incubation which were clearly separated from the liquid phase or the previously observed floating solids. Figure 3.23 shows a loose cap shaking flask culture (a) and a bead separated from the culture broth (b).

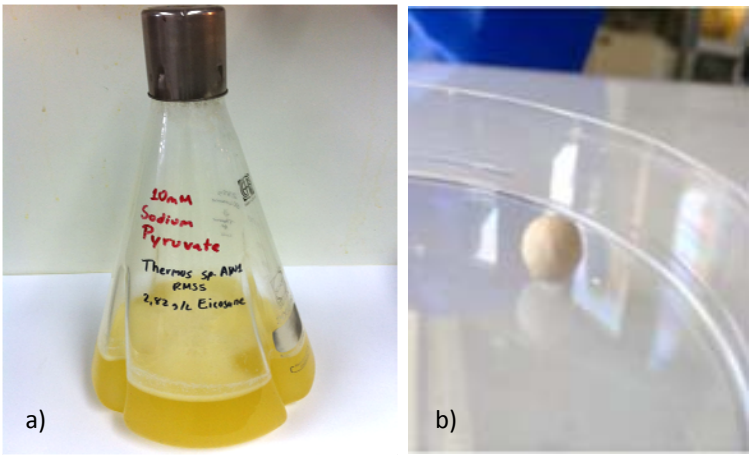
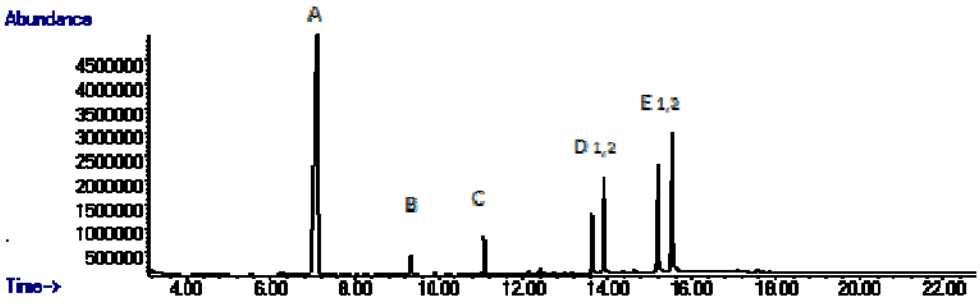


Figure 3.23 - (a) Culture of mutant strain RMS5 growing in mineral medium supplemented with sodium pyruvate and n-eicosane at 12 h of incubation. (b) A bead separated from this culture after 60 h incubation (observed in co-substrate fed-batch experiments only). Experiment was conducted in a 2 L loose cap shaking flask during 60 hours.

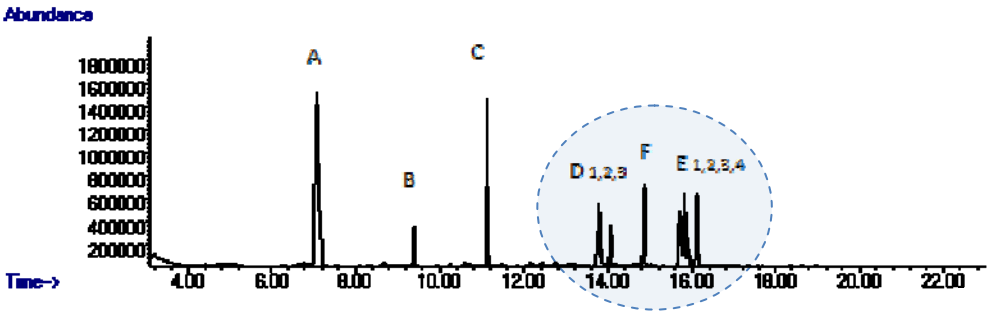
Samples taken during incubation were also solvent extracted and analyzed by GC-MS. A similar peak profile to those previously observed was found in the GC-MS chromatograms at early stages of the experiment (generally until 24 h). However, as the experiment progressed and the described beads appeared, chromatograms showed additional peaks. As in the case of sealed serum vials or screw caps shaking flasks, no correlation was found between peak intensities and incubation times, except for the decreasing alkane concentration.

Figure 3.24 (b) shows a chromatogram observed for the conversion of n-octadecane in a loose cap shaking flask after 48 h of incubation and it is compared to the chromatogram observed in a screw cap shaking flask after about 60 h of incubation (Figure 3.24 a).

a)



b)



c)

Peak	Retention Time [min]	Substance
A	7.09	n-Octadecane
B	9.38	Hexadecanoic acid, trimethylsilyl ester
C	11.13	Octadecanoic acid, trimethylsilyl ester
D1	13.70	2-Monopalmitin, trimethylsilyl ester
D2	14.04	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
F	14.80	Octadecanedioic acid, bis(trimethylsilyl) ester
E1	15.50	2-Monostearin trimethylsilyl ether
E2	15.90	Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester

Figure 3.24 - Peaks detected from the silylated solvent extracts from samples taken during cultivation of mutant strain RMS5 growing in mineral medium supplemented with 10 mM sodium pyruvate and n-octadecane: (a) After 60 h incubation in 1 L screw cap shaking flask; (b) after 48 h incubation in 2 L loose cap shaking flask; (c) peak identities determined by library match and later confirmed with reference substances. Peaks D3, E3 and E4 could not be identified.

Peak F in figure 3.24 (b) corresponded to 1,18-octadecanedioic acid, a bioconversion product of n-octadecane. This showed the ability of this mutant to produce α,ω-oxidized molecules from long-chain n-alkane. Further studies were performed with the strain to evaluate the bioconversion influencing factors and potential for the production of long-chain di-carboxylic acids. This is discussed in section 3.7.

Higher cell densities were observed in loose cap shaking flaks. In figure 3.25(a) growth curves of the wild-type strain, mutant RMS5 and a comparison to growth of this mutant in a screw cap flask are presented.

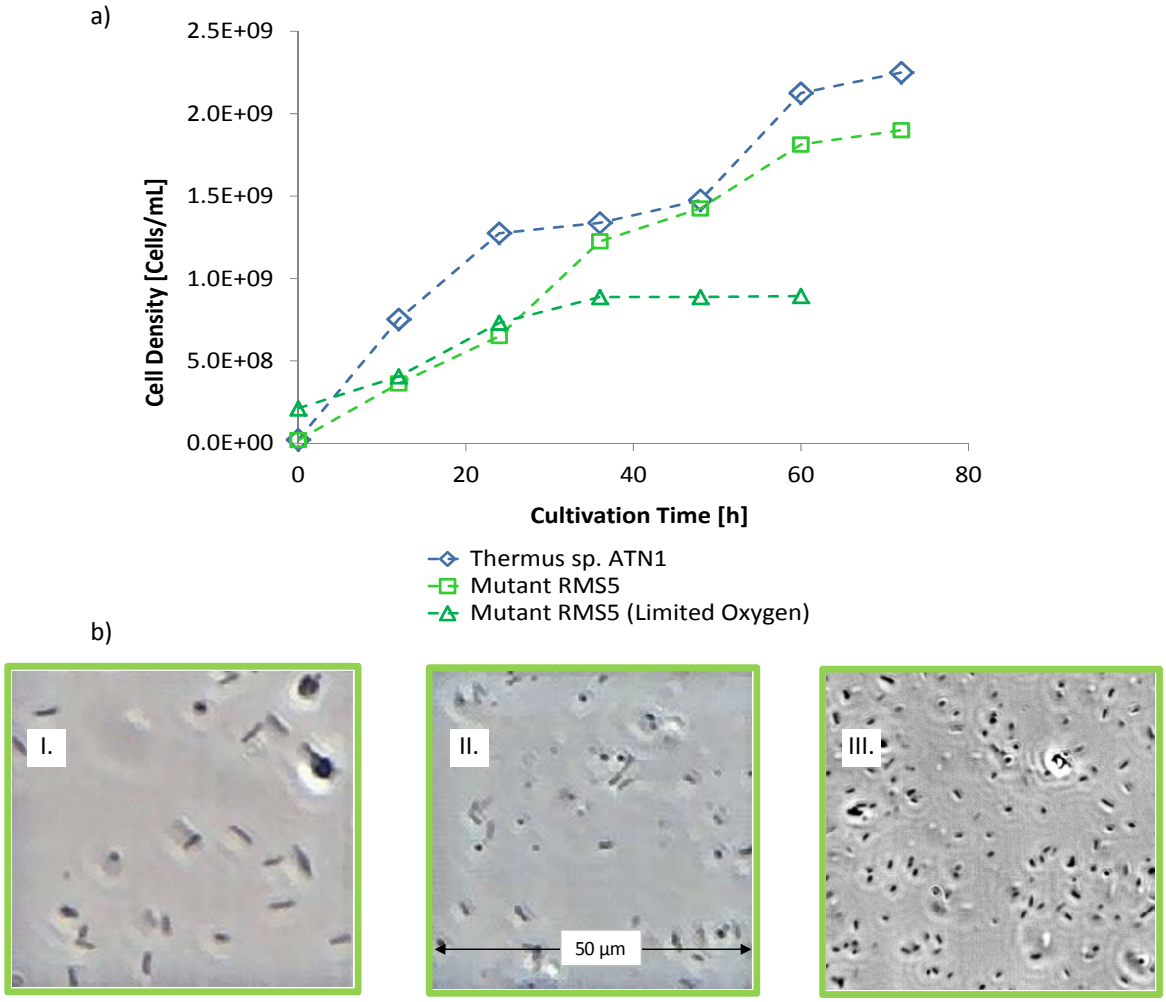


Figure 3.25 - (a) Growth of *Thermus sp.* ATN1 wild-type strain and mutant RMS5 in mineral medium supplemented with 10 mM sodium pyruvate (fed-batch, adjusted to 10 mM each 12 h until 36 h) and 1.27 g/L (5mM) n-octadecane. Experiments were conducted in 2 L loose cap shaking flasks during 72 hours. Growth curve of mutant RMS5 growing on sodium pyruvate and n-octadecane in screw cap shaking flask (from figure 3.21) is also shown for comparison. (b) Cell morphology observed during growth of the wild-type strain in the different growth phases: (I.) Rods - first exponential phase; (II.) Small coccobacillus – plateau; (III.) Coccobacillus – second exponential phase.

The growth curve of the wild-type strain in figure 3.25 (a), as in the case of sealed serum vials experiments, presented 2 defined exponential phases. Generation times were calculated to be 2.3 h for the first 12 h growth phase and about 23 h for the second phase observed after 48 h of incubation. The mutant strain RMS5 reached cell densities comparable to that of the wild-type strain after 36 h of incubation but the growth curve showed a flatter and longer exponential phase with a generation time calculated to about 9 h, 33% shorter than the generation time calculated for this mutant (13.5 h) when cultured in screw cap shaking flasks (limited oxygen) under comparable conditions, where the RMS5 mutant reached only half of the cell density observed with loose cap shaking flasks. It was assumed that increased oxygen availability enabled by improved gas exchange (because of the loose caps) was responsible for these changes.

Cell morphology development for the mutant RMS5 during bioconversion experiments was similar to that shown by the wild-type strain, except that for the mutant strain the change in morphology did not occur in two defined exponential phases and small coccobacillus were observed as early as 12 h after incubation (Figure 3.25 b). The wild-type strain and its mutants did not show this morphology change when cultured in complex media. Interestingly mutant RMS5 showed some filamentation in complex media, which might be an indication of unregulated expression of the alkane hydroxylase complex. A similar phenomenon was observed with the physiology of *P. putida* Gpo1 and *P. putida* GPo12 *alk* recombinants after induction of expression of the *alk*BFGHJKL genes on glucose as a carbon source (Chen *et al.*, 1996). It has been suggested that overproduction of the *alk*B hydroxylase, which is a membrane protein, may be responsible for the observed phenotype (Wentzel *et al.*, 2007).

3.5.2 Mutants obtained by gene disruption

AlkDH gene disrupted mutants incubated in loose cap shaking flasks reached lower cell densities compared to those of the wild-type strain and mutant RMS5 under similar cultivation conditions. This was not the case when screw cap shaking flasks were used during co-substrate fed-batch cultivation in the presence of n-alkanes. Figure 3.26 presents both cases for mutant DG11. The first case (a) can be explained by the mutation(s) that limit n-alkane metabolism in the mutant strain(s) and the favorable conditions for the wild-type strain to quickly consume the co-substrate followed by further growth on the alkane. The second case (b) results from a limited growth under restricted aeration which maintained co-substrate concentration sufficiently high to delay alkane metabolism in the wild-type strain whereas unregulated alkane metabolism continues in the mutant despite limited oxygen and the blocked AlkDH. Although cell densities were higher, observed wet cell weights were lower compared to the wild-type strain cultures.

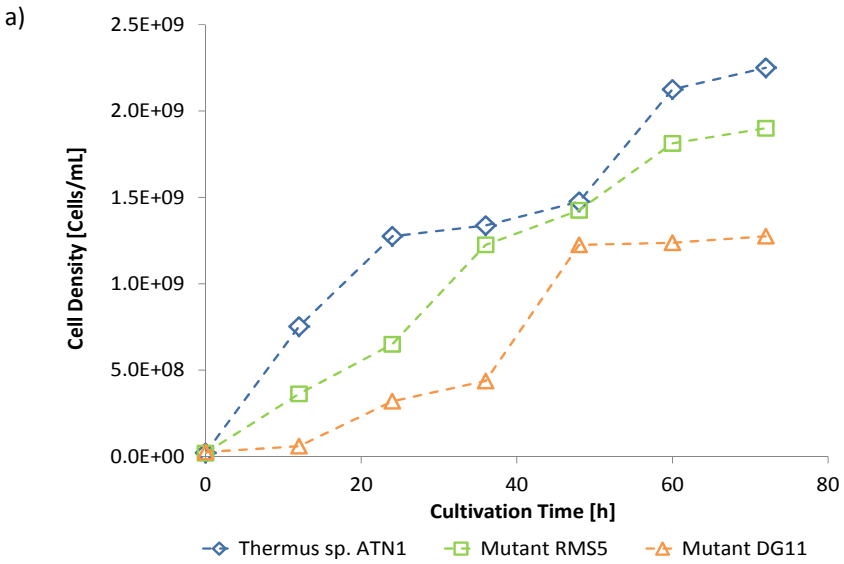


Figure 3.26 - (a) Growth of *Thermus sp.* ATN1 wild-type strain, mutants RMS5 and mutant DG11 in mineral medium supplemented with 10 mM sodium pyruvate (fed-batch, adjusted to 10 mM each 12 h until 36 h) and 1.27 g/L (5mM) n-octadecane. Experiments were conducted in 2 L loose cap shaking flasks for 72 hours.

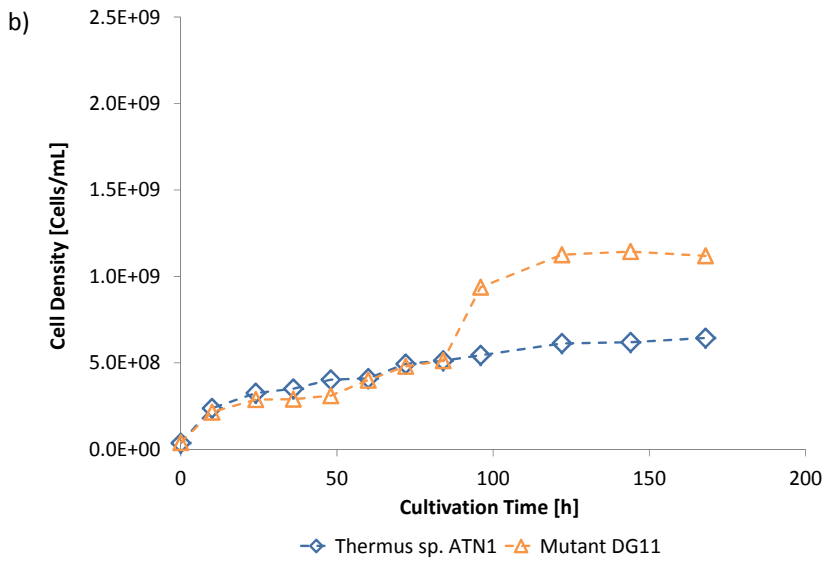


Figure 3.26 - (b) Growth of *Thermus sp.* ATN1 wild-type strain and mutant DG11 in mineral medium supplemented with 10 mM sodium pyruvate (fed-batch, adjusted to 10 mM each 12 h until 48 h) and 1.41 g/L n-eicosane. Experiments were conducted in 1 L screw cap shaking flasks for 168 hours.

The growth behavior of DG mutants, in the presence of n-alkanes, implies that TADH might not be the only enzyme capable to oxidize alkanols in this strain (see 3.4.5).

Cultures of mutants obtained by gene disruption showed also formation of beads (figure 3.23) and some material floating in the culture in physical appearance similar to the one found with random mutants under similar cultivation conditions. However, this material was found different in composition when compared to the composition observed with random mutants. Characterization results are shown in section 3.6. Samples from cultures of these mutants were also analyzed by GC-MS. A chromatogram of a sample taken at 60 h from a culture of mutant DG11 (growth curve in figure 3.26 - a) is presented in figure 3.27. Peaks similar to those for random mutants in limited oxygen cultures were found. Peak intensities for alkanes and glycerol mono esters appeared much higher compared to random mutant cultures and in general significantly lower alkane consumption levels were observed.

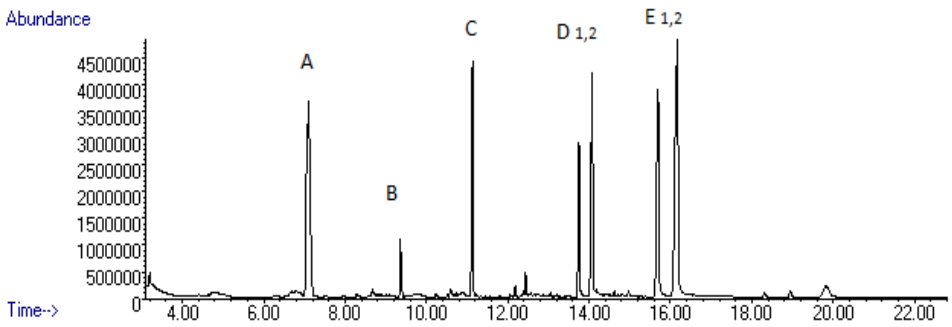


Figure 3.27 - Peaks detected from the silylated solvent extracts from samples taken during cultivation of gene disrupted mutant strain DG11 growing on 10 mM sodium pyruvate and n-

octadecane supplemented mineral medium (1 L) after 60 h incubation in 2 L loose cap shaking flask. Peak identities are the same as shown in figure 3.24.

3.5.3 Aldehyde detection in crude extract and resting cell experiments

These experiments were performed to elucidate bioconversion products from n-alkanes by random mutants without the possible influence of metabolites interfering during normal cell growth. It was expected to find aldehyde intermediates, since a qualitative test performed with the Purpald® reagent (see 2.6.1) on samples taken during RMS5 cultivation gave positive for colorimetric aldehyde detection.

A 20 % weight wet cell suspension from mutant RMS5 was used directly (resting cells) or cells were disrupted by sonication and centrifugation to prepare crude extract (see 2.4.1.). Substrate consumption and possible product formation were monitored at different incubation times. C14 and C16 n-alkanols were used for testing.

Decreasing alcohol concentration was monitored by GC-MS analysis of solvent extracts from crude extract incubation experiments (figure 3.28 a). Neither aldehyde metabolites nor carboxylic acid formation was observed in the corresponding GC-MS chromatograms.

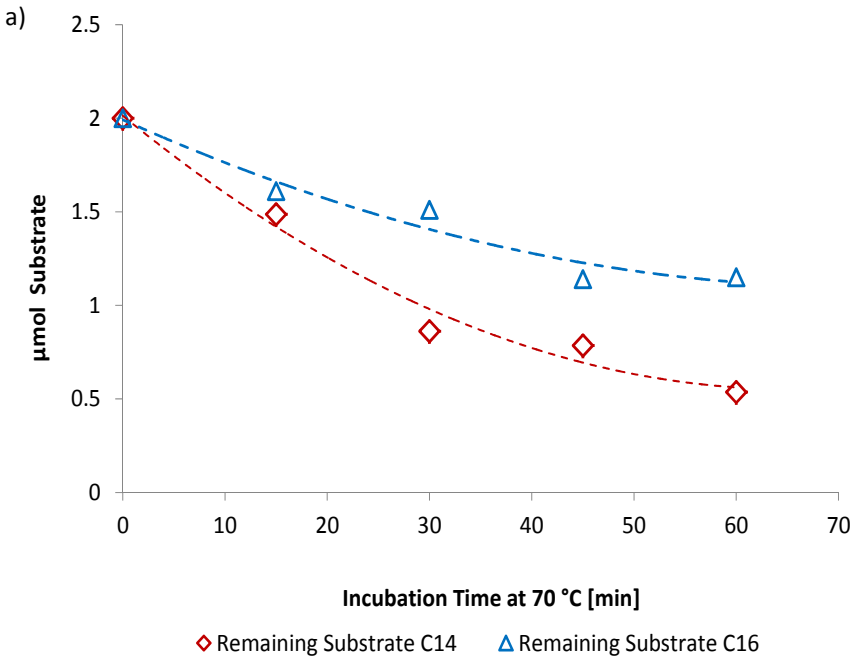


Figure 3.28 - (a) ADH activity with C14 and C16 alkanols in crude extract prepared from RMS5 mutant cultures. Alkanol consumption was determined by GC-MS analysis of solvent extracts (1:1) from crude extract experiments.

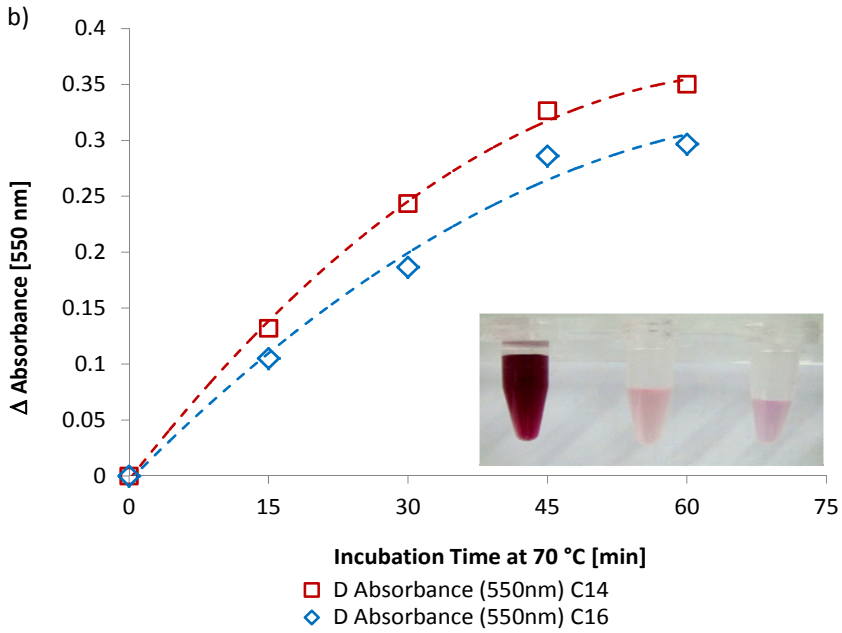


Figure 3.28 - (b) Absorbance at 550 nm after Purpald® reaction in resting cell suspensions incubated with alkanols. The Purpald® reaction was prepared from supernatant obtained of centrifuged resting cell suspensions after incubation. Experiments were carried out with 400 µL crude extract/resting cell suspension and 100 µL of NAD 50 mM in 2 mL Eppendorf tubes containing 2 µmol of alkanol. Samples were incubated at 70 °C and 500 rpm in a thermo mixer.

