

PRODUCTION OF MEDIUM- CHAIN α,ω -BIFUNCTIONAL MONOMERS FROM FATTY ACIDS AND n -ALKANES

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PRODUCTION OF MEDIUM-CHAIN α,ω -BIFUNCTIONAL MONOMERS FROM FATTY ACIDS AND n -ALKANES

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Thesis

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Chapter 1 Introduction

1.1. Transition to a sustainable industrial chemistry

Due to environmental concerns, the chemical industry is aiming to develop alternative, more sustainable production processes. Research efforts focus on finding processes that are mild, emit less greenhouse gases, minimize waste, and use renewable resources. The largest share of products coming from the chemical industry are used as building blocks for polymers, with a global revenue of 454 billion US \$ in 2011, and is estimated to reach 567 billion US \$ in 2017¹. These building blocks usually have a functional group (e.g. hydroxy, amine, carboxy) at the α and ω carbon atoms, and will henceforth be referred to as α,ω -bifunctional monomers (BMs). The functional groups of BMs can be joined together in a polymerization reaction to produce polymers. Among these BMs, α,ω -dicarboxylic acids and α,ω -diols are large volume building blocks. Their general structures are depicted in Figure 1.1.

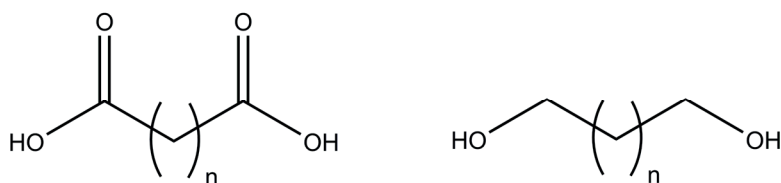


Figure 1.1. Structure of α,ω -dicarboxylic acids (left) and α,ω -diols (right)

1.2. α,ω -Dicarboxylic acids

1.2.1 Applications of dicarboxylic acids

α,ω -Dicarboxylic acids (DCAs) are found in many applications. These include polyamides, polyesters, coatings, lubricants and cosmetics. An example of a polymer produced from DCAs is nylon, which can be produced by polymerization of a DCA and a diamine. Industrial production of nylon 6,6 is mostly achieved by condensation of hexamethylene diamine and adipic acid (AA). Among DCAs, AA is produced most; its annual global production is prospected to reach 2.7 million tons in 2017². Other DCAs have significantly smaller production volumes³; due to the cumbersome production from petrochemical feedstocks.

1.2.2 Current state of DCA production

Unfortunately, current synthesis of DCAs is associated with a high energy requirement and high greenhouse gas emissions⁴. These adverse effects are a result of the application of petrochemicals as starting material. In conventional processes, petrochemicals have to undergo several energy-intensive conversions to yield a functional product.

A good example is the production process of AA. In the case of AA, benzene is commonly the starting material. Benzene, which is obtained by catalytic reforming of crude oil, is first converted to cyclohexane. Cyclohexane is then oxidized to produce ketone-alcohol oil (KA oil); a mixture of cyclohexanone and cyclohexanol. This mixture is treated with nitric acid, to yield AA, see Figure 1.2. The treatment with nitric acid results in release of nitrous oxide⁵, a gas that has a global warming potential of about 290 times more than CO₂⁶. As a result, AA production results in the emission of large quantities of greenhouse gas, ≥ 4 ton CO₂-equivalent per ton AA^{7,8}. Similar processes are applied for C8 and C12 DCA production, for which the ring structures have to be synthesized from 1,3-butadiene.

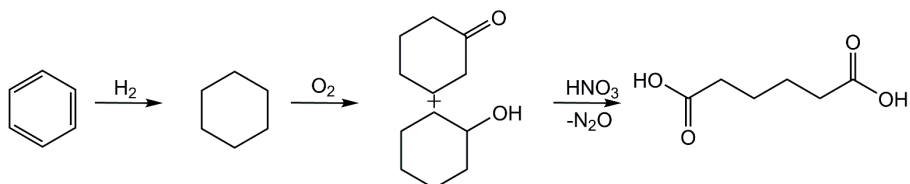


Figure 1.2. Simplified reaction scheme for adipic acid production from benzene

1.3. α,ω -Diols

1.3.1 Applications of diols

Diols are used in the synthesis of polyesters and polyurethanes⁹. A large market volume α,ω -diol is 1,4-butanediol, of which 1.8 million tons/year are produced¹⁰ in several ways. The most applied route is via acetylene. Other important routes start from propylene oxide or maleic anhydride¹¹.

Another important α,ω -diol is 1,6-hexanediol. The conventional production of this diol is achieved through hydrogenation of adipic acid^{12,13}. Hence, 1,6-

hexanediol production suffers from the same drawbacks as mentioned above for adipic acid production. Moreover, the necessary hydrogenation requires high hydrogen pressure and expensive catalysts¹⁴, which increases the energy demand of this process even further.

1.4. Biobased alternatives for medium-chain DCA production

The disadvantages associated with conventional DCA production have motivated researchers to develop more sustainable and mild processes. Fatty acids can be applied as a sustainable feedstock for DCA production, due to their high natural abundance. Mono-unsaturated fatty acids are the major constituent of vegetable oils and thus this process is based on a renewable feedstock. Unsaturated fatty acids can be cleaved across the double bond to yield a DCA and a fatty acid¹⁵. An example is the production of azelaic acid from oleic acid¹⁶. This process has a major drawback. The cleavage of the fatty acid is achieved *via* ozonolysis. Ozonolysis is associated with operational hazards and high energy input^{15,17}. Another drawback is the fact that this process yields fatty acids as a byproduct, if the market demand of that fatty acid is low.

This problem can be circumvented by using a biocatalyst. The long-chain fatty acid can be ω -oxidized to yield a long-chain DCA, which is subsequently β -oxidized. If the β -oxidation cycle is halted, medium-chain DCA can accumulate. Beardslee and Picataggio showed that adipic acid can be produced *via* this route, after removing the broad specificity acyl-CoA oxidase in *Candida tropicalis*¹⁸. A clear disadvantage of this process is that a large share of the carbon is lost in the β -oxidation process. Moreover, vegetable oils are important for the food chain. Therefore, direct ω -oxidation of medium-chain fatty acids (MCFAs) would be advantageous.

1.4.1 Medium-chain fatty acids as feedstock for DCA production

MCFAs are less abundant in nature. Plants mostly accumulate fatty acids with a chain length ≥ 12 . Production of DCAs $\geq C6$ has been achieved from glucose and glycerol as feedstock, but this has so far resulted in rather low product titers^{19,20}.

MCFAs are found in small quantities in palm kernel and coconut oil²¹. They are also produced as a byproduct in the ozonolytic process mentioned above^{15,22,23}. Another way of MCFA production, that does not interfere with the food chain, is chain elongation of volatile fatty acids from organic rest streams to MCFAs²⁴. Recently, improvements on this process were made, resulting in high production rates of hexanoate, heptanoate and octanoate^{25–27}. MCFA production based on this process is being commercialized by Chaincraft and Earth Energy Renewables. This makes MCFAs an interesting sustainable feedstock for medium-chain DCA production.

To convert MCFAs into DCAs, only the terminal methyl group has to be oxidized to yield DCAs. This terminal oxidation of fatty acids is shown in Figure 1.3B and is called ω -oxidation.

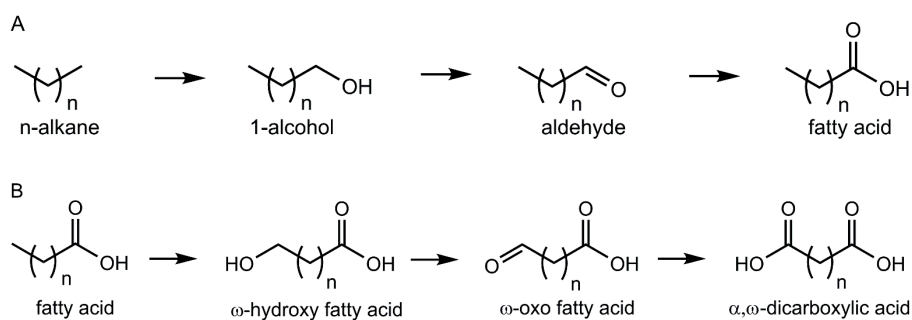


Figure 1.3. A: terminal oxidation of an n-alkanes to fatty acids. B: ω -Oxidation of fatty acids to α,ω -dicarboxylic acids.

n-Alkanes are also considered as a more sustainable feedstock, since energy-intensive cracking or thermal dehydrogenation would not have to be applied to introduce functional groups on the molecule²⁸. This is more challenging, since both termini of an n-alkane have to be oxidized to yield a BM, as illustrated in Figure 1.3.

1.5. Terminal C-H oxidation

The oxidation of carbon-hydrogen bonds of fatty acids and n-alkanes is not challenging *per se*. Doing this in a selective manner, however, is extremely challenging. This challenge is caused by the fact that C-H bonds adjacent to heteroatoms, e.g. oxygen, are weaker²⁹. It is thus difficult to stop the reaction

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after introduction of a heteroatom, since further oxidation to CO_2 and H_2O is favored³⁰. Furthermore, in most cases, the terminal oxygenate is the desired product. For DCAs and diols both termini are oxidized. What further complicates selectivity, is the difference in C-H bond strengths. The C-H bond strengths of a terminal methyl group are higher than those of a methylene group, see Table 1.1

Table 1.1. Bond dissociation energies of some C-H bonds at 298 K. Adapted from ^{29,31}

Bond type	Dissociation energy (kJ mole ⁻¹)
H-CH ₃	461.9±0.8
H-CH ₂ CH ₃	423.0±1.7
(CH ₃) ₂ CH-H	412.5±1.7
H-CH ₂ OH	402.1±0.8
H-CH(CH ₃)OH	389.1±4.2
H-COOH	401.7±4.2

This makes terminal oxidation difficult with traditional chemical approaches, since oxidation tends to occur where the C-H bonds are weaker. This results in poor selectivity²⁸, and thus the formation of byproducts. When DCAs or diols are the desired product, two oxygen functionalities have to be introduced. Reports of chemistry-based diterminal oxidation are rather scarce, since terminal oxidation in itself is already challenging. Nevertheless, organometallic C-H activation approaches based on late transition metals has resulted in terminal C-H bond oxidation with great selectivity. Noteworthy are two reports on diterminal oxidation from n-alkanes. A Shilov-chemistry based process for ethylene glycol production from ethane was developed³². A clear disadvantage of this process is the need for stoichiometric amounts of platinum. Later, adipic acid was produced from n-hexane with molecular sieves. Although the achievement of diterminal oxidation is impressive, the low selectivity is a clear drawback in this case. Hence, to date no economically feasible and selective process has been developed based on transition metals^{28,33,34}.

1.5.1 Enzymatic terminal C-H oxidation by CYP450 monooxygenases

In contrast to chemical catalysts, enzymes can achieve terminal oxidation with 100% selectivity. Furthermore, enzymes can ω -oxidize fatty acids with such high selectivity. A well-known class of enzymes that catalyze this reaction are the heme-containing cytochrome P450 (CYP450) monooxygenases from the CYP52 family, found in *Candida* species. Whole cells expressing these enzymes have been successfully applied for ω -oxidation of long-chain fatty acids and diterminal oxidation of n-alkanes, resulting in high titers of DCAs^{35–39}. *Candida tropicalis* is now applied as a production platform for commercial scale production of dodecanedioic acid from dodecanoic acid by Verdezyne⁴⁰.

1.5.2 Enzymatic terminal C-H oxidation by non-heme monooxygenases

Many bacteria have the ability to grow on n-alkanes as carbon source. The range of n-alkanes that can be utilized for growth is imposing; growth has been documented for methane (C1) up to tetracontane (C40)^{41–43}. Bacteria hydroxylate the alkanes and can then utilize them as carbon source. The enzymes responsible for the hydroxylation of the n-alkanes are monooxygenases. Among them, short to medium-chain monooxygenases have attracted much attention for their ability to convert alkanes to structural chemicals, thereby extending their use from fuel to a higher value product.

Pseudomonas putida GPo1 (also referred to as *Pseudomonas oleovorans*) is an example of an organism that can grow on n-alkanes as the sole carbon source. It was isolated in the '60s⁴⁴ and Peterson *et al.* identified that alkane oxidation depended on the activity of three proteins, an alkane hydroxylase, a rubredoxin and a rubredoxin reductase⁴⁵. Later, the genetics of alkane oxidation in *Pseudomonas putida* GPo1 were unravelled. Growth on medium-chain n-alkanes was possible by virtue of the OCT plasmid. On this plasmid, a regulatory locus and the *alk* operon were situated⁴⁶. The translation products of the *alk* operon were identified and functionally expressed in *E. coli*^{47–49}. An overview of the *alk* operon and regulon are shown in Figure 1.4⁵⁰. In the *alkBFGHJKL* cluster, all but one of the functional genes for alkane degradation are situated. In the *alkST* cluster, another functional gene *alkT* and transcriptional regulator *alkS* are situated. In absence of n-alkanes or the non-

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metabolizable inducer dicyclopropylketone (DCPK), AlkS represses its own transcription. In presence of n-alkanes or DCPK, AlkS drives its own expression, and induces expression from *PalkB*. The *alkBFGHJKL* gene cluster is under control of a single *PalkB* promoter.

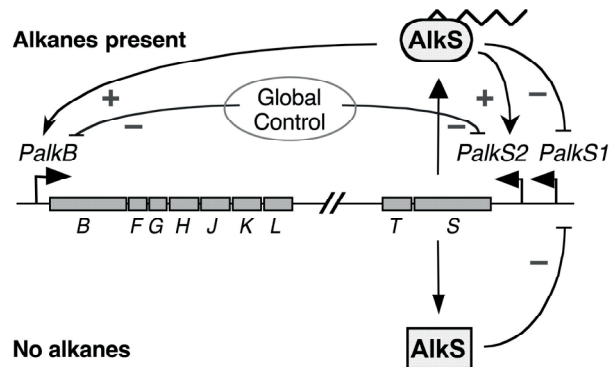


Figure 1.4. Overview of the genetics of the alkane degradation pathway in *Pseudomonas putida* GPo1. Picture taken from M. Alejandro Dinamarca *et al.* J. Bacteriol. 2003;185:4772-4778⁵⁰.

The functions of the translation products of the *alk* operon are shown in Figure 1.5. AlkB is a non-heme, di-iron alkane monooxygenase, responsible for terminal oxidation of n-alkanes⁵¹. AlkB is an integral membrane protein, which likely has 6 transmembrane helices, and may exist as a trimer⁵². AlkB inserts a single oxygen atom derived from molecular oxygen into the terminal carbon-hydrogen bond by a very reactive iron-oxo species⁵³. The electrons necessary for the creation of this reactive species are delivered by soluble rubredoxin AlkG. AlkF also codes for a rubredoxin, but is dispensable for alkane hydroxylation activity⁵⁴. Rubredoxin AlkG is reduced by soluble rubredoxin reductase AlkT, which is under control of the *PalkS* promoter. AlkT carries out this reduction at the expense of NADH. Thus, for terminal hydroxylation, the enzyme system AlkBGT is necessary. The primary alcohol produced by AlkB is then converted into an aldehyde by alcohol dehydrogenase AlkJ. AlkJ is a membrane-associated enzyme with a tightly bound FAD, that most likely reduces ubiquinone in the electron transport chain. As such, it has the ability to irreversibly oxidize alcohols⁵⁵. The aldehyde is oxidized by aldehyde dehydrogenase AlkH, and likely yields NADH^{54,56}. The fatty acid that is the result of AlkH activity is then converted to acyl-CoA by

AlkK, at the expense of hydrolysis of ATP to AMP^{48,57}. Acyl-CoA can enter the β -oxidation cycle, in which the acyl-CoA can be further utilized for energy and carbon supply. The role of outer membrane protein AlkL was recently also confirmed. This protein forms a pore in the outer membrane and facilitates uptake of apolar substrates^{58,59}.

E. coli expressing AlkB and the enzymes necessary to deliver reducing power has been applied for octanoic acid production from n-octane, but this process has never been commercialized^{60,61}.

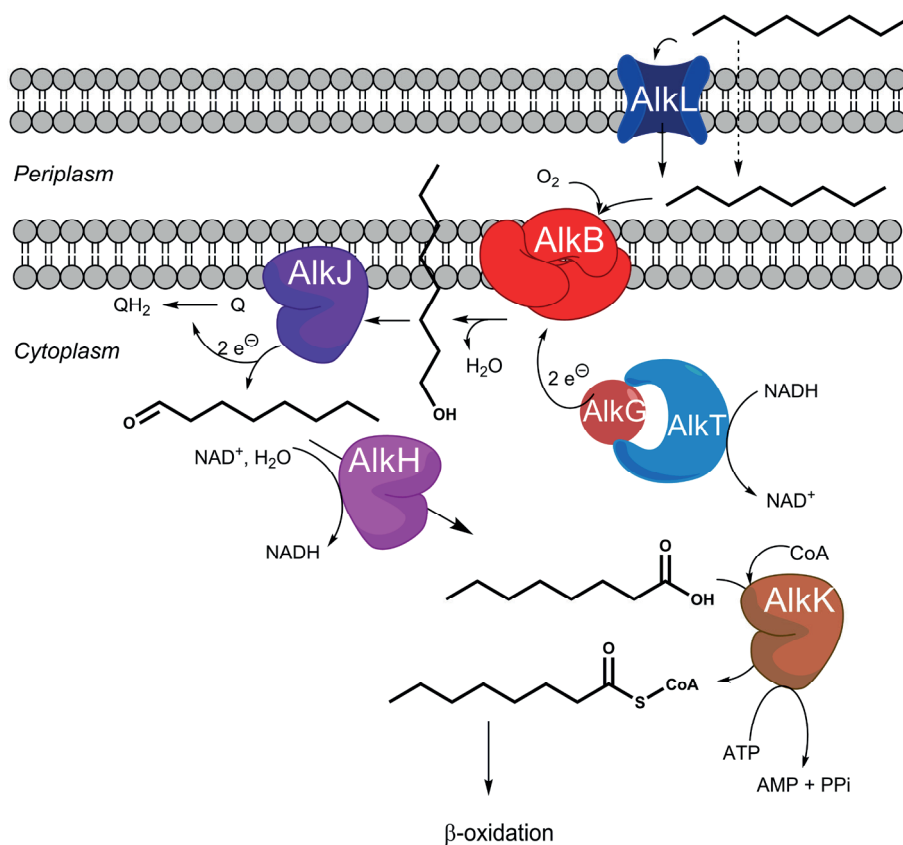


Figure 1.5. Alkane degradation pathway from *Pseudomonas putida* GPo1, with n-octane as model substrate. See text for explanation of the functions of the proteins.

CYP52 and AlkB differ greatly with respect to their substrate specificity. Members of the CYP52 family accept C10-C18 molecules^{35,62}, whereas the

specificity of AlkB ranges from C3-C14^{59,63,64}. AlkB further has the ability to hydroxylate branched and cyclic alkanes, and aromatic compounds⁶⁴. When non-natural substrates are used, monooxygenases can uncouple the oxidation of NADH and hydroxylation, resulting in the production of H₂O₂⁶⁵.

Hence, CYP450-based processes are restricted to production of long-chain DCAs. Long-chain DCAs can be used for specialty plastics, such as nylon-6,12. Besides the long-chain DCAs there is also a large industrial demand for medium-chain DCAs, such as adipic acid (C6), azelaic (C9) and sebacic acid (C10). Therefore, it is necessary to develop a biocatalytic process for these DCAs.

1.5.3 DCA production from MCFAs

The most straightforward option would be to copy the process of Figure 1.3, using medium-chain fatty acids (MCFAs), this would yield medium-chain DCAs. However, there are several challenges to be overcome.

Firstly, CYP450 enzymes are not efficient in ω -oxidizing MCFAs. Application of *Candida tropicalis* for direct ω -oxidation of MCFAs is thus not a feasible option. Other CYP450 enzymes are able to ω -oxidize MCFAs, such as CYP153A, FoCYP and CYP450-BM3 monooxygenases^{66–69}. However, those monooxygenases are not 100% selective and/or display low activity on MCFAs. The monooxygenase AlkB from *Pseudomonas putida* GPo1 is 100% selective, but also suffers from low activity on MCFAs^{19,64,65}.

Secondly, free fatty acids of medium chain length are toxic to microorganisms⁷⁰. This would be problematic if intact or growing cells are applied as biocatalyst. Hence, for medium-chain DCAs, a different process has to be developed.

1.6. Sustainable alternatives for diol production

Not many alternatives exist for the production of aliphatic α,ω -diols¹. Diols are more reduced compounds compared to DCAs, and probably this has complicated their synthesis from renewable resources. Nevertheless, carbohydrate-based processes are being explored.

1.6.1 Sustainable production of α,ω -diols from carbohydrates

For 1,4-butanediol, a carbohydrate based production platform with a genetically modified organism has been developed by Genomatica. This organism reduces C4 molecules from the TCA cycle in several steps to 1,4-butanediol.

A non-biocatalytic process was investigated for 1,5-pentanediol production from furfural. These strategies are not yet feasible, due to poor selectivity or high necessary loads of expensive catalysts^{71–73}. Also for 1,6-hexanediol a biobased process has been developed. This route proceeds *via* 5-hydroxy methyl furfural (HMF), which can be produced from glucose or fructose. HMF is hydrogenated in a multistep process to yield 1,6-hexanediol. Unfortunately, the hydrogenation of HMF results in the formation of a significant amount by-products and requires a high H₂ pressure^{2,74}.

1.6.2 α,ω -Diol production from n-alkanes

The starting materials for 1,4-butanediol production -acetylene, maleic anhydride or propylene oxide- are oxidized/unsaturated molecules produced from paraffins. In diol production energy-intensive hydrogenation steps are involved to reduce the intermediates to diols. This oxidation/reduction cycle is energy intensive. Hence, direct diterminal oxidation of n-alkanes to α,ω -alkanediols would have a great advantage over conventional processes regarding energy input. However, α,ω -alkanediol production from n-alkanes is extremely difficult. Conversion of n-octane to 1,8-octanediol has been achieved *via* biocatalysis, and thus under mild reaction conditions. The enzymes responsible for this conversion belong to the class of CYP153^{75–77}. Direct production from n-alkanes *via* (whole-cell) biocatalysis is thus possible in a one-pot synthesis under ambient conditions. Unfortunately, the activity of CYP153 enzymes is low, and the activity on the produced 1-alkanols is even lower. This has so far resulted in low 1,8-octanediol titers (~0.1 g/L) with rather high biomass concentrations (~28 g_{cdw}/L)^{1,75}. A major problem is the poor ω -oxidation activity of the monooxygenase, which also hampers efficient ω -oxidation of fatty acids.

1.7. Solving poor ω -oxidation on medium-chain substrates

Although monooxygenases are highly selective, the ω -oxidation activities on medium-chain fatty acids/alcohols are low. This is probably due to their polarity, which prevents substrates from efficient entry into the hydrophobic substrate channel. This has limited conversion of n-alkanes and fatty acids to DCAs, diols or ω -hydroxy fatty acids. In the case of AlkB, this was not the case when methyl esters of fatty acids were used as substrate. An *Escherichia coli* strain was created that expressed monooxygenase AlkB, along with rubredoxin AlkG and rubredoxin reductase AlkT that are necessary to deliver electrons. This strain efficiently ω -oxidized methyl esters of C5 to C12 fatty acids^{58,65,78}. The activity with methyl nonanoate as substrate was even higher than with the natural substrate, n-nonane. Besides the ω -alcohol, also the ω -aldehyde and carboxylic acid accumulated. Those products were produced by AlkB, indicating that overoxidation occurred. This could be a problem if one is aiming to produce (ω -)alcohols.

Two-liquid phase biotransformations with an *E. coli* strain expressing outer membrane protein AlkL besides AlkBGT yielded 49 g/L of ω -oxidized products with methyl dodecanoate as substrate. The products included the ω -alcohol, aldehyde and acid. AlkL has been shown in several reports to facilitate transport of hydrophobic molecules with a $\log P_{o/w} > 4$ ^{58,59,79}.

Esterification of the substrate thus facilitates ω -oxidation. Esters have several other advantages. Firstly, the esters are less toxic than MCFAs to microorganisms^{70,80}. MCFAs are toxic since they can passively diffuse into the cytoplasm. When fatty acids reach the cytoplasm, they release protons and thus the intracellular pH decreases. The proton import also impairs the proton motive force. Esters do not cause this effect.

Secondly, esters can be separated more easily from an aqueous broth. The esters of MCFAs have a low solubility, thus forming a second liquid phase in the biocatalytic process. The formed products dissolve well in this organic phase^{58,78}.

1.8. Biocatalytic ester production

Esters can be synthesized prior to a biotransformation process, for example *via* Fischer esterification. This method is effective, but commonly achieved with a strong oxidant, such as H_2SO_4 . Stoichiometric amounts of this strong oxidant make the process environmentally unfriendly. Much research is devoted to more benign methods of ester synthesis, for example with pincer complexes⁸¹.

More interesting is to let the biocatalyst convert the terminal oxygenate to an ester, so that esterification and ω -oxidation can be integrated into a one-pot synthesis. Biocatalytic ester production has been done before^{82–89}, but was never integrated with ω -oxidation.

In microorganisms, carboxylate esters are produced by condensation of a carboxylic acid and an alcohol, which results in the release of water (Figure 1.6). In an aqueous environment, such as inside the cell, the concentration of water approaches 55 M. Due to this high water concentration, the Gibbs free energy change of this reaction at physiological conditions ($\Delta G'^m$) is high (28.8 kJ/mol for ethyl acetate⁹⁰). This hampers synthesis of esters directly from a carboxylic acid and an alcohol. Microorganisms circumvent this by first converting the carboxylic acid to an acyl-CoA intermediate, at the expense of ATP. This acyl-CoA can also be produced by other means; an example is acetyl-CoA production from pyruvate by pyruvate dehydrogenase. This enables microorganisms to produce a vast array of esters. This feature has been utilized in the last decades to produce industrially relevant esters through biocatalysis^{82–89}. Esterification combined with monooxygenation could thus be a viable strategy to ω -oxidize MCFAs.

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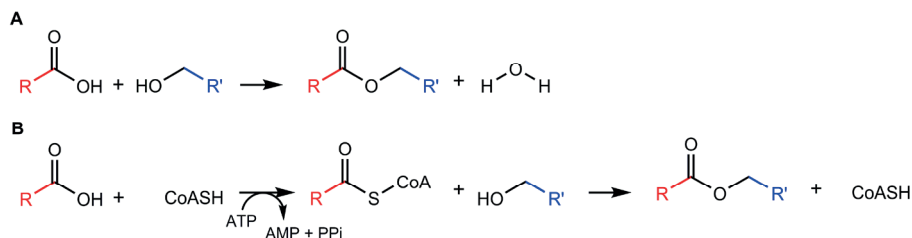


Figure 1.6. Carboxylate ester formation. The acyl chains are shown in red, the alkyl chains in blue. Reaction A: standard chemical route. Reaction B: biological route.

Biocatalytic production of methyl esters is possible using fatty-acid O-methyltransferase, but this has resulted in rather low product titers so far⁹¹. More promising are the alcohol-acyltransferases (AATs). AATs are responsible for the production of a plethora of esters. This includes tri-acylglycerides, wax esters and volatile esters. AlkB functions well with methyl esters, and is most active with methyl nonanoate as substrate⁵⁸, when expressed in *E. coli*. There are not many reports of AATs that accept methanol as substrate, and usually the AAT is not very active with this alcohol⁹². On the contrary, several AATs can synthesize ethyl esters^{84,86,93–95}. Of these AATs, AtfA and Eeb1 have a substrate specificity that enables short-chain alcohols to be coupled with medium-chain acyl-CoAs. Both enzymes have already been applied for (medium-chain) ester production in *E. coli*^{83–85,93}. Hence, converting the MCFA to an ester should be possible. However, it is not known how well AlkB functions with esters that have a longer alkyl chain.

1.9. Conversion strategy

AlkB thus far seems to be the most promising monooxygenase for ω -functionalization of esterified MCFAs. For production of commodity chemicals, high product titers should be reached, since the product value is low. Considering that AlkB is membrane-bound and that NAD(H) has to be recycled, whole-cell biocatalysis is necessary to achieve high product titers. In this setup, AlkB is most stable and NADH can be regenerated by the host.

Both the native host, *P. putida* GPo1, and *E. coli* have been used as whole-cell biocatalyst expressing the AlkBGT system for ω -oxidation of n-alkanes and fatty acid methyl esters^{49,78,96,97}. However, *E. coli* seems a more appropriate

host for the production of abovementioned compounds for several reasons. Firstly, *E. coli* expressing AlkBGT shows higher activities on n-alkanes and fatty acid methyl esters than *P. putida* GPo1⁵⁸. Secondly, *P. putida* GPo1 can utilize MCFAs and medium-chain n-alkanes for growth *via* β -oxidation, whereas the β -oxidation of *E. coli* is not induced by MCFAs⁹⁸.

Further conversion towards acids and/or esters is also necessary, increasing the amount of necessary proteins for the pathway. Considering all these factors, whole-cell biocatalysis is the most promising approach to produce BMs from MCFAs and n-alkanes.

1.10. Aim of this thesis

The aim of this thesis is to develop a method to produce α,ω -bifunctional monomers of C6 to C10 chain length directly from fatty acids and n-alkanes. This thesis focuses on the use of esters to improve ω -oxidation by monooxygenase AlkB *via* whole-cell biocatalysis with *E. coli*. Furthermore, it is investigated whether the esters can be produced by the whole-cell biocatalyst itself.

1.11. Thesis outline

In order to achieve efficient ω -oxidation, fatty acids or fatty alcohols have to be esterified. Preferably, the esters are generated by the biocatalyst itself, following the proposed pathway in Figure 1.7. This would reduce the amount of unit operations in the process. Before, AlkB was only tested with methyl esters of fatty acids. If those esters have to be synthesized *in vivo*, methanol would be necessary as alcohol donor. Methanol however, is not efficiently used by alcohol-acyltransferases that are necessary for the esterification. Furthermore, methanol is toxic, creating an operational hazard^{99,100}. In **chapter 2**, we tested if AlkB also ω -oxidizes medium-chain *ethyl* esters, in a whole-cell biocatalytic setup. Furthermore, we examined the effect of outer membrane protein AlkL on the ω -oxidation activity of whole-cells.

In **chapter 3**, we investigated whether the ω -alcohols of fatty acid esters, that are produced by AlkB, can be converted into mono-esterified dicarboxylic acids. The AlkBGT system was expanded with alcohol dehydrogenase AlkJ and aldehyde dehydrogenase AlkH.

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Esterification of the fatty acid *in vivo* would obviate the need of adding a fatty acid ester to the conversion medium. This could reduce the costs of the overall production process. We combined *in vivo* ester synthesis, by AlkK and AATs, and ω -oxidation in *E. coli*, to produce (esterified) medium-chain DCAs directly from MCFAs in **chapter 4**.

In chapter 4 ω -oxidation was combined by *in vivo* esterification. We also investigated if di-terminal oxidation of n-alkanes to dicarboxylic acids is possible using this approach. Furthermore, n-alkanes can in theory be used as substrate for α,ω -diol production if the primary alcohol is not further oxidized and also the ω -carbon atom is oxidized to the alcohol. In **chapter 5** we tested both concepts.

Finally, in **chapter 6** we present the general discussion of this thesis, focusing on key factors that need to be addressed to improve the processes.