Increasing absorbance at 550 nm was observed in the resting cell preparations with alkanols treated with the Purpald® reagent (figure 3.28 b). This indicated the presence of aldehydes, but they were not detected in sample solvent extracts analyzed by GC-MS. This could have been caused by inadequate sample derivatization for the employed GC-MS method.

3.6 Characterization of alkane bioconversion products (strain RMS5)

Several peaks were identified in GC-MS chromatograms that represent interesting alkane bioconversion products or metabolites by *Thermus sp.* ATN1 mutants. Mono and dicarboxylic acids as well as glycerol mono esters of fatty acids were detected free and/or bound to floating matter produced by cultures of mutant strain RMS5. Most interesting are α,ω-long-chain dicarboxylic acids since they fit with the objectives of this work and from these type of compounds carbon chains larger than 16 carbon atoms are not commercially available.

3.6.1 Free mono- carboxylic acids and glycerol esters

Solvent extracts from samples taken in all experiments in mineral media with n-alkanes showed peaks for free fatty acids and glycerol mono esters (as described in figure 3.19). These peaks

were not observed when the strains (wild-type and mutants) were growing in the absence of n-alkanes.

Free fatty acids detected corresponded to the alkanes used in the experiments. For instance, eicosanoic acid was detected in the samples when eicosane was used. However this was not the case for hexadecanoic and octadecanoic acid, these were present in all samples; although higher peak intensities were observed for these acids when either n-hexadecane (see figure 3.19) or n-octadecane were used (see figures 3.24 b and 3.27).

Glycerol mono esters of hexadecanoic and octadecanoic acids were found in all samples (with higher peak intensities for the octanoic acid mono esters of glycerol). It was demonstrated that these glycerol mono esters (and therefore some hexadecanoic and octadecanoic acid that could be trans-esterified during sample derivatization) are part of a surfactant-type substance that *Thermus sp.* ATN1 (and its mutants) produces when growing in the presence of long-chain n-alkanes. This biosurfactant was observed as an oily layer spread on the wall of incubation flasks or as a milky halo around colonies on mineral media plates. Section 3.7 describes observations and characterization results of this biosurfactant.

3.6.2 Floating solids composition (Retentate composition)

The floating solids observed in the experiments conducted in loose cap shaking flasks were isolated by filtration together with not converted alkane. Alkanes were separated by solvent extraction/washing. Remaining solids were hydrolyzed and solvent extracts of acidic solutions were derivatized (as trimethyl silyl or methyl esters) and analyzed by GC-MS. Mono and dicarboxylic acids were identified as part of these solids (see figure 3.30). Composition of the solids was carbon chain length dependent as shown in figure 3.29.

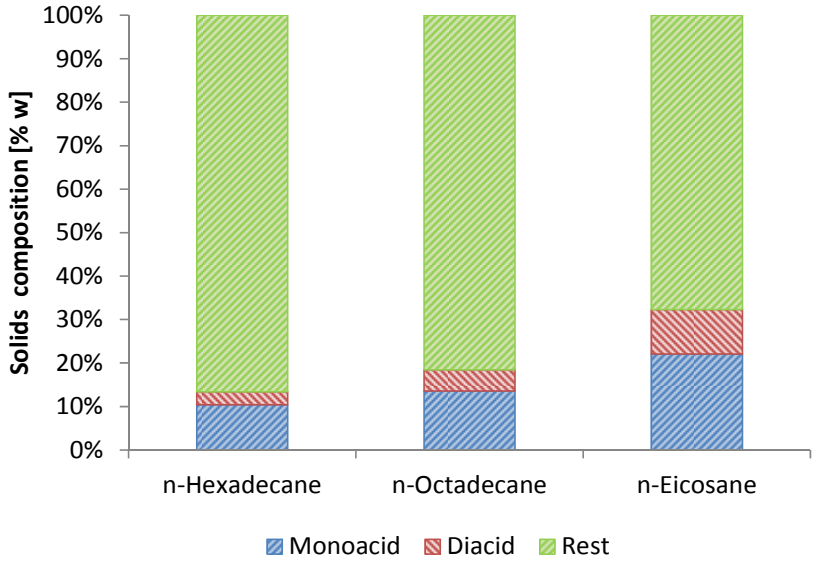


Figure 3.29 - Composition in % weight of mono and dicarboxylic acids in the floating matter isolated from loose cap shaking flask cultures of mutant RMS5 growing on mineral media with sodium pyruvate and n-alkanes.

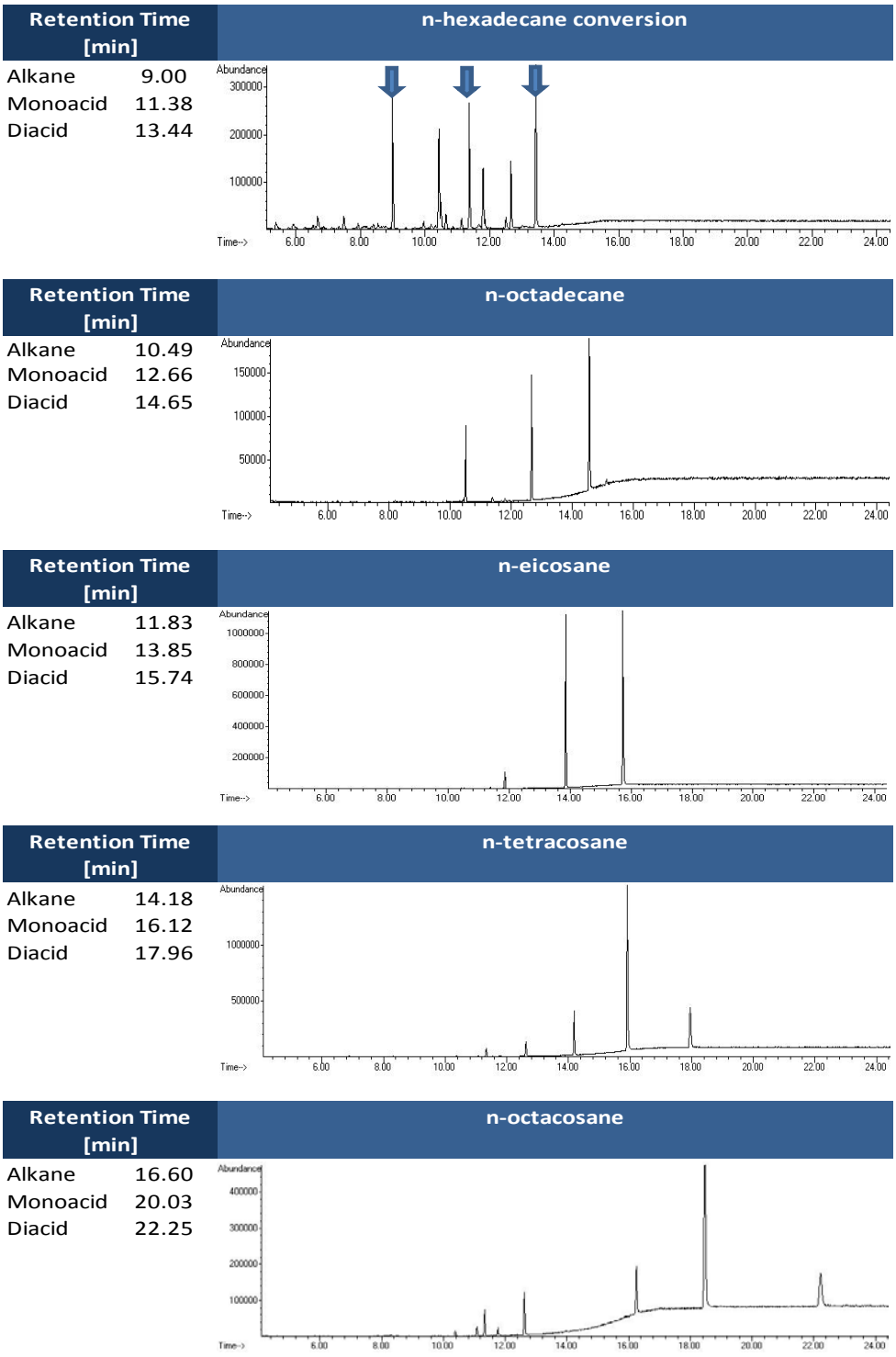


Figure 3.30 - Chromatograms of solvent extracts containing methyl esters of mono and dicarboxylic acids obtained from treatment of solids isolated from RMS5 mutant cultures growing on sodium pyruvate and different long-chain n-alkanes. Samples were prepared from few milligrams of solids (5-10) treated with 2% v/v methanolic sulphuric acid. Solids were re-suspended in 800 µl of the derivatizing reagent and incubated for 1 h at 80 °C. After ice cooling samples were extracted with 800 µl of n-heptane. 250 µl of the upper phase were transferred to micro-vials for GC-MS analysis. Mass spectra for mono and dicarboxylic methyl esters are shown in figure 3.32 (a) and (b) respectively.

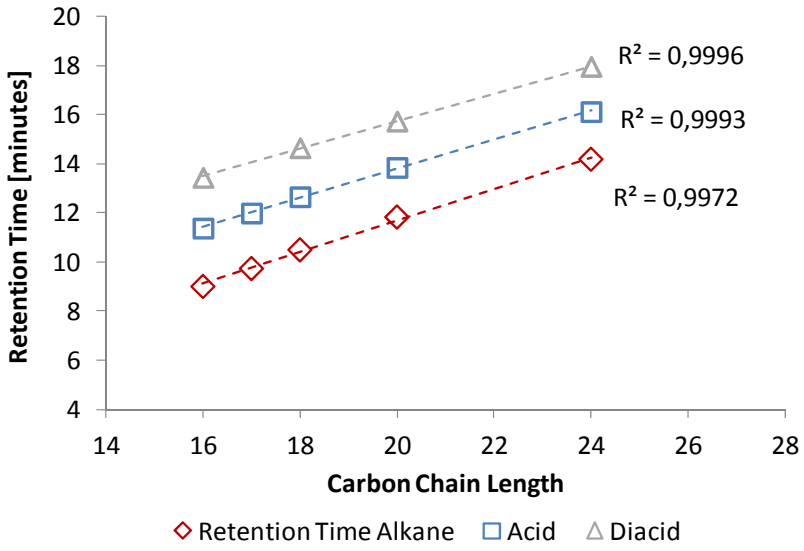


Figure 3.31 - Correlation of n-alkane, mono and dicarboxylic acids carbon chain length with retention times during GC-MS analysis.

Linearity in the correlation between carbon chain length and retention times (figure 3.31) as well as similar m/z patterns (figure 3.32) of observed peaks confirmed product identity for those substances whose reference was not available either physically at the laboratory or in the NIST mass spectra library.

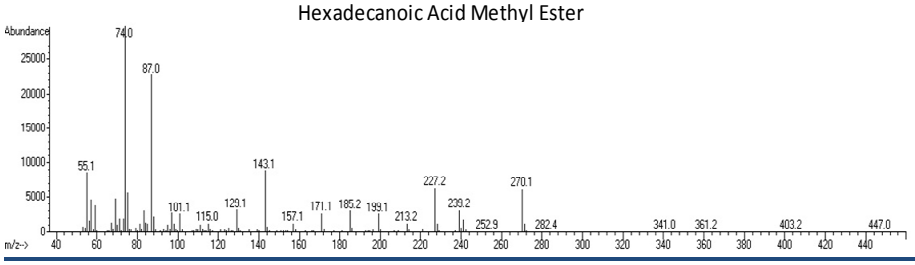
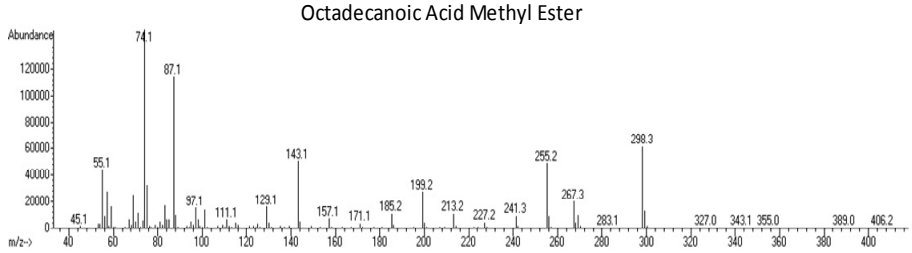
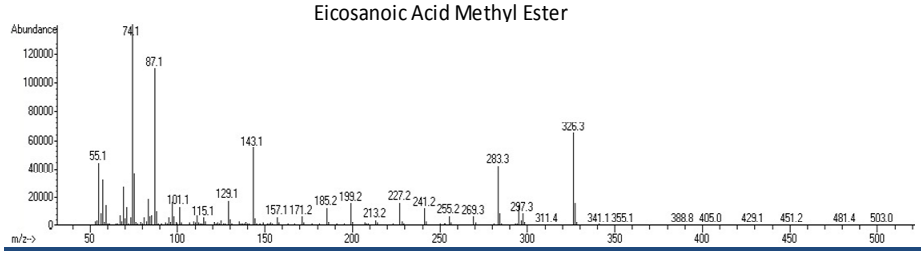
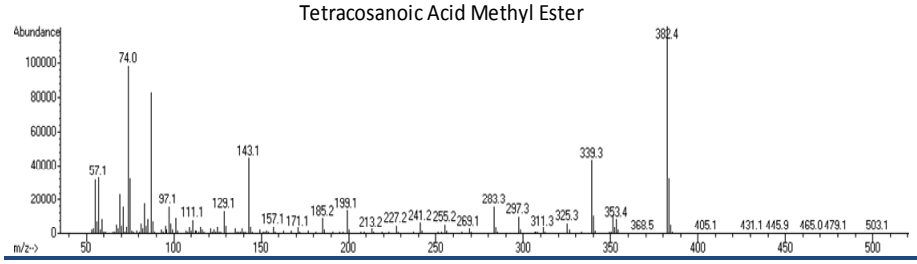
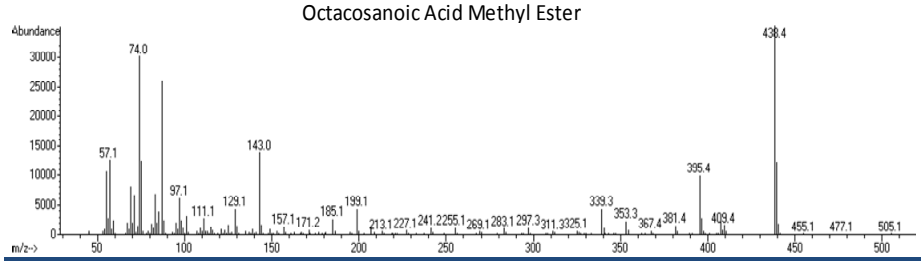
Product	m/z	Relative Abundance
Hexadecanoic Acid Methyl Ester 	55.0	27.1%
	74.1	100.0%
	87.1	79.3%
	143.1	34.9%
	227.2	36.4%
	270.2	37.5%
Octadecanoic Acid Methyl Ester 	55.1	29.3%
	74.1	100.0%
	87.1	75.1%
	143.1	30.1%
	255.2	28.9%
	298.3	37.2%
Eicosanoic Acid Methyl Ester 	55.1	30.9%
	74.1	100.0%
	87.1	78.1%
	143.1	38.8%
	283.3	29.6%
	326.3	45.8%
Tetracosanoic Acid Methyl Ester 	55.1	23.7%
	74.1	74.3%
	87.1	60.0%
	143.1	34.3%
	339.3	35.2%
	382.4	100.0%
Octacosanoic Acid Methyl Ester 	55.1	20.7%
	74.1	60.1%
	87.0	52.5%
	143.1	30.0%
	395.4	27.2%
	438.5	100.0%

Figure 3.32 - (a) Mass spectra for mono carboxylic methyl esters obtained from treatment of solids isolated from RMS5 mutant cultures growing on sodium pyruvate and different long-chain n-alkanes.

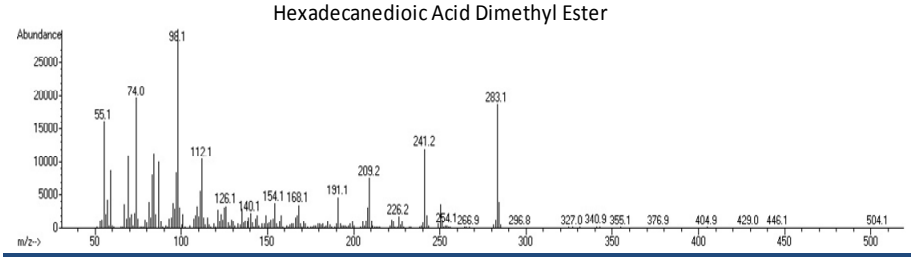
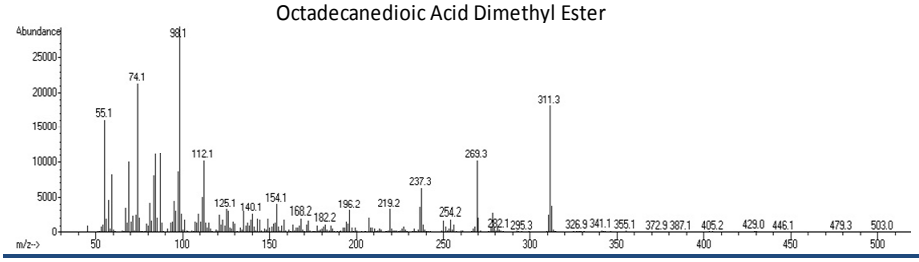
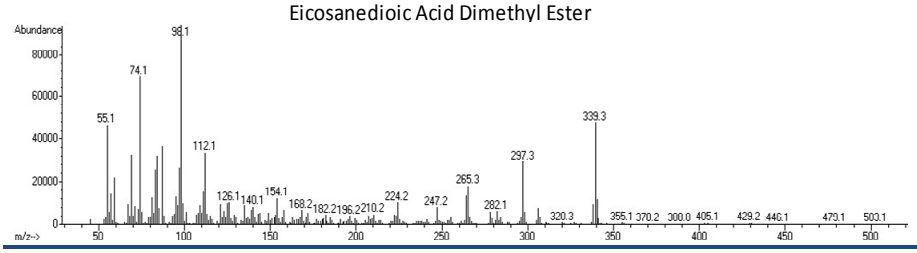
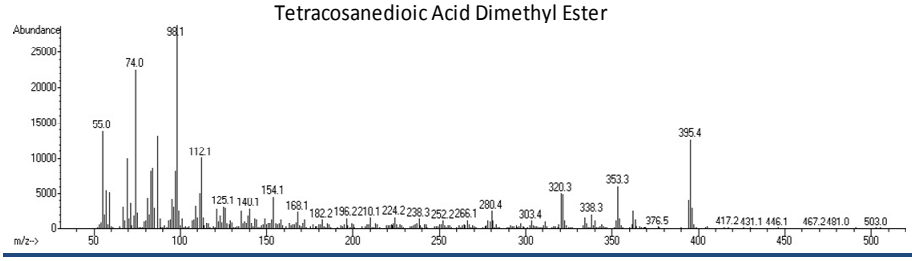
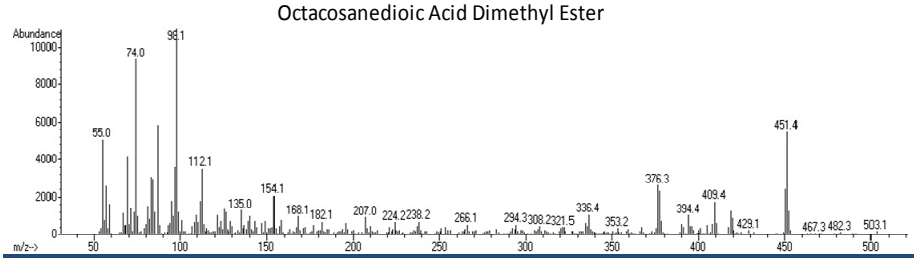
Product	m/z	Relative Abundance
Hexadecanedioic Acid Dimethyl Ester 	55.1	53.4%
	74.0	65.4%
	87.0	33.2%
	98.1	100.0%
	112.1	34.8%
	241.2	40.0%
	283.2	62.2%
Octadecanedioic Acid Dimethyl Ester 	55.1	50.7%
	74.1	72.7%
	87.1	36.8%
	98.1	100.0%
	112.1	36.5%
	269.2	50.5%
	311.3	86.7%
Eicosanedioic Acid Dimethyl Ester 	55.1	50.7%
	74.1	86.7%
	87.1	50.5%
	98.1	100.0%
	112.1	36.8%
	297.3	35.3%
	339.3	72.7%
Tetracosanedioic Acid Dimethyl Ester 	55.1	48.4%
	74.1	79.0%
	87.1	45.6%
	98.1	100.0%
	112.1	35.1%
	353.3	21.1%
	395.4	43.9%
Octacosanedioic Acid Dimethyl Ester 	55.0	45.7%
	74.0	85.3%
	87.1	51.0%
	98.1	100.0%
	112.1	32.0%
	409.4	15.7%
	451.4	50.2%

Figure 3.32 - (b) Mass spectra for dicarboxylic methyl esters obtained from treatment of solids isolated from RMS5 mutant cultures growing on sodium pyruvate and different long-chain n-alkanes.

3.6.3 Beads composition

Beads from approximately 1 to 5 mm diameter were formed during cultivation of random and gene disrupted mutants in loose cap shaking flasks when sodium pyruvate (fed-batch) was used as co-substrate in the presence of n-alkanes. These beads were ground, extracted and washed with MTBE over a filter to remove insoluble matter. Around 85 % weight of the material was soluble in MTBE. GC-MS analysis of the MTBE extract derivatized by silylation showed the same peaks as found in samples taken during cultivation in limited oxygen conditions but with a much higher proportion of fatty acid monoesters of glycerol (previously described in figure 3.24) in relation to alkane and free acids. Approximate composition of beads isolated from an RMS5 culture with sodium pyruvate and n-octadecane is presented in figure 3.33 (a).

During filtration, it was noticed that crystals were formed at the edges of the filter once solvent started to evaporate. A chromatogram of a silylated sample of these crystals (figure 3.33 b) showed that they consisted almost entirely of fatty monoesters of glycerol. It was assumed that the beads were formed because of high production of these substances during cultivation (associated to high production of biosurfactant, see 3.7).

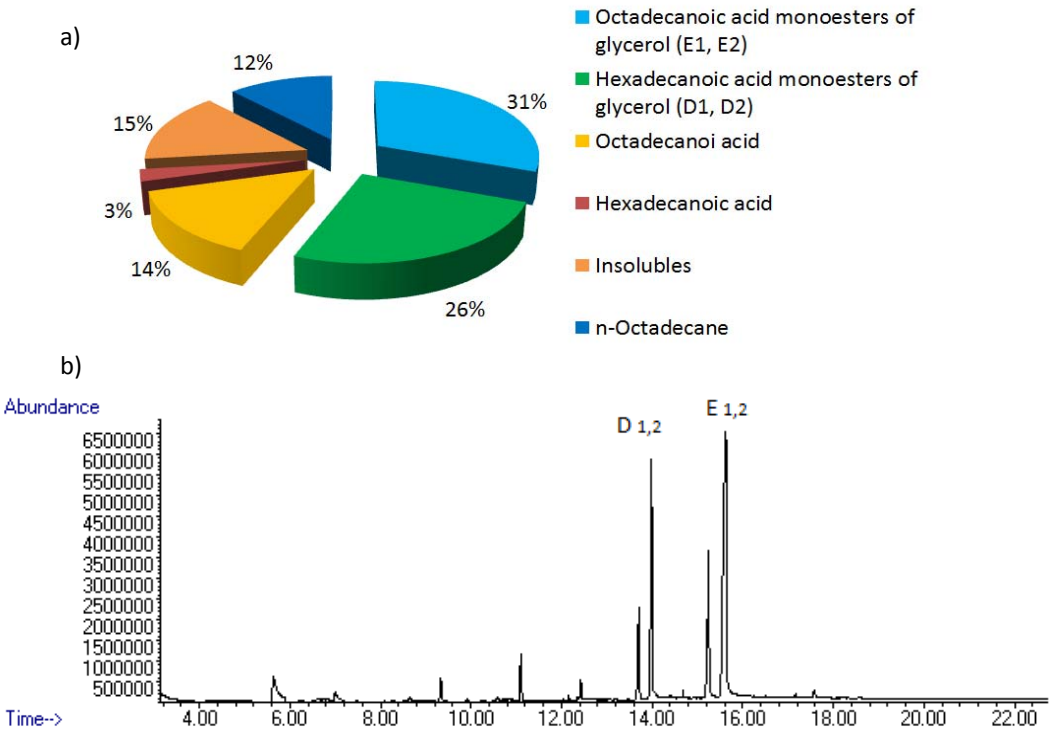


Figure 3.33 - (a) Composition of beads isolated from an RMS5 culture with sodium pyruvate and n-octadecane. Beads were ground and dissolved in MTBE prior derivatization (silylation) and analyzed by GC-MS. (b) Insoluble material in the beads (~ 15%) was separated by filtration; crystals formed at the edge of the filter were analyzed separately by GC-MS after silylation. Crystals were mostly fatty acid monoesters of glycerol.

3.6.4 Alkane bioconversion products by kanamycin resistant mutants

The GC chromatogram presented in figure 3.27 shows peaks that were common for all screened mutants obtained by gene disruption. No fatty alcohols or diols, nor DCA's were detected in any form (e.g. bound to floating solids) from bioconversion experiments with these strains. For free mono-carboxylic acids others than C16 and C18, only traces were observed in some chromatograms when substrates like n-eicosane were tested.

3.7 Surfactant production by *Thermus s*p. ATN1 and its mutants

An oily and soapy film spreading on the wall of incubation flasks was observed during growth on n-alkanes in mineral media with *Thermus sp.* ATN1 and its mutants. In addition, a milky halo appeared around colonies growing on n-alkanes on mineral media plates. As previously proposed by Otto (2001), it was assumed that, as reported for other hydrocarbon degrading bacteria, *Thermus sp.* ATN1 produces surfactant(s) to facilitate uptake of long-chain n-alkanes. Production of surface-active substances was monitored by the Methylene Blue method for Active Substances, MBAS.

Figure 3.34 shows MBAS absorbance at 625 nm vs. cell growth of the wild-type, RMS5 and DG11 mutant strains. Higher absorbance values were measured for samples taken from mutant cultures compared to those taken from wild-type strain cultures. It was concluded that the mutant strains showed higher surfactant production levels, possibly as a result of regulatory alterations of the alkane metabolism.

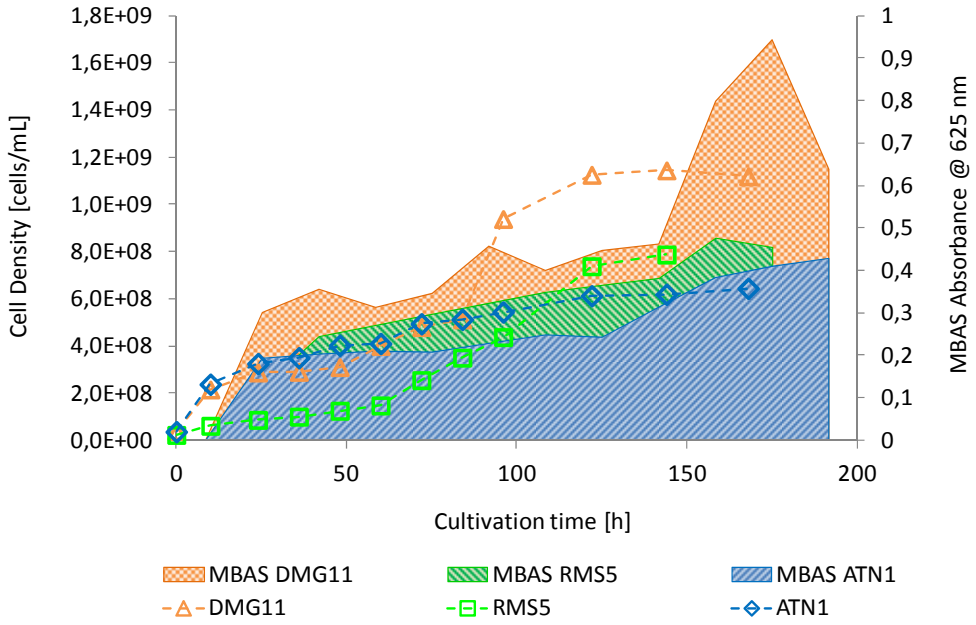


Figure 3.34 - Methylene Blue method for Active Substances (MBAS) absorbance results at 625 nm vs. cell growth for the wild-type, RMS5 and DG11 mutant strains of *Thermus sp.* ATN1. Strains were grown in mineral medium supplemented with sodium pyruvate and n-eicosane in screw cap shaking flasks (see figure 3.26 b).

3.7.1 Biosurfactant characterization

Biosurfactant was isolated and purified from processing 400 mL of cell-free culture broth obtained from a culture of mutant strain DG11 that resulted in 325 mg of crude lyophilized material (refer to 2.9.2). In contrast, only about 130 mg of crude surfactant were obtained from solvent extraction of 500 mL of cell-free broth from mutant strain RM5. Less than 30 mg were obtained under similar conditions with the wild-type strain.

For characterization experiments material isolated from the strain DG11 culture was used. The material appeared similar to the one obtained from the wild-type strain culture in FT-IR (ATR) comparison. IR spectra are shown in figure 3.42.

Characterization was performed in comparison to commercial surfactants (SDS and Triton X-100). Figures 3.35 shows MBAS absorbance of reference SDS solutions compared with those obtained from equal weight concentrations of the partly purified biosurfactant. Lower values were observed for the material obtained from *Thermus sp.* ATN1 mutant cultures suggesting that this material, if pure, has a higher molecular weight than SDS.

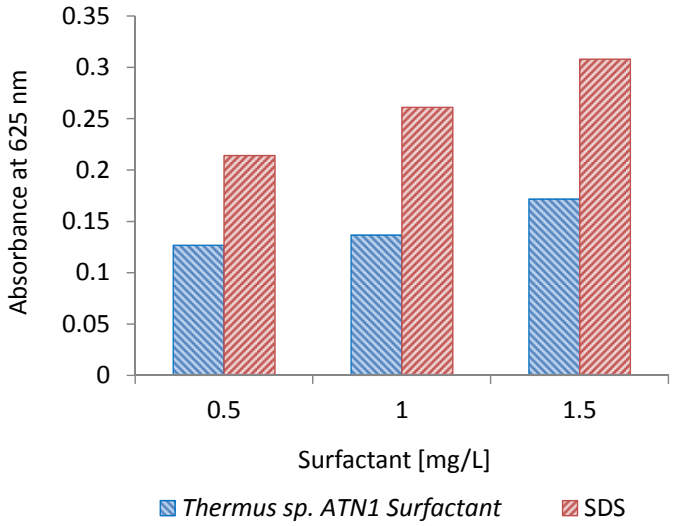


Figure 3.35 - MBAS absorbance at 625 nm of solutions from *Thermus sp.* ATN1 surfactant compared to equal concentrated solutions of SDS (weight). Lower absorbance values for the new surfactant suggest a higher molecular weight compared to SDS.

Figures 3.36 to 3.38 show characterization results according to Cirigliano and Carman (1984, 1985) using n-hexadecane to test emulsification activity and stability of the biosurfactant produced by *Thermus sp.* ATN1 in comparison to other known surfactants at different temperatures.

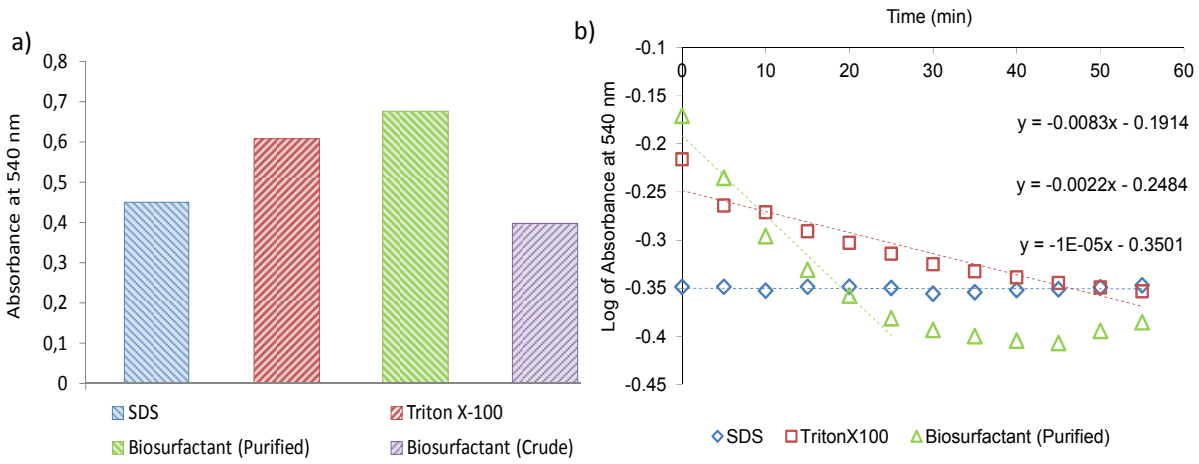


Figure 3.36 - (a) Emulsification activity with n-hexadecane (initial absorbance at 540 nm) at 20 °C for 1 g/L solutions of commercial surfactants and biosurfactant produced by *Thermus sp.* ATN1. (b) Emulsification stability curves at 20 °C for 1 g/L solutions of commercial surfactants and biosurfactant produced by *Thermus sp.* ATN1 with n-hexadecane.

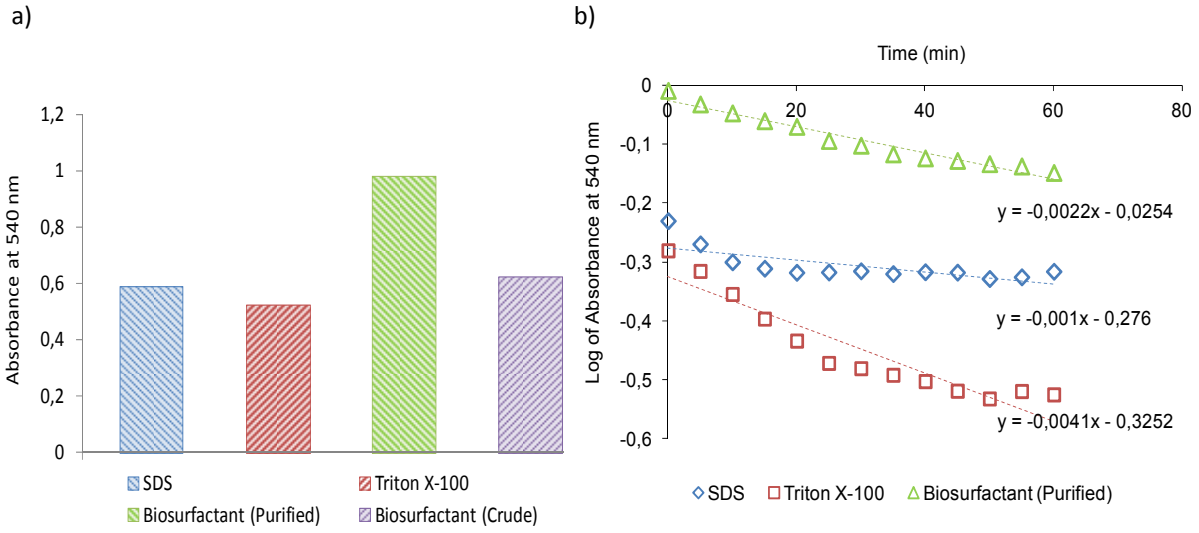


Figure 3.37 - (a) Emulsification activity with n-hexadecane (initial absorbance at 540 nm) at 50 °C for 1 g/L solutions of commercial surfactants and biosurfactant produced by *Thermus sp.* ATN1. (b) Emulsification stability curves at 50 °C for 1 g/L solutions of commercial surfactants and biosurfactant produced by *Thermus sp.* ATN1 with n-hexadecane.

The emulsification activity between commercially available surfactants (SDS, Triton X-100) and the biosurfactant produced by the mutant strains of *Thermus sp.* ATN1 were compared. Values were found higher for the purified biosurfactant at both tested temperatures (20 and 50 °C). However it was observed, that the crude biosurfactant showed less emulsification activity than SDS and Triton X-100 at 20°C, whereas it was higher at 50 °C. The purified biosurfactant showed a significantly higher emulsification activity at 50 °C compared with the commercial surfactants. A slightly lower emulsification activity at 70°C for the pure biosurfactant suggests that maximum is at 50 °C or between these two temperatures. Emulsification stability under tested conditions was found best at 50 °C for the pure biosurfactant, although it was slightly less stable than SDS at this temperature. At 20 °C the biosurfactant exhibited poor emulsification stability.

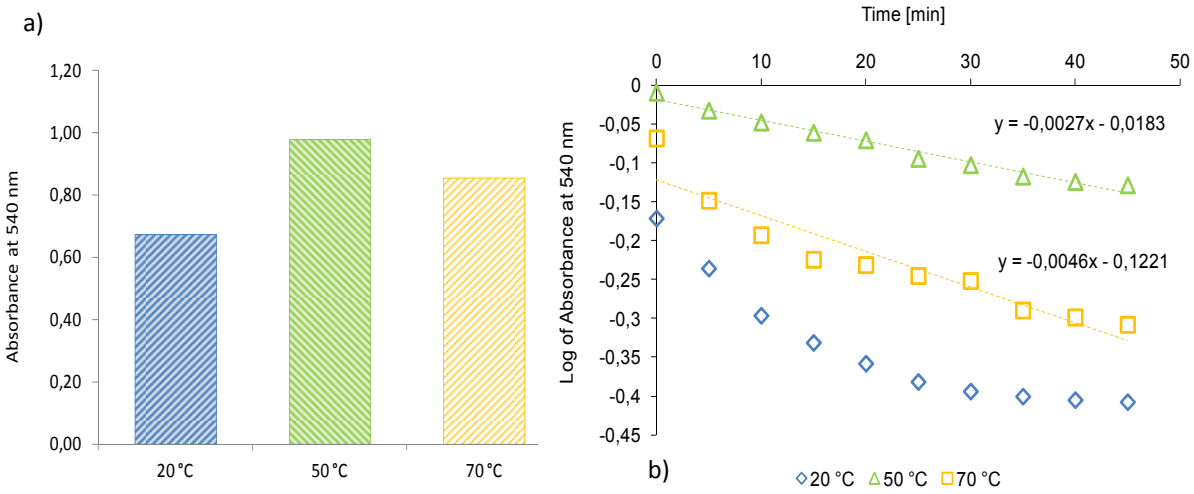


Figure 3.38 - (a) Emulsification activity with n-hexadecane (initial absorbance at 540 nm) at different temperatures for 1 g/L solution of biosurfactant produced by *Thermus sp.* ATN1. (b) Emulsification stability curves at different temperatures for the biosurfactant (purified) produced by *Thermus sp.* ATN1 with n-hexadecane.

3.7.2 Emulsification of n-alkanes due to biosurfactant

Cells were removed by centrifugation of broth culture samples taken at 12 h intervals during bioconversion of n-octadecane by the mutant strain RMS5 in screw cap shaking flaks for FT-IR offline measurements (with the ReactIR™ 45m equipment). Spectra of samples from a control culture without the alkane were subtracted from spectra taken from the test culture. Figure 3.39 presents overlapped spectra resulting from the calculation. It was observed that peaks typical for aliphatic saturated hydrocarbons at ~ 1470 and ~1380 cm^{-1} (1470 – 1430/1380 – 1370 Methyl C-H asymmetric/symmetric bend) increase with the cultivation time (whereas alkane is being consumed). It was assumed that the increase in peak intensity was caused by increasing emulsification of the alkane in the culture broth (increase in its apparent concentration). This can be attributed to the effect of a surfactant facilitating the build-up of an emulsion.

The effect of biosurfactant on alkane emulsification was also observed in the experiment to determine the apparent n-octadecane concentration in a culture broth where *Thermus sp.* ATN1-RMS5 had reached the stationary phase (see 2.9.4). After cells were removed and solids were filtrated at 4°C (including remaining solidified alkane), it was determined that permeate still contained 0.0039 g/L n-octadecane (originally added at 1.3 g/L). Reported solubility or accommodation of n-octadecane in water is in the order of 2.1 X 10^{-6} g/L. It was assumed that the apparent solubility of this alkane in water was increased 1900-fold due to the presence of biosurfactant facilitating the build-up of a stable micro-emulsion.

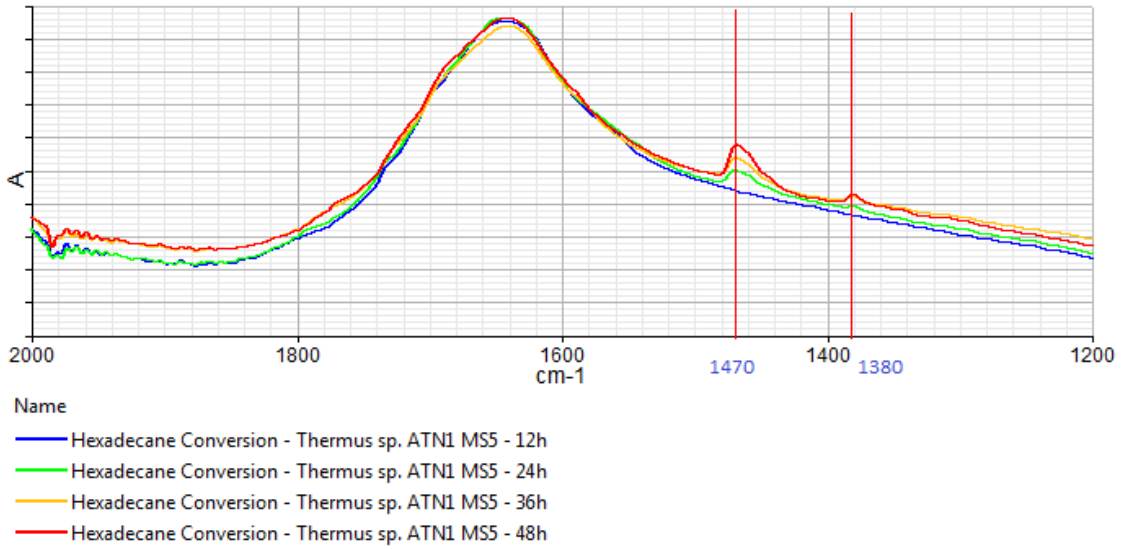


Figure 3.39 - Off-line FT-IR monitoring during cultivation of mutant strain RMS5 in mineral medium supplemented with 1.3 g/L n-octadecane and 10 mM sodium pyruvate. Peak intensities at ~1470 and ~1380 cm^{-1} (bands typical for saturated aliphatic hydrocarbons) indicate an increase on apparent alkane concentration in the culture broth with cultivation time. This was attributed to the effect of increasing biosurfactant concentration with cell density throughout the experiment (as observed during MBAS monitoring of the same culture).

3.7.3 Increasing long-chain n-alkane bioavailability for *Pseudomonas sp.* Strain 273 with the biosurfactant produced by *Thermus sp.* ATN1

As described in 2.9.5, *Pseudomonas sp.* Strain 273 was cultured in mineral media with n-hexadecane as sole carbon source and compared to cultures containing 1 g/L of the biosurfactant produced by *Thermus sp.* ATN1 and 1g/L SDS. Alkane concentration and cell density was monitored during these experiments and the results are presented in figure 3.40.

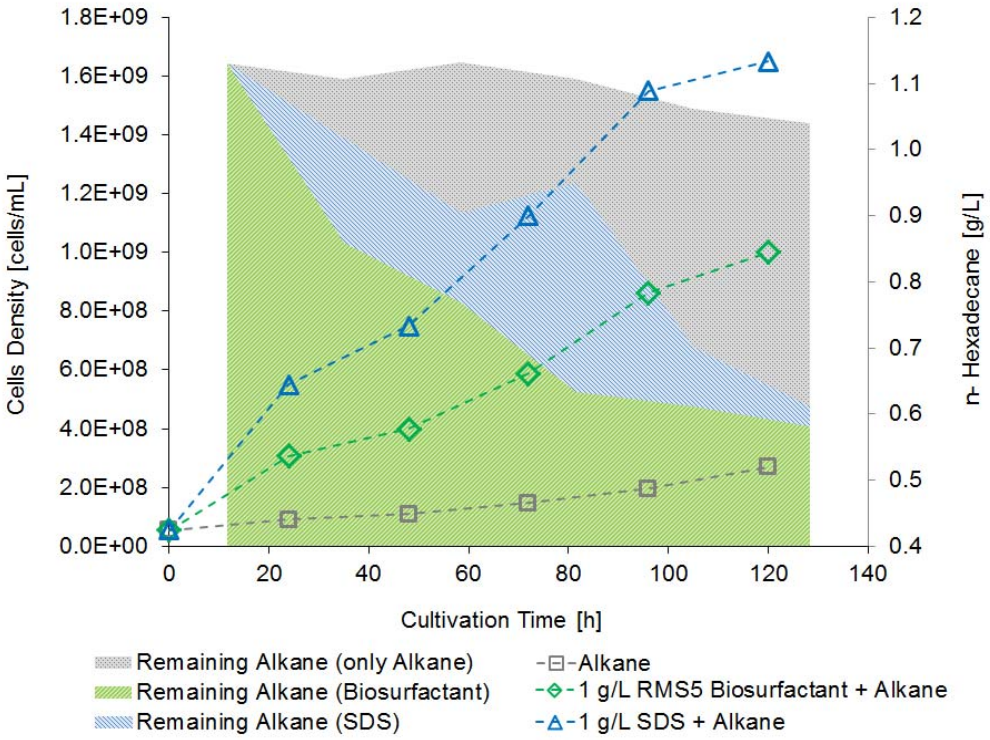


Figure 3.40 - Cell density and alkane concentration in cultures of *Pseudomonas sp.* strain 273 in mineral medium containing n-hexadecane as sole carbon source in comparison to cultures were 1/g L surfactants were added to increase alkane bioavailability. Experiments were carried out in sealed serum vials.

Wischnak *et al.* (1998) reported that the strain 273 is able to metabolize n-hexadecane. It can be observed that without surfactant growth is rather limited and alkane concentration is depleted slowly. The addition of SDS accelerates n-alkane depletion but also contributes to cell growth. Dodecanoic acid was found in the chromatograms during GC-MS analysis performed to determine alkane concentration in cultures containing SDS, implying that *Pseudomonas sp.* Strain 273 is metabolizing not only the alkane but also SDS. The addition of the *Thermus sp.* ATN1 biosurfactant had a stronger effect on alkane degradation compared to that of SDS. Lower cell densities with the biosurfactant also indicate that SDS contributed to cell growth in the form of carbon source. It cannot be excluded that the biosurfactant also contributed as a carbon source, but if it did, this was at a lower level compared to SDS.

3.7.4 Biosurfactant composition analysis

The biosurfactant material isolated from mutant strain cultures was analyzed to determine the nature of its hydrophilic and hydrophobic moieties. A positive result in the DNSA assay indicated that reducing sugars are part of the hydrophilic moiety, whereas preparation of trimethylsilyl and methyl esters revealed the presence of glycerol esters of hexadecanoic and octadecanoic acid. Although the substance(s) could be classified as glycosyl acylglycerol(s) it was not possible to determine whether these are the only building blocks in the molecule(s). Figure 3.41 presents GC-MS chromatograms for the analysis performed to determine the hydrophobic moieties.