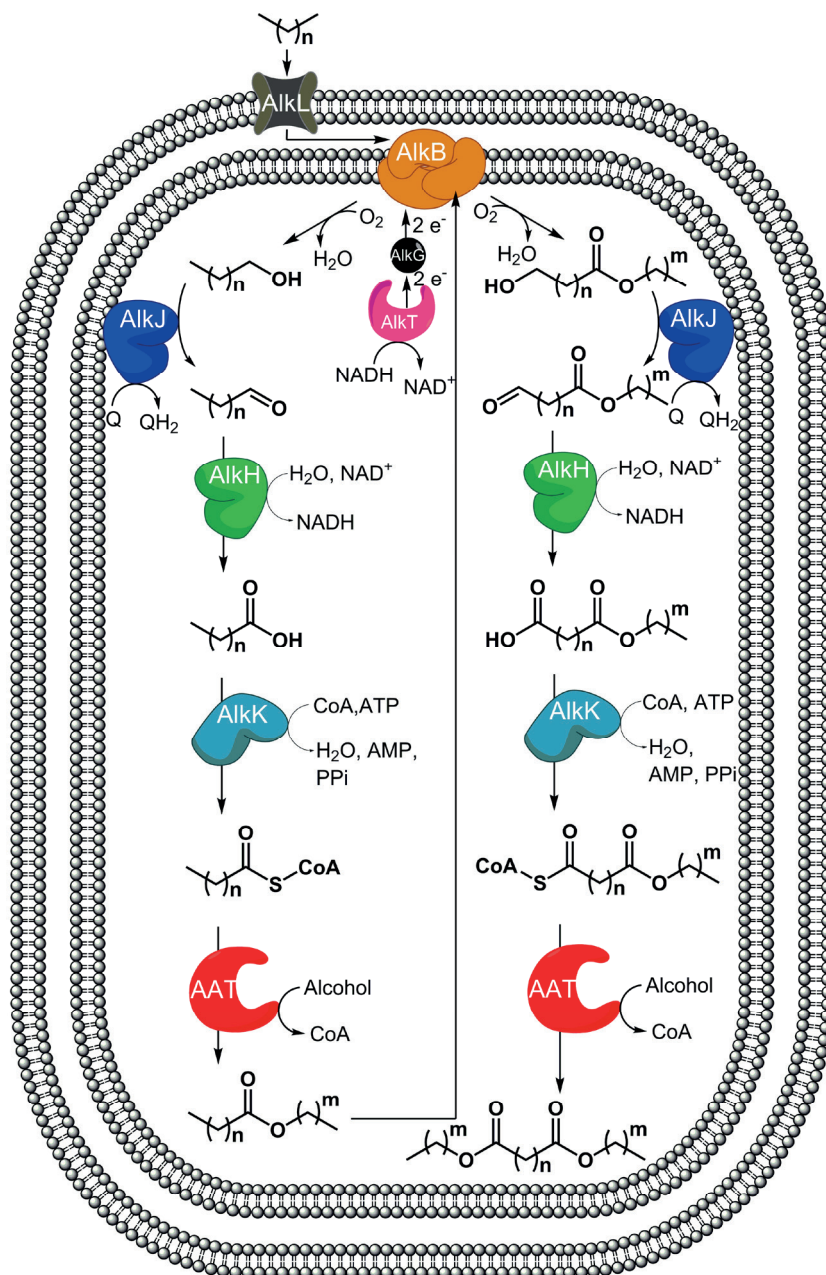
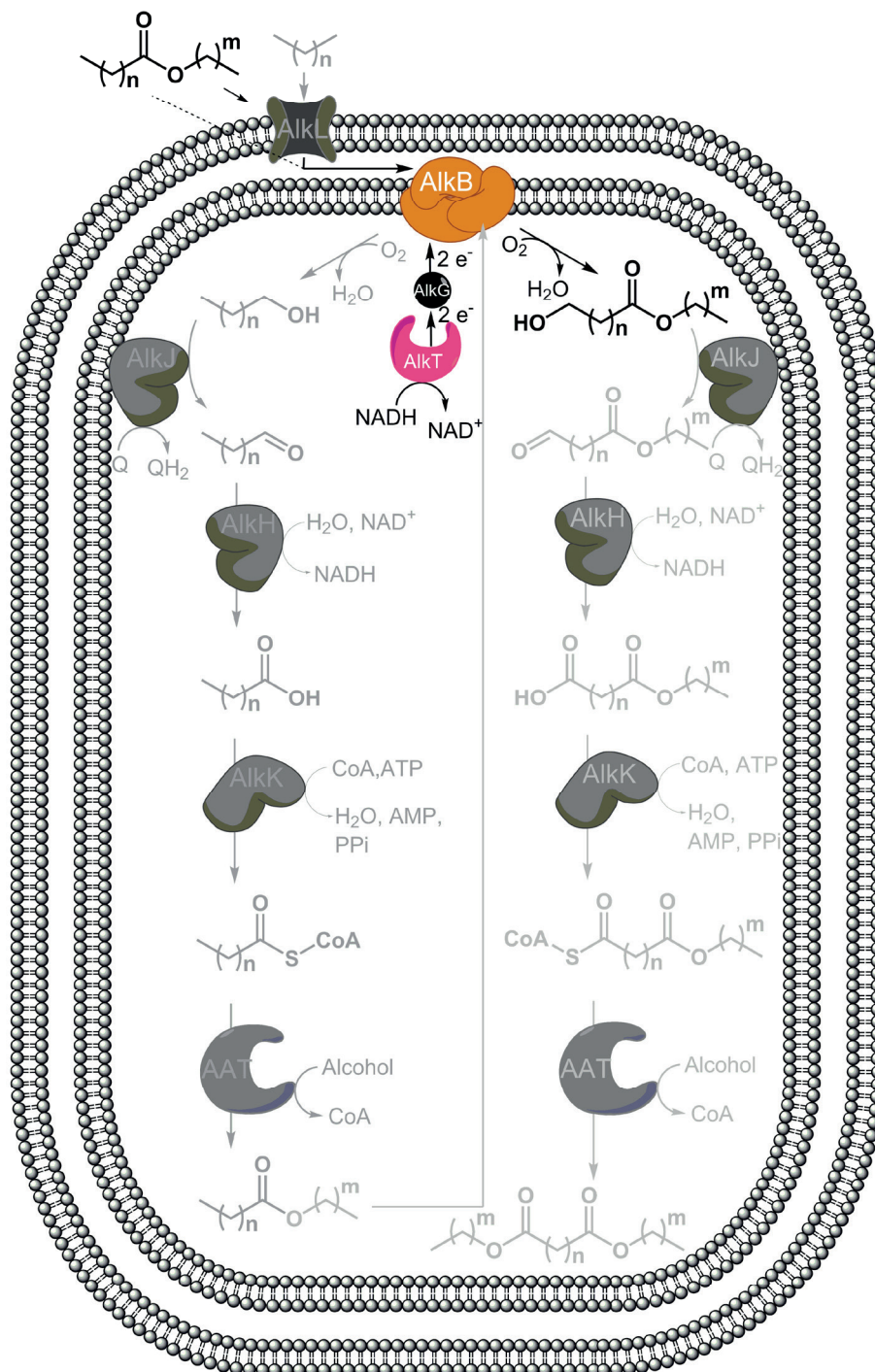


Figure 1.7. Proposed pathway for diterminal oxidation of n-alkanes and fatty acids in *E. coli*. AlkL facilitates transport. The AlkB_{GT}HJ proteins are responsible for terminal oxidation, AlkK and alcohol acyltransferases (AAT) for *in vivo* esterification



Chapter 2 Application of AlkBGT and AlkL from *Pseudomonas putida* GPo1 for Selective Alkyl Ester ω -Oxyfunctionalization in *Escherichia coli*

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2.1. Abstract

The enzyme system AlkBGT from *Pseudomonas putida* GPo1 can efficiently ω -functionalize fatty acid methyl esters. Outer membrane protein AlkL boosts this ω -functionalization. In this study it is shown that whole-cells of *E. coli* expressing the AlkBGT system can also ω -oxidize ethyl nonanoate (NAEE). Co-expression of AlkBGT and AlkL resulted in 1.7-fold higher ω -oxidation activity on NAEE. With this strain, initial activity on NAEE was 70 U/g_{cdw}, 67% of the initial activity on methyl nonanoate. In time-lapse conversions with 5 mM NAEE the main product was 9-hydroxy NAEE (3.6 mM), but also 9-oxo NAEE (0.1 mM) and 9-carboxy NAEE (0.6 mM) were formed. AlkBGT also ω -oxidized ethyl, propyl and butyl esters of fatty acids ranging from C6 to C10. Increasing the length of the alkyl chain improved the ω -oxidation activity of AlkBGT on esters of C6 and C7 fatty acids. From these esters, application of butyl hexanoate resulted in the highest ω -oxidation activity of 82 U/g_{cdw}. Co-expression of AlkL only had a positive effect on ω -functionalization of substrates with a total length of C11 or longer. These findings indicate that AlkBGT(L) can be applied as a biocatalyst for ω -functionalization of ethyl, propyl and butyl esters of medium chain fatty acids.

2.2. Importance

Fatty acid esters are promising renewable starting materials for the production of ω -hydroxy fatty acid esters (ω -HFAEs). These ω -HFAEs can be used to produce sustainable polymers. Chemical conversion of the fatty acid esters to ω -HFAEs is challenging as it generates by-products and needs harsh reaction conditions. Biocatalytic production is a promising alternative. In this study, biocatalytic conversion of fatty acid esters towards ω -HFAEs was investigated, using whole cells. This was achieved with recombinant *Escherichia coli* cells that produce the AlkBGT enzymes. These enzymes can produce ω -HFAEs from a wide variety of fatty acid esters. Medium chain length acids (C6 to C10) esterified with ethanol, propanol or butanol were applied. This is a promising production platform for polymer building blocks, that uses renewable substrates and mild reaction conditions.

2.3. Introduction

The global demand for polymers is expected to grow in the coming years¹, and thus also the need for sustainable polymer production processes. ω -Hydroxy fatty acids (ω -HFAs) and dicarboxylic acids (DCAs) are building blocks of polymers such as polyesters and polyamides^{1,101,102}. These compounds can be produced from medium-chain-length fatty acids (MCFAs) by oxidation of the terminal methyl group, a reaction called ω -oxidation¹⁰³.

A recent development is the production of fatty acids by processes using microbial chain elongation from organic waste material, yielding both odd and even chain length fatty acids ranging from C4 to C9^{25,27}.

Chemical ω -oxidation of non-activated sp^3 C-H bonds remains challenging due to the inert nature of these bonds. This results in poor selectivity^{28,104}. Biocatalytic ω -oxidation can be a solution for the terminal activation of fatty acid (esters). Several classes of enzymes can ω -oxidize terminal methyl groups⁴¹. A key advantage is that some of these enzymes only oxidize the terminal methyl group. CYP52 enzymes have been applied in whole-cell approaches to yield high titers of ω -oxidized product from (esterified) fatty acids, but this approach is limited to a chain length of \geq C12^{35,37}.

The alkane hydroxylase system AlkBGT from *Pseudomonas putida* GPo1 could be a more promising approach for ω -oxidation of medium chain length compounds. This system is composed of a monooxygenase (AlkB), a rubredoxin (AlkG) and a rubredoxin reductase (AlkT), all located on the *alk*-operon⁴⁶. AlkB was found to oxidize methyl groups of medium-chain alkanes and medium-chain fatty acids¹⁰⁵. AlkB has a remarkably relaxed substrate specificity. This enzyme can oxidize C5 to C16 alkanes, cycloalkanes, alkenes and thioethers^{59,64,106}.

Recently, this AlkBGT system has also been shown to efficiently catalyze the ω -oxidation of methyl esterified fatty acids. Methyl pentanoate to methyl dodecanoate were ω -oxidized by this system when expressed in *Escherichia coli*. The activity of AlkB was highest on methyl nonanoate, even higher than on n-octane and n-nonane. This activity declined strongly for methyl esters of fatty acids with a chain length shorter than 8⁶⁵. Outer membrane protein AlkL

was successfully applied to further increase the activity of *E. coli* expressing AlkBGT on larger, more hydrophobic molecules. The ω -oxidation activity of cells harboring AlkL on methyl dodecanoate was improved 28-fold in small scale assays, and 62-fold in a two-liquid phase setup^{58,78}.

Using an esterified fatty acid as substrate has several advantages over the use of free fatty acids. Firstly, the substrate does not dissolve well in water and forms a separate phase. The ω -oxidized product accumulates in the organic phase, which obviates the need of water removal for downstream processing⁵⁸. Secondly, esterified MCFAs might pose less toxicity problems to the cell compared to free MCFAs. Free fatty acids inhibit growth of a large variety of organisms⁷⁰. Medium chain fatty acids can freely diffuse through the membrane of *E. coli*¹⁰⁷, inhibit its growth and cause leakage of the membrane^{108,109}. But when methyl nonanoate or methyl dodecanoate was added to growing *E. coli* W3110 cultures, the growth rate only declined slightly⁵⁸. This suggests that esterified MCFAs are less toxic than free MCFAs. Thirdly, the broth does not need to be acidified for product recovery when acids are esterified, reducing the amount of chemicals needed for downstream processing.

Considering the broad substrate specificity of AlkB and the abovementioned advantages for the use of esterified fatty acids, it is also interesting to assess whether AlkBGT facilitates ω -oxidation of alkyl-esters with a longer alkyl chain. By increasing the length of the alkyl chain, the total length of the substrate increases. This might boost activity of AlkBGT on shorter chain fatty acid esters.

In this paper it was investigated in whole-cell experiments whether the AlkBGT system can also ω -oxidize ethyl, propyl and butyl esters of C6 to C10 fatty acids. Also, AlkL was applied to evaluate whether this could increase the ω -oxidation activity of whole cells.

2.4. Materials and methods

2.4.1 Plasmids, strains and chemicals

The plasmids used in this study are listed in Table 2.1. pCOM10_alkL and pGEc47 were kindly provided by Dr. Bruno Bühler. Plasmids pBT10 and pBTL10 were constructed as described in⁶⁵ and⁵⁸. *E. coli* TOP10 (Invitrogen™) was used for cloning purposes. *E. coli*-NEBT7 (New England Biolabs™) was used for conversion and toxicity studies.

Table 2.1. Plasmids used in this study

Plasmids	Characteristics	Reference
pCOM10_alkL	pCOM10 vector containing <i>alkL</i>	⁵⁸
pGEc47	<i>alk</i> operon on pLAFR1	46
pSTL	<i>alkTL</i> on pCOM10	This study
pBT10	<i>alkBGT</i> on pCOM10	This study, created as in ⁵⁸
pBTL10	<i>alkBGT</i> L on pCOM10	This study, created as in ⁵⁸

Chemicals were ordered with the highest purity available from the following vendors:

Sigma Aldrich: dodecane; nonanoic acid; methyl hexanoate, ethyl hexanoate, propyl hexanoate, butyl hexanoate; butyl octanoate; methyl nonanoate, ethyl nonanoate; butyl decanoate; 6-hydroxy ethyl hexanoate; 7-hydroxy ethyl heptanoate. Alfa Aesar: methyl octanoate, ethyl octanoate; methyl decanoate, ethyl decanoate, propyl decanoate; Merck: methyl heptanoate, ethyl heptanoate; Pfaltz and Bauer: propyl heptanoate; TCI: propyl octanoate; 9-hydroxy nonanoic acid methyl ester; LGC standards: butyl heptanoate; Santa Cruz Biotechnology: propyl nonanoate; TRC: 9-hydroxy nonanoic acid ethyl ester, 9-oxo methyl nonanoate, azelaic acid mono-ethyl ester.

2.4.2 Cultivation and gene expression for conversion studies

Cultivation was done in a rotary shaker at 250 rpm, 37 °C. *E. coli*-pSTL was grown overnight in 5 mL LB + 50 µg/mL kanamycin. The next day, 500 µL of the overnight culture was transferred to 50 mL mineral medium in a 300 mL shake flask. The mineral medium contained: 1X M9 salts, 0.5% glucose, 2 mM MgSO₄, 1 mL/L US^{Fe} trace elements¹¹⁰ and kanamycin. Cells were again

cultured overnight, and then inoculated into the same mineral medium to 0.05 g_{cdw}/L in 300 or 500 mL shake flasks. The culture was directly induced with 0.025% DCPK and incubated for 4 hours.

2.4.3 Toxicity studies

E. coli-pSTL was grown as above, except that the cells were not induced with DCPK. The overnight mineral medium culture was used to inoculate 250 mL of mineral medium to a density of 0.05 g_{cdw}/L. This culture was grown to a density of 0.18 g_{cdw}/L, and then divided into 20 mL cultures. Nonanoic acid or ethyl nonanoate were added and growth rates were determined by measuring the optical density at 600 nm. Experiments were done in duplicate.

2.4.4 Whole cell conversions

DCPK-induced cells were harvested by centrifugation at 4255 x g for 10 minutes and resuspended in 50 mM pH 7.4 phosphate buffer, containing 1% of glucose and 2 mM MgSO₄. Biomass concentration was set to 1 g_{cdw}/L. 1mL conversion tests were done in triplicate at 37 °C in a closed vessel at 250 rpm. Prior to the conversion, cells were incubated in the shaker for 5 to 10 minutes. The conversion was started by addition of 5 mM substrate from a concentrated stock in ethanol, the final concentration of ethanol in the reaction was 2.5 % v/v. Initial activities in units per gram cell dry weight were determined by quantification of oxidized product after 5 minutes of incubation, in which 1 U = 1 μ mol/min. Time-lapse tests were done similarly to the determination of the initial activities, except that 300 μ L of resting cell suspension was used and different incubation times were applied.

2.4.5 GC analysis

The whole reactions were extracted with 1:1 CHCl₃:MeOH containing 0.2 mM dodecane as an internal standard. The time-lapse samples were extracted by adding 200 μ L of the reaction to 800 μ L CHCl₃ containing 0.2 mM dodecane. For qualitative analysis samples were analyzed with a Thermo Scientific™ TRACE™ Ultra gas chromatograph coupled to a DSQII mass spectrometer. Quantitative analysis was done with an Agilent HP 6890 GC coupled to an FID. Response factors of chemicals that were not commercially available were based on structurally similar chemicals.

2.5. Results and discussion

2.5.1 Toxicity tests

First it was tested if ethyl nonanoate (NAEE) was indeed a better substrate than nonanoic acid (NA) by testing their effect on the growth rate. In Table 2.2 results of the toxicity study are shown.

Table 2.2. Growth rate of *E. coli*-pSTL in presence of NA or NAEE^a

Initial concn (mM)	NA		NAEE	
	μ (h ⁻¹)	Remaining concn (mM)	μ (h ⁻¹)	Remaining concn (mM)
0	0.52 ± 0	-	0.52 ± 0	-
1	0.55 ± 0	0.47 ± 0.03	0.37 ± 0.02 ^b	0.18 ± 0.06
10	-0.24 ± 0	7.14 ± 0.56	0.30 ± 0	8.77 ± 0.50

^aRemaining NA/NAEE was measured at the end of the culture phase. ^b A lag phase of 2 hours was observed

In the presence of 1 mM NA, the growth rate of *E. coli* was similar to the control, but growth ceased at a concentration of 10 mM. The growth rate was negatively affected by the presence of 1 mM NAEE, but cells still grew in presence of concentrations up to 10 mM. Because the growth rate of *E. coli* in presence of NAEE at 10 mM was higher, this was considered a more suitable substrate.

2.5.2 Whole-cell conversions with AlkBGT(L)

To verify functionality of the AlkBGT enzyme system with methyl nonanoate (NAME), resting cell conversions were done. *E. coli* was used carrying either the pBT10 vector, or the pBTL10 vector for the positive control. *E. coli*-pSTL was used as a negative control (Table 2.3)

Table 2.3. Specific activities for ω -oxidation of NAME and NAEE with different *E. coli* strains in a whole-cell bioconversion^a

Strain	Substrate	Specific activity (U/g _{cdw})	Ratio activity NAEE/NAME
<i>E. coli</i> -pSTL	NAME	No product	-
	NAEE	No product	
<i>E. coli</i> -pBT10	NAME	84 \pm 10	0.49
	NAEE	41 \pm 6	
<i>E. coli</i> -pBTL10	NAME	105 \pm 6	0.67
	NAEE	70 \pm 3	

^aApplied biomass concentrations: pSTL: 0.98 g_{cdw}/L, pBT10: 1.04 g_{cdw}/L, pBTL10: 0.96 g_{cdw}/L

Cells expressing AlkBGT yielded the product 9-hydroxy methyl nonanoate (9HNAME) from the substrate NAME, confirming the functionality of the AlkBGT enzyme system. The specific activity was 84 U/g_{cdw}. This activity was lower than the 104 U/g_{cdw} reported by Schrewe and colleagues⁶⁵, but in the same order of magnitude. The presence of outer membrane protein AlkL improved the ω -oxidation activity from 84 U/g_{cdw} to 105 U/g_{cdw}, a 1.3-fold improvement. This is in line with earlier findings, where the ω -oxidation activity on NAME increased from 104 U/g_{cdw} to 128 U/g_{cdw}, a 1.2-fold improvement⁵⁸.

The same strains were tested with nonanoic *ethyl* ester as substrate, to investigate the effect of using a longer ester length. In this case 9-hydroxy ethyl nonanoate (9HNAEE) was detected; indicating that AlkB is also active on NAEE, in both absence and presence of AlkL. The effect of increasing the alkyl chain length only led to a decrease in activity of 33% in cells co-expressing AlkL. As expected, the presence of AlkL does seem to increase the substrate availability of NAEE. The ω -oxidation activity of the pBTL10 strain was 1.7-fold higher than the activity of the pBT10 strain. The effect of AlkL is more evident for NAEE than NAME. The more apolar character of NAEE compared with NAME likely decreases transport into the cell and therefore the presence of AlkL has a more profound influence.

2.5.3 Whole-cell conversions time-lapse with pBT10 and pBTL10

We then explored production of ω -functionalized odd-chain esters with *E. coli*-pBT10/pBTL10 over time, in a time-lapse experiment. These tests were

done with NAEE as a substrate. The same experiment was done with NAME to compare ω -oxidation activity and the effect of a longer alkyl chain on this activity. Also, both pBT10 and pBTL10 strains were used to assess whether AlkL has a positive influence on yield.

The results of these conversions are shown in Figure 2.1. For all samples the presence of NAME was confirmed, except the samples at $t = 120$ min. This suggests that substrate became limiting after $t = 60$ min. Highest yields were reached with the pBTL10 strain that converted 4.4 mM NAME, nearly 90%, into ω -oxidized product. The pBT10 strain converted 3.9 mM NAME into ω -oxidized product, close to the amount converted by pBTL10. The presence of AlkL improved the whole-cell activity slightly, as tests with pBTL10 yielded higher product titers at $t = 30$ min compared with tests with pBT10.

The predominant product that was detected was 9HNAME, representing more than 85% of the ω -oxidized products. Under these conditions, the ω -aldehyde hardly accumulates (highest concentration measured was 0.14 mM). But AlkB does overoxidize the substrate, as about 0.5 mM of ω -acid of methyl nonanoate (9CNAME), was detected. This was comparable with findings in earlier published work⁶⁵. A recent publication by the same group reported the apparent K_s values for C12 oxygenation products⁷⁸, indicating that the apparent K_s for the aldehyde was lower compared to the alcohol. This might apply to C9 as well, resulting in higher activity for aldehyde oxidation and thus 9CNAME accumulation. Still the predominant product was the alcohol (alcohol: acid ratio of 100:17).

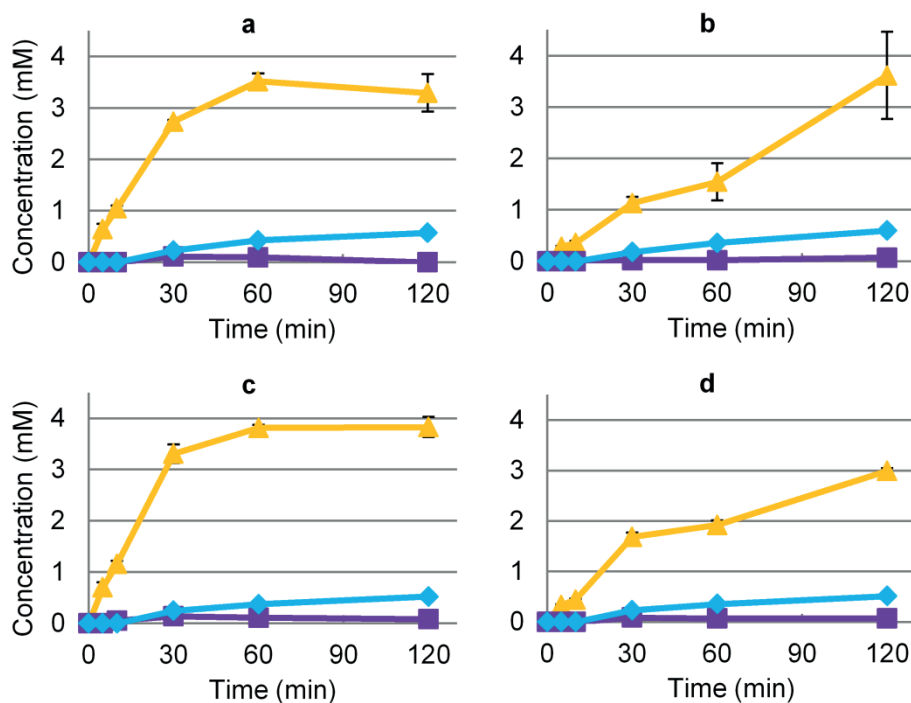


Figure 2.1. Resting whole-cell conversion time-lapse. (a) pBT10 strain plus 5 mM NAME; (b) pBT10 strain plus 5 mM NAEE; (c) pBTL10 strain plus 5 mM NAME; (d) pBTL10 strain plus 5mM NAEE. Diamonds, 9-hydroxy product; triangles, 9-carboxy product; squares, 9-oxo product. The applied biomass concentration was 1.1 gdcw/liter.

In the tests where NAEE was used as substrate, the initial activity is lower, corresponding with findings presented in Table 2.3

After 120 minutes however, the conversion with pBT10 yielded 4.3 mM oxidized product, nearly as much as the conversions with NAME. The ω -aldehyde did not accumulate above 0.1 mM. The alcohol:acid ratios after 120 minutes were 100:17 for pBT10 and pBTL10 tests, similar to conversions with NAME.

2.5.4 AlkB ω -oxidation of medium-chain esters with varying alkyl ester chain length

Knowing that AlkB has a very broad substrate specificity⁶⁴ and the fact that NAEE was also accepted, activity assays were done to see if this was also the

case for other acyl and alcohol chain lengths. Ethyl, propyl and butyl esters of C6 to C10 fatty acids were applied as substrates, with the exception of butyl nonanoate as this chemical was not available. The results are shown in Figure 2.2.

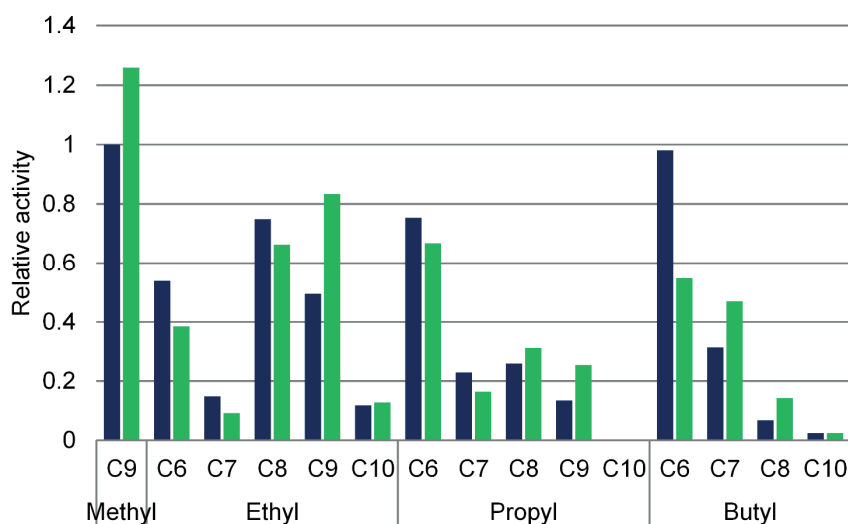


Figure 2.2. Oxidation activities of pBT10 (blue bars) or pBTL10 (green bars) strains on methyl nonanoate, ethyl, propyl, and butyl esters of C6 to C10 fatty acids, relative to the activity of the pBT10 strain on NAME. The tests with propyl heptanoate yielded multiple products, of which only ω -hydroxy propyl heptanoate was identified and used for activity calculations.

ω -Oxidation activity was detected for nearly all esters tested. Hydroxylation was only found to occur on the ω -position. These results indicate that the position of the ester group within the molecule does not strongly affect activity. Negative control experiments with the pLST strain on the different substrates did not yield products (data not shown). Activity on hexanoate and heptanoate esters could be greatly enhanced by increasing the alcohol length. Ethyl hexanoate and butyl hexanoate were ω -oxidized by the pBT10 strain at $45 \text{ U/g}_{\text{cdw}}$ and $82 \text{ U/g}_{\text{cdw}}$, respectively. The activity on butyl hexanoate was nearly as high as for the positive control NAME. These are both C10 molecules, but the position of the ester moiety is different. The comparable activities indicate that the total length of the ester substrate is more important than the position of the ester group. Apparently the polar nature of the ester bond

hardly influences the substrate binding in the hydrophobic binding pocket¹¹¹. Among the ethyl esters, a rather low activity was found for ethyl heptanoate. This phenomenon was also observed among methyl esters for methyl heptanoate⁶⁵. Still, the activity on this chain length could be enhanced by increasing the length of the alcohol group, as ethyl heptanoate was ω -oxidized by the pBT10 strain at 12 U/g_{cdw} and butyl heptanoate at 39 U/g_{cdw} by the pBTL10 strain. The tests with propyl heptanoate clearly yielded two products, but only the ω -hydroxy product could be identified. Both products gave similar peak areas in the chromatogram, suggesting that the by-product represents about half of the formed product. Only the ω -hydroxy product was used in activity determination.

For C8 and higher, the activity decreased with increasing ester length. This was also the case in tests with the pBTL10 carrying strain. So either AlkL is not efficiently transporting these molecules into the periplasmic space or AlkB is not very active on these esters. If the first explanation would apply, this effect would be contributed to the presence of the ester group and not to the length of the molecule as AlkL was shown to transport even hexadecane⁵⁹. Esters were also shown to be transported before, so this does not seem a likely explanation⁵⁸. When we analyzed the ratio of activities of pBTL10 to pBT10 strains per substrate, this explanation also did not seem to apply (Figure 2.3). Therefore, it is likely that AlkB activity is decreasing with these longer esters. In Table 2.4 the tested substrates with their structures are arranged based on the initial activities. It is also shown if AlkL improved the initial activity.

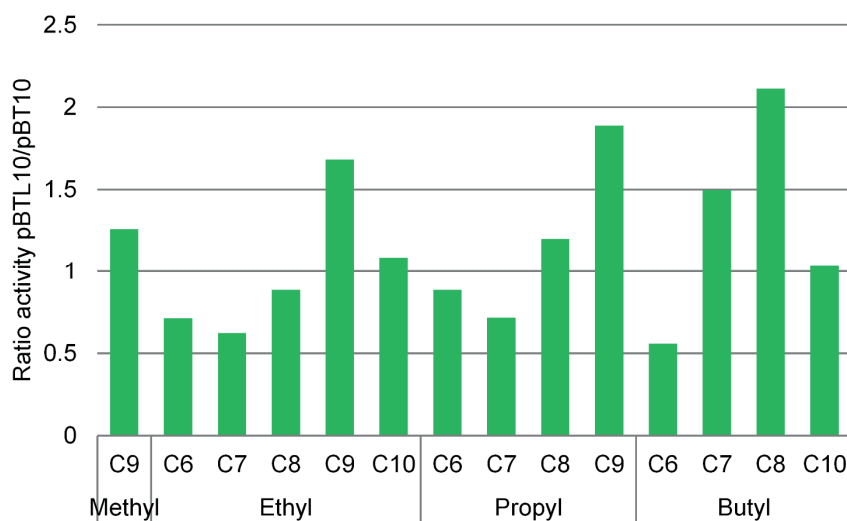


Figure 2.3. Ratio of activities of pBTL10 and pBT10 strains per substrate.

Table 2.4. Overview of tested substrates with confirmed activity of AlkBGT, arranged by initial activity (5 min)

Rank	Name	+/- AlkL ^a	Init. act. (U/g _{cdw})
1	Butyl hexanoate	-	82 ± 7
2	Ethyl nonanoate	+	70 ± 3
3	Propyl hexanoate	-	63 ± 7
4	Ethyl octanoate	-	63 ± 3
5	Ethyl hexanoate	-	45 ± 4
6	Butyl heptanoate	+	39 ± 4
7	Propyl octanoate	+	30 ± 0
8	Propyl nonanoate	+	21 ± 1
9	Propyl heptanoate	-	19 ± 1
10	Ethyl heptanoate	-	12 ± 0
11	Butyl octanoate	+	12 ± 4
12	Ethyl decanoate	+	11 ± 2
13	Butyl decanoate	+	2 ± 0

^a For each substrate, tests with and without AlkL were done, and the setup that yielded the highest activity was selected. -, the strain carried pBT10; +, the strain carried pBTL10.

2.5.5 When does AlkL have a beneficial effect?

It became clear that the tested substrates in this study were more efficiently ω -oxidized by the pBTL10 strain compared to the pBT10 strain when the total chain length (meaning the sum of carbon atoms in the acyl and the alkyl chain) exceeded 10. Only methyl nonanoate did not seem to follow this trend. This was also found to be the case in earlier work⁶⁵. Considering that the total length of the molecule was the most important factor, the ratio of pBTL10:pBT10 activities were plotted against the number of carbon atoms of the different substrates (Figure 2.4).