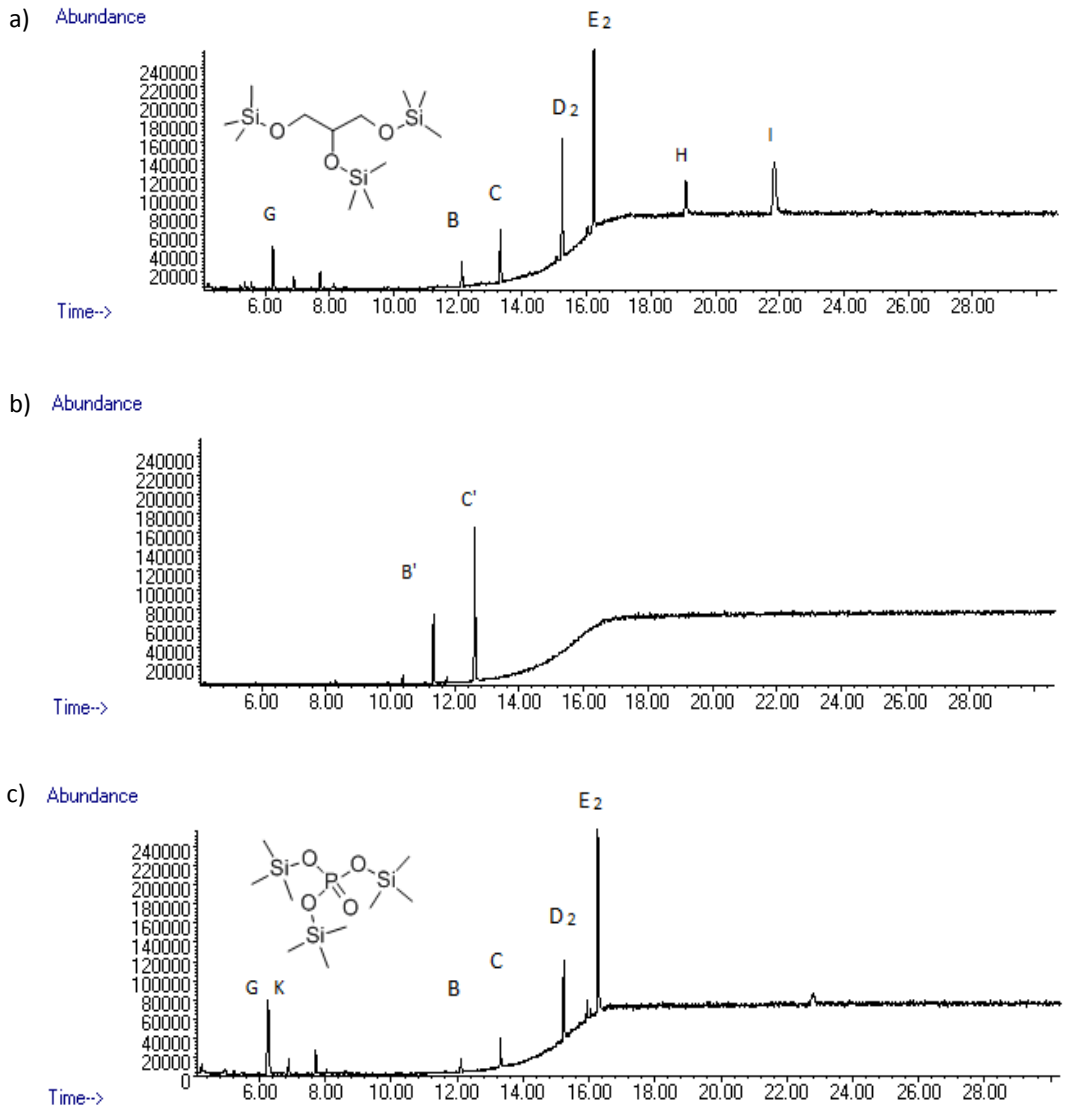


Figure 3.41 - GC-MS Chromatograms of biosurfactant samples obtained from mutant strains DG11 and RMS5. 1 mg aliquots were used for preparation of methyl esters and trimethylsilyl esters to determine hydrophobic moieties: (a) Trimethylsilyl derivatives of purified biosurfactant obtained from cultivation of mutant DG11; peak G corresponds to the trimethylsilyl derivative of

glycerol, peaks H and I were not identified, peaks B,C, D_2,E_2 identities were listed in figure 3.24., (b) Fatty acid methyl esters from processing DG11 mutant biosurfactant, (c) Trimethylsilyl derivatives of crude biosurfactant obtained from cultivation of mutant RMS5; peak K corresponds to tris-(trimethylsilyl) phosphate.

Calibration curves were prepared with fatty acid methyl monoesters (C16 and C18) to quantify the only peaks detected during methyl ester preparation from biosurfactant samples (figure 3.41-b). It was calculated that approximately 45% mass of the biosurfactant(s) material isolated from a DG11 mutant culture corresponds to glycerol esters as monoesters (peaks D2 and E2 in figure 3.41-a).

DNSA assay absorbance measurement against a calibration curve prepared with rhamnose resulted in a calculated value of about 40% mass of reducing sugars in the purified material. Sugars and acylglycerols added up about 85% total mass of this material.

Peaks H and I in figure 3.41(a) detected for the purified biosurfactant produced by the DG11 mutant were not identified in the material isolated from the RMS5 mutant 3.41(c). Also peak G in the chromatogram of the RMS5 mutant material appeared as a doublet (a second peak, K) not present in biosurfactant from mutant DG11. It must be noticed that biosurfactant from RMS5 mutant was obtained as a consequence of the solvent extraction procedure to recover n-alkanes and products (see 2.9.2) and was considered not pure. This crude material also contained about 37% reducing sugars and 40% acylglycerols. Phosphorous (<10 % w) was also found during elemental analysis of the crude material.

Additionally crude and pure biosurfactant were compared by FT-IR (ATR) analysis. Figure 3.42(a) presents the spectra comparison. Generally speaking materials appeared quite similar and it was assumed that the differences found were due to the crude material. The crude material was also compared to floating solids (see 3.6.2) appearing in the culture during alkane bioconversion by mutant RMS5, figure 3.42(b). Some pattern similarities were found in the region from 1700 to 1350 cm^{-1}. It is theorized that alkane bioconversion products by RMS5 mutant strain do react with material present in the crude biosurfactant (or with the biosurfactant) resulting in the floating solids observed during the experiments with this mutant strain for alkane bioconversion.

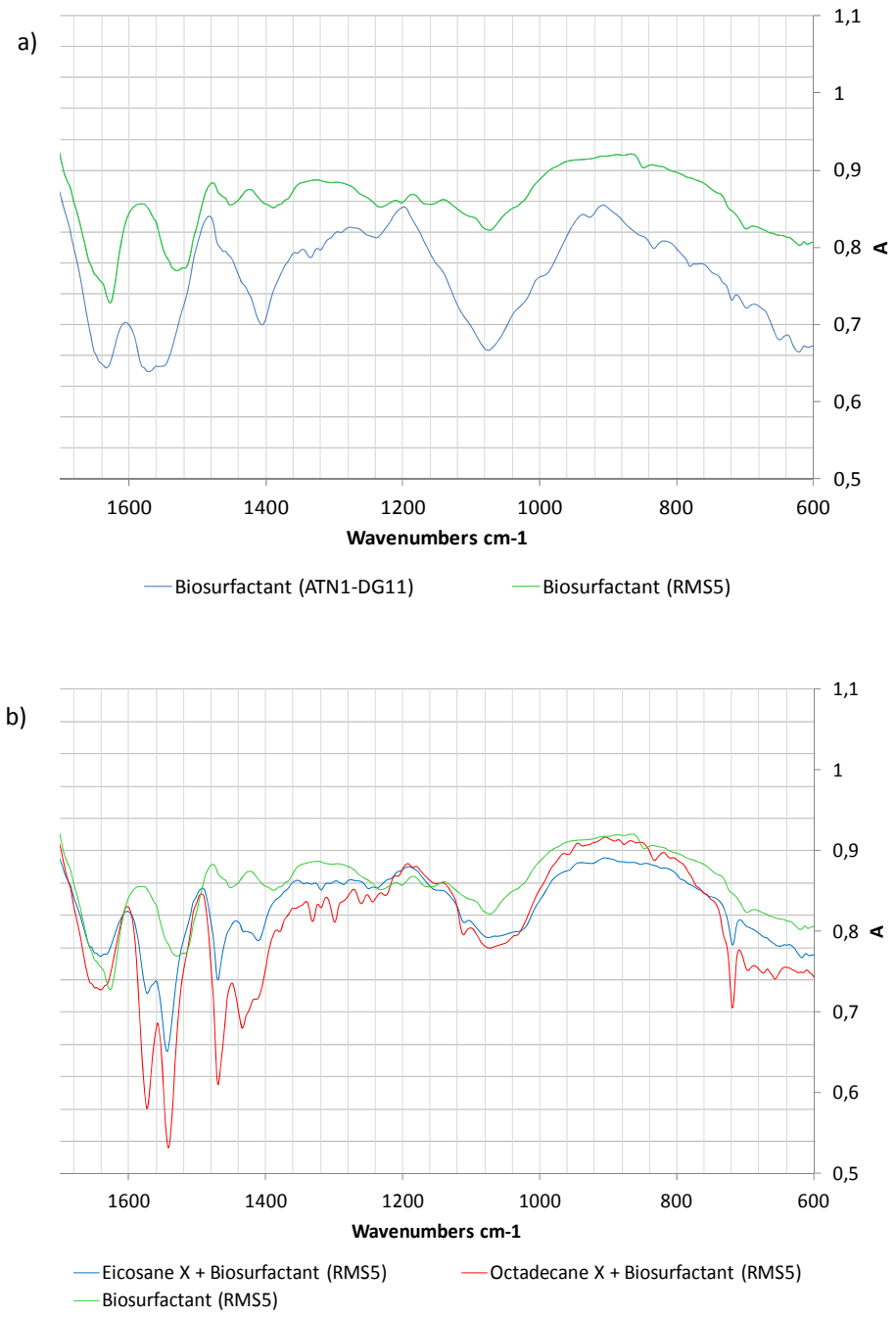


Figure 3.42 - FT-IR (ATR) comparisons of (a) crude biosurfactant isolated from a RMS5 mutant culture (green) and purified biosurfactant isolated from a DG11 mutant culture (blue); (b) crude biosurfactant (RMS5) and floating solids produced during bioconversion of n-octadecane (red) and n-eicosane (blue) removed from the culture by filtration in cold or decantation followed by solvent wash.

3.8 Bioconversion of long-chain n-alkanes by mutant strain RMS5

In order to understand culture conditions to maximize bioconversion, the influence of several factors on cell growth and alkane conversion results was studied.

As suggested in section 3.3 alkanes were not only converted but co-metabolized by mutant strain RMS5 (while growing in e.g. sodium pyruvate). This effect was particularly marked under aerobic conditions. How much alkane was used for cell growth or accumulated as conversion products (mono and dicarboxylic acids) was influenced by co-substrate choice, alkane chain length, alkane concentration and aeration.

Effect of substrate hydrocarbon chain length and its concentration on cell growth is shown in figures 3.43 and 3.44 respectively.

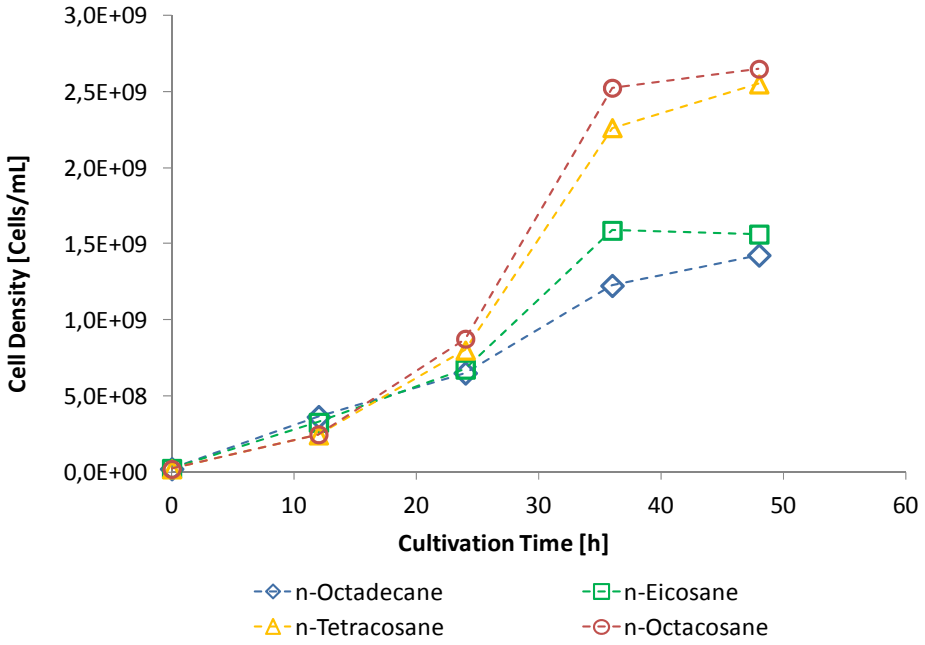


Figure 3.43 - Effect of hydrocarbon chain length on cell growth for mutant RMS5. Higher cell densities were obtained with longer n- alkanes at similar concentrations. Experiments were performed in loose cap shaking flasks; n-octadecane and n-eicosane were added at 10 mM whereas only 5 mM n-tetracosane and n-octacosane 5 mM were used.

In figure 3.44 it is illustrated that depletion of alkane (consumed + converted) was also dependent on the initial alkane amount and on cell density, both influencing n-alkane conversion.

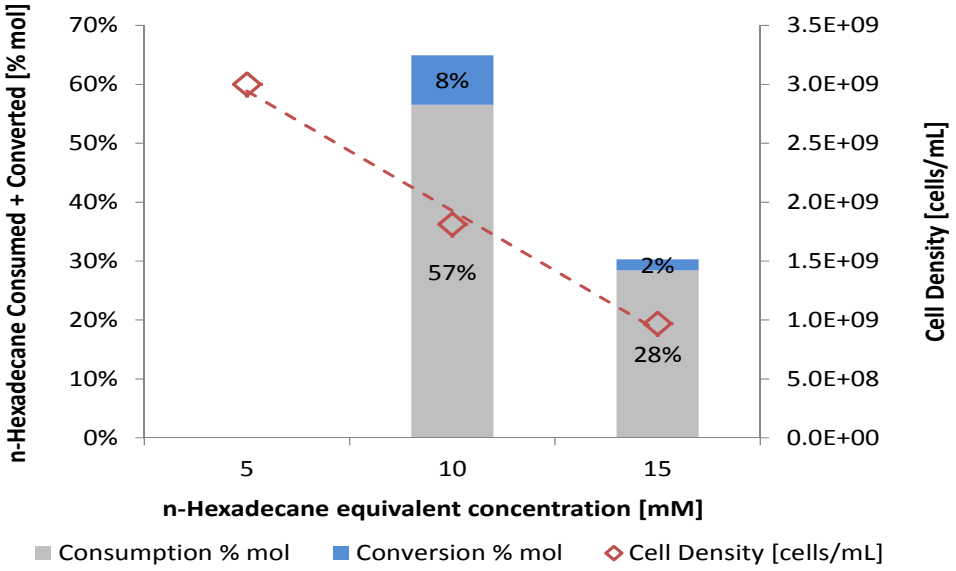
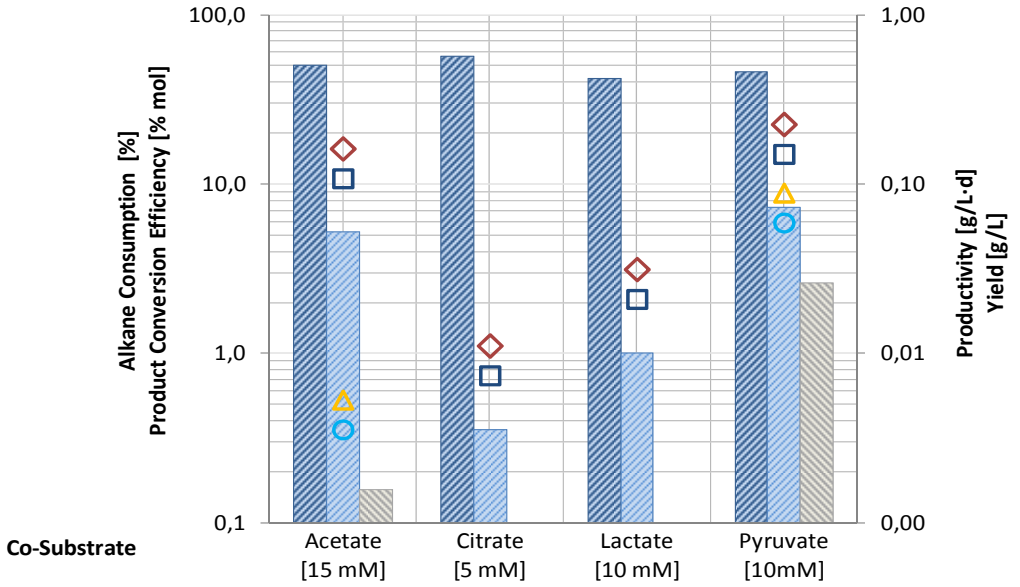


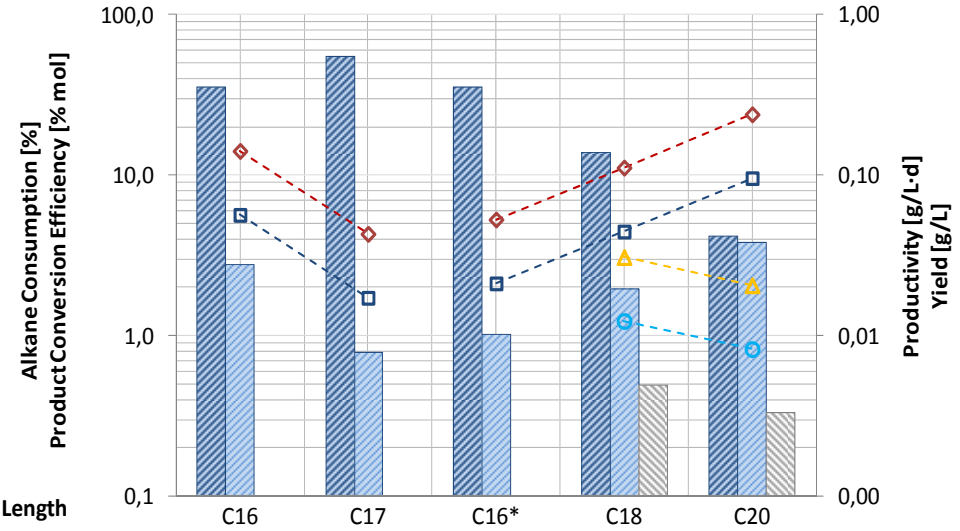
Figure 3.44 - Effect of n-alkane concentration on growth of mutant RMS5. Experiments were carried out in loose cap shaking flasks containing three different n-hexadecane concentrations. Incubation was run for 48 h with 5 and 10 mM n-hexadecane and for 60 h in the 15 mM experiment. Sodium pyruvate was used as co-substrate at equal dosages.



	Acetate [15 mM]	Citrate [5 mM]	Lactate [10 mM]	Pyruvate [10mM]
Consumption [%]	49,98	56,52	41,99	46,11
Conversion Efficiency [% mol] Acid	5,20	0,36	1,01	7,25
Conversion Efficiency [% mol] Diacid	0,16			2,60
Yield [g/L] Acid	0,16	0,01	0,03	0,23
Yield [g/L] Diacid	0,01	0,00	0,00	0,09
Productivity [g/L·d] Acid	0,11	0,01	0,02	0,15
Productivity [g/L·d] Diacid	0,004			0,06

Figure 3.45 - Co-substrate effect on n-eicosane conversion. Experiments were performed in loose cap shaking flasks with the indicated co-substrate concentration (no fed-batch) and 10 mM n-eicosane (2.82 g/L). Incubation was run at 70 °C and 120 rpm for 48 h. Conversions were calculated from GC-MS analysis of solvent extracts from cell free culture broth and floating solids hydrolyzed in acidic methanol (products detected as methyl esters).

Influence of co-substrate, n-alkane chain length and aeration (oxygen) on conversion, yield and productivities of mono and dicarboxylic acids are presented in figure 3.45 to 3.48. According to the results presented in figure 3.45, sodium pyruvate was chosen as the preferred co-substrate for bioconversion of long-chain n-alkanes to mono and dicarboxylic acids. α,ω-Dicarboxylic acids were also detected when sodium acetate was used as co-substrate but lower conversions where observed. No dicarboxylic acids were detected when sodium citrate or sodium lactate were used as co-substrates despite the formation of floating solids from whose hydrolysis only mono carboxylic acids were obtained.



Alkane Chain Length	C16	C17	C16*	C18	C20
Consumption %	35,38	55,39	35,38	13,94	4,19
Conversion Efficiency [% mol] Acid	2,75	0,79	1,03	1,96	3,83
Conversion Efficiency [% mol] Diacid				0,49	0,33
Yield [g/L] Acid	0,14	0,04	0,05	0,11	0,24
Yield [g/L] Diacid				0,03	0,02
Productivity [g/L·d] Acid	0,06	0,02	0,02	0,04	0,10
Productivity [g/L·d] Diacid				0,01	0,01

Figure 3.46 - Effect of n-alkane chain length on conversion under limited oxygen conditions. Experiments were carried out in screw cap shaking flasks with 10 mM alkane. Figures for C16* correspond to re-calculation from subtraction of n-hexadecanoic acid measured in culture of n-eicosane, assuming this was not a product of n-hexadecane conversion. For n-hexadecane and n-heptadecane conversion no diacids were detected.

Long-chain n-alkane conversion and uptake was dependent on hydrocarbon chain length. Whether in limited oxygen (screw caps) or aerobic (loose caps) experiments, alkane consumption (metabolized + only converted in %w) decreased with increasing hydrocarbon chain length (see figure 3.46) but also decreased with increasing initial alkane concentration (as in figure 3.44).

Figures 3.47 and 3.48 illustrate that the most influencing factor on n-alkane bioconversion by the RMS5 mutant was aeration. When experiments were performed in loose cap shaking flasks, aerobic conditions resulted in higher cell densities enabling higher alkane consumptions but also

accumulation of mono and dicarboxylic acids. However, initial n-alkane concentration and co-substrate selection also influenced strongly conversion, yields and productivities.

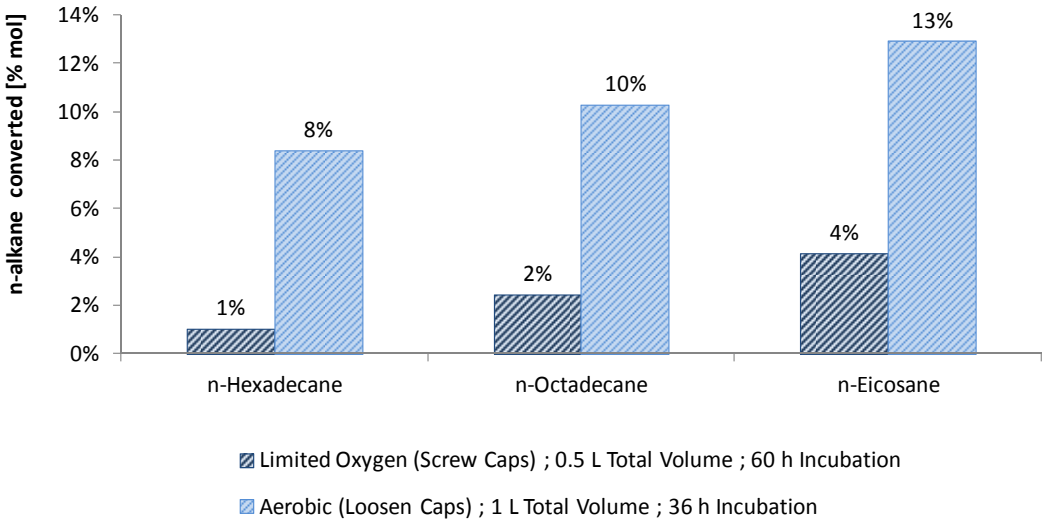


Figure 3.47 - Effect of aeration on conversion (acid and diacid products) of n-hexadecane, n-octadecane and n-eicosane. Experiments correspond to figures 3.46 and 3.49 in section 3.9.