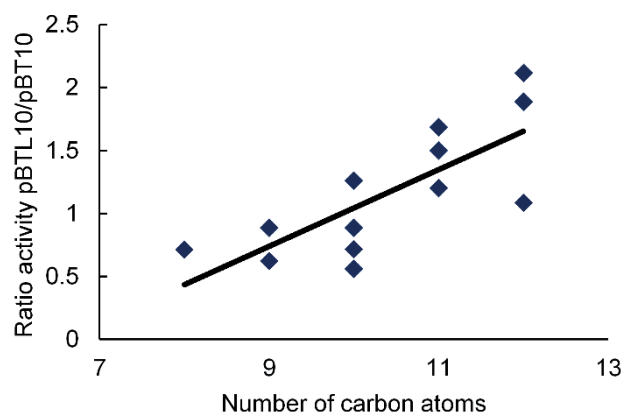


Figure 2.4. Ratio of activities of the pBTL10 and pBT10 strains plotted against the number of carbon atoms of the tested substrate. Butyl decanoate data were not used for this experiment, as the absolute activity was very low on this substrate.

For most substrates, the effect of AlkL was only beneficial when the number of carbon atoms exceeded 10. These substrates have a $\log P_{o/w}$ above 4. Substrates with a $\log P_{o/w}$ between 1 and 4 reach high concentrations within the membrane, but when $\log P_{o/w}$ exceeds 4 this is not the case, owing to low solubility¹¹². These data confirm that conversions with substrates having a $\log P_{o/w}$ higher than 4 suffer from poor mass transfer if AlkL is not present.

2.6. Conclusions

The AlkBGT-enzyme system was successfully applied for ω -oxidation of NAEE, and an activity of 67% compared to when NAME was used as substrate when

also AlkL was expressed. It has been shown before that AlkB overoxidizes the ω -hydroxy product of methyl esters^{58,65,78}. AlkB also overoxidizes NAEE to the acid product, after conversion of the hydroxy derivative towards the aldehyde. The aldehyde did not accumulate above 0.1 mM.

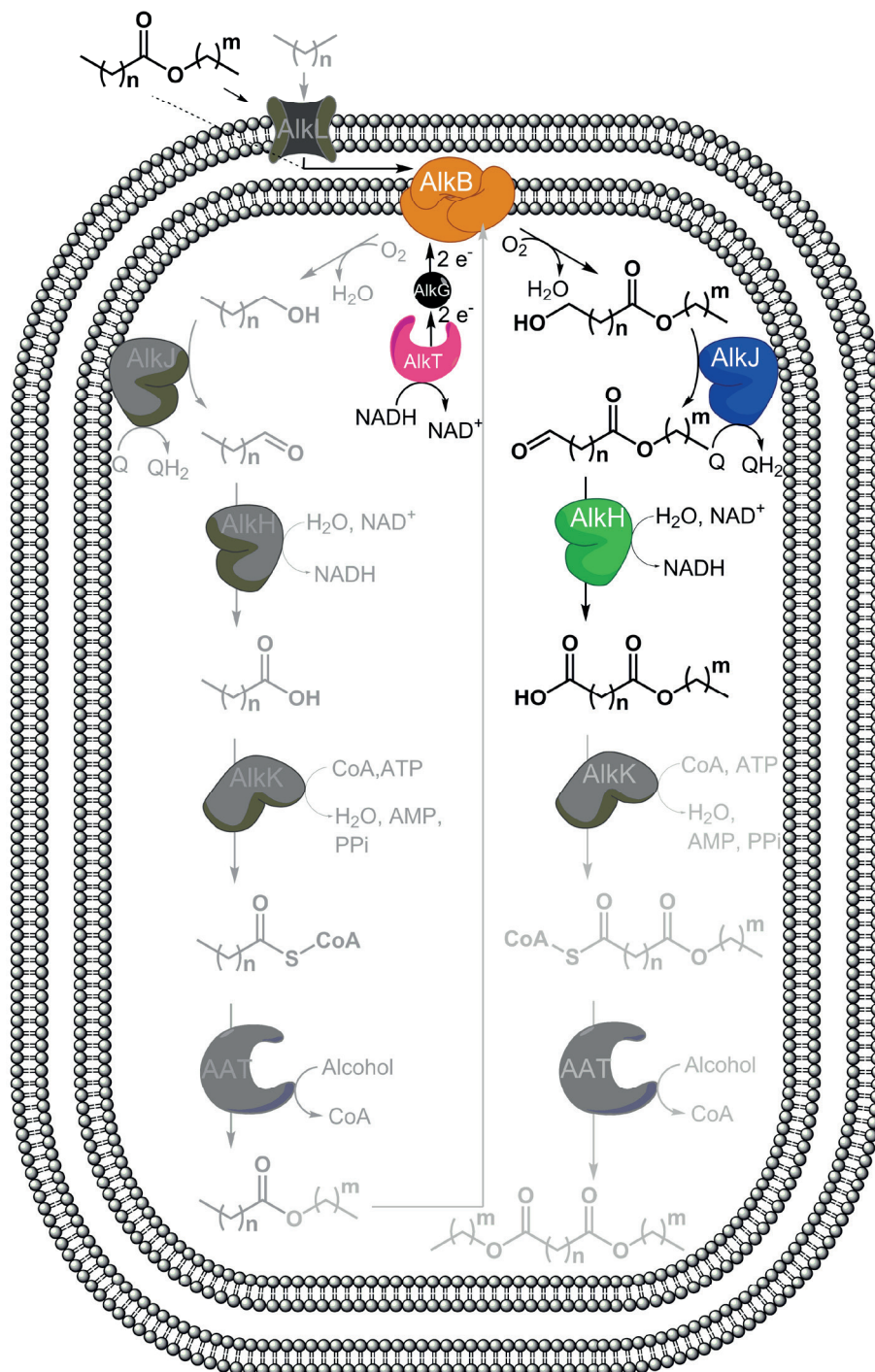
Also, propyl and butyl esters of medium chain fatty acids (C6-C10) were accepted as a substrate by AlkB. Furthermore, the activity of AlkB on esters of shorter fatty acids can be enhanced by using a longer alcohol donor for the ester substrate.

AlkL was shown to only have a positive effect on initial activity when the total number of carbon atoms was 11 or higher, with the exception of methyl nonanoate. This seems to correspond well with earlier findings, that solvents with a $\log P_{o/w}$ below 4 easily diffuse into the membrane and solvents with a $\log P_{o/w}$ above 4 do not^{58,112}.

These findings demonstrate the possibility of sustainable production of medium-chain ω -hydroxy esters with a chain length of C6 to C10. Substrates with a longer alkyl chain are also efficiently ω -oxidized, and increasing the alkyl chain resulted in high ω -oxidation activities on C6 and C7 esters.

2.7. Acknowledgements

The authors declare that there are no conflicts of interest. We would like to thank Dr. Bruno Bühler for supplying the pCOM10_alkL and pGEc47 plasmids, Dr. Elinor Scott for useful discussions and Jeroen de Jager for doing experiments.



Chapter 3 Expansion of the ω -Oxidation System AlkB_{GTL} of *Pseudomonas putida* GPo1 with AlkJ and AlkH Results in Exclusive Mono-esterified Dicarboxylic Acid Production in *E. coli*

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3.1. Abstract

The AlkB_{GTL} proteins coded on the *alk* operon from *Pseudomonas putida* GPo1 can selectively ω -oxidize ethyl esters of C6 to C10 fatty acids in whole-cell conversions with *Escherichia coli*. The major product in these conversions is the ω -alcohol. However, AlkB also has the capacity to overoxidize the substrate to the ω -aldehyde and ω -acid. In this study we show that alcohol dehydrogenase AlkJ and aldehyde dehydrogenase AlkH are able to oxidize ω -alcohols and ω -aldehydes of esterified fatty acids, respectively. Resting *E. coli* expressing AlkB_{GTHJL} enabled exclusive mono-ethyl azelate production from ethyl nonanoate, with an initial specific activity of 61 U/g_{cdw}. Within 2 hours this strain produced 3.53 mM mono-ethyl azelate, with a yield of 0.68 mol/mol. This strain also produced mono-ethyl dicarboxylic acids from ethyl esters of C6 to C10 fatty acids and monomethyl azelate from methyl nonanoate. Adding ethyl nonanoate dissolved in carrier solvent bis-(2-ethylhexyl) phthalate enabled an increase of product titers to 15.55 mM in two-liquid phase conversions. These findings indicate that *E. coli* expressing AlkB_{GTHJL} is an effective producer of mono-esterified dicarboxylic acids from fatty acid esters.

3.2. Introduction

Medium-chain α,ω -dicarboxylic acids (DCAs) are building blocks for polyesters, polyurethanes and polyamides. Adipic acid (AA), a C6 DCA, is produced from the petrochemical feedstock benzene. To produce AA, benzene is converted into a mixture of cyclohexanol and cyclohexanone. This mixture is oxidized with nitric acid to yield adipic acid with the concomitant emission of N_2O , a potent greenhouse gas^{2,5}

C8-C10 DCAs are produced from renewable feedstocks, such as oleic acid and ricinoleic acid, via ozonolysis^{16,101}. Although ozonolysis of unsaturated fatty acids is highly selective and effective, it suffers from several drawbacks. The applied ozone is highly toxic and associated with high explosion risks. Furthermore, the process needs a high energy input¹⁵. Finally, the cleavage of unsaturated fatty acids results in byproduct formation.

Direct biocatalytic conversion of medium-chain fatty acids (MCFAs) and their esters to the corresponding ω -carboxyl derivative could be a promising alternative production method. Several recent reports demonstrate that the AlkBGT system, part of the *alk* operon from *Pseudomonas putida* GPo1, can ω -oxidize esterified fatty acids of medium chain length when expressed in *Escherichia coli*. The primary product of the alkane monooxygenase AlkB is the alcohol, but it is also able to oxidize it further to the aldehyde and carboxylic acid, a process called overoxidation. Whole-cell conversions of nonanoic and dodecanoic methyl esters therefore yield the corresponding ω -alcohol, ω -aldehyde and ω -carboxylic acid^{58,65,78}.

However, the AlkBGT system is not efficient in the production of carboxylic acids. AlkB prefers esterified fatty acids or alkanes as substrate. This results in high titres of the ω -alcohol and low conversion rates towards the carboxylic acid. The *alk* operon of *P. putida* GPo1 also contains genes encoding alcohol dehydrogenase AlkJ and aldehyde dehydrogenase AlkH^{48,54}. Production of carboxylic acids should be more efficient with these enzymes, as the alcohol and aldehyde are natural substrates for AlkJ and AlkH, respectively. Furthermore, these enzymes do not require the input of oxygen and NADH, but instead generate reduced cofactors as ubiquinol and NADH (Figure 3.1).

Mono-esterified dicarboxylic acid production

This would shift the need of NAD^+ regeneration to regeneration of the ubiquinol. Ubiquinol is regenerated to ubiquinone in the electron transport chain under aerobic conditions.

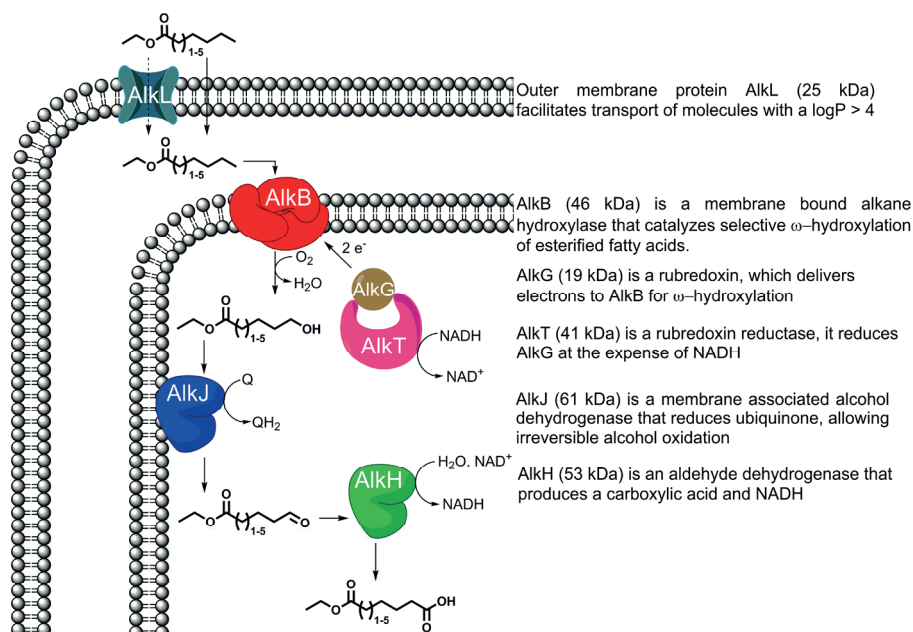


Figure 3.1. Production of C6 to C10 mono-ethyl DCAs from ethyl esters, catalyzed by AlkBGTJL

AlkJ reduces ubiquinone, ensuring irreversible alcohol oxidation⁵⁵. Recently, Schrewe and colleagues reported that AlkJ converts 12-hydroxy methyl dodecanoate to 12-oxo methyl dodecanoate. Expansion of the AlkBGT system with alcohol dehydrogenase AlkJ resulted in a shift of the product distribution towards the aldehyde and acid. Application of the carrier solvent bis(2-ethylhexyl) phthalate (BEHP) further steered the conversion towards the acid⁷⁸. The products accumulated in the organic phase, showing that they can leave the cell. Bowen *et al.* have also used AlkJ in combination with a set of aldehyde dehydrogenases in *E. coli*. They applied these enzymes for the production of α,ω -DCAs, from ω -hydroxy fatty acids that were produced *de novo* from glucose¹¹³. AlkJ was clearly the best performer among the tested alcohol dehydrogenases.

For further oxidation of the ω -aldehyde of alkyl esters, aldehyde dehydrogenase AlkH is a promising candidate as it converts medium-chain aldehydes to carboxylic acids. AlkH has been applied as part of the *alk*-operon for conversion of alkanes to fatty acids^{114,115}, but not for ω -oxidized alkyl esters.

Recently, we have demonstrated that *E. coli* expressing AlkBGT(L) can also ω -oxidize esters with an alkyl chain >1 ¹¹⁶. By increasing the alkyl chain length, the ω -oxidation activity on shorter fatty acid esters improved greatly. Outer membrane protein AlkL was beneficial when the sum of the alkyl and acyl chain length exceeded 10. In these conversions, the ω -alcohol was also the major product. The ratio of ω -alcohol: ω -acid was 100:17, and the ω -aldehyde was only detected in trace amounts. Alcohol dehydrogenase and aldehyde dehydrogenase activities are required to fully convert esterified fatty acids in mono-esterified dicarboxylic acids. The combined action of AlkJ and AlkH seem appropriate for this conversion, but have never been tested with ethyl esterified fatty acids before. Our aim is to investigate whether AlkJ and AlkH can improve production of the ω -carboxylic acid of ethyl-esterified fatty acids, and if application of carrier solvent BEHP can enhance production.

3.3. Results

Since AlkB can overoxidize ω -alcohols, it can impede the assessment of the performances of AlkJ and AlkH in the AlkBGT_{HJL} pathway. Therefore, we decided to test AlkJ and AlkH individually. This should allow us to compare AlkJ and AlkH activities with the activities of AlkBGT on the intermediates of the pathway. Conversion tests were done with resting *E. coli* cells, expressing these proteins. Activities of these cells are expressed in U/g_{cdw}, where 1 U equals 1 μ mol product formed per minute.

3.3.1 Testing functionality of AlkJ

We checked if AlkJ was functionally expressed in *E. coli* NEBT7 by testing the oxidation of 9-hydroxy ethyl nonanoate. Resting cells of *E. coli* pCOM10_alkJ and *E. coli* pCOM10_alkJL were incubated with 9-hydroxy ethyl nonanoate, see Figure 3.2a and b.

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Both strains produced 9-oxo ethyl nonanoate, indicating that AlkJ was functionally expressed and uses 9-hydroxy ethyl nonanoate as substrate. Co-expression of AlkJ and AlkL from pCOM10_alkJL resulted in lower activities (see Table 3.1), but the product titres reached after 2 h were similar (0.46 versus 0.51 mM). The conversion was incomplete; only 10% of the substrate was used, suggesting that an equilibrium was reached or that the enzyme is unstable.

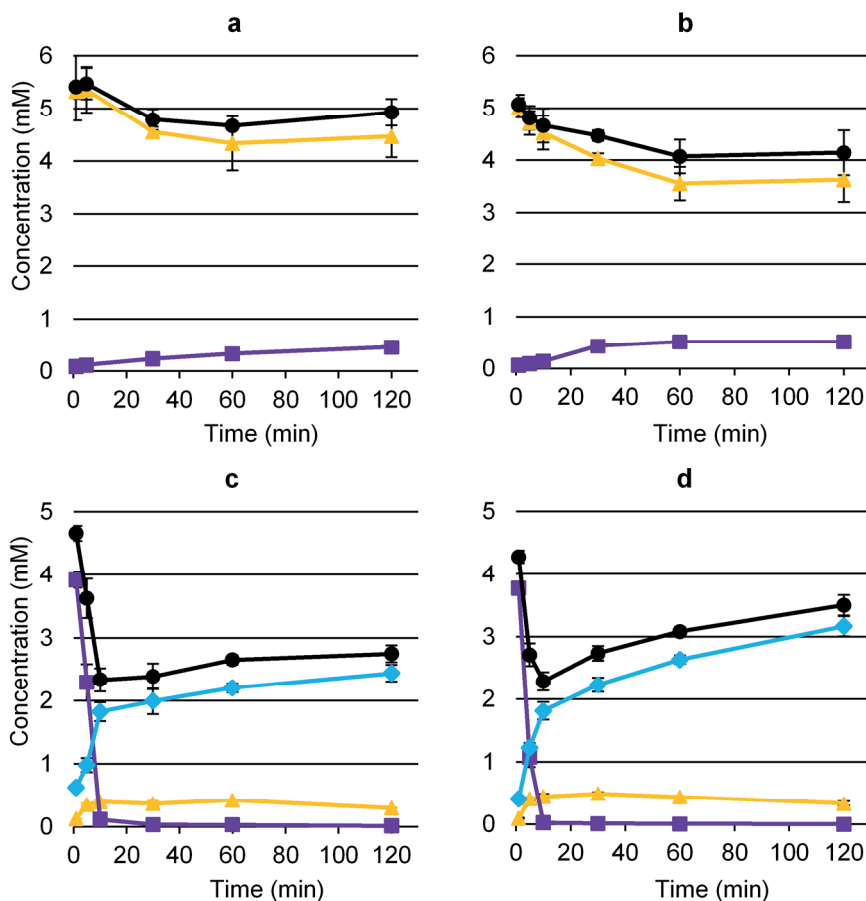


Figure 3.2. Resting cell conversions of 5 mM 9-hydroxy ethyl nonanoate (panel a and b) or 9-oxo methyl nonanoate (panel c and d) at 37 °C. Panel A: *E. coli* pCOM10_alkJ, 1.0 gcdw/L. Panel B: *E. coli* pCOM10_alkJL, 1.0 gcdw/L. Panel C: *E. coli* pCOM10_alkH, 1.1 gcdw/L. Panel D: *E. coli* pCOM10_alkHL, 1.1 gcdw/L. Triangles: ω -alcohol. Squares: ω -aldehyde. Diamonds: ω -acid. Circles: sum.

We performed the same conversions at 30°C, to see if this would improve the activity. This temperature corresponds to the optimal growth temperature of *Pseudomonas putida* GPo1. *E. coli* pCOM10_alkJ performed somewhat better at 30°C regarding initial activity (Table 3.1). The final product titres reached were lower (0.28 versus 0.46 mM; see Appendix Figure 3.4). *E. coli* pCOM10_alkJL, however, showed about fourfold higher initial activity at 30°C. Final product titres were also slightly higher at 30°C.

A control experiment was carried out at 30°C with *E. coli* pCOM10_alkL. This test confirmed that the production of 9-oxo ethyl nonanoate was due to the presence of AlkJ, as no 9-oxo ethyl nonanoate was produced by the pCOM10_alkL strain (Table 3.1; Appendix Figure 3.5).

Table 3.1. Initial (1 min) activities (U/g_{cdw}) of various resting *E. coli* NEBT7 strains in presence of 5 mM 9-hydroxy ethyl nonanoate (9HNAEE), 9-oxo methyl nonanoate (9ONAME) or ethyl nonanoate (NAEE). Negative values indicate the reverse reaction.

Strain	Substrate	T (°C)	Reaction		
			-CH ₃ -CH ₂ OH	→ -CH ₂ OH -CH=O	→ -CH=O → -COOH
pCOM10_alkL	9HNAEE	30	ND	ND	ND
	9ONAME	30	ND	-62±1	14±4
pBTL10	9HNAEE	30	ND	24±0	ND
		37	ND	30±1	27±8
	9ONAME	30	ND	-78±8	200±17
		37	ND	-31±3	101±6
	NAEE	30	51±1	ND	ND
		37	51±1	ND	ND
pCOM10_alkJ	9HNAEE	30	ND	102±6	ND
		37	ND	76±8	ND
pCOM10_alkJL	9HNAEE	30	ND	254±4	ND
		37	ND	61±2	ND
pCOM10_alkH	9ONAME	30	ND	-102±12	257±16
		37	ND	-116±22	594±12
pCOM10_alkHL	9ONAME	30	ND	-88±8	380±20
		37	ND	-88±7	383±17
pBGTHJL	9HNAEE	30	ND	227±10	58±11
	9ONAME	30	ND	-121±11	696±72
	NAEE	30	81±4	61±3	61±3

ND: not detected

3.3.2 Testing functionality of AlkH

With the same approach, we checked whether AlkH could be functionally expressed in *E. coli* NEBT7 at 37 °C. The substrate we used for these tests was 9-oxo methyl nonanoate, as 9-oxo ethyl nonanoate was not commercially available. *E. coli* pCOM10_alkH and *E. coli* pCOM10_alkHL catalysed both the reduction and oxidation of the substrate, but the oxidation activity was much higher (Figure 3.2C and D). At 30 °C, the oxidation activity of *E. coli* pCOM10_alkH was lower, but final product concentrations were similar (Table 3.1 and Appendix Figure 3.6). For *E. coli* pCOM10_alkHL, there were no differences between the two tested temperatures.

The carbon balances of these tests were not complete. We did not detect a by-product in these tests. The same experiment was carried out with *E. coli* pCOM10_alkL at 30 °C (see Table 3.1). This strain mainly reduced the substrate to the ω -alcohol. This indicates that a native *E. coli* enzyme was responsible for this reaction, also in strain pCOM10_alkH and pCOM10_alkHL. With these strains, a similar gap in the C-balance appeared. Volatility cannot be the cause of this gap, because no decrease in the concentration of 9-oxo methyl nonanoate occurred during abiotic incubations in resting-cell buffer (Appendix Figure 3.7). Also, the gap became smaller towards the end of the incubation, suggesting that the substrate was released again. These findings suggest that the substrate reversibly binds to the *E. coli* biomass.

3.3.3 Testing AlkB overoxidation capacity

To evaluate whether AlkJ/AlkH can contribute more to ω -acid production than AlkB, it is necessary to determine the overoxidation capacity of AlkB. AlkB overoxidizes ethyl nonanoate to 9-oxo ethyl nonanoate and mono-ethyl azelate¹¹⁶. We determined this overoxidation activity of whole cells expressing AlkB_{GTL} from the pBTL10 plasmid by the addition of the intermediates 9-hydroxy ethyl nonanoate and 9-oxo methyl nonanoate and measuring the initial activities (Table 3.1). *E. coli* pBTL10 oxidized 9-hydroxy ethyl nonanoate to 9-oxo ethyl nonanoate, with an initial activity of 24 U/g_{cdw} at 30°C. The activity of *E. coli* pCOM10_alkJL was about 10-fold higher (254 U/g_{cdw}). Further oxidation to mono-ethyl azelate was only detected after 5 min; at 37°C, this activity was higher. *E. coli* pBTL10 converted the aldehyde at much

higher rates, to both the alcohol ($78 \text{ U/g}_{\text{cdw}}$) and mono-methyl azelate ($200 \text{ U/g}_{\text{cdw}}$); at 37°C , those rates were lower. Still, *E. coli* pCOM10_alkHL produced mono-methyl azelate with a higher activity ($380 \text{ U/g}_{\text{cdw}}$).

3.3.4 Ethyl ester conversion with *E. coli* pBGTHJL

We then proceeded with whole-cell conversions using *E. coli* that expressed besides AlkBGL, also AlkJ and AlkH from the pBGTHJL plasmid. Tests with either 9-hydroxy ethyl nonanoate or 9-oxo methyl nonanoate were carried out, to verify functionality of AlkJ and AlkH when expressed as part of the AlkBGL pathway. *E. coli* pBGTHJL oxidized 9-hydroxy ethyl nonanoate to 9-oxo ethyl nonanoate with an initial activity of $227 \text{ U/g}_{\text{cdw}}$, which was nearly 10 times higher than the activity of *E. coli* pBTL10 (Table 3.1). The pBGTHJL strains oxidized 9-oxo ethyl nonanoate at $696 \text{ U/g}_{\text{cdw}}$; this activity was much lower with *E. coli* pBTL10 (Table 3.1). Both AlkJ and AlkH were thus functioning when expressed from the pBGTHJL plasmid.

Subsequently, we did conversions with ethyl nonanoate, using *E. coli* pBGTHJL (Figure 3.3). Conversions at 37°C pointed out that a large share of 9-hydroxy ethyl nonanoate was not converted to mono-ethyl azelate (alcohol:acid ratio 1:0.72). At 30°C however, no 9-hydroxy ethyl nonanoate was detected anymore after 2 h. Conversions of 5 mM ethyl nonanoate yielded 3.42 mM of mono-ethyl azelate, corresponding to a yield of 0.68 mol/mol. The intermediate products 9-hydroxy ethyl nonanoate and 9-oxo ethyl nonanoate did not accumulate above 0.15 mM. Moreover, at 2 h hardly any intermediate product accumulated. It is noteworthy to mention that the ester group was only hydrolysed to a very limited extent (0.07 mM after 2 h).

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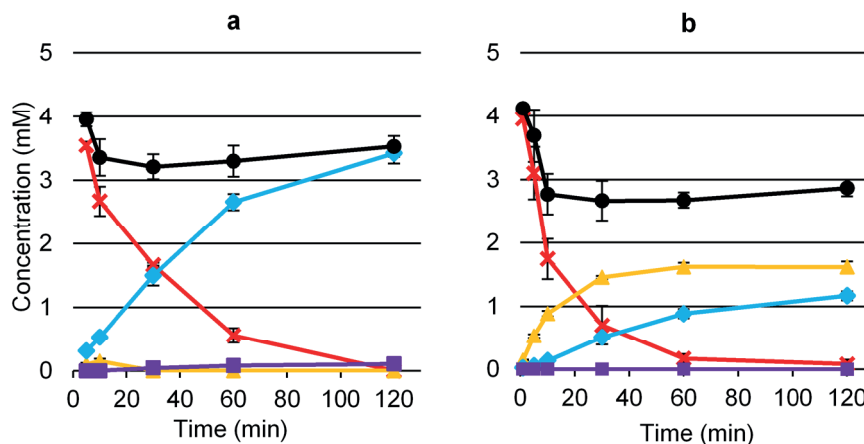


Figure 3.3. Whole-cell conversion of 5 mM ethyl nonanoate by *E. coli* pBGTHJL at 30 °C with 1.1 g_{cdw}/L biomass (a) and 37 °C with 1.1 g_{cdw}/L biomass (b). Crosses: ethyl nonanoate. Triangles: ω-alcohol. Squares: ω-aldehyde. Diamonds: ω-acid. Circles: sum.

After 2 h of incubation, the sum of products and substrate was 3.53 mM, 71% of the 5 mM substrate that was added. The rapid decrease in substrate in the first minutes of the conversion and the fact that ethyl nonanoate is volatile suggest that evaporation caused this gap. We set up a negative control experiment to test whether evaporation was the cause of this loss. The same set-up was applied, using 1.0 g_{cdw}/l *E. coli* pCOM10_alkL, a strain that is unable to ω-oxidize ethyl nonanoate. After 5 min of incubation, 52% of the added ethyl nonanoate was left, and after 2 h, only 23% (Appendix Figure 3.8). Ester hydrolysis occurred, but only 0.07 mM nonanoic acid was detected after 2 h. Evaporation thus caused the gap between added substrate and the sum of products and substrate after the conversion.

3.3.5 Substrate specificity

Ethyl esters of C6 to C10 fatty acids were also tested as substrate (Table 3.2). From all substrates, the pBGTHJL strain produced the corresponding mono-ethyl dicarboxylic acid. Also methyl nonanoate was tested, because the activity of AlkB on this substrate is the highest reported in the literature⁵⁸. The titre of mono-methyl azelate was comparable to the titre of mono-ethyl azelate. Product titres from other chain lengths were considerably lower than from methyl nonanoate and ethyl nonanoate. In samples with ethyl

heptanoate and ethyl octanoate, a large share of the added substrate was still present after 2 h. Conversions with ethyl heptanoate yielded clearly less product compared to the other substrates. Also the alcohol accumulated when ethyl esters of shorter fatty acids were used as substrate.

Table 3.2. Conversion of 5 mM C6 to C10 ethyl esters by *E. coli* pBGTHJL. Reactions were incubated for 2 hours at 30 °C, with 1.1 g_{cdw}/L biomass.

Substrate	Concentration (mM)			Substrate	Sum
	ω -alcohol	ω -aldehyde	ω -acid		
Ethyl hexanoate ^a	1.00±0.01	ND	1.39±0.09	0.33±0.01	2.72±0.10
Ethyl heptanoate	0.24±0.01	0.13±0.01	0.17±0.00	2.65±0.03	3.19±0.03
Ethyl octanoate	1.55±0.27	ND	1.00±0.36	1.69±0.92	4.23±0.80
Ethyl nonanoate	ND	0.07±0.01	3.46±0.14	0.13±0.03	3.66±0.10
Ethyl decanoate	ND	ND	1.49±0.10	0.18±0.04	1.67±0.07
Methyl nonanoate ^a	ND	ND	3.45±0.46	0.13±0.06	3.58±0.52

ND: not detected, ^a Tests were done in duplicate

3.3.6 Application of organic phase

For mono-ethyl azelate production, substrate concentrations appeared to be limiting after 2 h (Figure 3.3). The addition of 10 mM ethyl nonanoate instead of 5 mM ethyl nonanoate did not improve product titres (data not shown). The biocompatible organic solvent bis-(2-ethylhexyl) phthalate (BEHP) has been applied before to act as a substrate reservoir and product sink for similar bioconversions⁷⁸. We tested whether a two-liquid phase approach with BEHP as organic phase could enhance product yields. Ethyl nonanoate was tested as substrate and was added as a 25% (v/v) solution in BEHP (Table 3.3).

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Table 3.3. Resting *E. coli* pBGTHJL conversions of ethyl nonanoate in a two-liquid phase setup, with 25% ethyl nonanoate in BEHP as organic phase. 9HNAEE: 9-hydroxy ethyl nonanoate, MEA: mono-ethyl azelate, AzA: azelaic acid. The applied biomass concentration was 1.0 g_{cdw}/L.

Product	Phase	Product concentration (mM)	
		Incubation time (h)	
		2	18
9HNAEE	Aqueous	ND	0.04±0
	Organic	ND	2.12±0.23
MEA	Aqueous	1.35±0.13	1.63±0.08
	Organic	1.09±0.34	15.55±1.09
AzA	Aqueous	0.10±0.04	0.14±0.02
	Organic	ND	1.12±0.27
% Conversion		0.22	1.78

ND: not detected

In this set-up, mono-ethyl azelate was formed. These results indicate that *E. coli* pBGTHJL can efficiently convert the alcohol and aldehyde to the acid. Besides mono-ethyl azelate, also low amounts of azelaic acid were formed, indicating that *E. coli* pBGTHJL is able to hydrolyse the ester bond to a limited extent. After 2 h of incubation, this set-up resulted in formation of 2.54 mmol of product per litre of aqueous medium, less than the 3.53 mmol when the substrate was directly added. After 18 h however, the formed product amounted to 20.60 mmol (1.78% of substrate converted), and the majority of the products were carboxylic acids. Most of these acids were detected in the organic phase. The addition of ethyl nonanoate dissolved in BEHP thus decreased the productivity, but enabled higher final product titres. There was also a clear difference in the distribution of mono-ethyl azelate over the two phases at the two time points. At 2 h, the ratio organic:aqueous was 0.81:1, and at 18 h, this shifted to 9.54:1, meaning that at a later stage mono-ethyl azelate partitioned better in the organic phase. This was most likely due to a decrease in the pH of the aqueous medium, resulting in a higher concentration of protonated mono-ethyl azelate. This allowed accumulation of the acid in BEHP.

3.4. Discussion

AlkJ was applied before to catalyse the conversion of alcohols to aldehydes in *E. coli*^{78,113,117}. This is the first report of its functionality on 9-hydroxy ethyl nonanoate. Schrewe and colleagues reported that *E. coli* W3110 expressing AlkJ converted 12-hydroxy methyl dodecanoate to 12-oxo methyl dodecanoate, with an initial whole-cell activity of 78.9 U/g_{cdw} (measured at 5 min). The activity of *E. coli* NEBT7-pCOM10_alkJ on 9-hydroxy ethyl nonanoate we report here is 102 U/g_{cdw} (measured at 1 min), which is higher but in the same order of magnitude. The final product titres were lower; the system seems to reach equilibrium already at low titres of the aldehyde. We noticed that pCOM10-AlkL showed native 9-oxo methyl nonanoate activity, and apparently this activity interfered in the AlkJ assay. Initial activities of AlkJ were higher at 30 °C. AlkJ was shown before to have only 50% residual activity at 34 °C *in vitro*⁵⁵, suggesting that incubation at 37 °C greatly decreases the stability of AlkJ.