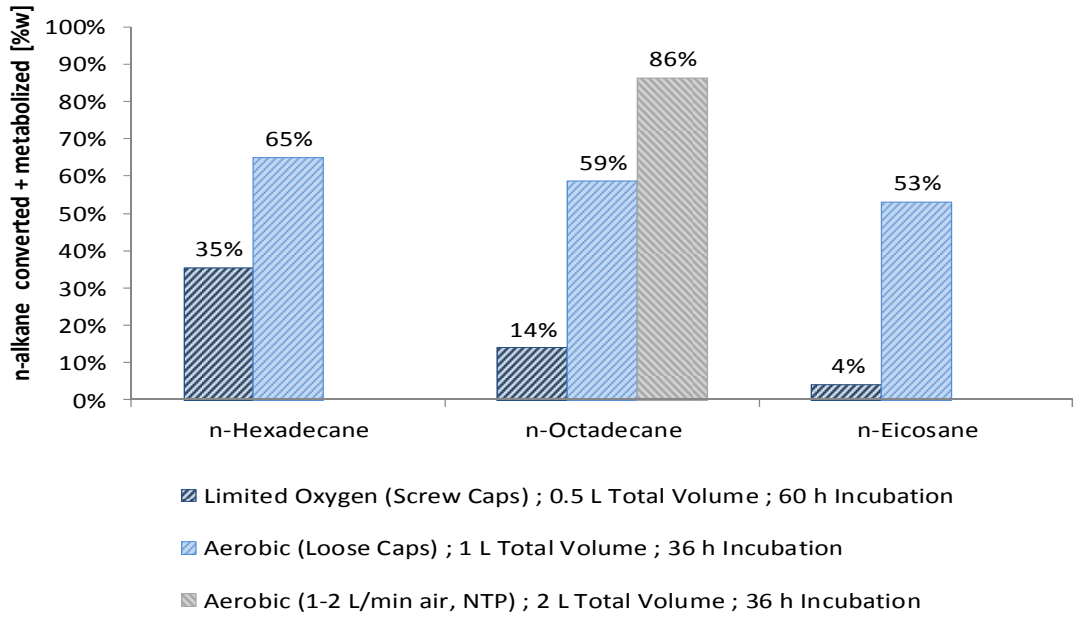


Figure 3.48 - Effect of aeration on total alkane consumption (converted + metabolized) for n-hexadecane, n-octadecane and n-eicosane. Data correspond to figures 3.46 and 3.49 except for the case of n-octadecane provided with active aeration, where air was supplied to the broth in a 5 L Schott bottle at 70 °C and 120 rpm during 36 h. Conversion could not be quantitatively determined and only traces of octadecanoic acid were detected. It is assumed most of the n-octadecane was metabolized in this case.

It must be noticed that most of the presented results correspond to single experiments, except for those presented for the limited oxygen conditions for n-hexadecane, n-octadecane and n-eicosane which were averaged results of two experiments which produced very similar results.

Figures for n-alkane consumption (not conversion) might be the result of combined consumption and evaporation in the aerobic cases and especially in the case were air was supplemented to the 2 L culture with n-octadecane. Otto (2001) found this effect when culturing the wild-type strain on n-eicosane in an aerated fermenter, although the real extent of evaporation was not quantifiable. For the experiments presented here a non-inoculated 500 mL shaking flask (with loose cap) containing 200 mL mineral medium with 2.82 g/L n-eicosane resulted in a concentration of 2.78 g/L n-eicosane after correction for volume evaporation after 36h of incubation (determined by solvent extraction of the whole end volume and GC-MS analysis). Since the difference to the initial concentration is not significant, it was assumed that evaporation with loose cap shaking flasks was not critical. This was not evaluated for lower molecular weight n-alkanes.

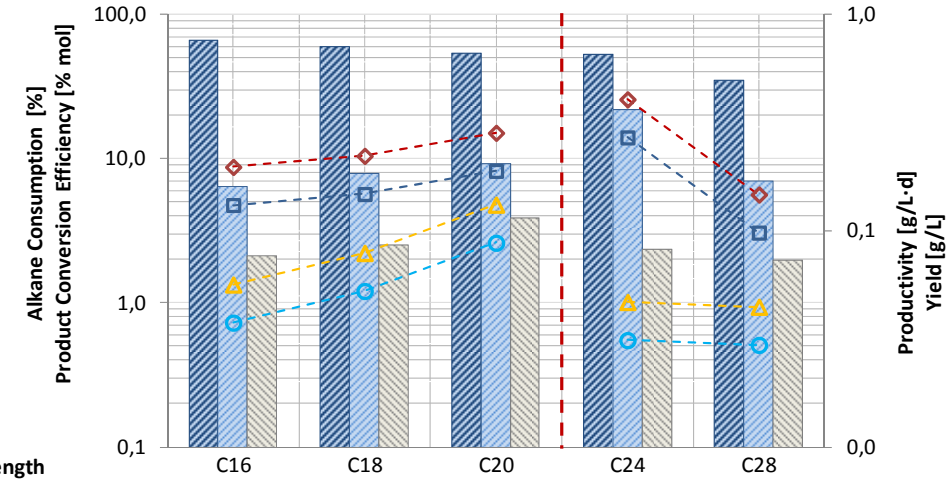
3.9 Production of long-chain α,ω-dicarboxylic acids by mutant strain RMS5

α,ω-Dicarboxylic acids are valuable monomers for production of polyesters, polyamides, and polyurethanes (Metzger, 2009). Long-chain α,ω-diacids are interesting building blocks because they can provide greater flexibility and strength than short-chain diacids due to their ability to bend, which minimizes breakage and reduces the number of links in a polymer. Thus, these compounds are attractive for the production of high performance polymers like polyamides/nylon. α,ω-dicarboxylic acids are also used in other applications where longer chain α,ω-diacids offer new possibilities for the manufacture of fragrances, cosmetics, powder coatings, corrosion inhibitors, lubricants additives, adhesives and also pharmaceuticals (Cathay Biotech, 2007).

As described in section 1.2.2, production of long-chain α,ω-diacids have been reported from several yeast strains (wild-type and mutant strain) using either fatty acids or aliphatic hydrocarbons as substrates. However the carbon chain length of these diacids is determined by substrates, which are liquid at yeast cultivation temperatures and reported diacids have been limited to chain lengths of C22 when produced from non-saturated substrates (olefins) and of C16 from saturated hydrocarbons. Authors have suggested the possibility to convert up to C22 saturated hydrocarbons using yeast, but this has not been reported (Schörken & Kempers, 2009).

By constructing and using mutants from *Thermus sp.* ATN1 it has been possible to demonstrate the production of long-chain α,ω-diacids up to a chain length of 28 carbon atoms by bioconversion of the corresponding aliphatic hydrocarbons. This is the first time that the bioconversion of aliphatic hydrocarbons of chain lengths larger than 16 carbon atoms with thermophilic bacteria to mono and dicarboxylic acids is reported. These results match with the objectives described within the scope of this project.

Figure 3.49 summarizes the results of experiments under aerobic conditions for the cultivation of mutant strain RMS5 growing in mineral media supplemented with sodium pyruvate in the presence of several long-chain n-alkanes.



Alkane Chain Length	C16	C18	C20	C24	C28
Consumption [%]	64,94	58,56	53,09	52,57	34,28
Conversion Efficiency [% mol] Acid	6,31	7,79	9,07	21,94	6,92
Conversion Efficiency [% mol] Diacid	2,08	2,51	3,85	2,35	1,96
Yield [g/L] Acid	0,20	0,22	0,28	0,40	0,15
Yield [g/L] Diacid	0,06	0,08	0,13	0,05	0,04
Productivity [g/L·d] Acid	0,13	0,15	0,19	0,27	0,10
Productivity [g/L·d] Diacid	0,04	0,05	0,09	0,03	0,03

Figure 3.49 - Effect of n-alkane chain length on conversion, yield and productivity of fatty acids and LCDAs under aerobic conditions. Experiments were carried out in 2 L shaking flasks with loose caps containing 1 L of mineral media with 10 mM equivalent alkane for C16 to C20 and 5 mM for C24 and C28. Sodium pyruvate was used as co-substrate (fed-batch at 10 mM until 24 h). Incubation was run at 70 °C and 120 rpm during 36 h. Conversions were calculated from GC-MS analysis of solvent extracts from cell free culture broth and floating solids hydrolyzed in acidic methanol (products detected as methyl esters).

Under the given experimental conditions it was possible to reach an alkane conversion efficiency of almost 22% for n-tetracosane (C24) to tetracosanoic acid and of almost 4% for n-eicosane (C20) to eicosanedioic acid.

As described in section 3.8, total alkane consumption decreased with increasing carbon chain length of the substrates and alkane conversion to mono and dicarboxylic acids tended to increase. This was not the case for bioconversion of n-octacosane, possibly because of its extremely limited water solubility or because its melting point (~65°C) was too close to the cultivation temperature.

Chapter 4

4. Discussion

4.1 Growth characteristics and inducible long-chain n-alkanes metabolism of *Thermus sp.* ATN1

Growth characteristics of *Thermus sp.* ATN1 in complex and mineral media with n-alkanes (C10 to C20) as sole carbon source were found similar to those previously reported by Otto (2001). In addition, the strain was able to grow quite well also on larger n-alkanes such as n-tetracosane (C24) and n-octacosane (C28).

New insights into the degradation of long-chain n-alkanes by this strain have resulted from studying growth in mineral media supplemented with two carbon sources: n-alkanes and a co-substrate. Under limited oxygen cultivation conditions, doubling times for the wild-type strain were significantly longer during the n-alkane utilization phase when compared to growth in mineral media supplemented only with n-alkanes. For instance, a doubling time of 85 h was observed during n-hexadecane utilization when 20 mM sodium citrate was initially added to the medium compared to 8.3 h when only n-hexadecane was used. This difference was particularly observable in sealed serum vials experiments, suggesting that oxygen starvation occurred during growth on the co-substrate and prior alkane utilization, resulting in a slower alkane uptake, which started only after the co-substrate was practically depleted.

These observations suggested that the alkane metabolism is not constitutive in the wild-type strain. Other carbon sources, like citrate or pyruvate, which are intermediates of the central carbon metabolism, were preferred as carbon sources over long-chain n-alkanes. The hydrocarbons are only metabolized if no other carbon source is available; indicating that alkane utilization by *Thermus sp.* ATN1 is inducible and regulated. Except for hydrocarbonoclastic bacteria, all other reported alkane degrading bacteria also exhibit an inducible alkane metabolism that obeys different regulatory mechanisms, almost particular to each species (Wentzel *et al.*, 2007; Harayama *et al.*, 2004; Yakimov *et al.*, 1998; 2007).

4.2 Mutants Construction

The construction of *Thermus sp.* ATN1 mutants with blocked expression of the AlkDH gene encoding the alcohol dehydrogenase (TADH) was proposed as a route to obtain long-chain compounds with alcohol groups at both ends of long-chain n-alkanes. The enzyme is suspected to be responsible for the second step in the alkane degradation pathway in this strain. These mutants should grow on a co-substrate as carbon and energy source while performing the alkane hydroxylation reaction by the still unknown alkane monooxygenase complex. Mutant construction was proposed with two different strategies, either by targeted gene disruption (a directed mutagenesis approach) or by classical random mutagenesis followed by selective screening techniques.

Having identified an inducible alkane metabolism in this strain, it was concluded that directed mutagenesis by disruption of AlkDH in the wild-type strain results in mutants without the ability to produce terminal oxidized products, because the alkane degradation pathway is not active while the strain grows on another substrate and once this catabolic pathway can be activated there is no other carbon source left for growth and NADH regeneration, required for the hydroxylation reaction.

Because the mechanisms and genes involved in the regulation of alkane degradation are still unknown for this species, it would have been necessary to elucidate them in order to perform the necessary targeted mutations (e.g. deletions) in the wild-type strain for the purpose of alkane bioconversion. In this case classical mutagenesis is a more attractive route to obtain mutants with the desired phenotype, given that an effective screening could be applied (see 3.2). Nevertheless, gene disruption remained an alternative for improvement of mutants obtained by random mutagenesis since the targeted mutant phenotype could not guarantee the pathway disruption required to obtain diol products (see table 2.8).

Several studies that have aimed to obtain terminal oxidized products from aliphatic hydrocarbons utilizing microorganisms have also relied on classical mutagenesis, particularly in the case of LDCA's. For instance mutants of *Candida sp.* obtained by this technique successfully increased yield and productivity of dicarboxylic acids from alkanes such as brassylic acid (DC13) from n-tridecane (Takagi *et al.*, 1990; Cao *et al.*, 2006; see table 1.4). This approach reached commercial ton scale production in the 1990's (Huf *et al.*, 2011). Another example is described in the patent document by Dahlstrom & Jaehning (1973) where mutants derived from *Corynebacterium sp.* strain 7E1C produced dicarboxylic acids (DC-12) in increased amounts compared to the wild-type strain (see 1.3.4).

4.2.1 Mutants obtained by classical random mutagenesis

Mutants exhibiting the desired phenotype were obtained (type 3, table 2.8). Selected mutant strains RMS2, RMS5, RMS17 and RMS26 could not grow on long-chain n-alkanes or in fatty alcohols as sole carbon source but on fatty acids, indicating that a mutation(s) preventing alkane utilization but not fatty acid utilization had occurred.

The alkane bioconversion screening tests carried out in sealed serum vials for these mutants indicated that at least with mutant strain RMS5 in contrast to the wild-type strain alkane depletion occurred simultaneously to co-substrate consumption (see 3.3). This suggests a constitutive expression of the alkane monooxygenase in this mutant. Some alkane depletion was also observed with mutant strain RMS17 in these tests. This suggested that the alkane degradation regulatory mechanisms have been altered in these mutants and that alkane bioconversion whilst growing on a second carbon source is possible. However, none of the desired alkane bioconversion products was detected by GC-MS for any of these mutants during this screening phase.

The only exception was the detection of C16 and C18 monocarboxylic acids matching the length of the alkanes used as substrates (C16 or C18), but these could not be fully explained as bioconversion products. Both monoacids (C16 or C18) where detected when only one of the corresponding alkanes was used as substrate and they were also detected, though at lower concentrations, in experiments with mutants RMS2 and RMS26 where alkane depletion was much lower under the screening conditions. These monoacids form also part of a biosurfactant that *Thermus sp.* ATN1 produces whilst growing on hydrocarbons and this biosurfactant is overproduced by the selected mutants (see 4.6).

Due to low alkane consumption, it was concluded that strains RMS2, RMS17 and RMS26 have suffered mutations that do not allow alkane bioconversion (e.g. damaged monooxygenase system or membrane transport mechanisms) or they simply kept a regulatory mechanism preventing simultaneous alkane and co-substrate utilization. Only mutant strain RMS5 was further studied.

4.2.2 Mutants obtained by gene disruption

It was not possible to detect formation of alcohols or diols by GC-MS analysis during the screening bioconversion experiments with the mutant strain RMS5. However, alkane depletion was observed during growth and similar cell densities where reached in the bioconversion and in control cultures without alkane, both containing an alternative carbon source (see figure 3.8). As the corresponding carboxylic acids were detected in culture broths from this strain, it was concluded that the long-chain alcohol dehydrogenase involved in alkane degradation was still active and alkanes could be further oxidized. Thus gene disruption of the known alcohol dehydrogenase gene (AlkDH) was performed in this mutant strain.

4.2.2.1 Gene disruption – AlkDHtKAT construction and transformation

Several challenges were faced in the construction of the truncated alcohol dehydrogenase DNA fragment (AlkDHtKAT) used for gene disruption. A double ligation of the AlkDH fragments in a linker ligation approach did not result in the expected product but in the AlkDH flanking regions ligated individually to the KAT gene. Possibly tertiary structural tension prevented the double ligation reaction. The AlkDH2-KAT and KAT-AlkDH3 products were amplified by PCR, purified and

successfully used as templates for an *incomplete polymerase sequence extension reaction* that generated the AlkDHtKAT construct used for the gene knock out.

In the work by Hashimoto *et al.* (2001) to disrupt the *trp E* gene in *Thermus thermophilus*, the DNA construct for the disruption was generated by inserting the target gene into a plasmid, from which after sub-cloning a fragment was excised to introduce a non-commercial kanamycin resistant marker, all favored by restriction sites in the *trp E* gene, convenient for a straight forward construction. With similar transformation results the DNA construct was generated using two-step PCR with homologous regions amplified directly from the strain genome using primers including a 20 bp common sequence to the kanamycin resistant marker that by overlapping with this gene allowed obtaining a complete construct from a subsequent PCR reaction.

Unfortunately, the restriction sites present in AlkDH were not suitable as to get flanking regions with sizes proposed by Hashimoto to target similar transformation efficiencies, and whenever a restriction site resulted in one flanking region, multiple or inexistent restriction sites made the other flanking region either to short or extremely long with the risk of still having and active AlkDH after recombination. Therefore, the construction of AlkDHtKAT was based on amplifying defined flanking regions by PCR as adopted by Fujiwara *et al.* (2006) but in a linker type ligation of these AlkDH flanking regions to the kanamycin resistance marker followed by *incomplete complementary sequence extension* that resulted in the targeted construct without the need of very long primers as those used by Hashimoto in their two-step PCR approach.

Transformation of AlkDHtKAT into the mutant RMS5 and the wild-type strain was performed with the construct as obtained, but also with the construct integrated into a plasmid. As reported by Hashimoto (2001), the transformation resulted in significantly higher number of kanamycin resistant clones when performed with the pUC19-AlkDHtKAT plasmid compared to using the AlkDHtKAT construct alone. The non-homologous regions in the plasmid might prevent DNA from being degraded by exonucleases in *Thermus sp.* ATN1 cells, enabling higher transformation/recombination efficiencies.

Interestingly, the sub-cloning of AlkDHtKAT (1.8 kb) into the cloning vector pUC19 resulted in an insert of only about 1.5 kb in the pUC19-AlkDHtKAT plasmid extracted from selected *E. coli* clones showing both, kanamycin and ampicillin resistance. The deleted region in the construct corresponds to the PslpA promoter integrated at the start of the KAT cassette used as reporter gene. It was not possible to amplify by PCR the KAT-AlkDH3 region of the construct from the plasmid isolated from the selected clones. A product from amplifying AlkDH2-KAT was obtained though. Several theories could explain this deletion: (a) The secondary structure within the AlkDH2-KAT portion of the insert might contain a hairpin loop section that was deleted after subsequent plasmid replication during clone cultivation caused by polymerase slippage and deleting bases on the KAT primers region (Viguera *et al.*, 2001); (b) the insert is unstable in *E. coli* DH5α as it may have been a substrate for recombinases (exonucleases) other than *rec*A, which is not present in this strain; (c) over-expression of the KAT cassette might be toxic for this strain and it responded by deleting a portion of the insert including the PslpA promoter.

4.2.2.2 Verification of genotype and phenotype of AlkDHtKAT-RMS5 mutants

Disruption of the AlkDH gene was successful (see 3.4). It was possible to observe kanamycin resistance in the RMS5 transformants (DG mutants) from which was not possible to amplify the AlkDH gene by PCR or, unlike the kanamycin resistance encoding gene (KAT), to detect this gene by southern blot hybridization. This indicated the KAT gene has been incorporated by homologous recombination affecting the chromosomal region that originally contained the AlkDH gene. It was however not possible to amplify the KAT cassette from genomic DNA extracted from these mutants with the primers originally used for PCR reactions during preparation of the AlkDHtKAT construct. It was assumed that a similar deletion to the one observed in the pUC19-AlkDHtKAT plasmid had occurred.

Kanamycin resistance indicated the KAT gene remained constitutively expressed in the selected mutants, either under the influence of the PslpA promoter or the AlkDH gene promoter. The later would be an indication that TADH is constitutively expressed in *Thermus sp*. ATN1 which is also the case for other long-chain alcohol dehydrogenases reported for strains able to degrade hydrocarbons such as *A. calcoaceticus* (Fox *et al.*, 1992) and *P. putida* (Gunt *et al.*, 1975).

The kanamycin resistant DG mutants were tested for aldehyde metabolites on mineral medium plates containing sodium pyruvate and 1-hexadecanol with the pararosaniline detection method (Conway *et al.*, 1987). Red coloration developed with all the screened mutants, indicating alcohol dehydrogenase activity. This phenotype contradicted the genotype screening results. This aldehyde detection method was most probably not discriminating for AlkDH gene disruption in this case. Kato *et al.*, 2001 reported that a high incubation temperature might result in an abiotic reaction causing background interference and false positive results. Subsequent TADH activity assay results (modified from Otto, 2001 and Hollrigl *et al.*, 2008) with crude extract from these mutants confirmed that TADH activity has decreased significantly (see 4.3).

4.3 Alkane dehydrogenase activity in *Thermus sp.* ATN1 mutants

Alcohol dehydrogenase activity in crude extract was tested for selected DG mutant cultures with 1-octanol as substrate, which was previously reported to be a suitable substrate for TADH (Hollrigl *et al.*, 2008). Test results showed significantly higher specific activity for the mutant strain RMS5 ($1.35x10^{-2}$ U/mg protein) compared to the tested transformant DG11 ($1.30x10^{-3}$ U/mg protein), indicating the AlkDH gene has been knocked out.

Again, and with the activity of TADH suppressed, it was not possible to detect formation of alcohols or diols during bioconversion screening experiments with the selected DG mutants. Moreover, long-chain alcohol dehydrogenase activity towards longer alkanols like 1-hexadecanol in crude extract experiments remained practically at the same specific level for the RMS5 mutant strain and the positive kanamycin transformants tested at around $1.0x10^{-2}$ U/mg (see 3.4.5).

Interestingly, Otto (2001) reported TADH activity towards long-chain 1-alkanols (C14 and C16) as quite low or undetectable. Hollrigl *et al.* (2008) reported TADH activities decreasing with alkane

chain increase for linear alcohols from 1-propanol (153%) up to 1-octanol (57%) in relation to that observed with cyclohexanol (100%). Activity results for recombinant/purified TADH reported in this work showed at least a 10 times higher specific activity of the enzyme with 1-octanol compared to 1-hexadecanol (8.97 vs. 0.87 U/mg).

These observations point to the conclusion that if TADH is involved in the degradation of long-chain n-alkanes in *Thermus sp.* ATN1, it is not the only enzyme capable to oxidize alcohols produced by this strain when growing on hydrocarbons. It is proposed the strain might possess another ADH showing specificity for long-chain 1-alkanols.

Existence of more than one alcohol dehydrogenase involved in alkane degradation has been reported for other species. Van Beilen *et al.* (1992) described the inducible ADH encoded by the *alk*J gene contained in OCT-plasmid in *P. putida* Gpo1 (*oleovorans*) as necessary for growth on alkanes only for mutants of the strain that lost constitutive chromosomal ADH activity, encoded in the *alcA* locus, confirming ADH redundancy previously described by Gunt *et al.* (1975). Fox *et al.* reported (1992) multiple alcohol dehydrogenase activities for *A. calcoaceticus* strain HOl-N. In this case the best characterized enzyme was an NADP+ dependent ADH that showed specific activity with long-chain substrates. Interestingly this dehydrogenase together with another also NADP+ dependent but more unstable ADH (not characterized) was accountable for about 90% of activity with 1-decanol as substrate whereas another NAD+ dependent ADH (not fully characterized) accounted for 10% of the activity in crude extract experiments prepared from cells grown on n-hexadecane. More recently Feng *et al.* (2007) reported the up-regulation of three putative ADH's in *G. thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir, when cells were grown on n-hexadecane, indicating expression of these ADH's is linked to alkane degradation.

The existence of another long-chain alcohol dehydrogenase in *Thermus sp.* ATN1 has not been confirmed.

4.4 Alkane bioconversion products by mutant strain RMS5

4.4.1 Fatty aldehydes (further oxidized to carboxylic acids)

As described in 3.5.3 aldehyde formation was qualitatively detected with the Purpald® reagent during growth of mutant RMS5 in alkane bioconversion experiments. Moreover, crude extract and resting cells experiments with 1-alkanols as substrates indicated ADH activity and formation of fatty aldehydes. These compounds were not detected by GC-MS and could not be isolated from culture broths. Certainly they were further oxidized under the experimental conditions (e.g. high temperature) either by the strain or abiotically.

Only fatty acids and esterified long-chain dicarboxylic acids (LDCAs) were identified and isolated from alkane bioconversion experiments with the mutant RMS5 (see 3.6). In contrast, LDCA's were not detected and fatty acids were found only at very low concentrations in bioconversion experiments with DG mutants.

This difference can be explained if involvement of TADH is considered in a preferred alkane uptake/degradation mechanism in the wild-type strain and assuming this pathway is defective at or before the aldehyde dehydrogenase step in the mutant RMS5 (e.g. product release by the membrane-bound TADH or transport into the cytoplasm). This results in the production of fatty aldehydes that can be further oxidized or that react with other cell products, like the biosurfactant produced by the strain and/or cell membrane components released outside the cell. Possibly LDCA's were formed because the terminal methyl group of the fatty aldehydes or fatty acids produced can also be exposed to the activity of alkane monooxygenase in *Thermus sp.* ATN1 at membrane level, implying this enzyme is a membrane-bound protein like the integral di-iron ω-hydroxylase *alk*B from *P. putida* Gpo1 (van Beilen *et al.*, 1994). This is also in line with the detection and isolation of mono and dicarboxylic acids from the cell-free floating material isolated from cultures of mutant RMS5 (see 3.6.2). On the other hand, AlkDH is not expressed in DG mutants and alkane degradation may occur via an ADH in the cytoplasm other than TADH (see 4.3) and subsequent β-oxidation, preventing formation of LDCA's and accumulation of fatty acids.

Assuming that similarly to the *alk*J ADH expressed by *P. putida* Gpo1 (van Beilen *et al.*, 1992), TADH is a membrane bound enzyme and its activity on larger substrates depends on its membrane binding, this explains why TADH did not show activity towards long-chain alcohols as isolated protein or in crude extract experiments.

One observation, which is in contradiction to the proposed alternative ADH is the fact that DG mutants are not able to grow on long-chain n-alkanes as sole carbon source. It could be argued that the degradation mechanism involving the proposed alternative ADH progresses at much lower rates compared to the route involving TADH. This would be supported by the lower alkane consumption levels observed with DG mutants compared to the wild-type strain and in contrast to the RMS5 mutant (figure 4.1). However, if similar alkane monooxygenase expression levels were obtained with RMS5 and DG mutants under comparable cultivation conditions, the step limiting conversion in the later would be the ADH step and it should result in the accumulation of alcohol intermediates, which was not the case. To reconcile this observation, it could be theorized that product repression limits the monooxygenase activity in *Thermus sp.* ATN1, thus accumulation of alkanols is not possible (see 1.3.2.3.2). Nevertheless the evidence for an alternative ADH is limited (except for the low activity from TADH with long-chain alcohols).

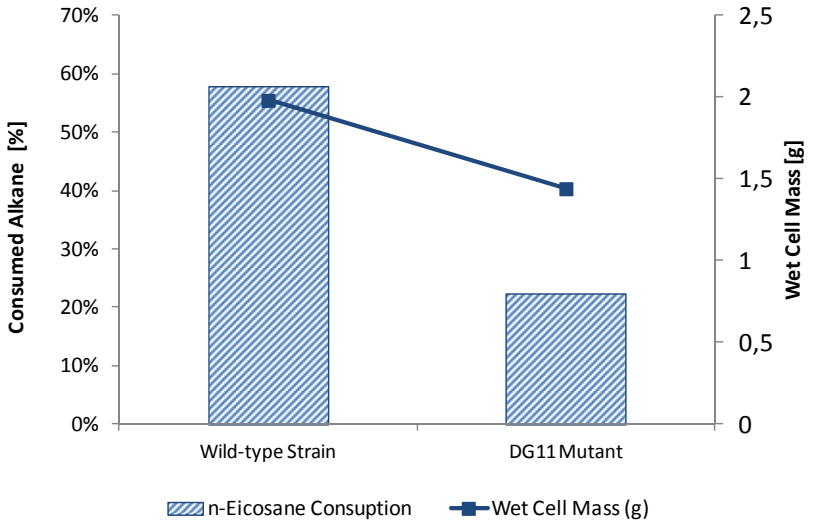


Figure 4.1 - Alkane consumption by the wild-type strain and DG11 mutant (AlkDH blocked) in relation to wet weight of harvested cells from experiments in mineral media with n-eicosane and sodium pyruvate as co-substrate for NADH regeneration.

4.4.2 Carboxylic acids by substrate over-oxidation

The absence of fatty alcohol products with the constructed mutants can be also and better explained if the alkane hydroxylase in *Thermus sp.* ATN1 is capable to over-oxidize alkanes to carboxylic acids. Over-oxidation is a known issue with monooxygenases. Several of them catalyze multiple oxidations of hydrocarbon substrates, which is a problem if a specific alcohol is the desired product. Such multiple oxidation activities may be due to a low (regio) specificity of the oxygenase resulting in oxidation at multiple sites or in over-oxidation of an alcohol product to the corresponding aldehyde, ketone, or acid. The later can occur if the primary product, the alcohol, can also bind as a substrate or is not released from the active site. Thus this mechanism cannot prevent over-oxidation (van Beilen *et al.*, 2003; Bühler & Schmid, 2004).

Some reports exist on over-oxidation of alkane substrates by monooxygenases. Scheller *et al.* (1998) reported the conversion of n-hexadecane to 1,16-hexadecanedioic acid catalyzed by the alkane monooxygenase system of the alkane degrader *Candida maltose* reconstituted from recombinant cytochrome P450 52A3 and the corresponding NADPH-cytochrome P450 reductase and without the action of an alcohol oxidase. Shet *et al.* (1996) reported oxidation of dodecanoic acid to the corresponding diacid with a purified recombinant fusion protein containing P450 4A1 and NADPH-P450 reductase.

Bacterial non-heme di-iron enzymes have also been reported to catalyze multiple oxidations and to over-oxidize alkane substrates. The alkane ω-hydroxylase from *P. putida* GPo1 was reported to catalyze the oxidation of terminal alkanols to alkanals (May & Katopodis, 1986). More recently, Grant *et al.* (2011) have reported the heterologous expression of this enzyme in *E. coli* performed

to increase total hydroxylase activity and yield of 1-dodecanol from n-dodecane, but the results indicated high over-oxidation of the alcohol and the acid was obtained in an 11:1 ratio to the preferred product, despite the fact that no alcohol dehydrogenase was involved in the biotransformation.

As in the case of many other monooxygenases it can be assumed that the alkane hydroxylase in *Thermus sp.* ATN1 has also the ability to oxidize its substrates further, especially if their interaction with the active site is sufficiently long and sufficient NADH is available for the reactions.

How does over-oxidation occur with the selected mutants in this work? Having lost the ability to grow in alkanes as sole carbon source, but being capable to convert and consume the alkanes in the presence of a co-substrate, these mutants must not have an active NADH regeneration mechanism when no other carbon source is present, as NADH required for the first step of the alkane catabolic pathway. The dehydrogenase step in this pathway (see figure 1.11) is defective (as could be for the RM5 strain) or is already blocked (as in DG11 strain). However, the catabolism of other substrates, through other pathways, makes NADH available for further oxidation reactions resulting in carboxylic acids (figure 4.2) that can be further catabolized by the β-oxidation pathway. This provides a solid explanation to the conversion and consumption of alkanes observed with mutant RMS5.

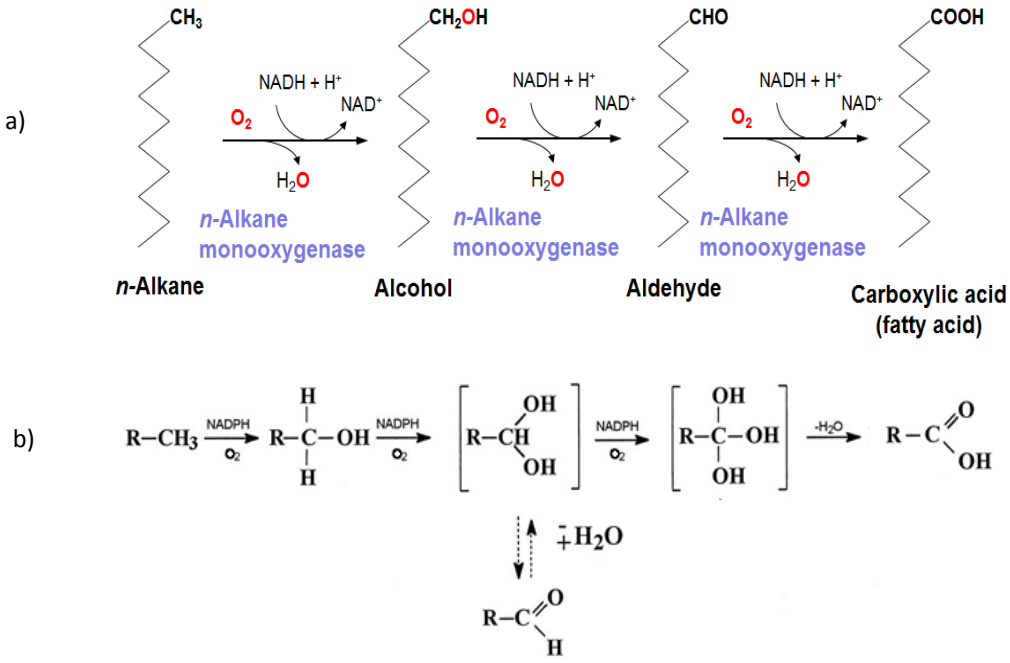


Figure 4.2 - Over-oxidation mechanism of n-alkanes by alkane monooxygenases provided sufficient reducing cofactor is available: (a) as proposed for *Thermus sp.* ATN1 mutants; (b) for a soluble Cytochrome P450 oxygenase (modified from Scheller et al.,1998).

It is not strange that these monooxygenases can introduce more than one oxygen atom at the same methyl group if the conditions are provided. After all, hydrogen atoms bound to the carbon carrying already the hydroxyl function are expected to be more reactive than those at neighboring methylene or other methyl groups.

4.4.3 Outlook for long-chain alkanols and α,ω - diols with *Thermus sp.* ATN1

It can be concluded that over-oxidation by the alkane hydroxylase complex has resulted in the formation of carboxylic acids, therefore, production of alkanols or diols with mutants of *Thermus sp.* ATN1 is not possible by simply disrupting ADH encoding gene(s) or by classical random mutagenesis in this strain.

It is remarkable that bacterial production of alkanols and α,ω-diols from n-alkanes has been only reported with recombinant monooxygenases. The hosts that have been employed harbor either the alkane hydroxylase system from *Pseudomonas putida* Gpo1 (Bosetti *et al.*, 1992; Grant *et al.*, 2011), the Cytochrome P450 monooxygenase complex from *Acinetobacter sp.* OC4 (Fujii *et al.*, 2006) and recently the long-chain alkane monooxygenase LadA from *Geobacillus thermodenitrificants* NG80-2 (Feng *et al.*, 2007; Dong *et al.*, 2012). With this approach expression of the alkane hydroxylase system is not repressed whilst the host grows on an alternative carbon source as no regulatory mechanism exists. Thus the effort needed to manipulate regulatory mechanisms is eliminated. Further oxidation and catabolism of the alcohol intermediates by the parent strain has been somehow prevented, since the necessary ADH's are not expressed (Bosetti *et al.*, 1992). In addition, over-expression of the monooxygenases has resulted in higher yield of the alcohol products, but particularly relevant is the fact that over-oxidation of the alcohol product to the carboxylic acid seems unavoidable and it is increased at higher expression levels as in the work reported by Grant *et al.*, 2011.

It is unclear how effective the recombinant approach is compared to block the through-conversion of the desired intermediate by mutagenesis of the wild-type strain for the purpose of obtaining long-chain alcohols. No other reports are available that describe the blockage of a long-chain ADH with the purpose of this thesis. However it has been discussed (A. Schmid, R. Müller, 2012) that other research groups have tried to obtain alcohol intermediates also by disrupting ADH's with little success, most probably for the same or similar reasons why it has not been possible to obtain them with *Thermus sp.* ATN1 mutants.

Still, if at the expense of over-oxidation, application of *Thermus sp.* ATN1 or its enzymes (or other alkane degraders) would be contemplated an alternative to obtain alcohols or diols, some aspects need to be addressed.

It should be considered that heterologous expression, especially in the case of membrane-bound enzymes, may lead to lower turnover numbers (k_{cat}) of the recombinant enzyme compared to the native form, requiring higher expression levels in the host strain to obtain comparable specific whole-cell activity like that in the wild-type strain. Duetz *et al.*, 2001 suggested this may be due to different membrane properties of the artificial host. As an example, Staijen *et al.*, 2000 reported the heterologous expression of the alkane hydroxylase complex from *P. putida* Gpo1 in *E. coli* W3110 at a maximal specific activity of 30 U/g dry w, which was practically identical to the specific activity of the wild-type strain despite the fact that the expression level of the enzyme was six fold higher in the recombinant host.

On the other hand, heterologous expression of the monooxygenase may be the only viable option if the wild-type strain does not grow well on minimal media, is a suspected or known pathogen, or can't be engineered either to avoid product consumption (van Beilen *et al.*, 2003) or to avoid repression of the hydroxylation step by one or more regulatory mechanisms. In addition, alkanols may be toxic for the cells when accumulated. For instance, an inhibitory effect of oxidized n-alkane derivatives on the expression of alkane hydroxylase *alk*M in *Acinetobacter baylyi* ADP1 has been noticed (Wentzel *et al.,* 2007) and antibacterial activities of long-chain alcohols have been studied extensively (Togashi *et al.,* 2007; Kabelitz *et al.,* 2003; Kubo *et al.,* 1995; Kato *et al.,* 1980).

Moreover, other challenges like strain sensitivity to hydrocarbons or toxicity of products (alkanols) can be addressed with more suitable hosts, e.g. tolerant to hydrocarbons solvents (Heipieper *et al.,* 2007).

Thus, recombinant expression of the alkane monooxygenase from *Thermus sp.* ATN1 or increased expression of this enzyme in ADH blocked mutants derived from the wild-type strain (self-cloning) could be alternatives to observe formation of long-chain mono and di-alcohols, but predominantly carboxylic acids. It is possible though that the alkane activation with this thermophilic monooxygenase at higher expression levels does not allow at all the production of alcohols from hydrocarbons unless the monooxygenase is engineered, but even then, enzyme turnover could be seriously compromised.

Further work to explore further possibilities with this strain or its mutants must involve the identification, sequencing and sub-cloning of the genes encoding the alkane hydroxylase function to promote its over-expression either in *Thermus* sp. ATN1, as done with *C. tropicalis* (Picataggio *et al.*, 1992) or in an heterologous host, together with the blockage of further catabolic steps to prevent product consumption, e.g. β-oxidation if LCDAs are to be obtained.

4.5 Production of LCDAs by mutant strain RMS5

As described (3.5 and 4.4), long-chain alkanes were converted by mutant RMS5 to the corresponding mono and dicarboxylic acids, possibly via aldehyde unspecific oxidation and/or over-oxidation. As introduced in 1.2.2, LCDAs are important industrial chemicals and this study has revealed that *Thermus sp.* ATN1 could be an alternative to produce higher LCDAs through biotransformation.

In this work the production of long-chain α,ω-diacids up to a chain length of 28 carbon atoms by bioconversion of the corresponding aliphatic hydrocarbons has been demonstrated. This is the first time that the bioconversion of aliphatic hydrocarbons of chain lengths with more than 16 carbon atoms with bacteria to mono and dicarboxylic acids is reported.

Figure 4.3 compares the results obtained with the mutant strain RMS5 for the biotransformation of alkanes to LCDAs under aerobic conditions as presented in section 3.9 with other microbial

studies referenced in table 1.4 and in section 1.3.4. From the latter only the reference by Picataggio *et al.* (1992), utilizing *C. tropicalis,* is known to have reached industrial scale production in China in a process operated by Cathay Industrial Biotech and developed by Cognis (Schörken & Kempers, 2009).

It is clear that results for production of larger LCDAs in shaking flask experiments with the mutants constructed during this work do not compete with the figures reported for DC14 production developed by Cognis. Nevertheless, several observations indicate that there is great potential for *Thermus sp.* ATN1 for production of LCDAs at levels that could compete with the *C. Tropicalis* based process to obtain the DC16 diacid and possibly lower diacids (DC12-DC15) as well as to broaden the spectra of commercially available LCDAs:

- Under aerobic conditions, the alkane consumption figures for the RMS5 mutant compared to cumulated acid products indicate that the later were also consumed during the bioconversion experiments via β-oxidation. For instance, n-octadecane consumption in shaking flask experiments reached 59% of the added substrate, whereas recovered mono and diacid products indicated a molar conversion of about 10.3% (see figure 3.49). In another example, consumption of n-octadecane reached 86% in only 36 h in an experiment where active aeration was provided (see figure 3.48). It is assumed that if the β-oxidation pathway is completely blocked in this mutant, molar conversions close to 100% can be obtained with longer incubation times and active aeration as in the case of *C. tropicalis* mutant H5343 where incubations were run for up to 160 h to obtain DC14 (reference 3 in table 1.4).

- It has been demonstrated that *Thermus sp.* ATN1 is suitable for strain engineering as it is capable to transform and recombine exogenous DNA. It should be possible to introduce multiple copies of the alkane hydroxylase complex encoding gene(s) to increase its expression level and the specific cell activity for long-chain alkanes, leading to increased LCDA specific productivities as in the case of *C. tropicalis* mutant AR40 (reference 5 in table 1.4). In addition, integration of an antibiotic resistance into the strain would provide unique characteristics for process control.

- Hill *et al.* (1986) described for *C. Tropicalis* mutant 7/34 that alkane conversion was observed increasing with decreasing substrate carbon chain length (reference 2 in table 1.4). Similarly alkane consumption with RMS5 was higher with shorter alkanes. With both strains, conversion efficiency and specific productivity were on a similar level with n-hexadecane as substrate, despite quite different total yield and productivity figures. It would be expected that with the mutations proposed above, *Thermus sp.* ATN1 would reach much higher conversions and productivities with n-hexadecane and shorter substrates, which may exceed what is achievable with the best engineered *C. tropicalis* AR40 reported by Picataggio *et al.* (1992).

- Commercial LCDA production is performed with engineered *C. tropicalis* only in China. This microorganism is classified as pathogenic in Europe and other countries (Huf *et al.,* 2011). Utilization of this microorganism would require a high security standard for an industrial process, making it difficult to establish and not competitive. *Thermus sp.* ATN1 does not suffer from this drawback.

- Process development for this thermophilic strain would offer some advantages (see 1.4) including less sensitivity to contamination at high process temperature, shorter incubation times due to higher reaction rates and simpler downstream processing as unconverted substrate can be easily separated at lower temperatures and as products are not soluble in water.

- The range of possible LCDA products from saturated hydrocarbons with this thermophile is much wider than that possible with mesophilic microorganisms. Solid hydrocarbons are not easily accessible to the microbial cells for uptake and catabolism and therefore not suitable for a biotransformation process. With *C. tropicalis* the substrate carbon chain length with paraffins is limited to 18 carbon atoms, since the melting point of n-octadecane is around 30 °C and its solubility is already quite low at the cultivation temperature of 32 °C. With cultivation temperatures close to 70 °C the carbon chain length can be of up to 32 carbon atoms as n-dotriacontane has a melting point at 69 °C. In addition, also larger non-natural long-chain mono-carboxylic acids could result as a valuable product.

- Availability of larger LCDAs would open new possibilities for existing and novel applications including new types of polymers (e.g. nylons), among others (see 1.2.2.1). This would incentivize research efforts to develop this novel process for the production of larger LCDAs.

- Long-chain mono and dicarboxylic acid production with an engineered *Thermus sp.* ATN1 mutant would not be limited to bioconversion of mineral oil or natural gas derived products, but also suitable to upgrade natural oil derived fatty acids (see figure 4.3). Having a process at high temperature would also be advantageous if the carboxylic acids are to be further converted by standard industrial processes to the respective alcohols or amines as such processes run normally at high temperatures.

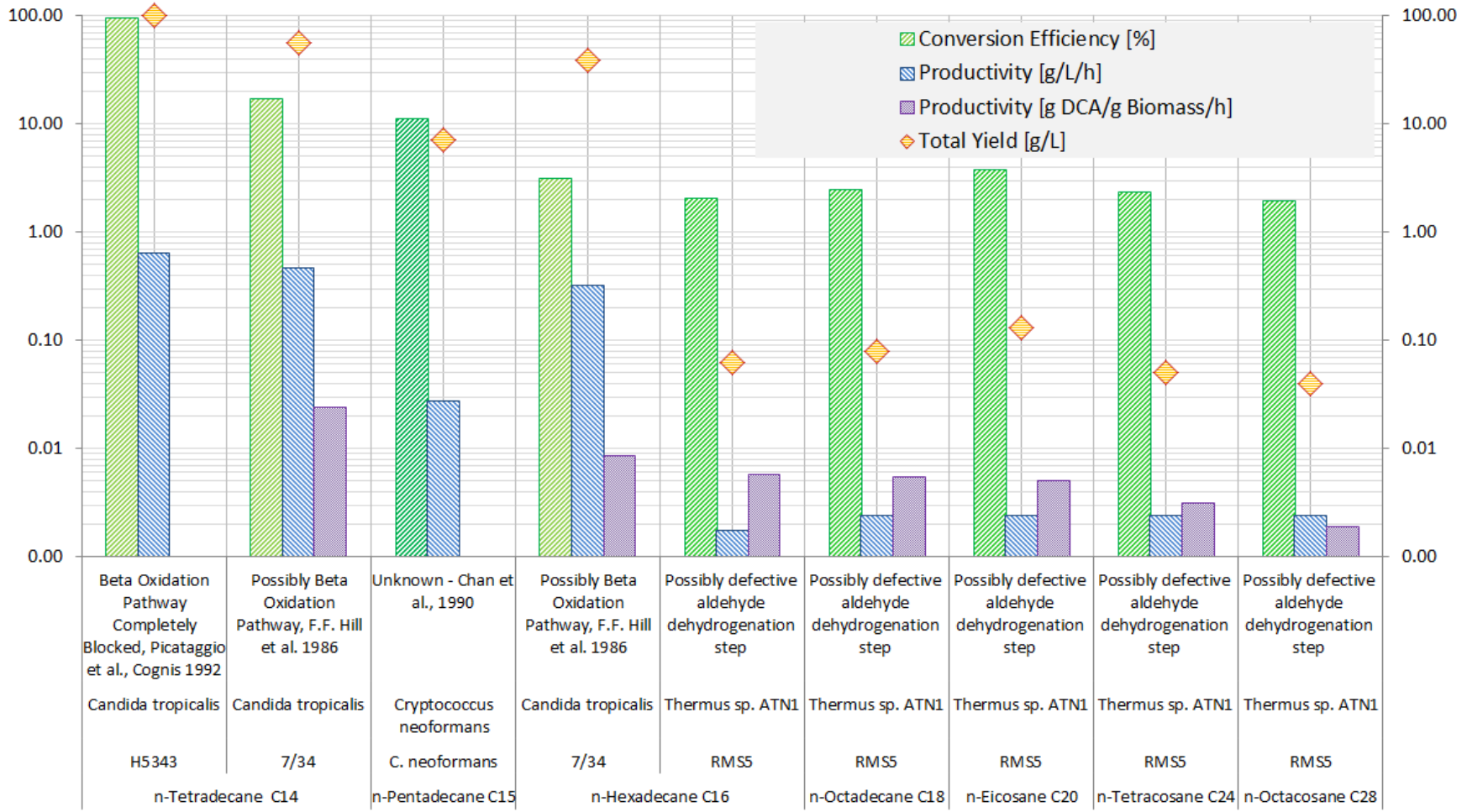


Figure 4.3 - LDCA production figures for bioconversion of long-chain n-alkanes with other microorganisms in comparison to figures obtained with mutant RMS5.