AlkH has only been applied before as part of the *alk* operon or modified versions of that operon, to our knowledge. Here, we have confirmed the aldehyde dehydrogenase activity of this protein, by the addition of 9-oxo methyl nonanoate to resting cells expressing AlkH. Among the tested strains in this study, *E. coli* expressing AlkH clearly shows the highest activity (257, 594 U/g_{cdw} when coexpressed with AlkL).

Coexpression of AlkJ and AlkH with AlkL improved whole-cell activities at 30 °C, but at 37 °C the activity actually decreased. Potentially, overexpression of AlkL at 37 °C causes misfolding of the protein. It has been shown before that high expression levels of AlkL can negatively affect biocatalyst performance⁵⁹.

We compared the activities of AlkJ and AlkH with the overoxidation activity of AlkB. Both the activities of AlkJ and AlkH were higher than activities of AlkB. Compared to results from similar tests with methyl dodecanoate and the corresponding ω -oxidized derivatives reported by Schrewe *et al.*⁷⁸, there were some clear differences. Firstly, in the study by Schrewe *et al.*, *E. coli* expressing AlkB oxidized 12-hydroxy methyl dodecanoate and 12-oxo methyl dodecanoate roughly at the same rate. Here,

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we describe that AlkB displays quite different activities on the alcohol and aldehyde of C9-esters. An activity of only 24 U/g_{cdw} was reported with 9-hydroxy ethyl nonanoate as substrate. Potentially this compound becomes too polar for the active site of AlkB due to the fact that the molecule is two carbon atoms shorter. The rate with 9-oxo methyl nonanoate was much higher, 200 U/g_{cdw}. This could be due to the fact that a methyl ester was the substrate. AlkB activities are higher on methyl nonanoate than on ethyl nonanoate^{58,116}; this might apply to the ω -aldehydes as well. Still, this would not explain the large differences between the rates of alcohol and aldehyde oxidation.

Secondly, the reduction of 9-oxo methyl nonanoate occurred at high rates. Host intrinsic reduction of 12-oxo methyl dodecanoate has been reported by Schrewe *et al.*, but at a significantly lower activity (~ 6 U/g_{cdw}), indicating that this activity is much higher for shorter chain lengths. This activity is most likely caused by an alcohol dehydrogenase, resulting in oxidation of NAD(P)H. This can result in a futile cycle, with a loss of energy as a consequence. We also observed the reduction of 9-oxo methyl nonanoate by *E. coli* pBGTHJL, when 5 mM 9-oxo methyl nonanoate was added as substrate. However, in tests where ethyl nonanoate was the substrate, both 9-hydroxy ethyl nonanoate and 9-oxo ethyl nonanoate concentrations remained low. This suggests that AlkH outcompetes the reductive activity under these conditions. To prove this however, more kinetic information of the involved enzymes would be necessary.

Because of the high activities of AlkJ and AlkH on ω -oxidized esters, we expected that a pathway consisting of AlkBGTHJL would outperform the AlkBGTL pathway concerning the yield of mono-ethyl DCA. Pathway AlkBGTHJL facilitated exclusive mono-ethyl DCA production from ethyl nonanoate and methyl nonanoate. This exclusive production was a clear improvement compared to earlier studies, wherein AlkBGTL or AlkBGTLJL enzymes were applied^{58,65,78,116}. Furthermore, mono-ethyl dicarboxylic acid production using AlkBGTHJL also requires less energy and oxygen input than when only AlkBGTL is applied. Complete oxidation to the acid *via* AlkB requires 3 NADH and 3 mol O₂, whereas with AlkBGTHJL this would yield a reduced

ubiquinone (assuming 100% coupling efficiency) and only require 1 mol O₂ for the ω -oxidation pathway⁷⁸ (Appendix Figure 3.9). The addition of AlkJ and AlkH also shifts the need of NAD⁺ recycling to ubiquinol recycling. This increases the need of O₂ by 0.5 mol to 1.5 mol O₂ in total and delivers ATP.

AlkH also accepts 9-oxo ethyl nonanoate, because high titres of mono-ethyl azelate were reached and there was no 9-oxo ethyl nonanoate accumulation. Hence, this pathway is a promising biocatalytic route for medium-chain α,ω -bifunctional monomers. The initial specific activity in the presence of glucose was 81 U/g_{cdw}, which is somewhat higher than the 70 U/g_{cdw} reported for ω -oxidation of ethyl nonanoate by *E. coli* expressing AlkBGLT at 37 °C¹¹⁶. The presence of AlkJ and AlkH caused low alcohol concentrations, which may have resulted in less competition for the active site of AlkB and thus allowed more ethyl nonanoate to be converted. The aldehyde concentration never exceeded 0.1 mM. This can be explained from the high activity of AlkH and to a lesser extent AlkB on this intermediate.

Hydrolysis of the ethyl ester bond only occurred to a limited extent in the tests without organic phase. The predominant product in these conversions is thus a mono-ester. This is not the case with *Candida tropicalis*, an industrially relevant dicarboxylic acid producer, because this strain hydrolyses the ester bond^{35,37}. Mono-esters can be useful starting materials for synthetic chemistry routes, such as high-yield di-ester production *via* Kolbe electrolysis¹¹⁸.

This pathway also accepted other chain lengths from C6 to C10. Hence, also the industrially relevant mono-ethyl adipate and mono-ethyl sebacate were formed. These could serve as precursors for the corresponding di-acids or di-esters. The product titres were lower with chain lengths other than C9. In the cases of ethyl hexanoate and ethyl octanoate, a large share of the products was the alcohol. Either the alcohol/aldehyde dehydrogenases are not active enough on these substrates, or *E. coli* NEBT7 has such high aldehyde reduction activity that it causes the alcohol to accumulate. If this is the case, knocking out the enzyme responsible for this reduction would be a necessity, although this enzyme can be essential. It must be noted that especially the ethyl esters of shorter fatty acids are volatile, which affects the obtained yields. Ethyl heptanoate does not seem to be a good substrate, as can be

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concluded from the low product titres. Methyl/ethyl heptanoate were tested before as substrates for the AlkBGT system and were also not ω -oxidized efficiently^{65,116}. The reason for this remains unknown.

When we applied BEHP containing a high concentration of ethyl nonanoate, the productivity declined. This lower productivity could have been a result of mass transfer limitation of ethyl nonanoate to the aqueous phase. Moreover, the conversions took place in a rotary shaker, which likely resulted in suboptimal mixing. Nevertheless, this set-up enabled much higher mono-ethyl azelate concentrations, with a low biomass concentration. The observed accumulation of mono-ethyl azelate in BEHP could facilitate downstream processing. *E. coli* NEBT7 has some esterase activity, as azelaic acid was accumulating in these tests. This phenomenon was observed before with *E. coli* W3110 pBTL10/pBTLJ10 with methyl dodecanoate as substrate⁷⁸.

3.5. Conclusion

The expansion of the AlkBGT pathway with AlkJ and AlkH in *E. coli* resulted in a biocatalyst that could efficiently ω -oxidize ethyl-esterified medium-chain fatty acids to yield mono-ethyl DCAs. Alcohol dehydrogenase AlkJ produces the aldehyde from 9-hydroxy ethyl nonanoate. Aldehyde dehydrogenase AlkH produces the carboxylic acid from 9-oxo methyl nonanoate. These enzymes enable *E. coli* to completely convert products from AlkBGT to the carboxylic acid, when methyl or ethyl nonanoate is used as substrate. *E. coli* expressing AlkBGTJL is thus a more efficient producer of mono-esterified DCAs compared to *E. coli* expressing AlkBGT or AlkBGTJL.

Mono-ethyl DCA production was also possible with other chain lengths, ranging from C6 to C10. This highlights the broad applicability of the AlkBGTJL pathway for (mono-ethyl) DCA production.

The addition of ethyl nonanoate as a 25% solution in carrier solvent BEHP boosted the production of carboxylic acids and can act as a biocompatible substrate reservoir.

This work demonstrates the possibility of producing (mono-esterified) DCAs from medium-chain esterified fatty acids directly. Application of whole cells

for multistep biocatalysis of chemically challenging reactions holds great promise. These findings could eventually lead to more sustainable production of industrially relevant DCAs.

3.6. Experimental procedures

3.6.1 Plasmids, strains and chemicals

The plasmids used in this study are listed in as used for conversion studies.

Table 3.4. *E. coli* TOP10 (Invitrogen™, Carlsbad, CA, USA) was used for cloning purposes. *E. coli*-NEBT7 (New England Biolabs™, Ipswich, MA, USA) was used for conversion studies.

Table 3.4. Plasmids used in this study

Plasmids	Characteristics	Reference
pCOM10_alkL	alkL on pCOM10	58
pGEc47	alk operon on pLAFR1	49
pSTL	alkTL on pCOM10	116
pCOM10_alkJ	alkJ on pCOM10	This study
pCOM10_alkJL	alkJL on pCOM10	This study
pCOM10_alkH	alkH on pCOM10	This study
pCOM10_alkHL	alkHL on pCOM10	This study
pBTL10	alkBGTL on pCOM10	116
pBGTHJL	alkBGTHJL on pCOM10	This study

3.6.2 Construction of plasmids

For construction of pSJ and pSH vectors, *alkJ* and *alkH* were amplified from pGEc47. These were ligated in digested pCOM10_alkL (digestion resulted in removal of *alkL*). *alkL* was then ligated into the digested pSJ and pSH vectors, to yield pSJL and pSHL.

pSTBGHJL was constructed as follows. The *alkBFGHJ* and *alkL* fragments were amplified from pGEc47, and the primers were designed in such a way that *alkL* ligated to a 3' overhang of the *alkBFGHJ* fragment. This fragment was ligated into a digested pSTL plasmid, to yield pSTBGHJL.

3.6.3 Chemicals

Chemicals were ordered with the highest purity available from the following vendors:

Sigma-Aldrich (St Louis, MO, USA): dodecane, tetradecane, ethyl hexanoate, ethyl nonanoate, 6-hydroxy ethyl hexanoate, 7-hydroxy ethyl heptanoate. Alfa Aesar: ethyl octanoate, ethyl decanoate, bis(2-ethylhexyl) phthalate, adipic acid mono-ethyl ester, pimelic acid mono-ethyl ester, suberic acid mono-ethyl ester, sebacic acid mono-ethyl ester. TRC (North York, ON, Canada): 9-hydroxy nonanoic acid ethyl ester, 9-oxo methyl nonanoate, azelaic acid mono-ethyl ester. Merck (Darmstadt, Germany): ethyl heptanoate.

3.6.4 Cultivation and bioconversions

Cultivation and bioconversions were carried out as described before¹¹⁶, except the temperature, which was set to 30 °C for most experiments.

Conversions with organic phase were carried out similarly, except that a solution of 25% ethyl nonanoate in bis-2-ethylhexyl phthalate was added to the reaction, in a 1:1 ratio. Furthermore, ethanol was not added. For sampling, equal volumes of the water phase and organic phase were withdrawn.

3.6.5 GC analysis

Aqueous samples were treated with 1% of an 85% phosphoric acid solution and then extracted with CHCl₃ containing 0.2 mM dodecane or tetradecane as an internal standard. Organic phase samples were diluted 40 times in CHCl₃ containing 0.2 mM tetradecane. Samples were analysed with and without derivatization with TMSH. For qualitative analysis, samples were analysed with a Thermo Scientific TRACE Ultra gas chromatograph coupled to a DSQII mass spectrometer. Quantitative analysis was carried out with an Agilent 6890 or 7890 GC coupled to an FID. Response factors of chemicals that were not commercially available were based on structurally similar chemicals.

3.6.6 Acknowledgement

This work was funded by BE-Basic (F01.006: medium-chain α,ω -dicarboxylic acids, production from non-food feedstock for biomaterials).

3.7. Appendix

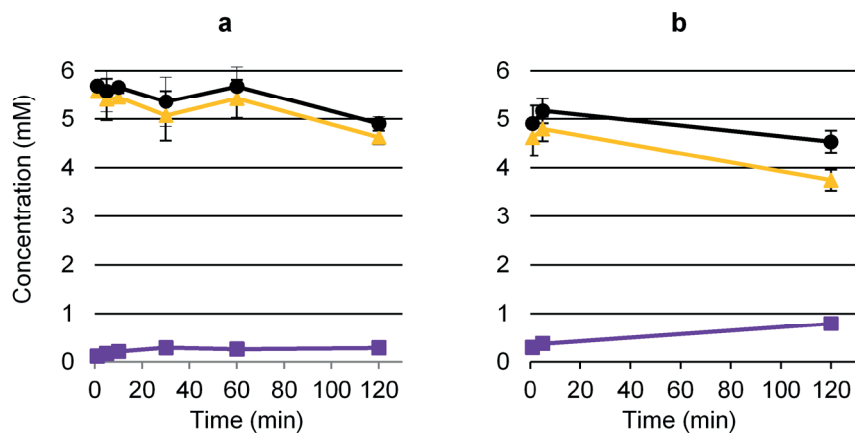


Figure 3.4. Resting cell conversions of 9-hydroxy ethyl nonanoate at 30 °C. Panel A: *E. coli* pCOM10_alkJ, 1.1 g_{cdw}/L. Panel B, *E. coli* pCOM10_alkJL 1.1 g_{cdw}/L. Squares: 9-oxo ethyl nonanoate. Triangles: 9-hydroxy ethyl nonanoate. Circles: sum.

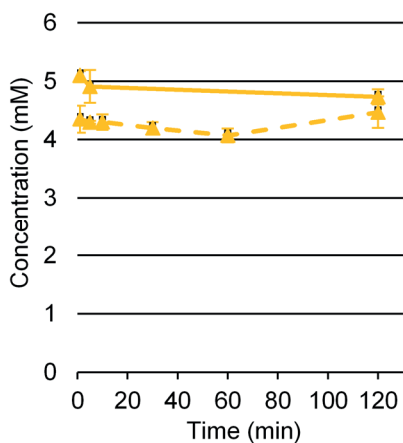


Figure 3.5. Incubation of 9-hydroxy ethyl nonanoate with 1.0 g_{cdw}/L of *E. coli* pCOM10_alkL (solid line), and without cells (dashed line). Triangles: 9-hydroxy ethyl nonanoate.

Mono-esterified dicarboxylic acid production

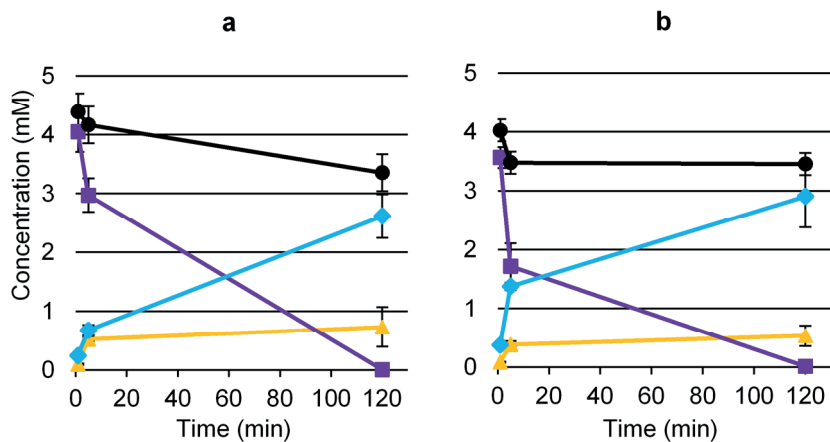


Figure 3.6. Resting cell conversions of 9-oxo methyl nonanoate at 30 °C. Panel A: *E. coli* pCOM10_alkH, 1.0 g_{cdw}/L. Panel B, *E. coli* pCOM10_alkHL 1.0 g_{cdw}/L. Squares: 9-oxo methyl nonanoate. Triangles: 9-hydroxy methyl nonanoate. Diamonds: mono-methyl azelate. Circles: sum.

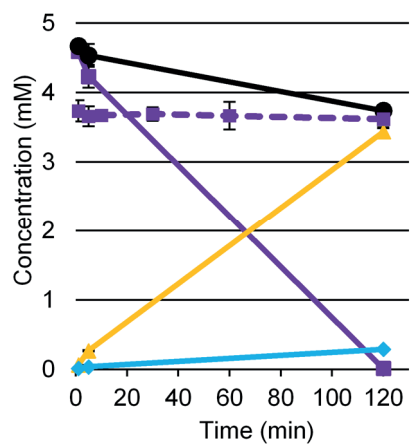


Figure 3.7. Incubation of 9-oxo methyl nonanoate with 1.0 g_{cdw}/L of *E. coli* pCOM10_alkL (solid lines), and without cells (dashed line). Squares: 9-oxo methyl nonanoate. Triangles: 9-hydroxy methyl nonanoate. Diamonds: mono-methyl azelate. Circles: sum.

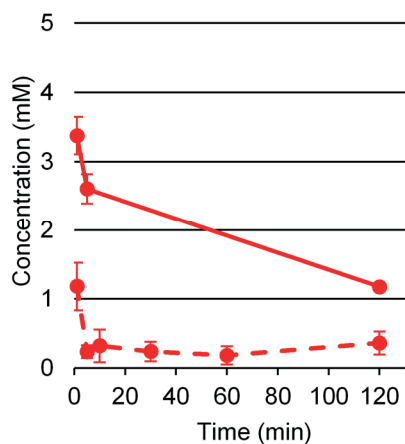


Figure 3.8. Incubation of ethyl nonanoate with 1.0 g_{cdw}/L of *E. coli* pCOM10_alkL (solid line), and without cells (dashed line). Crosses: ethyl nonanoate.

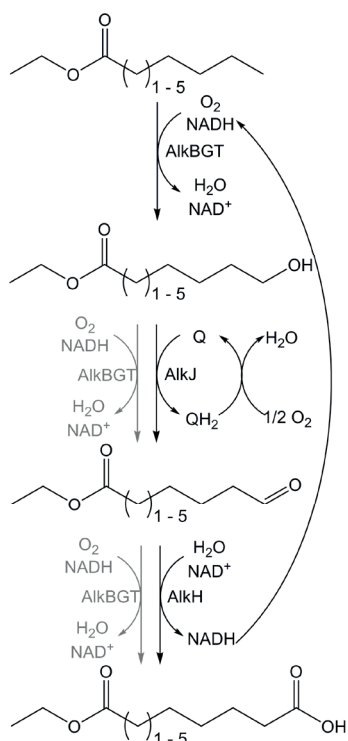
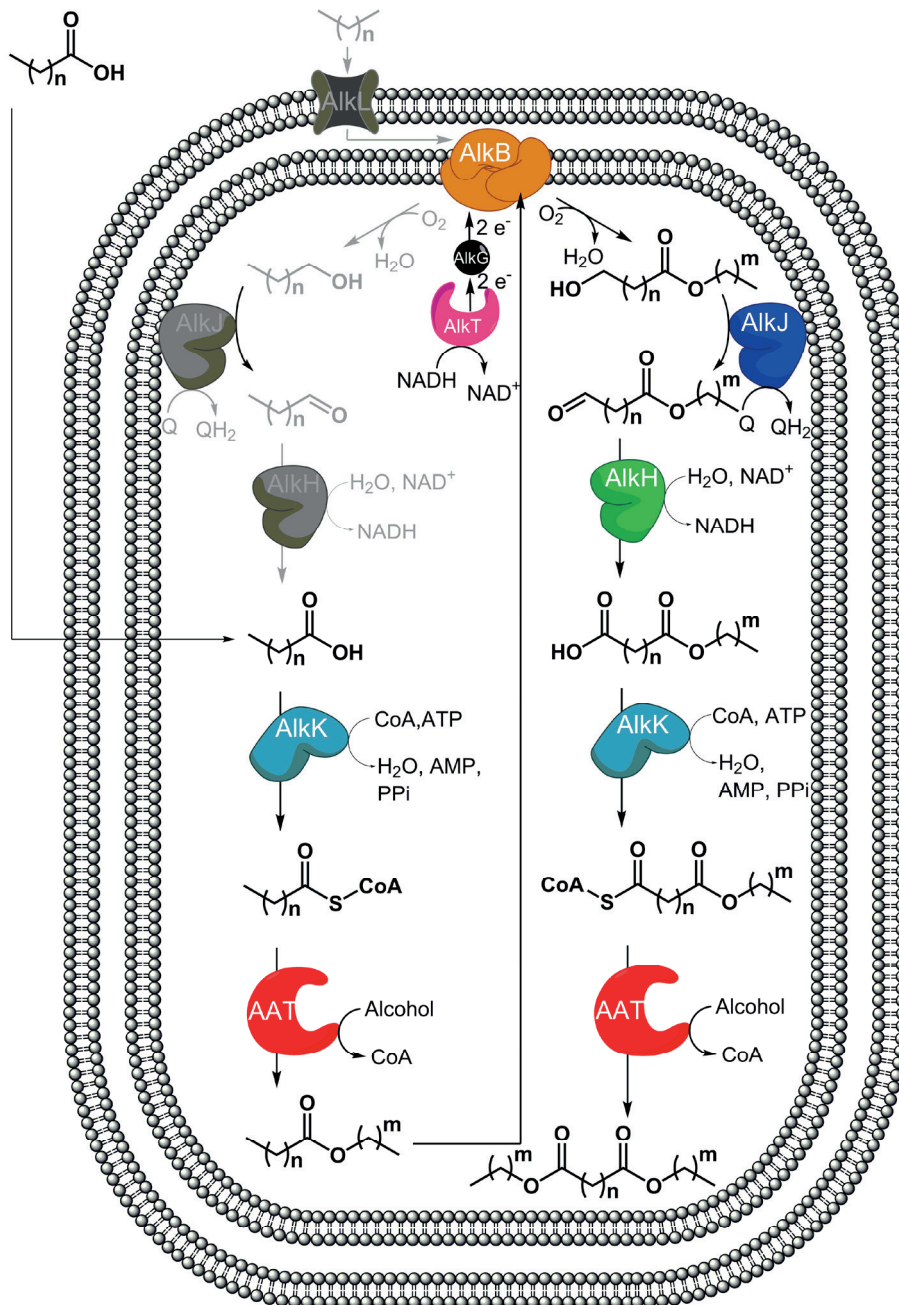


Figure 3.9. Comparison of the AlkBGT pathway with the AlkBGT (overoxidation) pathway, for ω-oxidation of ethyl esterified fatty acids. The overoxidation *via* AlkBGT is shown in grey.



Chapter 4 Combination of ester biosynthesis and ω -oxidation for production of mono-ethyl dicarboxylic acids and di-ethyl esters in a whole-cell biocatalytic setup with *Escherichia coli*

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Nuland, Y. M., Eggink, G., Weusthuis, R. A. "Combination of ester biosynthesis and ω -oxidation for production of mono-ethyl dicarboxylic acids and di-ethyl esters in a whole-cell biocatalytic setup with *Escherichia coli*".

4.1. Abstract

Medium chain length (C6-C12) α,ω -dicarboxylic acids (DCAs) and corresponding esters are important building blocks for the polymer industry. For DCAs of 12 carbon atoms and longer, a sustainable process based on monooxygenase catalyzed ω -oxidation of fatty-acids has been realized. For medium-chain DCAs with a shorter chain length however, such a process has not been developed yet, since monooxygenases poorly ω -oxidize medium-chain fatty acids (MCFAs). On the contrary, esterified MCFAs are ω -oxidized well by the AlkB_{GTHJ} proteins from *Pseudomonas putida* GPO1. Here we show that MCFAs can be efficiently esterified and subsequently ω -oxidized *in vivo*. We combined ethyl ester synthesis and ω -oxidation in one-pot, whole-cell biocatalysis in *Escherichia coli*. Ethyl ester production was achieved by applying acyl-CoA ligase AlkK and an alcohol acyltransferase, either AtfA or Eeb1. *E. coli* expressing these proteins in combination with the ω -oxidation pathway consisting of AlkB_{GTHJ}, produced mono-ethyl DCAs directly from C6, C8 and C9 fatty acids. The highest molar yield was 0.75, for mono-ethyl azelate production from nonanoic acid. Furthermore, di-ethyl esters were produced. Diethyl suberate was produced most among the di-ethyl esters, with a molar yield of 0.24 from octanoic acid. These results indicate that ω -oxidation of MCFAs to mono-ethyl DCAs *via* whole-cell biocatalysis is possible. This process could be the first step towards sustainable production of medium-chain DCAs and medium-chain di-ethyl esters.

4.2. Introduction

α,ω -dicarboxylic acids (DCAs) of medium chain length (C6-C12) and corresponding esters constitute an important share of building blocks for the polymer industry. However, their production from petrochemical resources using organic chemistry methods is associated with high gross energy requirements and severe greenhouse gas emissions¹⁰¹. This has directed research to alternative, more sustainable production processes. In the most promising alternative, fatty acids or their methyl esters are converted by microbial cells expressing an alkane 1-monooxygenase, sometimes assisted by an alcohol oxidase or dehydrogenase. This has resulted in high titers of DCAs or mono-esterified DCAs (MEDAs)^{18,35,78}.

The AlkBGT system from *Pseudomonas putida* GPo1 is especially suitable for the ω -oxidation of esterified medium chain length fatty acids¹¹⁶. Its main product is the ω -alcohol, and the ω -aldehyde and carboxylic acid are formed to a limited extent. Recently, we have expanded the AlkBGT system with alcohol dehydrogenase AlkJ and aldehyde dehydrogenase AlkH in *E. coli*, resulting in the exclusive and efficient production of MEDAs from esterified fatty acids¹¹⁹. However, the AlkBGT system is not efficient in ω -oxidizing non-esterified medium-chain fatty acids. So esterification of medium-chain fatty acids *in vitro* by organic chemical methods prior to the oxidation step is required, adding costs to the overall process.

The solution is straightforward: if fatty acids have to be used directly, the biocatalyst has to esterify the fatty acids first *in vivo*. *In vivo* esterification of fatty acids to ethyl esters for the sake of biodiesel production in *E. coli* has been shown before^{83,84}. This is done in two steps. First the fatty acid is converted to acyl-CoA by a fatty acid CoA ligase, after which the coenzyme A moiety is exchanged with an alcohol by an alcohol:acyltransferase. In earlier studies aiming at ethyl ester production in *E. coli*, the native fatty-acid CoA ligase was used. For the alcohol:acyltransferase reaction AtfA and Eeb1 have been applied^{83,84,93}. AtfA is known for its broad substrate scope, and can even synthesize wax di-esters^{87,99,120}. Eeb1 uses ethanol and medium-chain acyl-CoA as substrates⁹³.

Combination of ester biosynthesis and ω -oxidation

In this study, we investigated whether MEDAs can be produced directly from fatty acids. Two modules were used: an ω -oxidation module consisting of AlkBGTJHL and an esterification module consisting of an acyl-CoA ligase and an alcohol:acyltransferase. AlkK from *Pseudomonas putida* GPo1 was selected for acyl-CoA production from fatty acids, because we expect it to have the same broad substrate specificity as the enzymes encoded by AlkBGTJHL. AlkK has been shown to synthesize octanoyl-CoA from octanoic acid⁴⁸, but has not been tested with other substrates to our knowledge. AtfA or Eeb1 were selected to convert the generated acyl-CoA subsequently into ethyl esters^{84,93}. Together they realize the pathway depicted Figure 4.1A.

We also investigated if the esterification module may in principle be able to esterify the MEDAs into di-esterified DCAs (DEDAs, Figure 4.1B). DEDAs are less soluble in water which would be advantageous for product removal from the broth. Furthermore, DEDAs are beneficial for polymerization processes, since in the polymerization process volatile alcohols are released instead of water, shifting the equilibrium of the reaction to polymer formation.

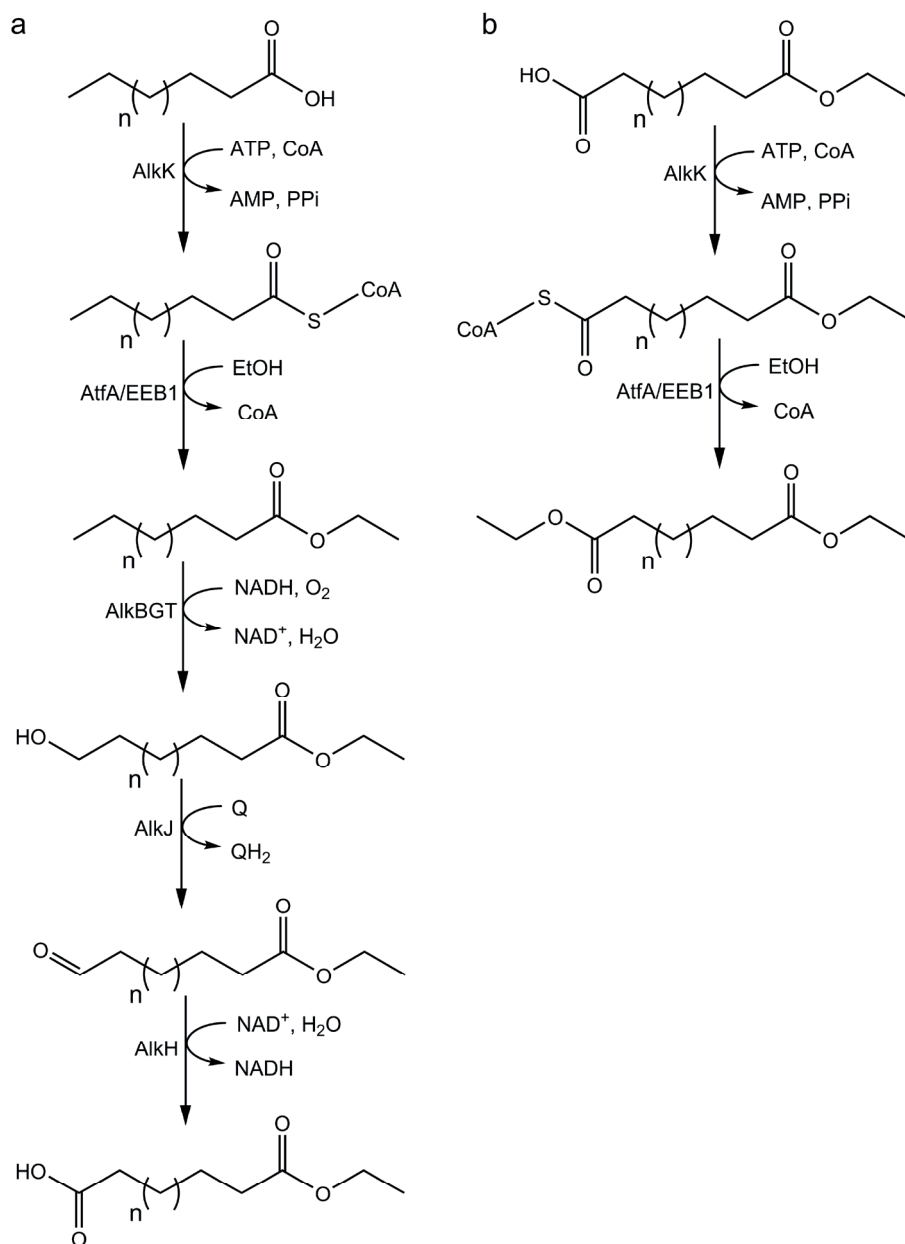


Figure 4.1. Panel A: Biocatalytic production of mono-ethyl dicarboxylic acids from fatty acids. Panel B: further conversion of mono-ethyl dicarboxylic acids to α,ω -diethyl esters

4.3. Materials and methods

4.3.1 Chemicals

The following chemicals were purchased with the highest purity available: hexanoic acid, octanoic acid, nonanoic acid, methyl nonanoate, ethyl nonanoate, ethyl hydrogen adipate, ethyl hydrogen pimelate, diethyl azelate and diethyl sebacate from Sigma; ethyl hydrogen suberate, ethyl hydrogen sebacate and diethyl suberate from Alfa Aesar; ethyl hydrogen azelate from TRC, diethyl adipate from Acros Organics; diethyl pimelate from Merck; dimethyl octadecanedioate from TCI; 0.2 M TMSH in MeOH from Macherey Nagel; Coenzyme A from VWR and ATP from GE Healthcare.

4.3.2 Strains and plasmids

For cloning purposes, *E. coli* competent TOP10 (Invitrogen™) cells were used. For conversion studies, *E. coli* competent T7 Express (New England Biolabs®) cells were used. Plasmids that were used are shown in Table 4.1.

Table 4.1. Plasmids used in this study

Plasmid	Description	Reference
pUC57- <i>alkKcodopt</i>	Codon optimized <i>alkK</i> in pUC57	This study
pUC57- <i>atfAcodopt</i>	Codon optimized <i>atfA</i> in pUC57	This study
pGEc47	Contains genes for growth on alkanes (<i>alkBFGHJKL</i> and <i>alkST</i>) in broad-host-range vector pLAFR1	⁴⁹
pET-Duet- <i>alkK</i>	codon optimized <i>alkK</i> under control of T7 promoter in MCS1 of pET-Duet	This study
pCOM10- <i>alkL</i>	Contains <i>alkL</i> in pCOM10, a broad host range alkane responsive vector	⁵⁸
pBGTHJKL- <i>atfA</i>	Complete <i>alk</i> -operon; codon optimized <i>atfA</i> under control of separate P_{alkB}	This study
pBGTHJKL- <i>eeb1</i>	Complete <i>alk</i> -operon; <i>eeb1</i> under control of separate P_{alkB}	This study
pE	<i>alkK</i> and <i>alkL</i> under control of P_{alkB} ; codon optimized <i>atfA</i> under control of separate P_{alkB} in pCOM10	This study
<i>palkKL</i>	<i>alkK</i> and <i>alkL</i> under control of P_{alkB} in pCOM10	This study
pSTL	<i>alkTL</i> in pCOM10	¹¹⁶
pBGTHJL	<i>alkBGTHJL</i> genes in pCOM10	¹¹⁹
pE-II	pE with pBR322 ori, Amp ^R	This study

4.3.3 Construction of vectors

For PCR reactions, Phusion polymerase (ThermoFisher Scientific) was used. *alkK* and *atfA* were codon optimized for *E. coli* and synthesised by GenScript®. These genes were delivered in pUC57 vectors. Codon optimized *alkK* was amplified from pUC57-*alkKcodopt* using primers 1 and 2. The product and pET-Duet™-1 (Novagen- EMD Millipore) were both digested with NdeI and XhoI; ligation resulted in generation of pET-Duet-*alkK*. Vector pBGTHJKL-*atfA* was constructed by a Golden Gate approach. pCOM10 was used as backbone, therefore pCOM10-*alkL* was digested with EcoRI and Sall. Primers were designed to generate three inserts, which were *alkBFGHJKL*, P_{alkB} and codon optimized *atfA*. *alkBFGHJKL* was amplified from pGEc47 with primers 3 and 4, P_{alkB} from pGEc47 with primers 5 and 6, codon optimized *atfA* from pUC57-*atfAcodopt* with primers 7 and 8. This yielded three amplicons that were digested with restriction enzymes that are listed in Table 4.2 and ligated. This resulted in ligation in the aforementioned order in pCOM10, which was possible due to the presence of 4 bp overhangs generated from BsaI digestion.

Table 4.2. Primers used in this study

Primer #	Sequence	Remarks
1	TAGCTCGCC CATATG CTGGGTCAAATGATG	NdeI site in bold
2	GTATCA CTCGAGT TATTCACACACCGATGAG	XhoI site in bold
3	AATTGGAG GAATTC CATATGCTTGAGAAACACAGAG	EcoRI site in bold
4	CACACCAGGTCTCA GTTT TAGAAAACATATGACG CACCAAGACT	overhang in bold, complementary to 5' <i>P_{alkB}</i>
5	CACACCAGGTCTCA AAACT ACCCGTAGGTGTAG TTGGCGCA	overhang in bold complementary to 3' <i>alkL</i>
6	CACACCAGGTCTCA ATTC AGAATTCTCCAATTTT ATTAAATTAGTCG	overhang in bold complementary to 5' <i>atfA</i>
7	CACACCAGGTCTCA GAAT GCGCCCGCTGCACCC	overhang in bold complementary to 3' <i>P_{alkB}</i>
8	GTATCT GTCGACT TAATTTGCCGTTTTGATATCTT C	Sall site in bold
9	TATAT CAATTG ATGTTAGGTCAGATGATGCGT	MunI site in bold
10	ATAT GGATCC TAATTTGCCGTTTTGATATC	BamHI site in bold
11	CACACCAGGTCTCA CACG TCGCCTCACATTGATG ATTAT	overhang in bold complementary to 5' <i>alkL</i>
12	CACACCAGGTCTCA CGTG ATGAGTTTTTCTAATT ATAAAGTAATCGCGATG	overhang in bold complementary to 3' <i>alkJ</i>
13	ACGCG TGCAC CTGCGACAGTGACAGACCTG	Sall site in bold
14	TAGTTT TGACAG CATGAGATTATCAAAAAGGATC	BsrGI site in bold
15	TATA CTCGAG ACTCAAAGGCGGTAATACG	XhoI site in bold

The pBGTHJKL-*atfA* vector was used for the construction of pE. Primers 9 and 10 were used to generate an amplicon from pBGTHJKL-*atfA* that contains *alkK* fused to *P_{alkB}-atfA*. This amplicon was digested with MunI and BamHI; backbone pCOM10-*alkL* was digested with EcoRI and BamHI. These two fragments were ligated to give pE. This vector was digested with EcoRI and ligated again, resulting in loss of 987 basepairs of *atfA* at the 5' end and giving *palkKL*. pSTBFGHJL was also created by the Golden Gate method. pSTL was

used as backbone, which was digested with EcoRI and Sall. *alkBFGHJ* was amplified from pGEc47 with primers 3 and 11. *alkL* was amplified from pGEc47 with primers 12 and 13. These two amplicons were ligated together with the pSTL digest to give pSTBFGHJL. pE was used to generate pE-II, that has a different *ori* and resistance marker. The pBR322-Amp^R cassette from pET-Duet was generated with primers 14 and 15. Both pE and the cassette were digested with BsrGI and XhoI. pE-II was created by ligation of those two fragments.

4.3.4 Qualitative AlkK assay

E. coli carrying pET-Duet-*alkK* or pET-Duet (empty vector control) was grown overnight in LB containing 100 µg/mL ampicillin, at 30 °C, 250 rpm. Of this culture, 250 µL was used to inoculate 50 mL of the same medium. Expression of AlkK was started at an OD_{600nm} of 0.3 with the addition of 0.4 mM of IPTG. This culture was incubated overnight at 20 °C. Cells were harvested and resuspended in a buffer consisting of 25 mM Tris pH 7.5, 2.5 mM EDTA and 1 % Triton-X100. Cells were then lysed with lysozyme and DNase I. The resulting mixture was centrifuged at 20.000 rcf for 15 min. The supernatant was loaded on a 10 kDa spin column to concentrate the protein. About 450 µg of this concentrate was added to a 5 min pre-incubated assay mixture consisting of 200 mM Tris pH 7.5, 12 mM MgCl₂, 10 mM ATP, 1 mM CoA, and 2 mM octanoic acid or ethyl hydrogen suberate. Reactions were done at 30 °C, and stopped by addition of 1:1 CHCl₃:MeOH, brief vortexing and transferring the mixture to liquid nitrogen. The aqueous phase was analyzed with LC-MS/MS with a Waters BEH C8 column coupled to an LCQ-Fleet.

4.3.5 Whole-cell conversions and GC analysis

Cultivation, conversions and GC analysis were done as described before¹¹⁶, except that induction with DCPK and the conversion were done at 30 °C. GC analysis of ω-acids was done after derivatization with TMSH.

4.4. Results

The ω-oxidation module system has been successfully tested before¹¹⁹. This system was expanded with AlkK and AtfA to realize the formation of esters.

First we tested if AlkK and AtfA were functionally expressed and able to esterify both fatty acids and mono-ethyl dicarboxylic acids.

4.4.1 Expression of AlkK

AlkK activity was determined by following the synthesis of the CoA esters from fatty acids and mono-ethyl diacids. The AlkK assay was performed qualitatively, since peak resolutions did not allow quantification. We tested functionality of AlkK in a cell-free extract assay, using octanoic acid, CoA and ATP in the assay mixture. The cell-free extracts were prepared from *E. coli* pET-Duet-*alkK* and *E. coli* pET-Duet (empty vector control). LC-MS analysis indicated that a product with an m/z of 892 was formed, see Figure 4.2a. This mass corresponds to the expected mass of octanoyl-CoA.

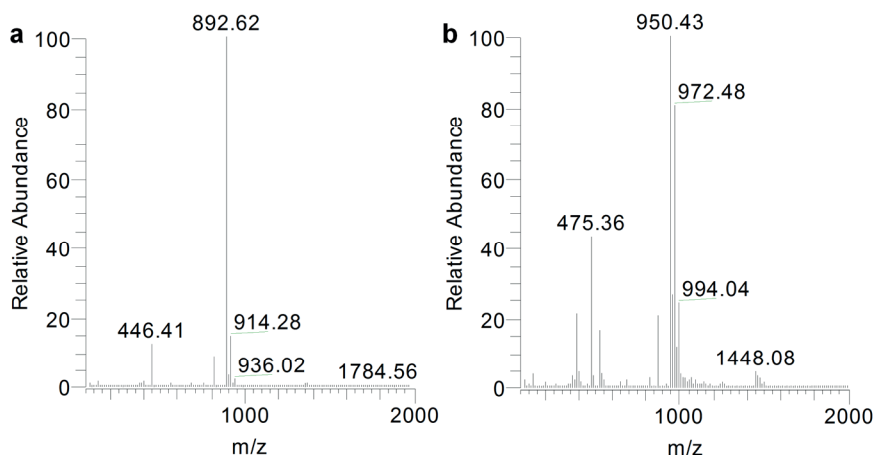


Figure 4.2. Panel a: mass spectrum of the formed product in incubations with cell free extract of *E. coli* pET-Duet-*alkK* and octanoic acid. The m/z of 892 corresponds to octanoyl-CoA. Panel b: mass spectrum of the formed product in incubations with cell free extract of *E. coli* pET-Duet-*alkK* and mono-ethyl suberate. The m/z of 950 corresponds to the CoA-ester of mono-ethyl suberate. The $M+22 \cdot n$ peaks indicate sodium adducts.

In a negative control experiment we added cell-free extract of *E. coli* carrying the empty vector. In this assay only trace amounts of octanoyl-CoA were detected, indicating that the formed octanoyl-CoA in the cell-free extract from *E. coli* pET-Duet-*alkK* was formed by AlkK. We repeated the assay, with mono-ethyl suberate as substrate, to check whether AlkK also has the ability

to generate the CoA-ester of mono-ethyl suberate. A product with m/z of 950 appeared (see Figure 4.2b). This was the expected mass for the CoA ester of ethyl hydrogen suberate. This implies that AlkK can also use mono-ethyl suberate as substrate.

4.4.2 Combined expression of AlkK and AtfA

CoA esters of mono-ethyl dicarboxylic acids are not available. We therefore tested AtfA activity by using a combined assay with whole-cells expressing both AlkK and AtfA. The tests were performed with induced, resting *E. coli* pE cells. The same setup was tested with *E. coli palkKL*, which served as negative control. Octanoic acid and nonanoic acid were applied as the substrate, added to 1 mM from a concentrated ethanol stock. This resulted in a final ethanol concentration of 2.5 % v/v. Results are shown in Table 4.3.

Table 4.3. Whole-cell conversion of fatty acids (1 mM) and mono-esterified dicarboxylic acids (5 mM) into ethyl esters and di-esters. Incubation was done with 1.0 g_{cdw}/L, and lasted for 2 h with fatty acids, 3 h for mono-ethyl dicarboxylic acids. ^aDetected product was methyl-ethyl azelate.

	<i>E. coli</i> pE	<i>E. coli palkKL</i>
	Ethyl ester/di-ethyl ester concn	Ethyl ester/di-ethyl ester concn
Octanoic acid	0.72 ± 0.03 mM	ND
Nonanoic acid	0.82 ± 0.12 mM	ND
Mono-ethyl adipate (C6)	0.17 ± 0.00 mM	<0.01 mM
Mono-ethyl pimelate (C7)	0.12 ± 0.00 mM	0.03 ± 0.00 mM
Mono-ethyl suberate (C8)	0.19 ± 0.02 mM	0.02 ± 0.00 mM
Mono-ethyl azelate (C9)	0.05 ± 0.00 mM	-0.02 ± 0.00 mM
Mono-ethyl sebacate (C10)	0.06 ± 0.01 mM	-0.01 ± 0.00 mM
Mono-methyl azelate (C9)	0.50 ± 0.03 mM ^a	NT

ND: not detected. NT: not tested

AtfA was functionally expressed, because ethyl octanoate was produced by resting cells expressing both AlkK and AtfA. Ethyl octanoate was not detected in cells expressing only AlkK and AlkL from the *palkKL* plasmid. Comparable

results were achieved with nonanoic acid as substrate. The maximum product titer of 0.82 mM was reached with this substrate. A small gap in the C-balance was observed, which was likely caused by evaporation of the volatile product and/or β -oxidation.

4.4.3 Testing the AlkK-AtfA esterification module for esterification of MEDAs

The same test was performed using 5 mM of C6-C10 MEDA instead of fatty acids using *E. coli* pE. Di-ethyl esters were produced from all chain lengths. The titers were rather low, and 4% of the MEDA was converted to di-ethyl ester at most. Especially with C9 and C10 chain lengths, conversion was poor. Mono-methyl azelate was clearly a better substrate, since incubations with this substrate yielded 0.50 mM of methyl-ethyl azelate, corresponding to a conversion of 10% of the added mono-methyl azelate. The incubations with mono-ethyl suberate, which is identical in length, yielded 0.19 mM. These findings suggest that the position of the ester group plays a role.

A contamination of di-ester was present in the MEDA stocks; this was confirmed by GC analysis of the pure stock. The impurity was measured at t_0 , this concentration was subtracted to evaluate which share of di-ester was formed by the cells.

4.4.4 Combining the esterification and ω -oxidation modules

Resting-cell conversions of fatty acids were done with *E. coli* pBGTHJKL-*atfA*, expressing both modules. The formed products and proposed pathways are shown in Figure 4.3a. The same conversion was done with *E. coli* pBGTHJL, expressing only the ω -oxidation module, to compare the ω -oxidation capacity (Figure 4.3b). *E. coli* pBGTHJL produced ω -hydroxy fatty acids and dicarboxylic acids, up to 0.30 mM after 19 h when 1 mM nonanoic acid was the substrate. Conversions with hexanoic acid did not yield ω -oxidation products. AlkB thus ω -oxidizes the fatty acids to a limited extent. Apparently, AlkJ and AlkH function poorly with ω -hydroxy fatty acids since the highest titer of dicarboxylic acid was 0.06 mM.

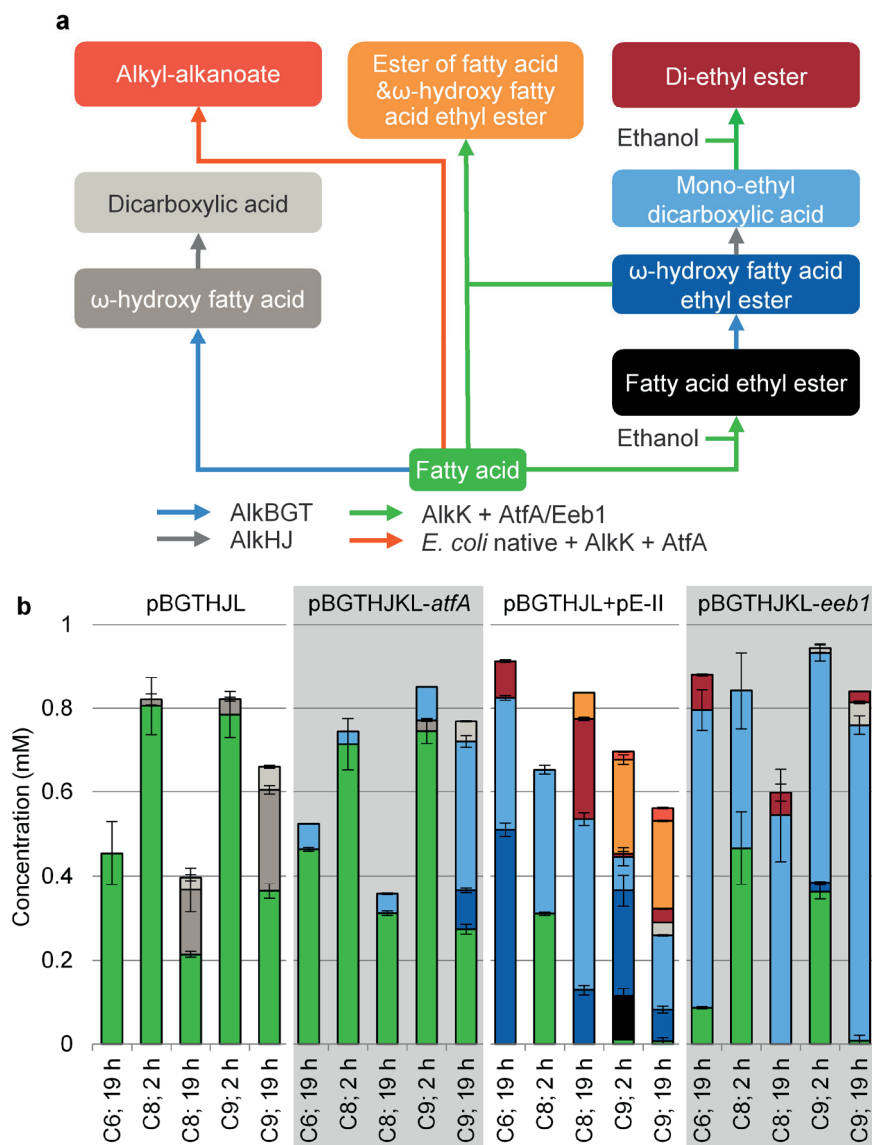


Figure 4.3. Panel a: legend indicating possible products from conversions of fatty acids with resting *E. coli* strains from panel b; with corresponding colors in both panels. The colors of the arrows indicate which enzymes catalyze the reactions. Panel b: resting cell conversion of fatty acids by various *E. coli* strains. Concentration of fatty acid was 1 mM, ethanol was added to 2.5%. C6, C8, C9 represent hexanoic, octanoic and nonanoic acid, respectively. Biomass concentrations were 1.0 g_{cdw}/L for all strains.

E. coli pBGTHJKL-*atfA* produced the ethyl esters of the added fatty acids (Figure 4.3b). These ethyl esters were ω -oxidized, resulting in production of 0.36 mM of mono-ethyl azelate after 19 h. This shows that further oxidation to the carboxylic acid is more efficient with ω -hydroxy fatty acid ethyl esters than with ω -hydroxy fatty acids. This is in line with earlier findings, where ethyl esters served as substrate¹¹⁹. In contrast to nonanoic acid, hexanoic and octanoic acid were not efficiently converted to MEDA. Conversions with these fatty acids yielded at most 0.06 mM mono-ethyl dicarboxylic acid, less ω -oxidized product than in conversions with *E. coli* pBGTHJL.

In general, a large share of the added fatty acids was not converted into product by *E. coli* pBGTHJKL-*atfA*. Intermediate esterified products did not accumulate in this strain, suggesting that ester synthesis limited higher product titers. Since *atfA* was equipped with its own P_{alkB} promoter, expression levels of this gene were high (Appendix Figure 4.5). On the contrary, AlkK is the penultimate gene in the *alk* operon, which resulted in low expression levels. In order to increase the titers, we switched the *ori* and resistance marker from pE to give pE-II, a plasmid that can be cotransformed with pBGTHJL. On this plasmid, AlkK has its own P_{alkB} promoter. *E. coli* transformed with pE-II produced 0.72 mM ethyl octanoate from 1 mM octanoic acid and thus performed similar to *E. coli* pE (data not shown). This plasmid was cotransformed with pBGTHJL, giving *E. coli* pBGTHJL + pE-II, which has higher expression levels of AlkK as indicated by SDS PAGE analysis (Appendix Figure 4.5).

The resulting strain clearly produced more ω -oxidized products, especially with hexanoic and octanoic acid as substrate (Figure 4.3b). In the tests with hexanoic acid, 0.91 mM of ω -oxidized product was formed in 19 h. This strain thus had a higher esterification capacity and as a result also further converted MEDAs to di-ethyl esters. Di-ethyl esters were formed from all the tested fatty acids. This means that the product went twice through the esterification pathway (constituted by AlkK and AtfA). The highest concentration of di-ethyl ester (0.24 mM) was detected in conversions with octanoic acid. GC-MS analysis pointed out that *E. coli* pBGTHJL + pE-II also produced side-products, the major one being the ester of nonanoic acid and 9-hydroxy ethyl

nonanoate, or ethyl 9-(nonanoyloxy)nonanoate (see Figure 4.6 for proposed pathway). Since this chemical was not commercially available, the quantification was done by taking the response factor of dimethyl octadecanedioate. Furthermore, nonyl nonanoate was produced, which is probably the result of nonanoic acid reduction to 1-nonanol. This is then esterified with nonanoyl-CoA to give nonyl nonanoate. Ethyl esters of *E. coli* native fatty acids were also detected, including: ethyl myristate, ethyl palmitate and ethyl oleate. These findings highlight the nonspecific nature of AtfA.

4.4.5 Investigating di-ethyl ester production

Di-ethyl ester production was less efficient than ethyl ester production. It seemed to occur when the fatty acids were depleted, suggesting that fatty acids are preferred over MEDAs as substrate by the esterification module. We therefore checked if di-ethyl ester production could be enhanced, by using ethyl nonanoate instead of nonanoic acid as substrate. Ethyl nonanoate is efficiently converted to the ω -acid in *E. coli* pBGTHJL¹¹⁹. The results are shown in Figure 4.4.

Combination of ester biosynthesis and ω -oxidation

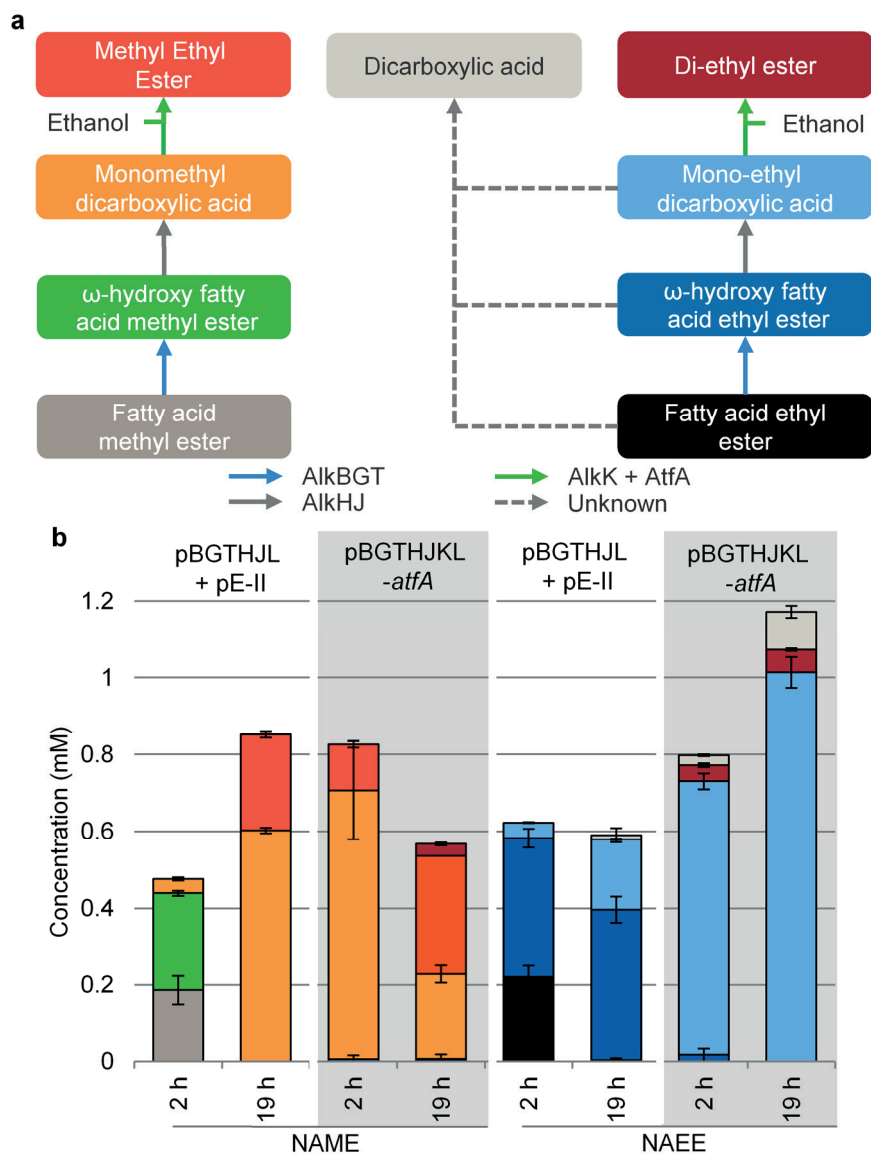


Figure 4.4. Panel a: possible products from conversions of fatty acid methyl/ethyl esters with resting *E. coli* strains from panel b; with corresponding colors in both panels. The colors of the arrows indicate which enzymes catalyze the reactions. Panel b: resting cell conversions of 1 mM methyl nonanoate (NAME) or 1 mM ethyl nonanoate (NAEE) by various *E. coli* strains. Ethanol was added to 2.5%. Biomass concentrations were 1.0 g_{cdw}/L for both strains.

In these tests, the di-ethyl azelate concentrations remained low as well, whereas mono-ethyl azelate was efficiently produced. This suggests that the substrate specificity of AlkK and AtfA causes the low titers. The product distribution of the conversions with *E. coli* pBGTHJL + pE-II and *E. coli* pBGTHJKL-*atfA* were clearly different. The pBGTHJL + pE-II strain has a high esterification activity, but a rather low ω -oxidation activity. The opposite is true for the pBGTHJKL-*atfA* strain. Since the P_{alkB} promoter is strong, the ability to synthesize proteins likely becomes limiting, which can cause the different behavior of the two strains. Since mono-methyl azelate seemed a better substrate for di-ester production (Table 4.3), methyl nonanoate was added to the same strains, see Figure 4.4.

In these tests, methyl-ethyl azelate was formed. The di-ester titers were clearly higher; the highest titer of 0.31 mM was reached by *E. coli* pBGTHJKL-*atfA*. This strain produced a limited amount of di-ethyl azelate as well, indicating that methyl ester hydrolysis had occurred, after which the molecule was esterified with ethanol. After 2 h of incubation, the product distribution in tests with *E. coli* pBGTHJL + pE-II was comparable for both methyl nonanoate and ethyl nonanoate. After 19 h however, the ω -hydroxy fatty acid ester was completely converted to ω -acid in the case of methyl nonanoate, which was not the case for the substrate ethyl nonanoate. This suggests that the oxygenates of the methyl ester are more efficiently further oxidized to the acid than those of the ethyl ester.

AtfA prefers longer alcohol and acyl-CoA chain lengths. It is also very nonspecific, which caused the accumulation of by-products as described above. Therefore, we switched to Eeb1 as an alcohol-acyltransferase to investigate whether the production of MEDA and potentially DEDA from fatty acids could be increased (Figure 4.3). *E. coli* pBGTHJKL-*eeb1* clearly produced more MEDA. In the conversions with nonanoic acid the highest amount of products accumulated (0.83 mM). Furthermore, 93 % of the product was efficiently ω -oxidized to the carboxylic acid. This strain was also more efficient, since more product was synthesized after 2 hours of incubation compared to conversions with other strains. DEDA concentrations were not significantly increased.

4.5. Discussion

Fatty acids were poorly oxidized by *E. coli* pBGTHJL. The highest concentration of ω -oxidized product was 0.30 mM after 19 h, with nonanoic acid as substrate. We show that this problem can be solved by first converting the fatty acids into ethyl esters.

We achieved medium-chain ethyl ester synthesis by using *E. coli* that expresses AlkK and AtfA or Eeb1. *In vivo* esterification of fatty acids results in products with a low solubility, which can facilitate product removal and product inhibition^{84,85,121}. Highest titer of ethyl ester was reached when nonanoic acid was applied as substrate, resulting in 0.82 mM of ethyl nonanoate from 1 mM nonanoic acid. AlkK also accepts mono-esterified dicarboxylic acids as substrate, which facilitated di-ester production.

This *in vivo* esterification was combined with ω -oxidation by AlkB^{GTHJL} to form mono-ethyl DCAs with a chain length of C6 to C10. Most successful was the conversion of 1 mM nonanoic acid by *E. coli* pBGTHJKL-*eeb1*, which produced 0.75 mM of mono-ethyl azelate. These titers are in the same order of magnitude as medium-chain dicarboxylic acid production from fatty acids in shake flask experiments reported before^{19,67,122}. To our knowledge however, this is the first report of the combination of ester biosynthesis and ω -oxidation. ω -Oxidation was also achieved without esterification, but this only yielded 0.30 mM ω -oxidized product. This process was also much slower than the process with esterification. The major products of ω -oxidation of fatty acids were ω -hydroxy fatty acids, even in presence of dehydrogenases AlkJ and AlkH. It has been shown before that ω -hydroxy fatty acids are not or poorly further oxidized by AlkB¹⁹. The limited amount of dicarboxylic acids in these tests could be products of dehydrogenase action, but the dehydrogenases are more efficient with esterified ω -hydroxy fatty acids. Esterification is thus a promising tool to enhance ω -oxidation of medium-chain fatty acids. Furthermore, there would be no need for esterification of the fatty acid prior to the conversion process, potentially increasing the cost-competitiveness.

Also α,ω -di-ethyl esters accumulated. Biocatalytic wax di-ester production has been reported before¹²⁰, but this study is the first to report on biocatalytic di-ethyl ester production. The highest α,ω -di-ethyl ester concentration was 0.24 mM (55 mg/L), which was reached by *E. coli* pBGTHJL + pE-II in conversions with octanoic acid. Production of di-esters was only detectable in the 19 h samples, which suggests that di-ester production only occurs when the fatty acids are depleted and thus no more competition occurs.

Di-ethyl ester synthesis was thus less efficient than ethyl ester synthesis. Since AlkK and AtfA or EEB1 were co-expressed, we were not able to determine which enzyme limits higher product titers. Direct addition of MEDAs to the conversion medium did not yield much more di-ethyl esters. In these test the highest di-ethyl ester concentration (0.19 mM) was reached with mono-ethyl suberate. Using mono-*methyl* azelate as substrate resulted in 2.6-fold higher product titers (0.50 mM), which suggests that the position of the ester moiety plays an important role. Similar differences were seen between conversions with methyl and ethyl nonanoate, since in the conversions with methyl nonanoate clearly more di-ester was produced, 0.34 mM from methyl nonanoate versus 0.04 mM from ethyl nonanoate. Production of a symmetric di-ester would however be more favorable, if the di-ester product is used for a polymerization reaction.

Besides the di-ethyl esters, also byproducts such as nonyl-nonanoate and ethyl 9-(nonanoyloxy)nonanoate were detected in nonanoic acid conversions with *E. coli* pBGTHJL + pE-II. For the production of dicarboxylic acid mono-/di-esters EEB1 seems a more promising candidate, since it prefers ethanol as the alcohol donor for the acyltransferase reaction. This results in less byproduct formation.

To our knowledge this is the first report on whole-cell biocatalytic di-ethyl ester production from fatty acids. Di-ethyl ester production has several advantages over dicarboxylic acid production. Firstly, di-ethyl esters have low solubilities, which could facilitate downstream processing. Secondly, di-ethyl esters can be applied directly as lubricants or plasticizers. Furthermore, if di-ethyl esters are polymerized with an alcohols or amines, ethanol is released from the reaction. If a dicarboxylic acid is used, water is released. Ethanol is

Combination of ester biosynthesis and ω -oxidation

easier to remove from the polymerization than water, and can be recycled in the di-ethyl ester synthesis. Unfortunately, the di-ethyl ester concentrations were lower than mono-ester concentrations. Hence, if one wants to improve the di-ester concentration, the specificity of the esterification enzymes AlkK and AtfA/EEB1 has to be improved. Application of an organic phase for *in situ* product removal could also facilitate higher product titers by shifting the equilibrium more to the product side. It could also alleviate potential toxicity/product inhibition issues.