One limitation for process development with *Thermus sp.* ATN1 is the substrate concentration in the culture broth. Experiments in loose cap shaking flasks showed that higher alkanes and lower concentrations are preferred as both have less impact on cell growth which favors higher molar conversion for each particular substrate (see figures 3.43 and 3.44). However even the highest alkane concentration tested in this study remains a limitation and impacts greatly bioprocess economics (see 1.2.2.5.2) as it results in a low product concentrations as seen in figure 4.2.

The toxic effect of hydrocarbons as a second liquid phase was also reported for the production of 1-octanol from n-octane with *P. oleovorans* by Schmid *et al.* (1998) and octanoic acid from n-octane with a recombinant *E. coli* strain by Favre-Bulle *et al.* (1993) and several related studies. As proposed by Bühler & Schmid (2004), substrate toxicity can be addressed by controlled substrate addition and by in-situ product removal such as: solid phase extraction, two-liquid phase concept or membrane-mediated product extraction. For solid phase extraction, immobilization via adsorption onto polymeric matrices such as ion-exchange or hydrophobic resins has been demonstrated for a large variety of compounds including antibiotics and fine chemicals (Bühler & Schmid, 2004). Immobilized cells have been investigated for continuous production of DC-15 with *Cryptococcus neoformans* where the diacid could be continuously removed from the culture broth and yield could be significantly increased (see 1.3.4). Thus several alternatives exist to explore the possibilities to use *Thermus sp.* ATN1 for the production of valuable LCDAs from renewable and mineral oil & gas resources (figure 4.4).

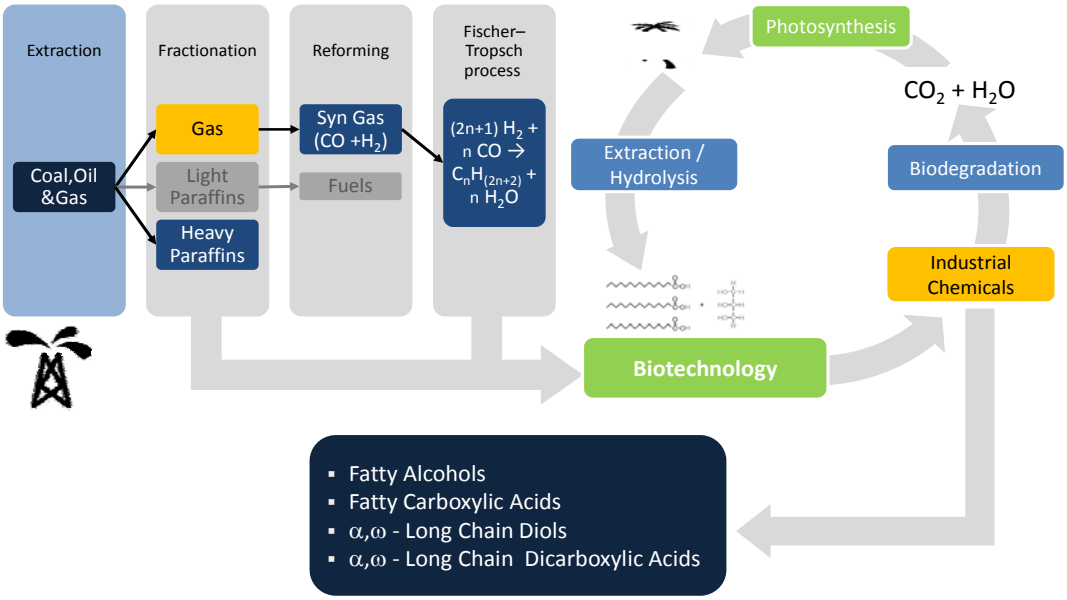


Figure 4.4 - Biotechnological approach to produce α,ω-oxidized chemicals from long-chain n-alkanes and natural derived fatty acids.

4.6 Biosurfactant production by *Thermus sp.* ATN1 and its mutants

Biosurfactant production is a common feature in alkane degrading bacteria and many species have been reported to produce surfactants while growing on hydrocarbons (Ron & Rosenberg 2002). *Thermus sp.* ATN1 and its mutants also produced surface active substances during cultivation in mineral media with n-alkanes (see 3.7.1). Production levels were monitored with the MBAS method. The oily and soapy film observed during these experiments was not present in control cultures without alkanes and the corresponding MBAS absorbance values were below the lowest levels observed in bioconversion cultures. Therefore and as observed by Otto (2001), it is proposed that the surfactant production is associated with hydrocarbon metabolism in this strain.

Production levels of biosurfactant by the wild-type strain were found the highest in the late stationary phase. A similar behavior was observed with the tested mutants but at higher levels and particularly distinctive was the high production levels by the AlkDH blocked mutant DG11 (see figure 3.34). Overproduction of biosurfactants during the stationary phase has been described for various *Pseudomonas spp.* (Desai & Banat, 1997) and overproduction by mutants of *P. aeruginosa* generated by random mutagenesis have been also reported (Tahzibi *et al.,* 2004). Several authors have highlighted the possibility to utilize bacteria overproducing biosurfactants to take advantages of these compounds over chemical surfactants (see 1.5), including bioremediation applications (Ron & Rosenberg, 2002).

An indirect indication of biosurfactant production was observed during off-line infrared measurements of culture broth samples (see figure 3.39). These measurements showed that, as the incubation progresses, alkane dispersion appears to improve in the media, since the intensity of bands at 1470, 1380 cm^{-1} increased. These bands are typical for aliphatic hydrocarbons and this behavior would indicate an increase in their apparent solubility in the bioconversion medium that can be explained by the effect of the biosurfactant. This observation also hints to substrate specificity, a feature of several high molecular weight bioemulsifiers (Ron and Rosenberg, 2002).

4.6.1 Biosurfactant composition and physiological role

In comparison to SDS, a larger molecular size for this new biosurfactant was expected. Lower MBAS absorbance readings were obtained for the purified biosurfactant solutions with equal weight concentrations of SDS indicating a higher molecular weight than that of SDS.

The materials isolated as biosurfactant from bioconversion cultures was partly characterized. Sugars and acylglycerols where found as building blocks in this substance(s) in the crude and purified material. In crude material, phosphate was found by GC-MS analysis. The presence of phosphorous was confirmed by elemental analysis. The purified biosurfactant(s) can be classified as glycolipid(s), but the crude material also contained phosphate esters from these or similar glycolipids. The presence of these type compounds in *Thermus sp.* ATN1 could be expected. Yang *et al*. (2004; 2006) and other authors have studied glycolipids and phosphoglycolipids from *Thermus spp.* and other thermophilic bacteria and suggested that their structure, composition and abun-

dance in the cell membrane is related to their biological role in ensuring the thermal stability of the cellular membrane in these microorganisms.

It has not been determined if the biosurfactant(s) from *Thermus sp.* ATN1 form originally part of the cell membrane. However it can be theorized that at least the phosphoglycolipids found in the crude material could have originated from an unstable cell membrane as a result of the interaction with a non-polar phase like the alkanes in the medium. Since the crude material, where phosphates were also detected, was obtained from a single solvent extraction step and floating material between the solvent and aqueous phases during the extraction was also collected with the solvent fraction.

Interestingly, the crude material was only obtained as such from mutant RMS5 bioconversion cultures and not from the AlkDH gene disrupted mutant DG11 from which the purified form was obtained. FTIR comparison of both materials showed some differences (figure 3.42-a) that can be possibly explained by the presence of the phosphorylated glycolipids. On the other hand, FTIR material comparison of floating solids (3.6.2) vs. the crude biosurfactant (figure 3.42-b) showed some similarities. It is proposed that partly oxidized alkane molecules (e.g. aldehydes) could have reacted with cell membrane components like complex phosphoglycolipds resulting in the unstable membrane and the floating solids and the phosphated compounds detected in crude biosurfactant observed only in the RMS5 bioconversion cultures.

In the case of the glycolipids isolated as pure biosurfactant, it is also possible that they are overproduced by the strain to maintain membrane stability or reconstitute it while growing on hydrocarbons. At the same time these compounds could contribute to the strain survival as they can facilitate hydrocarbons uptake (see 4.6.3). This could also explain why higher production levels where observed during the stationary phase, because membrane disruption of dying cells may contribute to the surface active substances determined by the MBAS method.

4.6.2 Biosurfactant characterization

As reported in section 3.7.1, characterization of this biosurfactant was performed with methods modified from Cirigliano and Carman (1984, 1985) and in comparison to commercial surfactants. It was surprising that compared to SDS and Triton X-100 the purified biosurfactant from *Thermus sp.* showed higher emulsification activities at both tested temperatures while the emulsion stability with n-hexadecane showed the poorest behavior at 20 °C and just comparable to SDS at 50 °C.

Both, high emulsification activities towards n-hexadecane and poor emulsification stability at 20 °C can be well explained from the chemical composition of the isolated biosurfactant. It is expected that the acyl hydrophobic moieties provide high specificity of this biosurfactant for aliphatic hydrocarbons resulting in higher emulsification activities compared to the other tested surfactants. Poor emulsification stability can be explained by agglomeration that can be predicted for higher hydrocarbons at lower temperatures resulting from Van der Waals forces in the interactions between n-hexadecane and/or biosurfactant molecules.

4.6.3 Increasing hydrocarbon bioavailability by addition of the biosurfactant from *Thermus sp.* ATN1

Results in section 3.7.3 clearly illustrate the effect of the biosurfactant from *Thermus sp*. ATN1 and SDS on increased hydrocarbon bioavailability for the growth of *Pseudomonas sp.* strain 273. Significant n-hexadecane degradation and cell growth were observed at early incubation stages for the cultures where either the biosurfactant or SDS were added.

Growth rate was higher in the medium with the alkane dispersed by SDS, but alkane degradation rate was lower than that for the culture to which the purified biosurfactant had been added as dispersant. The detection of dodecanoic acid by GC-MS analysis of samples obtained from the SDS containing culture supported the explanation that the bacteria also used SDS as carbon source.

Surfactants can enhance bioavailability, and if biodegradable, they may serve as a primary substrate for a pollutant that can be then co-metabolized, as it appeared to be the case for the SDS containing culture. In this sense, biodegradability of the surfactant may result in limited biodegradation of the hydrocarbons if the surfactant is preferentially degraded or if as a result essential nutrients are depleted. In addition, rapid degradation of the biosurfactant will reduce its bioavailability-enhancing effects (Parales *et al.*, 2008; Volkering *et al.*, 1998)

It is unclear if the biosurfactant from *Thermus sp*. ATN1 also served as carbon source for *Pseudomonas sp.* strain 273*,* but the differences with the SDS containing culture in alkane degradation and cell growth rates suggest that if this was the case, it was not a preferred substrate.

In the work by McKew *et al.* (2007), addition of a biosurfactant or nutrients produced no effect in hydrocarbon degradation experiments at early stages of cell growth. However greater degradation at early stages was observed in experiments where both nutrients and biosurfactant were supplemented suggesting that not only nutrients are a limiting factor, but that degradation is co-limited by hydrocarbon bioavailability. In the nutrient-only experiments by McKew *et al.* (2007) degradation of n-alkanes increased after several days to levels similar to experiments amended with both nutrients and bioemulsifier, possibly because the degrading bacterial community produced their own surfactants resulting in increased hydrocarbon bioavailability.

In our case, the control culture containing only the alkane as carbon source exhibited little depletion at the end of the incubation and showed limited cell growth. It is known that this strain can also grow on n-alkanes as sole carbon source (Wischnak *et al.*, 1998) but in this experiment alkane limited bioavailability combined with oxygen limitation (sealed flasks) could have delayed alkane uptake.

By increasing hydrocarbon bioavailability by addition of either the biosurfactant or SDS, the lag phase of microbial growth was shortened and alkane degradation accelerated. This demonstrates that the biosurfactant produced by *Thermus sp.* ATN1 could be used as a bioavailability enhancer for hydrocarbon biodegradation applications involving also mesophilic microorganisms.

5. Summary

Selective and efficient oxidation/hydroxylation of hydrophobic substrates such as mineral oil hydrocarbons remain an attractive route for production of high value chemicals. Long-chain alcohols and dicarboxylic acids (LCDAs) are important industrial chemicals mainly used as intermediates for additives applied in valuable products like cosmetics, washing detergents, paints, coatings, lubricants and polymers. These chemicals are industrially produced either from petro-chemical or renewable feed-stocks generally by chemical processing and only a reduced range of products is available. Biocatalysis based production of these chemicals has mainly remained at a research/exploratory stage.

A limited number of biocatalysts oxidizing long-chain n-alkanes have been studied. Typically, hydroxylation reactions have been studied only for the formation of mono-alcohols whereas terminal oxidation has involved formation of mono and dicarboxylic acids from aliphatic hydrocarbons with up to 18 carbon atoms.

In an attempt to produce α,ω-hydroxylated/oxidized compounds from long-chain n-alkanes, mutants of the thermophilic bacterium, *Thermus sp.* ATN1, capable to degrade these compounds at 70°C by terminal oxidation, have been constructed by classical random mutagenesis. Selected mutants were not capable of growing on alkanes as sole carbon source. To determine whether these mutants contained a disrupted alkane catabolic pathway, they were screened for alkane bioconversion metabolites in mineral media supplemented with an alternative carbon source. In contrast to the wild-type strain, these mutants converted alkanes constitutively. It was possible to detect aldehyde intermediates and to isolate long-chain mono and dicarboxylic acids from the bioconversion broths.

In addition, the gene AlkDH encoding the alcohol dehydrogenase (TADH) suspected to be involved in the long-chain alkane's metabolism in this strain was truncated with a kanamycin resistance gene by homologous recombination in the random mutants. Significantly reduced TADH activity was observed with 1-octanol in the crude extract obtained from these AlkDH gene truncated mutants (AlkDHtKAT, DG11 strain) in comparison to crude extract activity from the random mutants (RMS5 strain), indicating that AlkDH was effectively disrupted. However, α,ω-hydroxylated products were not obtained and further oxidation of 1-hexadecanol was still observed in the crude extract from AlkDHtKAT mutants. Either over-oxidation of the alcohol products by the alkane monooxygenase complex in this strain or the presence of another alcohol dehydrogenase could be responsible for these results. Substrate over-oxidation is proposed as the best explanation for the observed results. Over-oxidation is a known issue in the application of monooxygenases and, in the case of the mutants constructed in this study, it indicates that complete terminal oxidation

reactions are favored over hydroxylation reactions making the production of alcohol products by the described approach practically impossible.

On the other hand, the constructed mutants from *Thermus sp.* ATN1 are capable to produce α,ω-oxydized products from long-chain n-alkanes. In this work the production of long-chain α,ω-diacids (LCDAs) with a carbon chain length of up to 28 carbon atoms by bioconversion of the corresponding aliphatic hydrocarbons has been demonstrated. Saturated substrates with up to 32 carbon atoms or more can be converted by these mutants. This is the first known report on bioconversion of aliphatic hydrocarbons of chain lengths larger than 18 carbon atoms to mono and dicarboxylic acids with a thermophilic bacterium.

The broad range of LCDA products obtained with these mutants could be produced for new types of polymers, adhesives, lubricant additives, pharmaceuticals and other novel applications.

LCDAs have been obtained under aerobic conditions in shaking flask experiments with conversion and productivity levels interesting for further study. An indication of optimal culturing conditions to maximize growth/substrate conversion in terms of aeration, co-substrates and working concentrations is provided by this work. Analytical methods have also been developed or adapted to monitor the bioconversion as well as the production of a biosurfactant that *Thermus sp*. ATN1 produces when growing on hydrocarbons.

As basis for further development work, it has been also demonstrated that the strain can be engineered as it can naturally transform and incorporate exogenous DNA. Thus, LCDAs productivity could be enhanced by blocking the β-oxidation pathway and introducing multiple copies of the putative long-chain alkane monooxygenase complex in the wild-type strain or in the mutants constructed in this study, as it has been done with *Candida sp.* for the production of LCDAs. Hydrolysis products from natural fats and oils could also be used as substrates.

In addition, *Thermus sp.* ATN1 offers several advantages over other microorganisms for the production of LCDAs. The strain is non-pathogenic and its thermophilic nature provides unique characteristics for process control to avoid culture contamination. The integration of an antibiotic resistance into the strain has been also demonstrated.

Finally, the production of a biosurfactant by this thermophilic strain and its mutants as well as the isolation and characterization of the biosurfactant are described. This biosurfactant showed advantages over commercial surfactants like SDS and Triton X-100 in the characterization tests, especially at high temperatures. The use of this biosurfactant as hydrocarbon bioavailability enhancer was demonstrated in the utilization of n-hexadecane by a mesophilic strain.

6. References

Anastas, P.T. and Warner, J.C., 1998. Green Chemistry: Theory and Practice, Oxford University Press, New York.

Anastas, P.T. and Zimmerman, J.B., 2003. Design through the twelve principles of green engineering. Environ. Sci. Technol., 37(5), 95A-101A.

Aparna, A., Srinikethan, G. and Hegde, S., 2011. Effect of addition of biosurfactant produced by *Pseudomonas sps*. on biodegradation of crude oil. 2nd International Conference on Environmental Science and Technology, IPCBEE, IACSIT Press, Singapore, 6, 71-75.

Baptist, J.N., Gholson, R.K. and Coon, M.J., 1963. Hydrocarbon oxidation by a bacterial enzyme system: I. Products of octane oxidation. Biochim. Biophys. Acta, 69, 40-47.

Barkay, T., Navon-Venezia, S., Ron, E.Z. and Rosenberg, E., 1999. Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier Alasan. *Appl. Environ. Microbiol*., 65(6), 2697-2702.

Bell, G.H., 1973. Solubilities of normal aliphatic acids, alcohols and alkanes in water. Chem. Phys. Lipids, 10(1), 1-10.

Borjana, K.T., George, R.I., and Nelly, E.C., 2002. Biosurfactant production by a new *Pseudomonas putida*. Z. Naturforsch., 57, 356-360.

Bosetti, A., van Beilen, J.B., Preusting, H., Langeveen, G and Witholt, B.,1992. Production of primary aliphatic alcohols with a recombinant *Pseudomonas* strain, encoding the alkane hydroxylase enzyme system. Enz. Microb. Technol., 14(9), 702-708.

BP Statistical Review of World Energy, June 2010.

Brackmann, B., Cognis GmbH; Hager, C.D., Sasol GmbH.,2004. The statistical world of raw materials, fatty alcohols and surfactants. CD Proceedings 6th World Surfactant Congress CESIO, Berlin Germany, June 21-23; Paper 112.

Briton, L.N., 1984. Microbial degradation of aliphatic hydrocarbons in: Microbial degradation of organic compounds. Gibson, D.T., Marcel Dekker, New York, 89-129.

Bühler, B. and Schmid, A., 2004. Process implementation aspects for biocatalytic hydrocarbon oxyfunctionalization. J. Biotechnol., 113, 183–210.

Cameotra, S.S. and Singh, P., 2009. Synthesis of rhamnolipid biosurfactant and mode of hexadecane uptake by *Pseudomonas species*. Microb. Cell Fact., 8:16.

Casali, N. and Preston, A., 2003. *E. coli* plasmid vectors: methods and applications. Humana Press, New Jersey.

Cathay Industrial Biotech, 2011. Initial Public Offering, IPO S-1 filing form with the US Securities and Exchange Commission, SEC to enter the NASDAQ stock exchange.

Cathay Industrial Biotech Website, 2011. http://www.cathaybiotech.com/en/products/dibasic.

Chan, E-C. and Kuo, J., 1997. Biotransformation of dicarboxylic acid by immobilized *Cryptococcus* cells. Enzyme Microb. Technol., 20 (8), 585-589.

Chan, E-C., Kuo, J., Lin, H-P. and Mou, D-G., 1990. Stimulation of n-alkane conversion to dicarboxylic acid by organic-solvent- and detergent-treated microbes. Appl. Microbiol. Biotechnol., 34(6), 772-777.

Chen, Q., Janssen, D.B., and Witholt, B., 1995. Growth on octane alters the membrane lipid fatty acids of *Pseudomonas oleovorans* due to the induction of *alk*B and synthesis of octanol. J. Bacteriol., 177, 6894-6901.

Chen, Q., Janssen, D.B., and Witholt, B., 1996. Physiological changes and *alk* gene instability in *Pseudomonas oleovorans* during induction and expression of *alk* genes. J. Bacteriol., 178, 5508-5512.

Chemical Market Associates, Inc., CMAI, 2010. Long-chain dicarboxylic acids market report.

China Chemical Reporter, CCR, 2011. Long-chain dicarboxylic acids market outlook.

Christie, W.W., 1993. Preparation of ester derivatives of fatty acids for chromatographic analysis in: Advances in Lipid Methodology-Two. Ed. W.W. Christie, Oily Press, Dundee

Cirigliano, M.C., and Carman, G.M., 1984. Isolation of a bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol., 48(4), 747-750.

Cirigliano, M.C., and Carman, G.M. ,1985. Purification and characterization of Liposan, a bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol., 50(4), 846-850.

Conway, T., Sewell, G.W., Osman Y.A. and Ingram L.O., 1987. Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis.* J. Bacteriol., 169(6), 2591-2597.

Dahlstrom, R.V., Jaehnig, J.H., E.I. du Pont de Nemours and Company, 1973. Process for the biological production of alpha, omega-alkanedioic acid. US Patent Document US3773621.

Desai, J.D. and Banat, I.M., 1997. Microbial production of surfactants and their commercial potential. Microb. Mol. Biol. Rev., 6(1), 47-64.

Duetz, W.A., van Beilen, J.B. and Witholt, B., 2001. Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis. Curr. Opin. Biotech., 12, 419-425.

Eastcott, L., Shiu, W.Y. and Mackay, D., 1988. Environmentally relevant physical-chemical properties of hydrocarbon: a review of data and development of simple correlation. Oil Chem. Pollut. 4, 191-216.

Espada, J.J., Coutinho, J.A.P. and Peña, J.L., 2010. Evaluation of methods for the extraction and characterization of waxes from crude oils. Energy & Fuels, 24,1837-1843.

Feitkenhauer, H., 1998. Biodegradation of aliphatic and aromatic hydrocarbons at high temperatures: Kinetics and applications. Process engineering. Technical University Hamburg-Harburg. Master's thesis.

Feitkenhauer, H., Müller, R. and Markl, H., 2003. Degradation of polycyclic aromatic hydrocarbons and long-chain alkanes at 60–70 °C by *Thermus* and *Bacillus spp*. Biodegradation, 14, 367-372.