4.6. Appendix

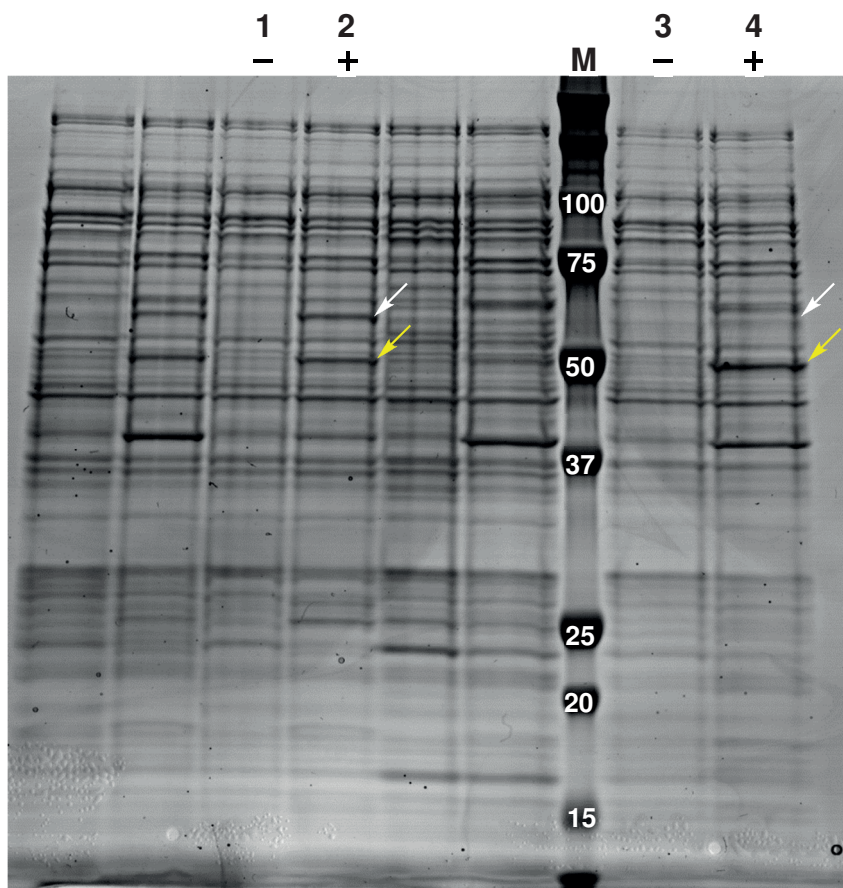


Figure 4.5. SDS PAGE analysis of non-induced (-) and DCPK-induced(+) *E. coli* carrying: pBGTHJL + pE-II (lane 1 and 2), pBGTHJKL-*atfA* (lane 3 and 4). Lane M is Precision Plus Protein™ Standards (Biorad), in white are the masses in kDa. White arrows indicate AlkK, yellow arrows indicate AtfA.

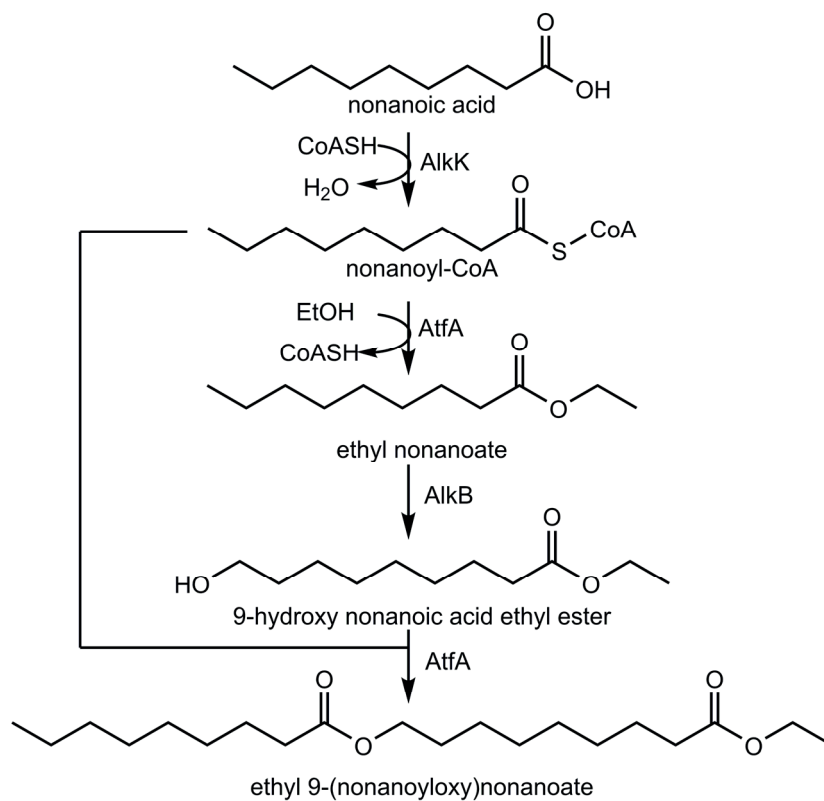
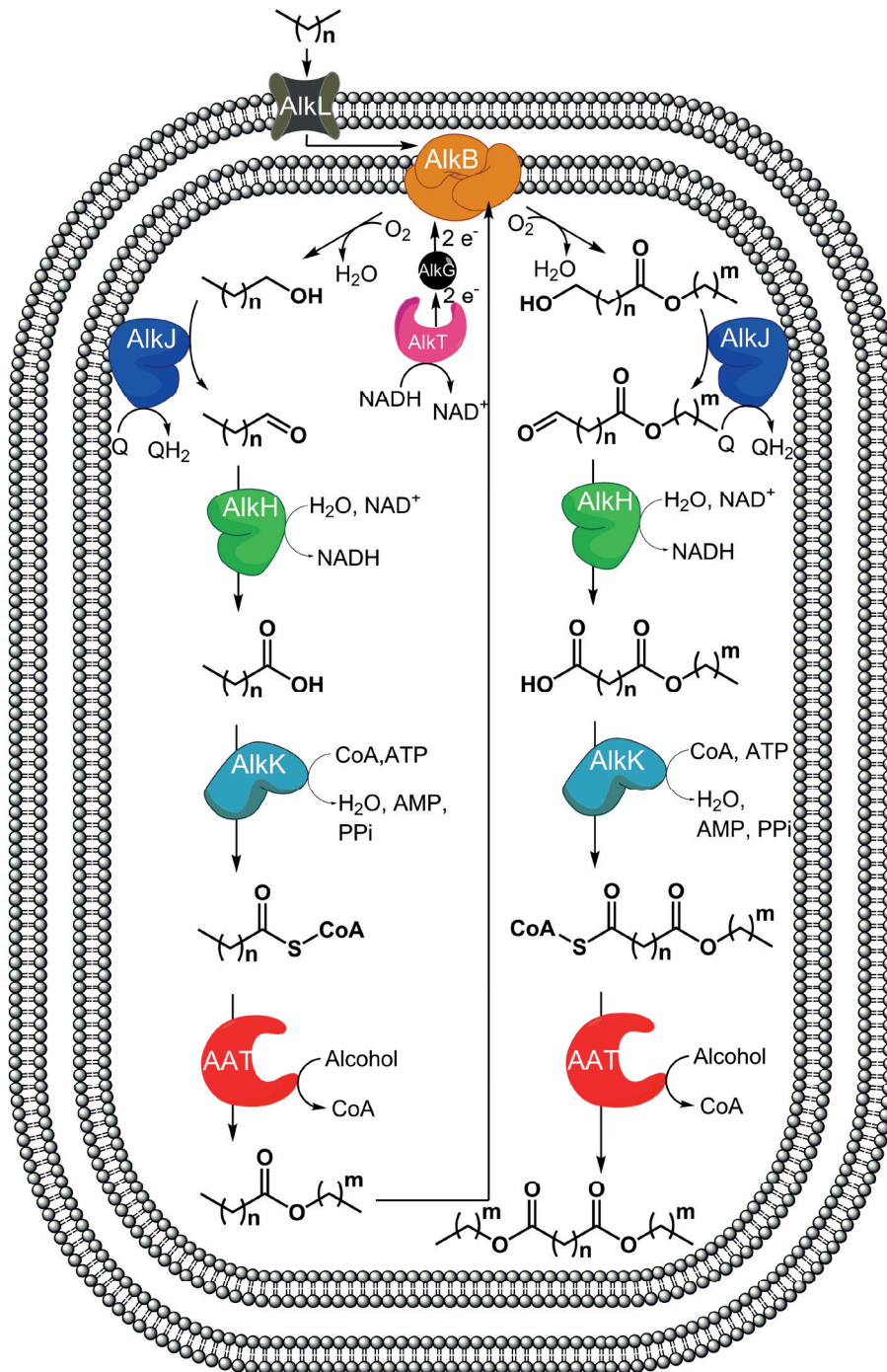
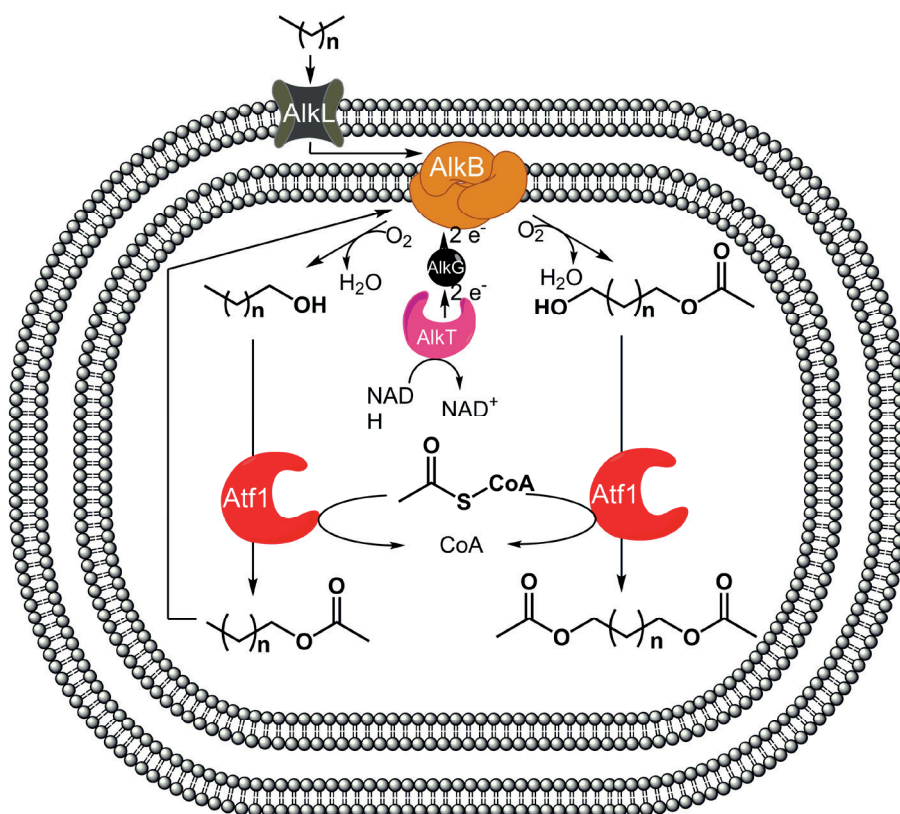


Figure 4.6. Proposed pathway for ethyl 9-(nonanoyloxy)nonanoate production.



Chapter 5 Combining *In Vivo* Esterification and ω -Oxidation Enables Production of Esters of α,ω -Diols and α,ω -Dicarboxylic Acids From n-Alkanes in a Whole-Cell Biocatalytic Setup



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5.1. Abstract

Direct and selective terminal oxidation of medium-chain n-alkanes is a major challenge in chemistry. Efforts to achieve this have so far resulted in low specificity and overoxidized products. These issues make diterminal oxidation even more challenging. Biocatalytic oxidation of medium-chain n-alkanes on the other hand is highly selective. However, it also results in overoxidation. Moreover, diterminal oxidation of medium-chain n-alkanes is not successful. Hence, α,ω -bifunctional monomers are mostly produced from olefins using energy intensive, multi-step processes. New approaches to solve this challenge are necessary.

Here we show that combining biocatalytic oxidation with esterification drastically increases diterminal oxidation and reduces overoxidation. This methodology allowed us to convert medium-chain n-alkanes into α,ω -diacetoxyalkanes and esterified α,ω -dicarboxylic acids. We achieved this in a one-pot reaction with resting-cell suspensions of genetically engineered *Escherichia coli*.

This approach allows to circumvent energy-intensive, multistep processes currently applied for the production of bifunctional monomers.

5.2. Introduction

Diterminal oxidation of abundantly available, inexpensive medium-chain n-alkanes to valuable medium-chain α,ω -diols and α,ω -dicarboxylic acids is highly desirable. Many medium-chain α,ω -oxygenated products are commodities with large market volumes, such as adipic acid, of which 2.7 million tons are produced annually². Those α,ω -oxygenated compound are produced in multistep, energy intensive processes. Direct, diterminal oxidation of n-alkanes could be more sustainable process. There are several chemical means to terminally oxidize alkanes, *via* organometallic C-H activation or by heterogeneous catalysis^{28,30}. However, due to the relative inertness of terminal C-H bonds, alkane oxidation results in subterminal oxidation and is therefore challenging^{30,123}. The application of molecular sieves has improved terminal oxidation^{124,125}, but these conversions are still far from commercial application. Synthesis of alkanols or alkanediols poses another challenge, because the hydroxy groups are easily overoxidized to aldehydes and carboxylic acids.

Therefore, α,ω -bifunctional medium-chain monomers are often produced from olefins. Olefins are obtained from energy-intensive cracking processes and subsequently have to undergo multi-step conversions to yield a bifunctional monomer. Direct diterminal oxidation of medium-chain n-alkanes would circumvent those problems and create a paradigm shift, resulting in a more sustainable petrochemistry¹²⁶.

Monooxygenases such as CYP52 have been applied for terminal oxidation of long-chain alkanes with 100% selectivity and high conversion efficiencies^{35,65,78}. This has resulted in production of high titers of α,ω -bifunctional monomers *via* whole-cell biocatalysis. Terminal oxidation of medium-chain n-alkanes is possible by applying the AlkB monooxygenase from *Pseudomonas putida* GPo1, but this results in overoxidation of the substrate to carboxylic acids^{59,115}. Furthermore, no diterminal oxidation of n-alkanes by AlkB has been reported. Ω -oxidation of medium-chain fatty acids was possible but only to a limited extent^{19,66,67,69}.

Ω -oxidation by AlkB is however efficient with esterified fatty acids as substrates^{65,116}. We realized that combining the ω -oxidation of medium-chain n-alkanes to acids with the subsequent *in vivo* esterification of the acids to ethyl esters could improve diterminal oxidation. In a similar fashion, esterifying fatty alcohols *in vivo* with acetate could be used to enable their ω -oxidation. Moreover, esterified alcohols and alkanediols would be protected against overoxidation.

We investigated whether combining terminal methyl group oxidation by AlkB with *in vivo* esterification using *E. coli* as chassis could improve diterminal oxidation of alkanes and reduce overoxidation. The final products we aimed for were acetoxy esters of α,ω -alkanediols and ethyl esters of α,ω -dicarboxylic acids.

5.2.1 Module design

Six *Escherichia coli* strains were developed to be equipped with modules for monooxygenation, dehydrogenation and esterification (Figure 5.1). An overview of the genetic constructs used to create these strains are shown in Figure 5.2, most strains have already been applied in Chapters 2 – 4, under different designations. All *E. coli* strains possess the monooxygenation module, consisting of AlkB_{GTL} from *Pseudomonas putida* GPo1. AlkB is a non-heme di-iron monooxygenase, responsible for the hydroxylation of terminal methyl groups⁴⁹. AlkB overoxidizes this alcohol to the aldehyde and acid. AlkG and AlkT are involved in the transfer of electrons from NADH to AlkB. AlkL, an outer membrane protein, improves uptake of n-alkanes (Figure 5.1)⁵⁹.

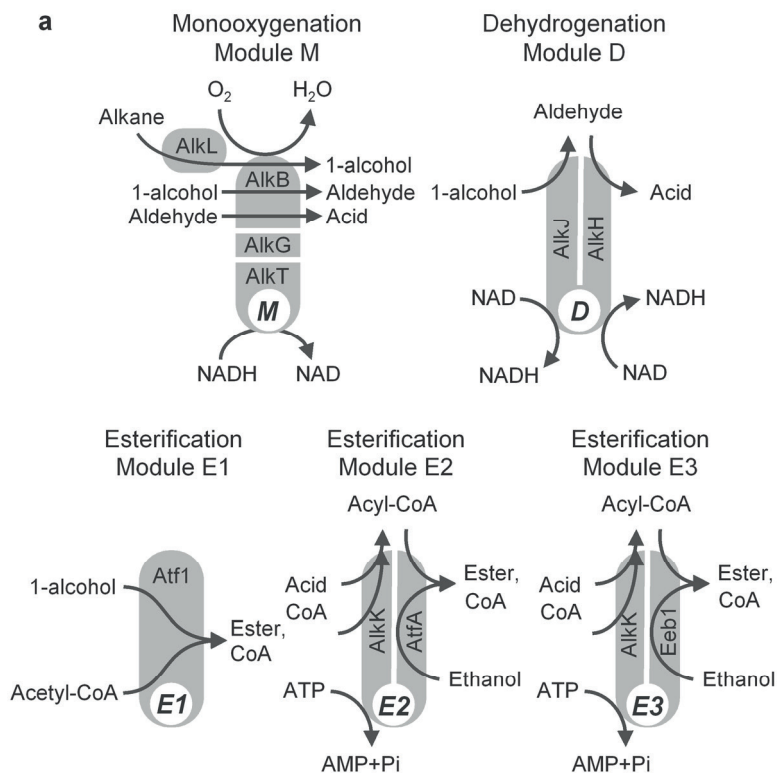


Figure 5.1. Function of the different modules applied in this study. (a) The monooxygenation module (abbreviated M), consists of AlkBGL. AlkL is an outer membrane protein that improves transport of hydrophobic molecules. The dehydrogenation module (D), consists of AlkJ and AlkH. The esterification module 1 (E1) consists of Atf1, esterification module E2/E2* of AlkK and AtfA, esterification module E3 of AlkK and Eeb1.

For production of acetate esters of α,ω -diols, we added esterification module E1. This module consisted of the alcohol acetyltransferase (AAT) Atf1, which can convert the formed 1-alcohol together with acetyl-CoA, derived from central carbon metabolism, to an acetoxalkane.

For the production of esterified diacids, we added the dehydrogenation module D and one of the E2 or E3 esterification modules. The dehydrogenation module consisted of the alcohol dehydrogenase AlkJ and the aldehyde dehydrogenase AlkH, both from *Pseudomonas putida* GPo1. Like AlkB from module M, it oxidizes alcohols to acids, but is more efficient with

respect to cofactor utilization and oxygen consumption¹¹⁹. We designed E2 and E3, based on acyl-CoA ligase AlkK (*P. putida* GPo1) and the AATs AtfA (*Acinetobacter baylyi*) and Eeb1 (*Saccharomyces cerevisiae*). AlkK activates the carboxy groups with CoA, and the AATs transesterify acyl-CoAs with externally supplied ethanol.

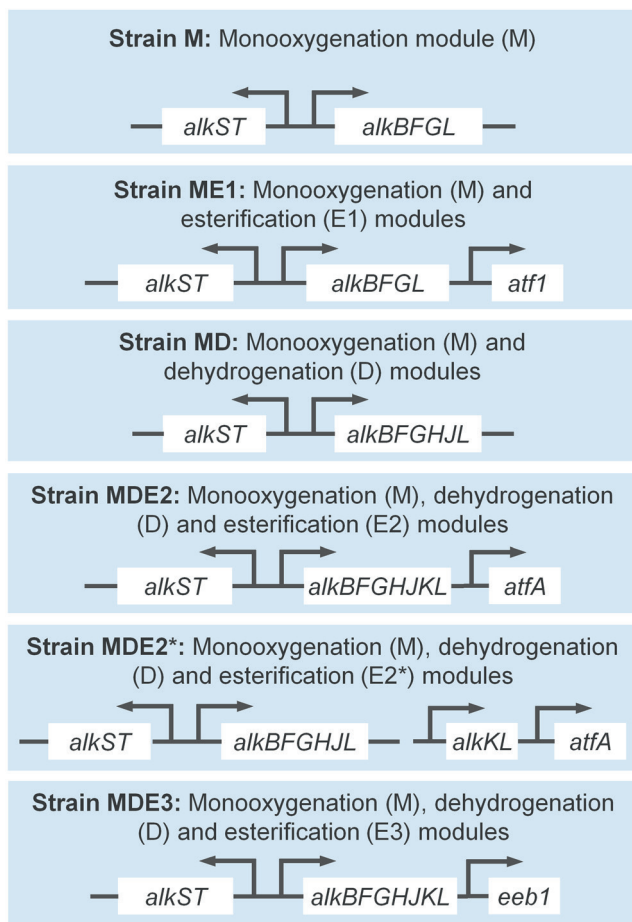


Figure 5.2. The strains tested in this study, with the organization of the different modules on the plasmid(s) they carry. Arrows pointing to the left are promoter P_{alkS} , those pointing to the right are promoter P_{alkB} . *AlkS* codes for a transcriptional regulator. *AlkF* codes for a non-functional rubredoxin.

Table 5.1. Strains used in this study

Strain	Plasmid(s)	Background strain	Reference
M	pBGTL	NEBT7 (BI21 derivative)	¹¹⁶
ME1	pBGTL- <i>atf1</i>	„	This study
MD	pBGTHJL	„	¹¹⁹
MDE2	pBGTHJKL- <i>atfA</i>	„	This study
MDE2*	pBGTHJL + pE-II	„	This study
MDE3	pBGTHJKL- <i>eeb1</i>	„	This study

5.3. Results

5.3.1 Conversion of n-alkanes to α,ω -diacetoxyalkanes

The extent of overoxidation and diterminal oxidation of n-alkanes by AlkB was tested with strain M (Figure 5.3a,c). Tests were done in tightly capped tubes, with 1 g_{cdw}/L resting *E. coli* cells fed with 1 % v/v of n-alkanes of different chain lengths. Glucose served as an energy source. Samples were taken after 19 hours incubation. At that time glucose was still present and the absence of fermentation products indicated that no oxygen limitation occurred (data not shown). Overoxidized products -fatty acids and ω -hydroxy fatty acids- formed at least 95 % of the total products. Minor amounts of 1-alcohols were detected.

Esters of α,ω -diols and α,ω -dicarboxylic acids from n-alkanes

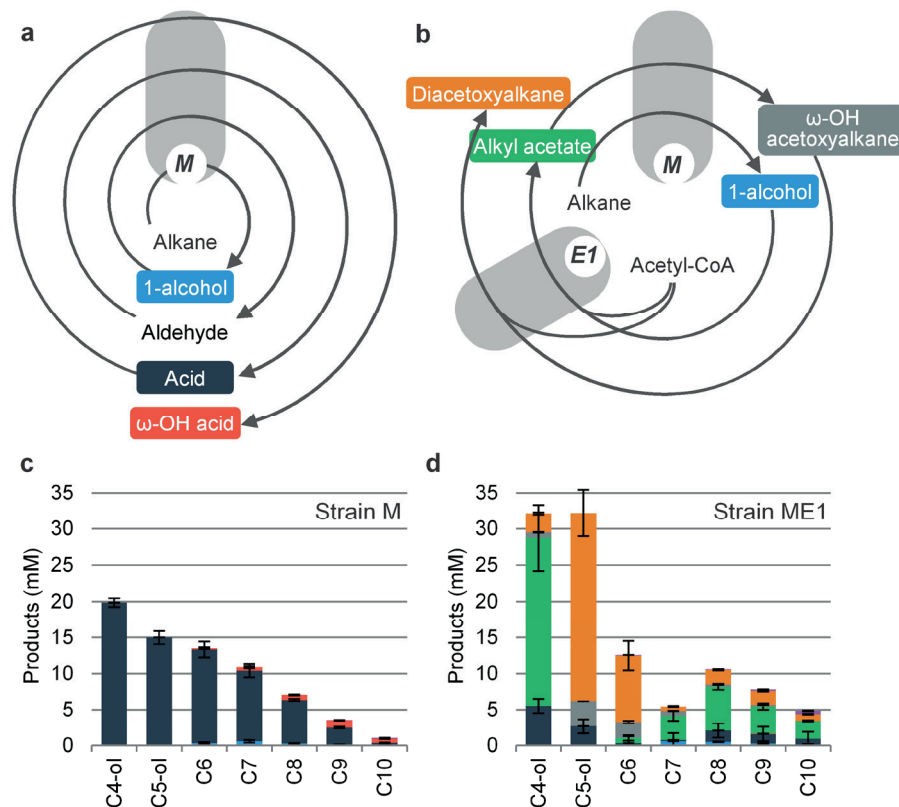


Figure 5.3. Fate of n-alkane conversions by 1 g_{cdw}/L resting cells of *E. coli* M (panel a) or *E. coli* ME1 (panel b). (c-d) Products of 19 h conversions of 1 % pure n-alkane of C6-C10 chain lengths or 0.3% n-butanol/n-pentanol by *E. coli* M (c) or *E. coli* ME1 (d). The colors of the products in panels a-b correspond to the colors in panels c-d. Purple bars from panel d refer to ω -acetoxy fatty acids.

To produce acetate esters of α,ω -diols, we added esterification module E1 (Figure 5.3b,d). Strain ME1 produced mainly alkyl acetates from n-heptane to n-decane. These alkyl acetates were products of transesterification of the 1-alcohols, generated by AlkB and acetyl-CoA. All alkyl acetates produced from the different alkane chain lengths were ω -oxidized, as in most samples ω -hydroxy acetoxyalkanes were detected. Although it is known that AlkB accepts a wide range of aliphatic substrates⁶⁴, no reports have been made that it can ω -hydroxylate alkyl acetates. Surprisingly, these ω -hydroxy alkyl acetates were again transesterified with acetyl-CoA to yield α,ω -diacetoxyalkanes.

Both ω -hydroxy acetoxyalkanes and α,ω -diacetoxyalkanes can serve as precursor for α,ω -diols.

Diacetoxyalkanes were detected by GC-MS for all chain lengths investigated. When n-hexane served as a substrate, 1,6-diacetoxyhexane was the major product after 19 h incubation, with a concentration of 9.20 mM. Furthermore, 2.23 mM 6-hydroxy hexyl acetate accumulated. So in total, 91 mol % contained two terminal hydroxy groups.

The experimental set-up did not allow the addition of smaller alkanes due to their low boiling points. Still, we wanted to see if the system could be used to produce acetate esters of 1,4-butanediol and 1,5-pentanediol. Therefore, we added 0.30 % v/v 1-butanol and 1-pentanol instead. Butyl acetate was produced successfully (23.22 mM), and served as substrate for 4-hydroxy butyl acetate (0.74 mM) and diacetoxybutane production, which accumulated to 2.65 mM. 1-pentanol was very efficiently converted into 1,5-diacetoxypentane (Figure 5.3), which accumulated to 26.01 mM. Thus, with this platform also precursors for short-chain α,ω -diols can be produced. The titers of 1,4-diacetoxybutane were much lower than of the 1,5-diacetoxypentane titers. Butyl acetate was present in high concentration, so apparently the activity of AlkB towards this substrate is low.

Overoxidation only occurred to a limited extent, due to the presence of the esterification module (Figure 5.4); 1-alcohols were overoxidized to fatty acids, but also ω -hydroxy acetoxyalkanes were overoxidized to ω -acetoxy acids. The highest percentage of overoxidized products was 26 mol % in reactions using n-decane, while experiments performed with n-hexane resulted in only 3 mol % overoxidized products. Thus, converting the products into acetate esters efficiently protects the molecule from overoxidation. For n-alkanes <C9, also more diterminal oxidation occurred, since up to 92 mol % of the product was diterminally oxidized in case of n-hexane.

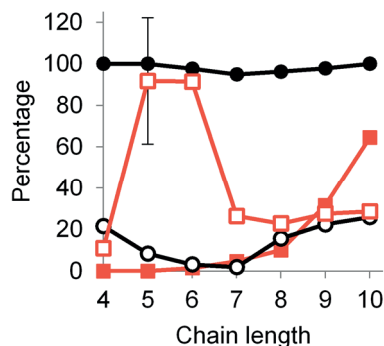


Figure 5.4. Percentage of total product that was overoxidized (black lines), percentage of total product that was diterminally oxidized (red lines) in n-alkane, n-butanol and n-pentanol conversions by *E. coli* M or *E. coli* ME1. Filled symbols correspond to strain M, open symbols to strain ME1.

Since diacetoxyalkane production from n-hexane was quite efficient, we added n-hexane dissolved to 10 % in a carrier solvent. The organic phase served as substrate reservoir and product sink, and was added with an organic:aqueous phase ratio of 1:10 (Figure 5.5). The overall product titers increased; 429 mM product accumulated in the organic phase. Thus, 56 % of the added n-hexane was converted into product. The amount of product with two alcohol moieties was 218 mM (43 g/L), 51 % of the total product. The selectivity of 1,6-diacetoxyhexane production was 47 % (defined as $[\text{product}]/[\text{total product}]$).

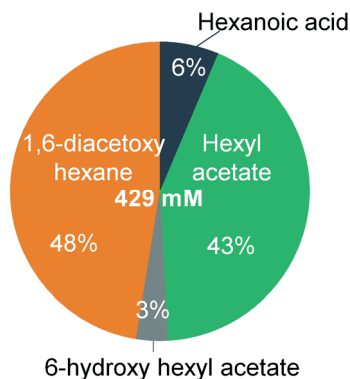


Figure 5.5. Conversion of n-hexane, by resting cells of *E. coli* ME1 (1 g_{cdw}/L), added as 10 % v/v solution in bis(2-ethylhexyl) phthalate. The ratio of organic phase to aqueous phase was 1:10, conversions lasted 31 h.

5.3.2 Conversion of n-alkanes to esterified α,ω -dicarboxylic acids

We tested MD and MDE2 strains with 5 mM n-alkane as substrate, and added 2.5 % ethanol for the esterification reaction. Strain MD produced fatty acids from all n-alkanes (Figure 5.6a,c). Diterminal oxidation occurred to a limited extent for C7-C10 n-alkanes, since small amounts of ω -hydroxy fatty acids accumulated. In C8-C10 n-alkane conversions, traces of dicarboxylic acids (≤ 0.04 mM) were formed.

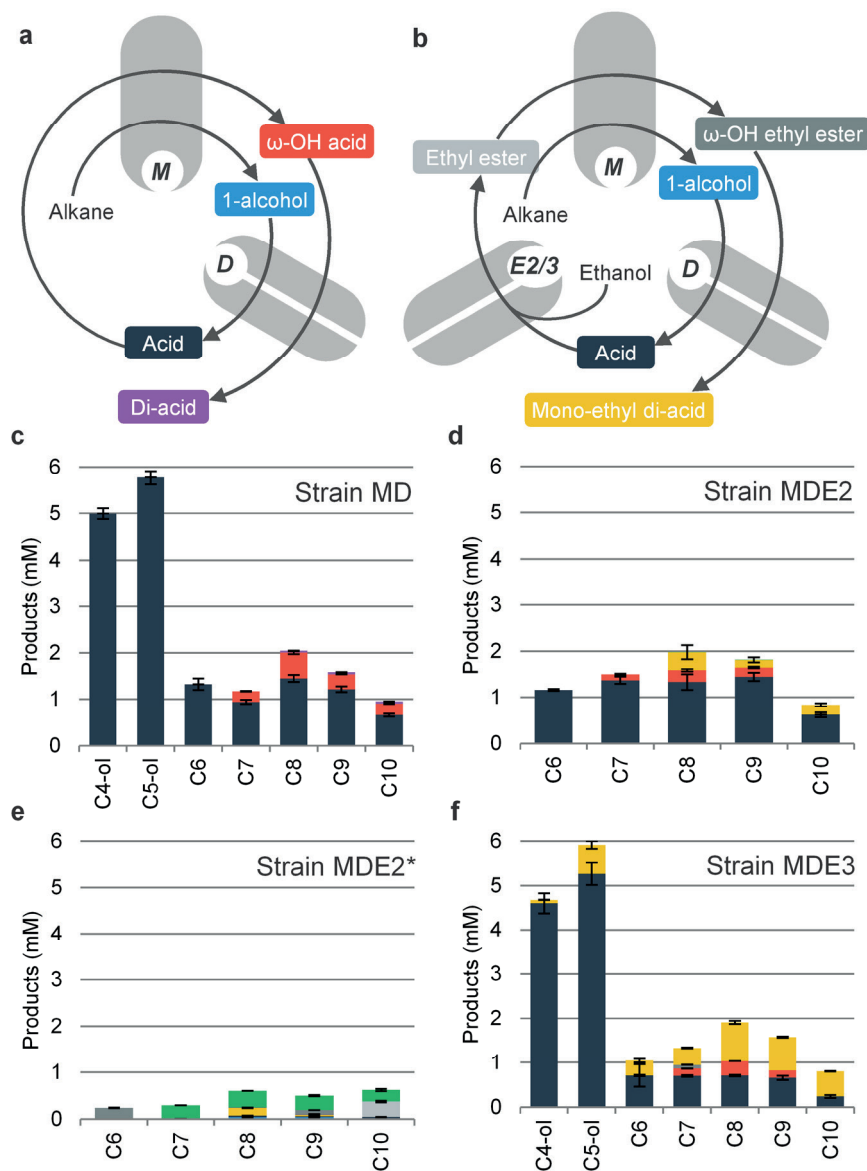


Figure 5.6. Conversion of 5 mM n-alkanes or 5 mM 1-alcohols by MD(E) strains (1 g_{cdw}/L), in presence of 2.5 % ethanol. (a) Fate of n-alkane conversion with *E. coli* MD. (b) Fate of n-alkane conversion with *E. coli* MDE2/MDE2*/MDE3. The colors of the products in a and b correspond to the colors in c-f. Green bars correspond to alkyl alkanoates, formed by esterification of 1-alcohol and fatty acids formed from the n-alkane. Products of 19 h conversions of pure n-alkane of C6 to C10 chain lengths or n-butanol/n-pentanol by *E. coli* MD (c), MDE2 (d), MDE2* (e) or MDE3.

Strain MDE2 produced mono-ethyl dicarboxylic acids (Figure 5.6b,d), showing that esterification was successful. No fatty acid ethyl esters accumulated, indicating that they were efficiently converted to mono-ethyl dicarboxylic acids. This strain produced up to 0.39 mM mono-ethyl dicarboxylic acid. Strain MD produced at most 0.04 mM dicarboxylic acid. Hence, complete oxidation to the ω -acid was more efficient in presence of an esterification module. AtfA also coupled 1-alcohols, that accumulated from n-alkane hydroxylation, to acyl-CoA. This resulted in alkyl alkanoate production. Furthermore, ω -hydroxy fatty acids accumulated, indicating that the pathway from Figure 5.6a is still active.

Strain MDE2 produced mostly acid, indicating that the esterification module was not very effective. Therefore strain MDE2* was constructed, aimed at higher expression levels of AlkK, to increase the activity of the esterification module. MDE2* produced mostly esters (Figure 5.6e), but it was less efficient regarding terminal oxidation. This resulted in lower product titers, and a high concentration of alkyl alkanoates. This shows that tuning the activity of the enzymes is important for module performance.

To investigate whether the monooxygenase module can ω -oxidize the alkyl alkanoates we added n-alkanes without ethanol (Figure 5.7). More alkyl alkanoates were formed, maximally 1.61 mM from n-heptane. Strains MDE2 and MDE2* could terminally oxidize hexyl hexanoate. Both strains produced a mixture of di-hexyl adipate and 6-hydroxyhexyl hexanoate hexyl ester. Strain MDE2 also accumulated a mixture of mono-hexyl adipate and 6-hydroxyhexyl hexanoate. These mixtures were quantified as the sum as gas chromatography did not allow sufficient separation of these compounds. However, it was possible to identify the different compounds with GC-MS. Hence, also without external alcohol addition, it is possible to di-functionalize n-hexane, yielding precursors for α,ω -dicarboxylic acids and ω -hydroxy fatty acids directly from n-hexane.

Esters of α,ω -diols and α,ω -dicarboxylic acids from n-alkanes

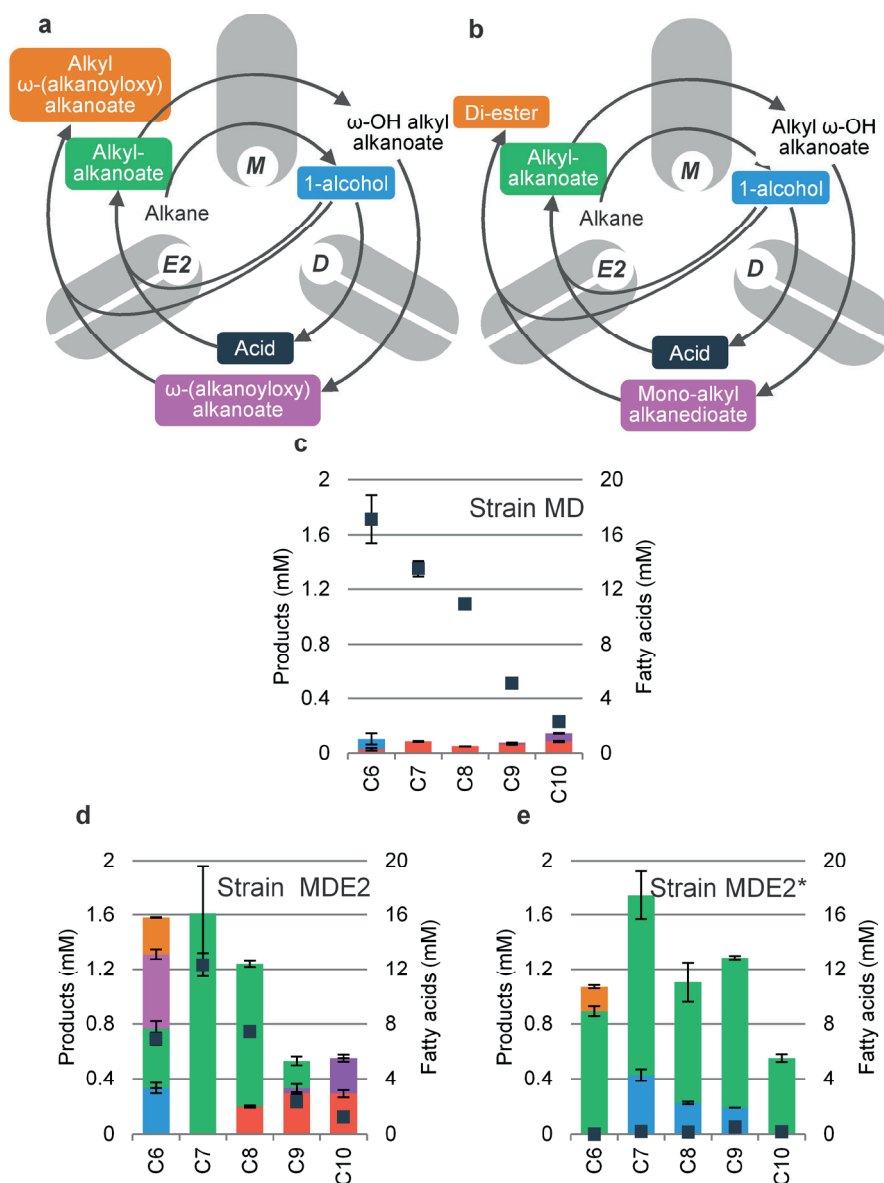


Figure 5.7. Fate of n-alkane conversion in absence of ethanol with *E. coli* MDE2/MDE2*, when AlkB oxidizes the terminal position of the alkyl group (a) or the terminal position of the alkanoyloxy group (b). The colors of the products in a and b correspond to the colors in c-e. (c) Products of 19 h conversion with 1 % n-alkanes by *E. coli* MD (see Fig 3a for pathway). (d) Products of 19 h conversion with 1 % n-alkanes by *E. coli* MDE2; red bars correspond to ω -hydroxy fatty acids, purple to α,ω -dicarboxylic acids. (e) Products of 19 h conversion with 1 % n-alkanes by *E. coli* MDE2*.

Without addition of ethanol, the formed alkyl alkanoates from C7-C10 did not undergo further ω -functionalization. Hence, here it is necessary to add ethanol and to avoid alkyl alkanoate formation to diterminally functionalize C7-C10 n-alkanes. To avoid alkyl alkanoate formation, we created strain MDE3. This strain expresses Eeb1 as alcohol acyltransferase. MDE3 did not produce alkyl alkanoates (Figure 5.6f), since Eeb1 is specific for short alcohols^{85,93}. Ethyl ester synthesis was more efficient, and as a consequence more mono-ethyl dicarboxylic acid accumulated, up to 0.87 mM from n-octane. Titters of products that contain two carboxylate moieties thus improved ~ 22 -fold. This strain also produced 0.36 mM mono-ethyl adipate from n-hexane, a product which was not produced by MDE2/2*. We also tested MD and MDE3 with 1-pentanol and 1-butanol as substrate. MD only produced fatty acids. MDE3 produced 0.65 mM mono-ethyl glutarate from 1-pentanol, and trace amounts of mono-ethyl succinate (0.08 mM). This indicates that ethyl butyrate and ethyl valerate were formed and subsequently ω -oxidized to add another carboxyl group.

Strain MDE3 produced more diterminally functionalized product than strain MD for all tested chain lengths (Figure 5.8a). In most cases, MDE3 also performed better than MDE2 and MDE2* with respect to diterminal functionalization. MD produced small amounts of dicarboxylic acid, indicating that AlkJ and AlkH are poorly or not accepting ω -hydroxy fatty acids as substrate. Esterification improved the conversion towards two carboxylic acid functionalities, since the mole percentage of (mono-ethyl) dicarboxylic acid increased from maximally 5 % with MD to 60 % with MDE3 (Figure 5.8b).

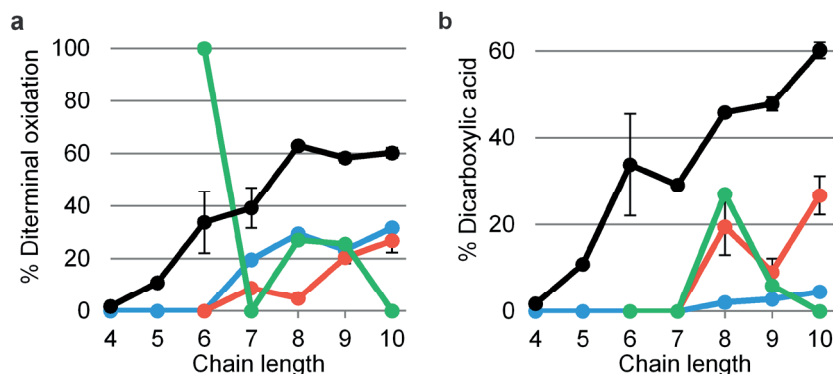


Figure 5.8. (a) Percentage of total product that was diterminally oxidized in tests with 5 mM n-alkane/1-butanol/1-pentanol in presence of 2.5 % ethanol. (b) Percentage of total product that contained two terminal carboxylic functionalities in the same tests. Blue line: strain MD. Red line: MDE2. Green line: MDE2*. Black line: MDE3.

Diterminal oxidation preferentially occurred after esterification, since 1-butanol, 1-pentanol and n-hexane were diterminally oxidized by MDE3, but not by MD. Furthermore, MDE3 produced more diterminally oxidized product than MD.

Although esterification improved the diterminal oxidation, titers were much lower than with diacetoxyalkane production. Higher product titers are most likely hampered by the low intrinsic activities of AtfA¹²⁷ and Eeb1⁹³, which are much lower than the activity of Atf1¹²⁸. Before any process conditions are optimized for higher product titers, this issue has to be addressed, e.g. by protein engineering or screening enzymes with higher alcohol acyltransferase activity.

5.4. Discussion

With these experiments we have shown for the first time that it is possible to convert medium-chain n-alkanes in a mild one-pot process, using air as oxidant, into mono- and di-alkyl dicarboxylic acids, diacetoxyalkanes and ω -acetoxy fatty acids. By combining the terminal oxidation step with esterification, up to 92 mol % of the formed products was diterminally oxidized. Clearly, the concept of combining ω -oxidation and esterification to improve the terminal functionalization of n-alkanes is a success. The fact that

also n-butanol and n-pentanol could be converted into esters of diols and dicarboxylic acids is promising for production of short-chain monomers.

Diterminal oxidation of medium-chain n-alkanes to yield the actual α,ω -diols has been achieved before^{75–77}, with CYP153 monooxygenases. Unfortunately, product titers are generally low and overoxidation also occurs. The technique presented in this chapter could offer a solution to these issues.

The overall conversion depends on the concerted action of 5 enzymes to make the diol esters, and 8 enzymes to make the dicarboxylic acid esters, supported by the cellular machinery to supply intermediates and cofactors. Tuning of all activities is necessary to achieve more efficient conversions. Furthermore, recovery of the diterminal oxidized alkanes from the organic phase has to be studied, maybe in combination with the polymerization reaction. Many improvements have to be made before this concept can be used in a commercial, industrial application. A model of all the aspects of this complex interplay will have to be made as an aid in further improving the process and to enable a comparison of the economics of the envisioned process with those of the conventional production processes.

The technology presented in this study offers a solution to the challenge of selective (di)terminal oxidation of medium chain n-alkanes and may contribute to more sustainable production processes for an array of bifunctional monomers.

5.5. Materials and methods

Table 5.2. Plasmids used in this study

Plasmid	Description	Reference
pUC57- <i>atfA</i> codopt	Codon optimized <i>atfA</i> in pUC57	This study
pET-Duet- <i>eeb1</i>	<i>eeb1</i> in pET-Duet	This study
pGEc47	<i>alkBFGHJKL</i> and <i>alkST</i> in pLAFR1	49
pCOM10- <i>alkL</i>	Contains P_{alkB} : <i>alkL</i> in pCOM10, a broad host range alkane responsive vector	58
pSTL	<i>alkT</i> and P_{alkB} : <i>alkL</i> in pCOM10	116
pBGTL	P_{alkB} : <i>alkBFGL</i> and <i>alkST</i> in pCOM10	116
pET9a:Atf1-opt	Codon optimized Atf1 in pET9a	Provided by A. Kruis, Microbiology, Wageningen UR. Codon opt <i>atf1</i> according to ⁸⁵
pSKL- <i>atf1</i>	P_{alkB} : <i>alkKL</i> , P_{alkB} :codon optimized <i>atf1</i> in pCOM10	This study
pBGTL- <i>atf1</i>	P_{alkB} : <i>alkBFGL</i> , P_{alkB} :codon optimized <i>atf1</i> and <i>alkST</i> , in pCOM10	This study
pE	P_{alkB} : <i>alkKL</i> , P_{alkB} :codon optimized <i>atfA</i> in pCOM10	This study
pE-II	P_{alkB} : <i>alkKL</i> , P_{alkB} :codon optimized <i>atfA</i> in pCOM10 with pBR322 ori, Amp ^R	This study
pBGTHJL	P_{alkB} : <i>alkBFGHJL</i> and <i>alkST</i> in pCOM10	119
pBGTHJKL- <i>atfA</i>	P_{alkB} : <i>alkBFGHJKL</i> ; P_{alkB} : <i>atfA</i> and <i>alkST</i> in pCOM10	This study
pBGTHJKL- <i>eeb1</i>	P_{alkB} : <i>alkBFGHJKL</i> ; P_{alkB} : <i>eeb1</i> and <i>alkST</i> in pCOM10	This study

Table 5.3. Oligonucleotides used in this study

#	Sequence
1	TAGCTCGCC CATATG CTGGGTCAAATGATG
2	GTATCA CTCGAG TTATTACACACCGATGAG
3	AATTGGAG AATTC CATATGCTTGAGAAACACAGAG
4	CACACCAGGTCTCA GTTTT AGAAAACATATGACGCACCAAGACT
5	CACACCAGGTCTCA AAACT ACCCGTAGGTGTAGTTGGCGCA
6	CACACCAGGTCTCA AATTC AGAATTCTCCAATTTTATTAAATTAGTCG
7	CACACCAGGTCTCA GAAT GCGCCCGCTGCACCC
8	GTATCT GTCGAC TTAATTTGCCGTTTTGATATCTTC
9	TATAT CAATTG ATGTTAGGTCAGATGATGCGT
10	ATAT GGATCC TTAATTTGCCGTTTTGATATC
11	CACACCAGGTCTCA CACGT CGCCTCACATTGATGATTAT
12	CACACCAGGTCTCA CGTG ATGAGTTTTTCTAATTATAAAGTAATCGCGATG
13	ACG CGT CGACCTGCGACAGTGACAGACCTG
14	CGAATTC ATGAACGAAATCGACGAAAAGAATCAAG
15	CGGATCC TTACGGACCCAGCAGCAGTGCCTTATAG
16	TAGTTTGTACAGCATGAGATTATCAAAAAGGATC
17	TATACTCGAGACTCAAAGGCGGTAATACG
18	CACACCAGGTCTCAGAATGTTTCGCTCGGGTTACTATC
19	GTATCTGTCGACTATAAACTAACTCATCAAAG

5.5.1 Plasmid constructions

atfA was codon optimized for *E. coli* and synthesised by GenScript®. This gene was delivered in pUC57 vectors. Vector pBGTHJKL-*atfA* was constructed by a Golden Gate approach. pCOM10 was used as backbone, therefore pCOM10-*alkL* was digested with EcoRI and Sall. Primers were designed to generate three inserts, which were *alkBFGHJKL*, P_{alkB} and codon optimized *atfA*. *alkBFGHJKL* was amplified from pGEc47 with primers 3 and 4, P_{alkB} from pGEc47 with primers 5 and 6, codon optimized *atfA* from pUC57-*atfA*codopt with primers 7 and 8. This yielded three amplicons, *alkBFGHJKL* was digested with EcoRI & Bsal, P_{alkB} fragment with Bsal, and *atfA* fragment with Bsal and Sall. Backbone and the three inserts were ligated in the aforementioned order to give pBGTHJKL-*atfA*, which was possible due to the presence of 4 bp overhangs generated from Bsal digestion. The same approach was applied for pBGTHJKL-*eeb1*, where the *atfA* fragment was switched with an *eeb1* fragment that was amplified with primers 18 and 19 from pET-Duet-*eeb1*.

The pBGTHJKL-*atfA* vector was used for the construction of pE. Primers 9 and 10 were used to generate an amplicon from pBGTHJKL-*atfA* that contains *alkK* fused to P_{alkB} -*atfA*. This amplicon was digested with MunI and BamHI; backbone pCOM10-*alkL* was digested with EcoRI and BamHI. These two fragments were ligated to give pE.

To change the ori and resistance marker of pE, a PCR was done on pET-Duet as a template, with primers 16 and 17 to generate the pBR322-Amp^R cassette. This cassette was digested with BsrGI and XhoI, and transferred to pSKL-*atfA*, which was digested with the same restriction enzymes. Ligation of this yielded pE-II.

pSTBFGHJL was also created by the Golden Gate method. pSTL was used as backbone, which was digested with EcoRI and Sall. *alkBFGHJ* was amplified from pGEc47 with primers 3 and 11. *alkL* was amplified from pGEc47 with primers 12 and 13. These two amplicons were ligated together with the pSTL digest to give pSTBFGHJL.

Vector pSKL-*atf1* was constructed by ligating an EcoRI and BamHI digested amplicon, generated from pET9a:Atf1-opt with primers 14 and 15, into EcoRI and BamHI digested pE. For production of pBGTL-*atf1*, the *alkL-atf1* fragment was generated by digestion of pSKL-*atf1* with Sall. This fragment was ligated in Sall-digested pBTL10, to generate pBTL10-*atf1*.

5.5.2 Cultivation and gene expression

For strains with two plasmids 25 μ g/mL kanamycin and 50 μ g/mL ampicillin was added to the medium. Strains were inoculated from glycerol stocks stored at -80 °C in LB containing the appropriate antibiotic(s) and incubated overnight at 30 °C in a rotary shaker set to 250 rpm. The overnight culture was diluted 100 times in M9* mineral medium, M9 medium containing 0.5 % glucose and 1 mL/L trace elements US^{Fe 110}. This culture was again incubated overnight. The next day, this second preculture was used to inoculate M9* mineral medium to an OD_{600nm} of 0.167. This culture was directly induced with 0.025 % v/v dicyclopropylketone to induce recombinant gene expression.

After 4 h induction at 30 °C, 250 rpm, the cells were harvested by centrifugation for 10 min. at 4255 x g.

5.5.3 Conversions

The cell pellet was resuspended in resting cell buffer, which contained 1 % glucose, 2 mM MgSO₄, and 50 mM KPi pH 7.4. Of this resting cell suspension, 0.5 mL was transferred to a pyrex tube. After addition of substrate, tubes were tightly capped with a PTFE cap and transferred to a rotary shaker, set to 30 °C, 250 rpm. The whole reaction was stopped for sampling. Reactions were stopped by addition of 1 % v/v of concentrated phosphoric acid and immediate transfer of the reaction to ice.

5.5.4 GC sample preparation

All compounds were analyzed with GC, except butyrate and valerate, which were analyzed with HPLC. Reactions were extracted with CHCl₃ containing 0.2 mM tetradecane or 0.2 mM dodecane as internal standard. The extraction was done for ~5 min. with a rotator. For conversions containing a carrier solvent or bulk n-alkane phase, the organic phase was sampled directly and diluted 20-40 times in CHCl₃ + internal standard. The samples were derivatized with 10% v/v of a 0.2 M TMSH solution in MeOH. Qualitative analysis was done with GC-MS, quantitative analysis with GC-FID.

5.5.5 GC-MS

GC-MS analysis was done with a Trace GC Ultra coupled to a DSQII mass spectrometer. 1 µL sample was injected in splitless mode, with the inlet set at 350 °C. The temperature program was as follows: 50 °C hold 3 min., 7.5 °C/min ramp to 350 °C, hold for 10 min.

5.5.6 GC-FID

GC-FID analysis was done for all compound except butyrate and valerate, with a 7890A (Agilent). 1 µL sample was injected in splitless mode, with the following temperature program: 50 °C hold 1 min., 15 °C/min to 180 °C, 7 °C/min to 230 °C, 30 °C/min to 350 °C hold 3 min. Quantification was done by using available standards. If standards were not commercially available, quantification was done on basis of structurally related compounds.

Esters of α,ω -diols and α,ω -dicarboxylic acids from n-alkanes

5.5.7 HPLC

HPLC analysis was done for butyrate and valerate quantification, with an Agilent 1260 Infinity UPLC, equipped with a 30 cm Rezex ROA column (Phenomenex), operated at a flowrate of 0.5 mL/min with 5 mM H₂SO₄ as running buffer. The RI detector was used for quantification of analytes.



Chapter 6 Discussion

6.1. Emerging sustainable processes for production of commodity chemicals

In the fields of green chemistry and industrial biotechnology, much research is devoted to the development of sustainable production processes for α,ω -bifunctional monomers (BMs). In order to develop sustainable processes, carbohydrates or plant oils are often selected as starting material. This has resulted in commercial development of renewable production processes for several commodities, such as succinic acid by Reverdia and Succinicity, 1,3-propanediol by DuPont Tate & Lyle, 1,4-butanediol by Genomatica, 2,5-furandicarboxylic acid by Corbion and Avantium, azelaic acid by Novamont and dodecanedioic acid by Verdezyne.

For adipic acid, glucose-based production processes were investigated. A well-known example is the production of *cis,cis*-muconic acid, which can be hydrogenated to yield adipic acid¹²⁹. This process is specific for a chain length of C6, and still requires a high glucose input (3.3 kg glucose per kg of *cis,cis*-muconic acid). Another promising route to adipic acid, is *via* dodecanoic acid, which can be ω -oxidized to dodecanedioic acid, similarly to the Verdezyne process. Dodecanedioic acid can then be β -oxidized partially, to yield adipic acid¹⁸. Palm oil fatty acid distillate is used, a byproduct of the palm oil industry. Palm oil mostly consists of fatty acids \geq C12, meaning that at least 50 % of the carbon is lost in this process. Direct ω -oxidation of fatty acids or diterminal oxidation of n-alkanes is thus an interesting alternative, since this in theory would be a more efficient process.

6.2. Possible feedstocks

In this thesis we investigated medium-chain fatty acids (MCFAs) and n-alkanes as feedstocks for α,ω -dicarboxylic acids (DCAs) and α,ω -diols. MCFAs can be produced from acidified organic waste, by a process called chain elongation^{24,26,27,130}. This process is being commercialized by ChainCraft and Earth Energy Renewables. Since organic waste is an abundant and inexpensive resource, this technology could be an interesting alternative to edible feedstocks. In first step of the process, organic waste is converted into volatile fatty acids, such as acetate and butyrate. In the second step of the process, a microbial community dominated by *Clostridia* elongates the fatty acid with

supplied ethanol to MCFAs. Hence, besides the fact that this feedstock is partly generated from waste, it is a renewable substrate. Compared to a process based on non-renewable feedstocks, this process could have a better emission profile. Furthermore, the feedstock price can be more stable than petroleum-based feedstocks, of which the prices are volatile.

Another emerging technology that delivers bio-based chemicals is fermentation of syngas. Syngas can be obtained from gasification of recalcitrant portions of residual biomass. Fermentation of syngas can lead to a variety of functional chemicals^{131,132}. Lanzatech has claimed that *via* this route a myriad of chemicals can be produced¹³². Among these are fatty acids and primary alcohols, such as butyrate and 1-butanol. Combining this technology with the pathways presented in this thesis, opens up novel routes to important commodity chemicals such as succinic acid and 1,4-butanediol.

n-Alkanes are abundant and cheap molecules. However, they are difficult to selectively functionalize, which prevents their direct usage other than for fuel. To introduce functional groups, n-alkanes are mostly cracked at high temperatures, and the resulting olefins or oxygenates are used for BM production in multi-step processes. Direct conversion of the n-alkanes could significantly reduce energy consumption for BM production¹³³. Furthermore, both fatty acids and n-alkanes can in theory be converted to a BM with a molar yield of 1. The only necessary reactions are oxidation of the α and ω -position when n-alkanes are the starting material, and in case of fatty acids only ω -oxidation is necessary.

Unfortunately, MCFAs are difficult to ω -oxidize^{19,60,66,67,69,114,115}. Terminal oxidation of n-alkanes is possible, but subsequently ω -oxidation has to take place, so the same problem arises as with MCFAs as substrate.

An interesting alternative is to integrate the synthesis of fatty acids and subsequent ω -oxidation. This should be achievable within a single host, which means that only one reactor is necessary to form BMs from carbohydrates or glycerol. Recently, *de novo* synthesis of fatty acids and fatty alcohols by microbes has received much attention^{84,134}, which can be achieved by

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interfering with the native fatty acid biosynthesis or by reversal of the β -oxidation cycle.

Fatty acid production has been combined with *in vivo* esterification^{83,84,89}. Furthermore, ω -functionalization of *de novo* produced fatty acids has been investigated, both for medium-chain¹⁹ and long-chain¹¹³ ω -hydroxy fatty acids/ α,ω -dicarboxylic acids. Interestingly, *de novo* production of esterified fatty acids and ω -oxidation have not been combined yet. It would be worthwhile to investigate whether the combination of the two concepts results in more efficient BM production from carbohydrates. Reversal of the β -oxidation cycle would then be the most interesting route to produce esters, since this proceeds *via* CoA-ligated intermediates and not *via* intermediates ligated to acyl-carrier protein, as in normal fatty acid synthesis. Acyl-CoA's can serve directly as substrate for the acyltransferase reaction, and free fatty acids do not have to be ligated by an acyl-CoA ligase such as AlkK that was applied in this thesis. By proper selection or engineering of the alcohol-acyltransferase, the chain length of the BM can be tailored.

Hence, many feedstocks can be used for the technology presented in this thesis. Life cycle assessments with the different feedstocks have to be done to identify the most sustainable option.

6.3. Solving the problem of medium-chain ω -oxidation

To ω -oxidize MCFAs, monooxygenase AlkB was selected to facilitate the first step in the ω -oxidation process. The enzymatic activity of AlkB was already described in the '60s⁴⁴. In the '80s the *alk* operon from *Pseudomonas putida* GPo1 was deciphered and expressed in *E. coli*^{46,48,49}, but only recently the excellent ω -oxidation activity of AlkB on fatty acid methyl esters was described⁶⁵. This aroused our interest and inspired us to explore whether (biological) esterification can improve whole-cell biocatalytic BM production.

It was only investigated before how well AlkB functions with methyl esters, not with ethyl or longer esters. We show in chapter 2 that a wide variety of esters can be ω -oxidized by AlkB. Ethyl nonanoate was efficiently converted to the corresponding ω -alcohol by *E. coli* expressing AlkB_{GTL}. Furthermore, by varying the chain length of the alkyl group, ω -oxidation activities could be

improved for the shorter fatty acids. The presence of a relatively polar ester group did not affect the activity of AlkB much.

Expansion of this system with alcohol dehydrogenase AlkJ and AlkH enabled complete conversion of the ω -alcohol to the carboxylic acid (chapter 3). Furthermore, the initial ω -oxidation activity increased from 51 to 81 U/g_{cdw}. Hence, the AlkB_{GTHJL} system is a suitable system for this type of conversions.

Since *in vivo* methyl ester synthesis has not been efficient⁹¹ so far, we focused on producing ethyl esters in chapter 4. *In vivo* ester production has gained a lot of interest, with the intention to produce biodiesel or fuels from fatty acids or carbohydrates^{83–85,88,89,135,136}. AtfA and Eeb1⁹³ were applied to produce a variety of esters, including ethyl esters. These proteins need an acyl-CoA molecule and an alcohol to form esters. We applied AlkK from *Pseudomonas putida* GPo1 for the conversion of fatty acids into acyl-CoAs. This enzyme was selected since it allows *P. putida* GPo1 to grow on medium-chain n-alkanes, by converting fatty acids into acyl-CoA, which are subsequently degraded in the β -oxidation cycle. We have proven the functionality of AlkK on octanoate and mono-ethyl suberate *in vitro* (chapter 4).

At most 0.75 mM mono-ethyl DCA accumulated from fatty acids, obtained with 1 mM octanoic acid conversions by *E. coli* expressing AlkB_{GTHJKL} and Eeb1. From n-alkanes this was maximally 0.87 mM, in 5 mM n-octane conversions. These product titers are still too low for an industrial application. Reasons for the low titers are yet unknown. Product inhibition is a possible cause, aggravated by the presence of 2.5 % ethanol. The physiology of the host can also be compromised by the expression of ten heterologous proteins, of which multiple proteins are membrane bound or associated. Expression of the acyltransferase(s) in *Pseudomonas oleovorans*, the native host of the *alk* operon, could result in a more stable phenotype. If this bacterium were to be used as a host, its β -oxidation should be blocked or knocked out.

Production of alkanediol in the form of α,ω -diacetoxylalkane was much more efficient; up to 203 mM accumulated in the organic phase, with an organic:aqueous phase ratio of 1:10 (chapter 5). The organic phase consisted of 10 % n-hexane in bis-(2-ethylhexyl) phthalate (BEHP). Overoxidation was

limited by efficient esterification by Atf1. This suggests that the alcohol leaves the active site of AlkB before overoxidation can take place. For short-chain diacetoxyalkane production, however, the pathways have to be improved. An overview of all the used substrates and products is shown in Figure 6.1.

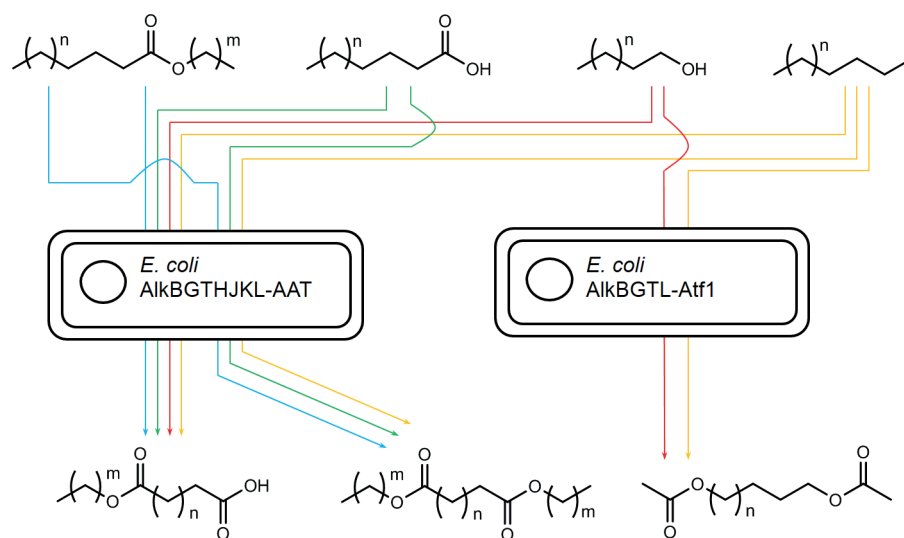


Figure 6.1. Substrates used and products obtained in this thesis, via the DCA pathway (AlkBGTJJKL-AAT) or the diol pathway (AlkBGTl-Atf1).

With *E. coli* expressing AlkBGTl and Atf1, also esterified ω -hydroxy fatty acids can be produced. Methyl nonanoate was converted by this strain to 9-acetoxy methyl nonanoate, showing that also this type of alcohol formed by AlkB can be protected from further oxidation (unpublished data).

6.4. Pathway improvement

All reactions of the designed pathways have been shown to function, and a multitude of novel products and routes are demonstrated in this thesis. However, productivities and final product titers need to improve for these processes to become feasible. This section deals with potential improvements on the metabolic pathway level that should be investigated.

6.4.1 Selection of the monooxygenase

AlkB is most active on medium-chain substrates. The activity of AlkB on shorter fatty acids was improved by using a longer alcohol in the ester group (Chapter 2). A disadvantage of using longer alcohols are their higher costs compared to methanol and ethanol. Furthermore, AlkB might ω -oxidize the alkyl chain, resulting in a mixture of products. Thus from an industrial point of view, a monooxygenase that has a higher activity on shorter molecules would be more interesting for short-chain bifunctional monomers. Butyl acetate and ethyl butyrate were still ω -oxidized by AlkB, but with very low activities (chapter 5). Butane monooxygenases are interesting candidates, since they catalyze the terminal hydroxylation of C3 to C6 n-alkanes^{137–139}. A disadvantage of butane monooxygenase is that also subterminal hydroxylation was reported¹³⁹. Hence, AlkB might still be a more suitable option, because it also has the ability to hydroxylate propane and n-butane¹⁴⁰. AlkB has also been evolved for higher activity on shorter alkanes, while retaining terminal selectivity⁶³. This would be especially useful for chain lengths \leq C6.