Feng, L., Wang, W., Cheng, J., Ren, Y., Zhao, G., Gao, C., Tang, Y., Liu, X., Han, W., Peng, X., Liu, R. and Wang, L., 2007. Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. Proc. Natl. Acad. Sci. U.S.A., 104(13), 5602-5607.

Ferrell, W.J., Radloff, J.F. and Jackiw, A.B., 1969. Quantitative analysis of free and bound fatty aldehydes: optimum conditions for p-nitropenylhydrazone formation. Lipids, 4(4), 278-282.

Fitzgerald, G. and Williams, L.S., 1975. Modified penicillin enrichment procedure for the selection of bacterial mutants. J. Bacteriol., 4345-4346.

Fox M.G.A., Dickinson, F.M. and Ratdledge C., 1992. Long-chain alcohol and aldehyde dehydrogenase activities in *Acinetobacter calcoaceticus* strain HOl-N. J. Gen Microbiol., 138, 1963-1972.

Franchini, A.G., Witholt, B. and Rojo, F., 2004. Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis*. Environ. Microbiol., 6, 264-273.

Fujii T., Narikawa T., Sumisa F., Arisawa A., Takeda K. and Kato J., 2006. Production of α,ω-Alkanediols using *Escherichia coli* expressing a Cytochrome P450 from *Acinetobacter sp.* OC4. Biosci. Biotechnol. Biochem., 70(6), 1379-1385.

Fujiwara, K., Tsubouchi, T., Kuzuyama, T. and Nishiyama, M., 2006. Involvement of the arginine repressor in lysine biosynthesis of *Thermus thermophiles*. Microbiology, 152, 3585-3594.

Gartshore, J., Lim, Y.C. and Cooper, D.G., 2000. Quantitative analysis of biosurfactants using Fourier Transform Infrared (FT-IR) spectroscopy. Biotechnol. Lett., 22, 169-172.

Ghisalba, O., Meyer, H.P. and Wohlgemuth, R., 2010. Industrial Biotransformation. Encyclopedia of Industrial Biotechnology, Bioprocess, Bioseparation, and Cell Technology, edited by Michael C. Flickinger; John Wiley & Sons, Inc.

Grant, C., Woodley, J.M. and Baganz, F., 2011. Whole-cell bio-oxidation of n-dodecane using the alkane hydroxylase system of *P. putida* GPo1 expressed in *E. coli*. Enzyme Microb. Technol., 4, 480-486.

Hao, R., Lu, A. and Wang, G., 2004. Crude-oil-degrading thermophilic bacterium isolated from an oil field. Can. J. Microbiol., 50, 175-182.

Harayama, S., Kasai, Y., and Hara, A., 2004. Microbial communities in oil-contaminated seawater. Curr. Opin. Biotechnol., 15, 205-214.

Hashimoto, Y., Yano, T., Kuramitsu, S. and Kagamiyama, H., 2001. Disruption of *Thermus thermophilus* genes by homologous recombination using a thermostable kanamycin-resistant marker. FEBS Lett., 506, 231-234.

Hatti-Kaul, R., Törnvall, U., Gustafsson, L. and Börjesson, P., 2007. Industrial biotechnology for the production of bio-based chemicals - a cradle-to-grave perspective. Trends Biotechnol., 25(3), 19-124.

Heipieper, H.J., Neumann, G., Cornelissen, S. and Meinhardt, F., 2007. Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Mini-Review. Appl. Microbiol. Biotechnol., 74, 961-973.

Hill, K., Cognis GmbH, 2007. Industrial development and application of biobased oleochemicals. Pure Appl. Chem., 79(11), 1999-2011.

Hirsinger, F., 1998. Comparing oleochemical and petrochemical surfactants with the life cycle inventory, LCI method. Chim. Oggi., 16, 62-68.

Hirsinger, F., Schick, K.P. and Stalmans, M., 1995. A life-cycle inventory for the production of oleochemical raw materials. Tenside Surf. Det., 32, 420-432.

Hirsinger, F., Schick, K.P., Shul, W. and Stalmans, M. 1995. A life-cycle inventory for the production of detergent-grade alcohols. Tenside Surf. Det., 32, 398-410.

Hoshino, T., Yoshino, Y., Guevarra, E.D., Ishida, S., Hiruta, T., Fujii, R. and Nakahara, T., 1994. Isolation and partial characterization of carotenoid underproducing and overproducing mutants from an extremely thermophilic *Thermus thermophilus* HB27. J. Ferment. Bioeng., 77(2),131-136.

Hou, C.T., 2006. Biotransformation of aliphatic hydrocarbons and fatty acids. In: Biocatalysis and Biotechnology for Functional Foods and Industrial Products. Edited by Hou, C.T. New York, NY:CRC Press., 227-265.

Houston, J.H., 2006. Petro - oleo battle engaging in higher alcohols market. Colin A. Houston & Associates. Inc., http://www.colin-houston.com

Huf, S., Krügener, S., Hirth., T, Rupp, S. and Zibek, S., 2011. Biotechnological synthesis of long-chain dicarboxylic acids as building blocks for polymers. Review article. Eur. J. Lipid Sci. Technol., 113(5), 548-561.

Julsing, M.K., Cornelissen, S., Bühler, B. and Schmid, A., 2008. Heme-iron oxygenases: powerful industrial biocatalysts? Curr. Opin. Chem. Biol., 12(2), 177-186.

Itoh, S., Honda, H., Tomita, F. and Suzuki, T., 1971. Rhamnolipids produced by *Pseudomonas aeruginosa* grown on n-paraffin. J. Antibiot., 14, 855-859.

Itoh, S. and Suzuki, T., 1972. Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in n-paraffin utilizing ability. Agric. Biol. Chem., 36, 2233-2235.

Jones, T. J. and Esposito, M. F., 2000. An assay evaluation of the methylene blue method for the detection of anionic surfactant in urine. J. Ana. Toxic., 24, 323-327.

Joyner, E.A.L., 1999. Gene Targeting: A Practical Approach. 2nd Ed., Oxford University Press, New York.

Kabelitz, N., Santos, P.M., Heipieper, H.J., 2003. Effect of aliphatic alcohols on growth and degree of saturation of membrane lipids in *Acinetobacter calcoaceticus*. FEMS Microbiol. Lett., 220, 223-227.

Kato, T., Miyanaga, A., Haruki, M., Imanaka, T., Morikawa, M. and Kanaya, S., 2001. Gene cloning of an alcohol dehydrogenase from thermophilic alkane-degrading *Bacillus thermoleovorans* B23. J. Biosci. Bioeng., 91(1) 100-102.

Kato, N. and Shibasaki, I., 1980. The antimicrobial characteristics of 1-alkanols. J. Antibact. Antifung. Agents, 8, 325-331.

Kester, A. S. and Foster, J. W., 1963. Diterminal oxidation of long-chain alkanes by bacteria. J. Bacteriol., 85, 859-869.

Kobashi, N., Nishiyama, M. and Tanokura, M. ,1999. Aspartate kinase-independent lysine synthesis in an extremely themophilic bacterium, *Thermus thermophilus*: Lysine is synthesized via α-aminoadipic acid not via diaminopilemic acid. J. Bacteriol., 1713-1718.

Koch, A.K., Kappeli, O., Fiechter, A. and Reiser, J., 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J. Bacteriol., 173, 4212-4219.

Koyama, Y., Hoshino, T., Tomizuka, N. and Furukama, K., 1986. Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus spp*. J. Bateriol., 338-340.

Kubo, I., Muroi H., Kubo A., 1995. Structural functions of antimicrobial long-chain alcohols and phenols. Bioorg. Med. Chem., 3(7), 873-880.

Labinger, J.A. and Bercaw, J.E., 2002. Understanding and exploiting C-H bond activation. Nature, 417, 507-514.

Lamprecht, W. and Heinz, F., 1984. Ed. in Methods of enzymatic analysis (Bergmeyer, H. U., ed.) 3rd ed., 6, 570-577. Verlag Chemie, Weinheim , Deerfield Beach , Basel.

Leahy, J.G. and Colwell, R.R., 1990. Microbial degradation of hydrocarbons in the environment. Microbiol. Rev., 54, 305-315.

Lebeault, J. M., Roche, B., Duvnjak, Z. and Azoulay, E., 1998. Isolation and study of the enzymes involved in the metabolism of hydrocarbons by *Candida tropicalis*. Arch. Microbiol., 72(2), 140-153.

Liese, A., Seelbach, K., Wandrey, C., 2006. Industrial biotransformations. 2nd ed. Weinheim: Wiley-VCH Verlag GmbH & Co KGaA.

Little, J.L., Eastman Chemical Company, 1999. Artifacts in trimethylsilyl derivatization reactions and ways to avoid them. J. Chromatogr. A, 844(1-2), 1-22

Little, J. L., 2003. Artifacts in trimethylsilyl derivatization reactions and ways to avoid them (update). Eastman chemical company, Kingsport, TN 37662-5150, USA.

Lynd, L.R., Wyman, C., Laser, M., Johnson, D. and Landucci, R., 2005. Strategic biorefinery analysis: Review of existing biorefinery examples. National Renewable Energy Laboratory, U.S. Department of Energy.

Madigan, M.T., Martinko, J.M., Parker J., 2004. Brock Biology of Microorganisms. 10th Edition, Pearson Education, Madrid, 151-157.

May, S.W., Katopodis, A.G., 1986. Oxygenation of alcohol and sulphide substrates by a prototypical non-haem iron monooxygenase: catalysis and biotechnological potential. Enzyme Microb. Technol., 8, 17-21.

McCoy, M. ,2005. A tale of two raw materials. Chemical & Engineering News, 83 (4), 21-22.

McDaniel, R., Behrouzian, B., Clark, L., Hattendorf, D. and Valle, F.; Codexis, Inc., 2011. Production of fatty alcohols with fatty alcohol forming Acyl-CoA reductases, FAR. Patent Application Publication US 2011/0000125A1.

McKew, B.A., Coulon, F., Yakimov, M.M., Denaro, R., Genovese, M., Smith, C.J., Osborn, A.M., Timmis, K.N. and McGenity, T.J., 2007. Efficacy of intervention strategies for bioremediation of crude oil in marine systems and effects on indigenous hydrocarbonoclastic bacteria. Environ. Microbiol., 9 (6), 1562-1571.

Meinhold, P., Peters, M.W., Hartwick, A., Hernandez, A., Arnold, F.H., 2006. Engineering Cytochrome P450 BM3 for terminal alkane hydroxylation. Adv. Synth. Catalysis, 348, 763-772.

Meintanis, C., Chalkou, K.I., Kormas, K.A. and Karagouni, A.D., 2006. Biodegradation of crude oil by thermophilic bacteria isolated from a volcano island. Biodegradation, 17(2), 105-111.

Metzger, J.O. and Bornscheuer, U., 2006. Lipids as renewable sources: current state of chemical and biotechnological conversion and diversification. Appl. Microbiol. Biotechnol., 71,13-22.

Metzger, J.O., 2009. Fats and oils as renewable feedstock for chemistry. Eur. J. Lipid Sci. Technol., 111(9), 865-876.

Meyer, A. and Panke, S., 2008. Genomics in Metabolic Engineering and Biocatalytic Applications of the Pollutant Degradation Machinery in Microbial Biodegradation: Genomics and Molecular Biology ed. by Eduardo Díaz. Caister Academic Press. Chapter 12, 359-387.

Mobley, D.P., 1999. Biosynthesis of long-chain dicarboxylic acid monomers from renewable resources. Final technical report for the United States Department of Energy under cooperative agreement No. DE-FC36-95G010099. GE Corporate Research and Development.

Moreno, R., Zafra, O., Cava, F. and Berenguer, J., 2003. Development of a gene expression vector for *Thermus thermophilus* based on the promoter of the respiratory nitrate reductase. Plasmid, 49(1), 2-8.

Müller, R., Antranikian, G., *et al.*, 2008. Script of Practical course in Technical and Environmental Microbiology, Technical University Hamburg-Harburg, WS 2008/09.

Müller, R., Antranikian, G., Maloney, S., Sharp, R., 1998. Thermophilic degradation of environmental pollutants. Adv. Biochem. Eng. Biotechnol., 61, 155-169.

Olsen, C., 1971. Enzymic fluorometric micromethod for the determination of acetoacetate, b-hydroxybutyrate, pyruvate, and lactate. Clin. Chim. Acta, 33, 293-300 .

Organisation for Economic Co-operation and Development, OECD, 2001. The application of biotechnology to industrial sustainability. OECD Publ., http://www1.oecd.org/publications/e-book/9301061e.pdf

Otto, K., 2001. Characterization of a thermostable alkane oxidizing system. Technical University Hamburg-Harburg. Master's thesis.

Para, J.L., Guinea, J., Manresa, M.A., Robert, M., Mercade, M.E., Comelles, F. and Bosch, M.P., 1989. Chemical characterization and physicochemical behavior of biosurfactants. J. Am. Oil Chem. Soc., 66, 141-145.

Presents, Z. ,2000. All about fatty alcohols: Saturated fatty alcohols. CONDEA

Primrose, S.B., Twyman, R.M., 2006. Principles of Gene Manipulation and Genomics, Chapter 8. Changing genes: site-directed mutagenesis and protein engineering. Blackwell Publishing.

Rahman, K. S. M. and Gakpe, E., 2008. Production, characterisation and applications of biosurfactants – Review. Biotechnology, 7(2), 360-370.

Rambeloarisoa, E., Rontani, J.F., Giusti. G., Duvnjak, Z. and Bertrand, J.C., 1984. Degradation of crude oil by a mixed population of bacteria isolated from sea-surface foams. Mar. Biol., 83, 69-81.

Rojo, F. ,2009. Minireview: Degradation of alkanes by bacteria. Environ. Microbiol., 11(10), 2477-2490.

Rosenberg, E. and Ron, E.Z., 1999. High- and low-molecular-mass microbial surfactants. Appl. Microbiol. Biotechnol., 52, 154-162.

Rowlands, B., 2011. Industrial Strain Improvement: Optimisation of Classical Screening Techniques. Bukisa.com: http://www.bukisa.com/articles/453711_industrial-strain-improvement-optimisation-of-classical-screening-techniques#ixzz1rX7krzvk.

Rubin, D., Rubin, E.J., Century Laboratories, Inc., 1990. Tumor treatment process and composition. US Patent Document US4895874.

Rybak, A. and Meier, M.A.R., 2007. Cross-metathesis of fatty acid derivatives with methyl acrylate: Renewable raw materials for the chemical industry. Green Chem., 9, 1356-1361.

Sabirova, J.S., Ferrer, M., Regenhardt, D., Timmis, K.N., and Golyshin, P.N., 2006. Proteomic insights into metabolic adaptations in *Alcanivorax borkumensis* induced by alkane utilization. J. Bacteriol., 188, 3763-3773.

Samorski, M., Dierker, M., Cognis IP Management GmbH., 2010. Method for the production of unsaturated alpha, omega dicarboxylic acids and/or unsaturated alpha, omega dicarboxylic acid diesters. Patent Application Publication: WO2010020368.

Sang-Cheol, L., 2007. Characterization of new biosurfactant produced by *Klebsiella sp.* Y6-1 isolated from waste soybean oil. Biores. Tech., 99, 2288-2292.

Scheller, U., Zimmer, T., Becher, D., Schauer, F. and Schunck, WH., 1998. Oxygenation cascade in conversion of n-alkanes to α,ω-dioic acids catalyzed by Cytochrome P450 52A3. J. Biol. Chem., 273(49), 32528-32534.

Schirmer, A., Rude , M.A. and Brubaker, S., LS9, Inc., 2009. Methods and compositions for producing hydrocarbons. Patent Application Publication: WO2009140695.

Schirmer, A. and Rude, M.A., 2009. New microbial fuels: a biotech perspective. Curr. Opin. Microbiol., 12, 274-281.

Schmid A., Dordick J., Hauer B., Kiener A., Wubbolts M. and Witholt, B., 2001. Industrial biocatalysis today and tomorrow. Nature, 11, 258-268.

Schmid, A., Sonnleitner, B., Witholt, B., 1998. Medium chain length alkane solvent-cell transfer rates in two-liquid phase, *Pseudomonas oleovorans* cultures. Biotechnol Bioeng., 60(1), 10-23.

Schneiker, S., Martins dos Santos, V.A., Bartels, D., Bekel, T., Brecht, M., Buhrmester, J., *et al.*, 2006. Genome sequence of the ubiquitous hydrocarbon-degrading marine bacterium *Alcanivorax borkumensis*. Nat. Biotechnol., 24, 997-1004.

Schörken, U. and Kempers, P., Cognis GmbH, Düsseldorf, Germany, 2009. Review article - Lipid biotechnology: Industrially relevant production processes. Eur. J. Lipid Sci. Technol., 111, 627-645.

Shet, M.S., Fisher, C.W., Holmans, P.L., Estabrook, R.W., 1996. The omega-hydroxylation of lauric acid: oxidation of 12-hydroxylauric acid to dodecanedioic acid by a purified recombinant fusion protein containing P450 4A1 and NADPH-P450 reductase. Arch. Biochem. Biophys., 330, 199-208.

Shiio, I. and Uchio, R., 1971. Microbial production of long-chain dicarboxylic acids from n-alkanes. Part I. Screening and properties of microorganisms producing dicarboxylic acids. Agric. Biol. Chem., 35, 2033-2042.

Soberón-Chávez, G., Lépine, F. and Déziel, E., 2005. Mini-review: Production of rhamnolipids by *Pseudomonas aeruginosa*. Appl. Microbiol. Biotechnol., 68, 718-725.

Sonnleitner, B. and Fiechter, A., 1983. Advantages of using thermophiles in biotechnological processes: expectations and reality. Trends Biotechnol., 1(3),74-80.

Staijen, I.E., van Beilen, J.B., Witholt, B., 2000. Expression, stability and performance of the three-component alkane monooxygenase of *Pseudomonas oleovorans* in *Escherichia coli*. Eur. J. Biochem., 267, 1957-1965.

Stalmans, M., Berenbold, H., Berna, J.L., Cavalli, L., Dillarstone, A., Franke, M., Hirsinger, F., Jansen, D., Kosswig, K., *et al.*, 1995. European life-cycle inventory for detergent surfactants production. Tenside Surf. Det., 32, 84-108.

Steen, E.J., Kank, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., B. del Cardayre, S. and Keasling, J.D., 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature, 463, 559-563.

Straathof, A.J.J., Panke, S. and Schmid, A., 2002. The production of fine chemicals by biotransformations. Curr. Opin. Biotech., 13, 548-556.

Tahzibi, A., Kamal, F. and Assadi, M.M., 2004. Improved production of rhamnolipids by a *Pseudomonas aeruginosa* mutant. Iran. Biomed. J., 8(1), 25-31.

Tani, A., Ishige, T., Sakai, Y. and Kato, N., 2001. Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter sp.* strain M-1. J. Bacteriol., 183, 1819 -1823.

Thomas, S.M., DiCosimo, R. and Nagarajan, V., 2002. Biocatalysis: applications and potentials for the chemical industry. Trends Biotechnol., 20, 238-242.

Togashi, N., Shiraishi, A., Nishizaka, M., Matsuoka, K., Endo, K., Hamashima, H. and Inoue, Y., 2007. Antibacterial activity of long-chain fatty alcohols against *Staphylococcus aureus*. Molecules, 12, 139-148.

Urlacher, V.B., Girhard, M., 2012. Cytochrome P450 monooxygenases: an update on perspectives for synthetic application. Trends Biotechnol., 30(1), 26-36.

U.S. Department of Energy, Chemical Industry Vision 2020 Technology Partnership and Oak Ridge National Laboratory, 2007. Alternative, renewable and novel feedstocks for producing chemicals. Study report.

van Beilen, J.B., Duetz, W.A., Schmid, A. and Witholt, B., 2003. Practical issues in the application of oxygenases. Trends Biotechnol., 21(4), 170-177.

van Beilen, J.B., Eggink, G., Enequist, H., Bos, R. and Witholt, B., 1992. DNA sequence determination and functional characterization of the OCT-plasmid-encoded *alk*JKL genes of *Pseudomonas oleovorans*. Mol. Microbiol., 6(21), 3121-3136.

van Beilen, J.B. and Funhoff, E.G., 2007. Alkane hydroxylases involved in microbial alkane degradation. Appl. Microbiol. Biotechnol., 74, 13-21.

van Beilen, J.B., Kingma, J. and Witholt, B., 1994. Substrate specificity of the alkane hydroxylase system of *Pseudomonas oleovorans* GPo1. Enzyme Microb. Technol., 16, 904-911.

van Beilen, J.B., Li, Z., Duetz, W.A., Smits, T.H.M. and Witholt B., 2003. Diversity of alkane hydroxylase systems in the environment. Review. Oil Gas Sci. Technol., 58(4), 427-440.

van Beilen, J.B., Marin, M.M., Smits, T.H.M., Roethlisberger, M., Franchini, A.G., Witholt, B. and Rojo, F., 2004. Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis*. Environ. Microbiol., 6, 264-273.

van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Röthlisberger, M., and Witholt, B., 2001. Analysis of *Pseudomonas putida* alkane degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk*-genes. Microbiology 147, 1621-1630.

van Beilen, J.B., Wubbolts, M.G. and Witholt, B., 1994. Genetics of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation, 5, 161-174.

van Hamme, J.D., Singh, A. and Ward, O.P., 2003. Recent advances in petroleum microbiology. Microbiol. Mol. Biol. Rev., 67,503-549.

Viguera, E., Canceill, D. and Ehrlich S.D., 2001. In vitro replication slippage by DNA polymerases from thermophilic organisms. J. Mol. Biol., 312, 323-333.

Volkering, F., Breure, A.M. and Rulkens, W.H., 1998. Microbiological aspects of surfactant use for biological soil remediation. Biodegradation, 8, 401-417.

World Business Council for Sustainable Development (WBCSD), 2000. Eco-Efficiency: Creating more value with less impact. Report.

Wentzel A., Ellingsen T., Kotlar H., Zotchev S. and Throne-Holst, M., 2007. Bacterial metabolism of long-chain n-alkanes. Appl. Microbiol. Biotechnol., 76, 1209-1221.

Wischnak, C., Löffler, F.E., Li, J., Urbance, J.W. and Müller, R., 1998. *Pseudomonas sp.* strain 273, an aerobic alpha, omega-dichloroalkane degrading bacterium. Appl. Environ. Microbiol., 64(9), 3507-3511.

Yang, F.L., Lu, C.P., Chen, C.S., Chen, M.Y., Hsiao, H.L., Su, Y., Tsay, S.S., Zou, W. and Wu, S.H., 2004. Structural determination of the polar glycoglycerolipids from thermophilic bacteria *Meiothermus taiwanensis*. Eur. J. Biochem., 271, 4545–4551.

Yakimov, M.M., Golyshin, P.N., Lang, S., Moore, E.R.B., Abraham, W.R., Lunsdorf, H. and Timmis, K.N., 1998. *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. Int. J. Syst. Bacteriol., 48, 339-348.

Yakimov, M.M., Timmis, K.N. and Golyshin, P.N., 2007. Obligate oil-degrading marine bacteria. Curr. Opin. Biotechnol., 18, 257-266.

Yang, Y.L., Yang, F.L., Jao, S.C., Chen, M.Y., Tsay, S.S., Zou, W. and Wu, S.H., 2006. Structural elucidation of phosphoglycolipids from strains of the bacterial thermophiles *Thermus* and *Meiothermus*. J. Lipid Res., 47, 1823-1832.

Zhu, Y., Patel, J., Mujcinovic, S., Jackson, W.R. and Robinson, A. J., 2006. Preparation of terminal oxygenates from renewable natural oils by a one-pot metathesis-isomerisation-methoxycarbonylation-transesterification reaction sequence. Green Chem. 8, 746-749.