6.4.2 The effect of alcohol and aldehyde dehydrogenases

In principle, AlkB is able to overoxidize the alcohol to the aldehyde and finally the carboxylic acid. This would cost 3 oxygen and 3 NADH, since the conversion from methyl group to carboxylic acid requires three times the action of AlkB. However, production with alcohol dehydrogenase AlkJ and aldehyde dehydrogenase AlkH is more efficient in three respects. Firstly, the conversion is quicker, resulting in higher productivity and lower intermediate concentrations. In conversions where AlkJ and AlkH were also present, alcohol and aldehyde hardly accumulated (Chapter 3, 4 and 5). Secondly, uncoupling of AlkB might occur when it further oxidizes alcohols and aldehydes, since they are not the natural substrates. This could mean that some of the NADH is wasted to generate H_2O_2 . Thirdly, the pathway actually delivers energy to the host, instead of costing 3 NADH, see Figure 6.2. NADH that was invested for the terminal hydroxylation (first step), can be regenerated by the action of AlkH. AlkJ has tightly bound FAD as cofactor, and is associated with the membrane. It funnels electrons to the electron transport chain by reducing ubiquinone⁵⁵. This results in irreversible alcohol oxidation⁷⁸, which is desirable

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for DCA production. Recently, AlkJ was also identified as the best among 5 alcohol dehydrogenases for C12 and C14 DCA production with *E. coli*¹¹³. The ubiquinol generated by AlkJ can be oxidized in the electron transport chain, which yields ATP. This ATP can be utilized by the host for maintenance purposes, or in the further conversion of fatty acids into acyl-CoAs, which costs 2 ATP equivalents.

The oxidation of ubiquinol requires 0.5 mol of O₂, which brings the oxygen demand for production of carboxylic acids to 1.5 mol O₂. If the process is based on AlkBGT only for the terminal oxidation, the oxygen demand is 3 mol O₂ (Figure 6.2). Considering that oxygen transfer plays an important role on industrial scale (see also section 6.5.3), reducing the oxygen demand is beneficial.

AlkJ has been applied before for conversion of 12-hydroxy dodecanoic acid methyl ester into 12-oxo dodecanoic acid methyl ester, together with AlkBGT^{78,141}. AlkJ has received attention since it can produce the terminal aldehydes that can be converted to amines by ω -transaminases^{117,141}. In two-liquid phase conversions with AlkBGTJL, the aldehyde and alcohol intermediates still accumulated. AlkH was not included in these reports, and should be incorporated in case the carboxylic acid is the desired product or intermediate. Furthermore, the dehydrogenases can keep the concentration of terminal alcohols and aldehydes low as shown in chapter 3, alleviating potential product inhibition of these intermediates.

In chapter 4 and 5 AlkJ and AlkH were also tested as part of the AlkBGTJL pathway on fatty acids and n-alkanes. Dicarboxylic acid titers were very low in these tests, and ω -hydroxy fatty acids accumulated. It seems that medium-chain, non-esterified ω -hydroxy fatty acids are poorly accepted by AlkJ and/or AlkH. For conversion of ω -hydroxy fatty acids to DCAs of medium chain length, ChnD (6-hydroxyhexanoate dehydrogenase) and ChnE (6-oxohexanoate dehydrogenase) are good candidates. These enzymes are involved in the cyclohexanol metabolism of *Acinetobacter* sp.¹⁴², in which cyclohexanol is converted to adipate. Their activity has already been demonstrated for DCA production of C6 to C14 chain lengths^{19,113}. Activity of ChnE was also

demonstrated with methyl 6-oxohexanoate¹⁴², indicating that esters are likely to be accepted as well.

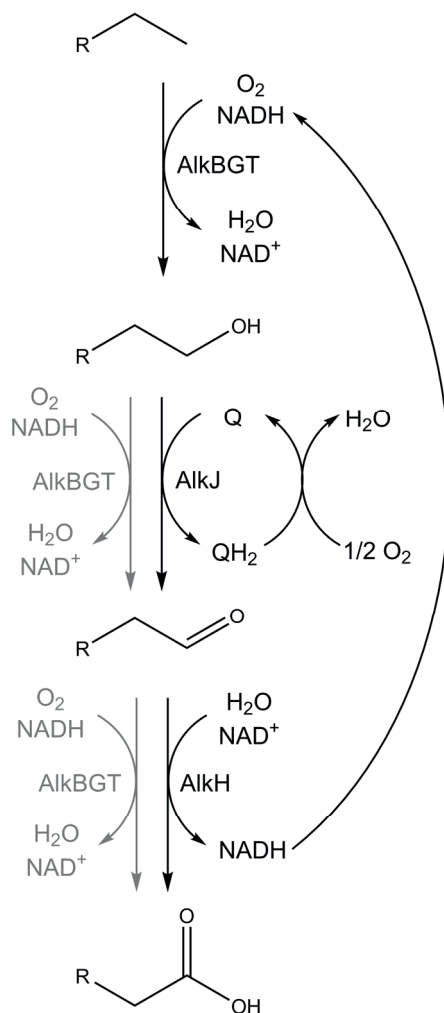


Figure 6.2. Terminal methyl group oxidation towards the carboxylic acid. In grey is the route via AlkBGT, in black the route via AlkBGT, AlkJ, and AlkH.

6.4.3 Considerations on *in vivo* esterification

For DCA production, esterification was the main limitation. In general, the intermediate fatty acid ethyl ester did not accumulate in whole-cell bioconversions. This indicates that ω -oxidation is efficient, and esterification

is the key factor limiting higher mono- and di-ethyl ester titers. Esterification is achieved in two steps: conversion of carboxylic acids to acyl-CoA and transesterification of acyl-CoA with an alcohol.

6.4.3.1 Acyl-CoA synthesis

For the acyl-CoA synthesis we have applied acyl-CoA ligase AlkK. We have not screened other acyl-CoA ligases. AlkK has been tested in more detail with fatty acids $\leq C8$ and hydroxy fatty acids. AlkK prefers octanoate as a substrate, although the specificity is rather broad. Acetate, propionate and butyrate are also converted into acyl-CoA. Besides fatty acids, AlkK also forms 3-hydroxy butyryl-CoA, 3-hydroxy octanoyl-CoA and 4-hydroxybutyryl-CoA⁵⁷.

Hence, the mono-esters that were formed in chapter 4 and chapter 5 could have been the result of esterification of hydroxy fatty acids or dicarboxylic acids that were produced by ω -oxidation of fatty acids. However, since AlkK prefers alkanates, that were present throughout those biotransformations, ω -oxidation has probably mostly occurred after esterification. Mono-esterified DCAs were not tested with AlkK by Satoh et al.⁵⁷, but our qualitative tests with cell-free extracts of *E. coli* expressing AlkK indicated that less CoA-ligated product was formed from mono-ethyl octanedioic acid than from octanoate (unpublished data). Though one should bear in mind that mono-ethyl octanedioate has a longer chain length than octanoate, which could have affected the activity as well. Nevertheless, the lower activity on mono-ethyl DCAs could be a factor causing the low titers of di-(ethyl) esters.

A more detailed analysis of this enzyme and other acyl-CoA ligases is necessary to tune enzyme specificity and conversion rate. For di-ethyl ester production, experiments were done with a coupled whole-cell assay, in which both AlkK and an alcohol acyltransferase were expressed. We have therefore not been able to identify which enzyme limits the di-ester production. Assays with the purified proteins could give more insight. However, the activity of AlkK reported in literature on octanoate was 7 U/mg⁵⁷, which leads us to suspect that the alcohol-acyltransferases (< 0.3 U/mg, see below) are the bottleneck.

The production host, *E. coli* also possesses enzymes that can form medium-chain acyl-CoA from alkanoates, FadD and FadK. FadD is involved in producing long-chain acyl-CoAs, but still has some activity with hexanoate and octanoate¹⁴³. FadK is only expressed under anaerobic conditions, and displays the highest activity towards octanoate among C6-C18 fatty acids¹⁴³. However, both proteins have lower activities than AlkK. This is not surprising, since the native host of AlkK is able to grow on medium-chain n-alkanes and medium-chain fatty acids by virtue of AlkK among others. *E. coli* does not have this ability under aerobic conditions. Hence, if the activity of AlkK is uncompromised by being expressed in *E. coli*, it seems a more appropriate enzyme.

Another interesting way of producing the acyl-CoAs, when alkanes or alcohols are the starting material, is by an acetylating aldehyde dehydrogenase. This enzyme catalyzes the conversion of aldehydes to acyl-CoAs, and reduces NAD^+ . This could save 2 ATP in the pathway, since the acyl-CoA ligase reaction is not necessary anymore. However it would be challenging to stop the ω -oxidation process at the aldehyde stage.

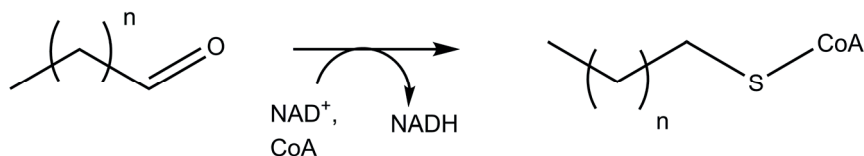


Figure 6.3 Reaction catalyzed by an acetylating aldehyde dehydrogenase

6.4.3.2 Alcohol acyltransferase selection

Alcohol acyltransferase (AAT) AtfA is highly unspecific, which can have an influence on the fitness of the production host. We found hexyl esters of C14 to C18 fatty acids in n-hexane conversions with *E. coli* AlkB^{GTHJKL}-AtfA (unpublished data). Hence, the produced 1-hexanol is not only coupled to hexanoyl-CoA, but also to *E. coli*'s native fatty acids. This results in a mixture of products that can have an influence on downstream processing and the cellular architecture. Nevertheless, this enzyme is promising for the production of short waxes, such as hexyl hexanoate and octyl octanoate. This esterification process is mild and does not need expensive catalysts, such as

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ruthenium complexes¹⁴⁴. These esters can be applied as lubricants, or hydrolyzed to give primary alcohols and fatty acids.

Hexyl hexanoate was also further terminally oxidized, resulting in the production of diterminally oxidized molecules. The rationale behind this process resembles that of a patent filed by Evonik Industries¹⁴⁵, except that in the Evonik process three separate process steps are necessary to obtain the same type of product. Integration of the process into a one-pot synthesis could greatly reduce capital and operating expenses. The diterminal oxidation only occurred with hexyl hexanoate, not with longer esters. For these chain lengths, a CYP450 monooxygenase like CYP52 from *Candida tropicalis* would be an interesting option, since it produces DCAs from C12-C18 n-alkanes and fatty acids. However, both AlkB and CYP52 are membrane-bound monooxygenases, from prokaryotic and eukaryotic origin. It would thus be quite a challenge to achieve stable expression of both monooxygenases in the same host. CYP153 is a soluble monooxygenase that can be expressed in *E. coli* and could potentially take over the role of CYP52⁶⁹.

Alcohol acyltransferase Eeb1 has a narrower substrate range. It is known for the ability to couple ethanol to acyl-CoAs⁹³, although it also accepted n-butanol⁸⁵. This enzyme allowed us to produce mono- and di-ethyl esters more specifically (Chapter 4 and 5). A closely related enzyme Eht1, also produced ethyl esters. In 2006 however, it was shown to favor production of short-chain ethyl esters⁹³. In 2014, Knight and coworkers contradicted these findings and showed that this enzyme has a quite similar substrate range as Eeb1. Eht1 is thus an AAT that is worthwhile to test in combination with the AlkB^{GTHJKL} pathway.

Both AtfA and Eeb1 have the ability to form di-esters. In a two-liquid phase setup, di-esters would partition better into the organic phase. Furthermore, di-esters are more valuable products than dicarboxylic acids. The ester group can be beneficial for polymerization reactions, and di-esters can also be applied as lubricants.

Although Eeb1 and Eht1 are more specific towards short-chain alcohols, their stability might be an issue when *E. coli* is the production host. Both enzymes

resided in membrane fractions of *Saccharomyces cerevisiae* after cell disruption⁹⁴, thus protein stability in *E. coli* is probably low.

Esterification activity of cells only expressing AlkKL and AtfA are in the order of 7 U/g_{cdw} (1 U equals 1 μmol/min). The activity of Atf1 was much higher for (di-)acetoxyalkane production, where esters accumulated and hardly any alcohols or fatty acids were detected. The activity of Atf1 was determined from 1-pentanol conversions and was about 57 U/g_{cdw} (unpublished data). This higher activity could be a result of higher acyl-CoA concentrations in the case of acetoxyalkane production. Acetyl-CoA is present in high concentrations in the cell and this intermediate does not have to be produced by an acyl-CoA ligase. For ethyl ester synthesis medium-chain acyl-CoAs have to be synthesized, by AlkK. AATs commonly have thioesterase activity, so the formed acyl-CoA can be hydrolyzed again to yield free fatty acids and CoA. Furthermore, Eeb1 is known to have esterase activity. For AtfA such activity is not known, but for both enzymes this putative activity on ethyl esters should be investigated. In literature, an activity of 0.3 U/mg was reported for AtfA¹²⁷ and $3 \cdot 10^{-3}$ U/mg for Eeb1⁹³, indicating that the activity of the alcohol-acyltransferases is intrinsically low. Atf1 was reported to have an activity up to 190.4 U/mg¹²⁸. Potentially protein engineering of AtfA/Eeb1 could yield a protein that has a higher activity, such as Atf1.

An alternative way of producing the esters should also be considered. Esters can be produced extracellularly during the conversion process, for example by lipase CALB^{146,147}. The necessary alcohol can be added or produced by ω-oxidation of n-alkanes.

6.4.4 Availability of cofactors

Although there is no net consumption of CoA in the esterification process, it might be one of the metabolites that create the bottleneck in esterified DCA production. In *E. coli*, the concentration of free CoA can be low compared to the acetyl-CoA concentration when grown on glucose^{148,149}. Potentially there is not enough free CoA available to generate acyl-CoA. Using growing cells or an *E. coli* strain with a feedback resistant pantothenate kinase could shed a light on this, since this strain produces more CoA due to the fact that feedback inhibition by CoA is abolished or limited¹⁵⁰. Alternatively, expressing a

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different pantothenate kinase, which increases overall CoA-levels, could improve the production of acyl-CoA¹⁵¹.

In the case of the diol pathway, no acyl-CoA ligase activity is necessary. This saves 2 ATP. For each diacetoxylalkane produced, two acetyl-CoAs are required. Therefore, this process benefits from high intracellular acetyl-CoA concentrations. Glycolysis until acetyl-CoA delivers 2 ATP and 4 NADH. NADH is necessary to drive the monooxygenase reaction. Thus, for diacetoxyl alkane production 2 NADH has to be invested, leaving 2 NADH and 2 ATP for the host.

In chapter 5, ~600 mM acetoxyl esters were detected in the organic phase (organic phase:aqueous phase ratio of 1:10). In tests with 25 % n-hexane in BEHP this value was 782.4 mM (data not published). The latter test contained 55.50 mM glucose. Glucose was depleted, meaning that 70 % of the acetyl-CoA that was derived from glucose was converted into acetoxyl esters. So 30 % of the consumed glucose was not associated with product formation. Increasing the glucose concentration to 2 % slightly improved product titers, so higher product titers are limited by other factors (unpublished data). Once those factors are identified, testing the bioconversion process in a bioreactor setup would be the next step. Coupling efficiency can be an important factor that increases the demand of glucose.

If the coupling efficiency is 100 %, 1 mol NADH is consumed per mol monooxygenase product. With non-natural substrates, or in absence of substrates, uncoupling can occur. The monooxygenase then produces H₂O₂, which is toxic for the host. Uncoupling has been observed *in vitro* with AlkB⁶⁵. Uncoupling could also be a major factor that causes the large difference between cofactor regeneration and product formation, that was observed for monooxygenase-based styrene epoxide production¹⁵². In this report, cofactor regeneration rate was around 3 times higher than product formation rate. The coupling efficiency was not determined in this thesis, but should be done in order to determine what share of the cosubstrate glucose is wasted to generate H₂O₂. Tailoring the ratio between AlkB and its reducing partners (AlkG and AlkT) could result in a better coupling efficiency. If a non-native substrate is used, poor binding of the substrate in the active site of the monooxygenase occurs. This problem can be tackled by protein engineering.

6.5. Scale up considerations

In order to further boost the production of diacetoxyalkanes and esterified DCAs, the supply of extra carbon source, oxygen and pH have to be controlled. Monooxygenase-based processes are applied on large scale, but this mostly involves production of fine chemicals¹⁵³. There are several factors that hamper oxygenase based bulk chemical production processes.

6.5.1 Productivity

Compared to traditional chemistry approaches, enzyme and whole-cell based processes generally have low conversion rates. The activities of monooxygenases are low compared to other enzymes. The maximum *in vitro* activity of AlkB has been measured at 5.2 U/mg⁵¹, with n-octane as substrate. In this thesis, initial activities in the range of 41-105 U/g_{cdw} were reached. This corresponds to ~2.5-6 mM/g_{cdw}/h. For a product with a molecular mass of 200 Da, this means that initial productivities of 0.5-1.2 g/g_{cdw}/h are reached. Whole-cell biotransformations with *E. coli* expressing AlkBGTJL or AlkBGTJL have been conducted in bioreactors, for the conversion of methyl dodecanoate into terminally oxyfunctionalized products^{58,78}. The maximum productivity reached with resting cells was 4.10 g/L/h, with 6.3 g_{cdw}/L of *E. coli* cells. These productivities are in the range of industrially relevant processes, such as 1,3-propanediol production¹⁵⁴, and demonstrate that AlkB in principle has high enough activity when expressed in *E. coli*. However, in these processes, the oxygenation activity was not sustained for a long time, especially with the shorter substrate methyl nonanoate.

In this thesis, the biocatalyst not only has to terminally oxidize aliphatic substrates. It also has to esterify terminal oxygenates. In the conversions where dicarboxylic acids were the intended products, the esterification activity was much lower than the activity of AlkB (~7U/g_{cdw}). This drastically lowers the productivity, and more efficient alcohol:acyltransferases should be screened or engineered to boost the (ethyl) ester synthesis.

In the conversions where diols were the intended products, esterification did not seem to limit the productivity of the system. Moreover, high activities were sustained over a long period with n-hexane; after 19 h, the AlkB activity

was 70 U/g_{cdw} and the activity of Atf1 62 U/g_{cdw} (for diterminal functionalized products 1 μ mol formed per minute equals 2 U). The proteins are thus quite stable, and in principle it should be possible to reach industrially relevant productivities if mass transfer is not limiting.

6.5.2 Product inhibition

Many of the substrates, intermediates and products involved in this thesis have the potential to be inhibiting at concentrations below the concentrations that are necessary for a feasible process. This inhibition is largely caused by the partitioning behavior of the compounds. Compounds that have a logP_{o/w} in the range of 2-4 can passively diffuse over the membrane of bacteria^{112,155}. The membrane functions are affected in several ways. The proton motive force can be diminished and membrane proteins can be affected¹⁵⁶. It is thus key to keep the aqueous concentration of the inhibitory compounds low. Addition of a carrier solvent can greatly reduce toxicity of compounds within the hazardous logP_{o/w}-range¹⁵⁷. The carrier solvent bis-(2-ethylhexylphthalate (BEHP) was applied in this thesis, and clearly improved product titers. By doing so, explosion hazards are also minimized¹⁵³. This carrier solvent was applied before for production of high concentrations of styrene oxide, by *E. coli* expressing xylene monooxygenase^{158,159}. However, there are many more carrier solvents that are compatible with a whole-cell process based on *E. coli*. Determining the partition coefficient and inhibitory concentrations of the compounds involved could aid in the assessment which solvent should be chosen. Ideally, a carrier solvent is non-inhibitory to the host, and has properties that are different from the product. Those different properties can then be used for efficient downstream processing, after which the relatively pure carrier solvent can be recycled in the next biotransformation.

6.5.3 Oxygen transfer

In a standard stirred tank reactor, an oxygen transfer rate of 90 mmol/L/h should be attainable¹⁶⁰, this corresponds to 1500 U/L in the aqueous phase. As an example, the product 1,6-diacetoxylhexane would then be produced at 18 g/L/h. On liter scale, higher productivities (~1800 U/L) have been reached by *E. coli* expressing styrene monooxygenase for styrene epoxide production¹⁶¹. The maximum specific activity reached by the *E. coli* strain was

56 U/g_{cdw}. The strain equipped with pBTL10-Atf1 reached a monooxygenase activity of 70 U/g_{cdw} for n-hexane oxidation, which is in the same order of magnitude. However, one should bear in mind that production of diacetoxyalkanes and di-esters are multistep processes in which the enzymes are involved in two cycles. Hence, the productivity of the final product will be lower.

Higher biomass concentrations could solve these issues. But at high biomass concentrations, the oxygen demand in the reactor vessel increases. Oxygen is also necessary for host specific processes, such as oxidative phosphorylation. This is necessary to generate ATP for maintenance purposes; an important factor to consider, since maintenance requirements in whole-cell biotransformations are elevated¹⁵². In a growing cell setup, the oxygen demand would be even higher, owing to higher oxidative phosphorylation demands.

6.5.4 Downstream processing

Another important factor to consider is downstream processing (DSP). Free polycarboxylic acids, such as adipic acid and dodecanedioic acid, can be crystallized to facilitate their recovery^{162,163}. This is not easily achieved with di-esters. For di-esters, that mostly accumulate in the organic phase, distillation could be a viable strategy. This has been shown for the recovery of styrene epoxide, dissolved in BEHP¹⁵⁹. BEHP has a high boiling point (460 °C), which would be an advantage in such process, and can be recycled if impurities are sufficiently removed¹⁶¹. Impurities such as alkyl acetates should be relatively easy to recover and can be considered as a valuable byproduct, since they are important flavor/fragrance molecules.

Regarding DSP, the production of di-esters is an advantage. Di-esters accumulate efficiently in the organic phase, whereas free medium-chain dicarboxylic acids and diols remain mostly in the aqueous phase. If distillation would be applied as purification method, there would be no need to distill large amounts of water in the case of di-esters.

6.6. Economic considerations

Commodity chemicals, such as the chemicals discussed in this thesis, are large volume, cheap materials. The costs of commodities is strongly influenced by the cost of the feedstock. For some α,ω -dicarboxylic acids (DCAs), the conventional feedstocks are cheap, cyclic aromatic hydrocarbons. For example, adipic acid (C6) is produced from benzene and dodecanedioic acid (C12) from cyclododecatriene. Other DCAs are produced from fatty acids, such as ricinoleic acid.

Among these DCAs, adipic acid is the cheapest. Its price was ~1500 €/ton in 2012¹⁶⁴. The other dicarboxylic acids are considerably more expensive, due to the more expensive feedstocks and extra process steps. The diols are mostly produced by hydrogenation of dicarboxylic acids, so their prices are higher.

The feedstocks that were applied in this thesis are divided in two categories, renewable feedstocks and petrochemical feedstocks. The fatty acids from chain elongation are brought to the market at prices ≤ 3000 €/ton (Niels van Straalen, director Chaincraft, personal communication, April 20, 2017). Adipic acid production from hexanoic acid is thus not feasible yet, due to the feedstock price. n-Hexane however, is much cheaper (~450-600 €/ton¹⁶⁵). Hence, adipic acid production from n-alkanes might be more feasible until the chain elongation technology is matured and delivers cheaper MCFAs to the market.

An economic evaluation has been made for the production of 1-octanol from n-octane in a whole-cell biotransformation¹⁶⁶. The cost of 1-octanol produced in this theoretical process is around 7 \$/kg. Of this cost, about 70 % was determined by variable costs. Conservative input parameters were used, such as the specific activity that was set at an average of 100 U/L. We have shown prolonged hydroxylation activity of 70 U/L, with 1 g_{cdw}/L with n-hexane as substrate. The reaction rate could be increased with higher biomass concentrations, which would significantly reduce medium costs. A higher specific activity of the biocatalyst would require less nutrient input per gram of product. The authors indicate that with a productivity of 500 U/L at 10 g_{cdw}/L, medium costs could be reduced by 2.5 \$/kg. If such condition can be

realized with the strains presented in this thesis, (di)alcohol production could become cost-competitive. Though, one should bear in mind that downstream processing could be more costly for di-acetoxyalkanes. Furthermore, for diterminal oxidation, two hydroxylation events have to take place per product.

A more recent economic assessment was done for the conversion of styrene to (*S*)-styrene oxide, another monooxygenase based process. The authors reached a productivity of 1800 U/L, and medium costs were 2.9 \$/kg of (*S*)-styrene oxide. This figure could be lower in the diol process, owing to a lower price of n-alkanes and a lower cost of glucose¹⁶¹.

Besides fatty acids or alkanes, cosubstrates glucose and ethanol are necessary. Glucose is needed for biomass production, and the demand for glucose can be reduced if the biomass can be reused. Glucose is also necessary for maintenance requirements of the biocatalyst. The maintenance requirements during biotransformations will likely be higher than under normal conditions, due to the presence of toxic intermediates and products. For the bioconversion only a limited amount of glucose is necessary, since the pathway that was designed in this thesis to generate mono-/di-ethyl DCAs is energy neutral if 100 % coupling efficiency is reached by AlkB. Ethanol is another feedstock, necessary as an alcohol donor for ester production. Ethanol can in principle be recycled if esterified DCAs are hydrolyzed/processed. The applied ethanol concentration was 2.5 %, of which only a fraction was utilized. A more detailed study would be necessary to assess what ethanol concentration results in highest biocatalyst activities. Furthermore, the final concentration of ethanol after the bioconversion process should be as close to 0 as possible, so that no ethanol is lost in the process.

In chapter 5, diacetoxyalkanes are produced from n-alkanes by whole-cells expressing AlkB_{GTL} and Atf1. These compounds can be hydrolysed to give diols and acetic acid. Furthermore, 1,4-diacetoxybutane can be used to produce tetrahydrofuran¹⁶⁷. The biocatalytic process for di-acetoxyalkane production presented in this thesis requires more glucose compared to the di-ethyl ester synthesis. The acetate group is derived from acetyl-CoA,

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produced in *E. coli*'s glycolytic pathway. Two moles of acetic acid are thus bound, that come from the catabolism of glucose. The glucose demand is also higher because no dehydrogenases are involved in this pathway. Acetoxy esters that can be produced with this pathway also have opportunities. Concentrated acetic acid from this process could be considered as a biobased alternative to conventional production of acetic acid. Acetic acid is mostly produced by *via* carbonylation of methanol¹⁰, only a fraction of acetic acid production is microbial¹⁶⁸. Perhaps more interesting is the recycling of acetic acid in the production process of the diacetoxyalkanes. Acetate can be converted to acetyl-CoA by the production host, at the expense of ATP. Acetyl-CoA can then be utilized for the production of acetoxy esters.

The diols that can be obtained from diacetoxyalkane hydrolysis can also be efficiently converted to diamines¹⁶⁹. This could provide greener routes to bulk commodities to another important class of bulk chemicals. An important diamine is 1,6-hexamethylenediamine, with an annual production of about 1.4 million tons/year¹⁷⁰.

Acetoxyalkanes, the precursors for diacetoxyalkanes, accumulated in these conversions as well. All these acetoxyalkanes are interesting products for the chemical industry. In general, acetate esters have a pleasant smell and are applied in the flavor and fragrance industry. They can also be used as solvent, either directly or after hydrolysis to the primary alcohol. Currently, medium chain alcohols are produced by the Ziegler process, starting from ethylene. Also, α -olefins are used as a starting material, in oxo synthesis processes. The biocatalytic route of alcohol production presented here could be more sustainable and cost-effective than current medium-chain alcohol production. For example, for 1 mole of 1-octanol, 4 moles of ethylene (obtained from cracking process) and an oxidant are required. In the biocatalytic process, octane can be used directly as substrate. Furthermore, this process would be highly selective, whereas the Ziegler process results in a wide range of alcohol chain lengths¹⁷¹.

6.6.1 High-value products

The acetate esters that were produced in this thesis were of simple nature. The concept of acetate ester production to protect alcohols from

overoxidation could potentially also be exploited for the production of more complex and/or chiral molecules. AlkB can hydroxylate a wide variety of molecules⁶⁴, such as ethylbenzene. The product phenylethanol contributes to the smell of roses and can be used as a starting material for styrene. Phenethyl acetate is also known for its aroma and production of this molecule by Atf1 has been shown before⁸⁵. This indicates that Atf1 has a broad specificity as well, and that the combination of AlkBGT and Atf1 are interesting candidates for production of specialty chemicals.

6.7. Outlook

This thesis has been the first to combine *in vivo* ester production with ω -oxidation. Combining the two processes enhanced the terminal functionalization. This has opened new routes to production of BMs in a mild way. C4 to C10 chain lengths were tested and all yielded BMs. This indicates the broad applicability of the tested production platforms. The problem of overoxidation has been greatly reduced and efficient diol production with monooxygenases is now possible.

These findings could be a first step to a more sustainable production of α,ω -bifunctional monomers.

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Summary

In **chapter 1**, we give an introduction to bifunctional monomers that play an important role in the chemical industry. Briefly, the conventional production processes of α,ω -dicarboxylic acids and α,ω -diols are discussed. Strategies for more sustainable alternatives for production of medium-chain bifunctional monomers are discussed. Monooxygenase-based processes seem promising, if the problem of poor diterminal oxidation capacities of monooxygenases is solved. Esterification could be a tool to solve this problem.

In **chapter 2** we have investigated the ω -oxidation activities of *E. coli* expressing AlkBGT or AlkBGTL, with various esters having an alkyl chain >1 . These strains were able to ω -oxidize ethyl, propyl and butyl esters of C6-C10 fatty acids. Using esters with a longer alkyl chain enhanced ω -oxidation activities for C6 and C7 fatty acids. The major products were ω -hydroxy fatty acid esters, but over oxidation to the aldehyde and carboxylic acid also occurred. AlkL improved whole-cell ω -oxidation activities for substrates with a $\log P_{o/w}$ above 4.

Since the major products were ω -hydroxy fatty acid esters in chapter 2, we investigated further conversion of these compounds to mono-esterified dicarboxylic acids in **chapter 3**. Alcohol dehydrogenase AlkJ and aldehyde dehydrogenase AlkH were functionally expressed in *E. coli*. AlkJ is functional with 9-hydroxy ethyl nonanoate as substrate, AlkH is functional with 9-oxo methyl nonanoate. Expansion of the AlkBGTL system with AlkJ and AlkH yielded strain *E. coli* AlkBGTHJL. This strain accumulated mono-ethyl azelate exclusively from ethyl nonanoate. Adding the substrate dissolved in a carrier solvent increased final product titers.

Subsequently, we investigated if *in vivo* esterification could enhance the ω -oxidation of AlkB in **chapter 4**. *E. coli* expressing AlkBGTHJL can ω -oxidize octanoate and nonanoate, but not efficiently. When acyl-CoA ligase AlkK and acyltransferase AtfA or Eeb1 were also expressed, ω -oxidation was more efficient. Furthermore, complete oxidation to the carboxylic acid was much

more efficient when also *in vivo* esterification was achieved. Also di-ethyl esters were produced, meaning that esterification occurred twice.

Since ω -oxidation of fatty acids was improved with *in vivo* esterification in chapter 4, we were interested to investigate whether this system could also work with n-alkanes in **chapter 5**. Mono-esters of dicarboxylic acids were produced from n-alkanes by *E. coli* expressing AlkB^{GTHJKL} and either AtfA or Eeb1. Starting from n-alkanes would also allow production of alcohols if overoxidation could be prevented. Application of a different alcohol acyltransferase (Atf1), limited the overoxidation by AlkB. ω -Oxidation of the formed ester resulted in the production of ω -alcohols, which were again esterified by Atf1.

Chapter 6 is the general discussion of this thesis, which evaluates the combination of esterification and terminal oxidation. Suggestions for improvements of the biocatalytic pathway are provided and critical factors for experiments in bioreactors are identified.

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*...Stay with me
Sweet lucky lady
Don't you ever leave me
Honey Bee*

About the author

Youri van Nuland was born on the 17th of April, 1989 in Lelystad, The Netherlands. He obtained his gymnasium diploma in 2007 from the Oelbert gymnasium in Oosterhout. In 2007 he started studying for his bachelor in biotechnology at Wageningen University. He started with his master in cellular and molecular biology in 2010. He did his master thesis at the laboratory of Systems and Synthetic Biology, titled 'Screening for lipoxygenases in fungi and transformation into fungal production strains'. After this thesis he participated in the iGEM competition with the first iGEM team of Wageningen University in 2011, part of a minor thesis. Then he joined the Rumen Microbiology group of AgResearch in Palmerston North (New Zealand), for his internship, titled 'Investigation into the evolution, biochemistry and structural biology of pseudomurein cell wall biosynthesis in thermophilic methanogens'. He obtained his MSc degree in 2012 and started his PhD project in december 2012. Currently, he is working as postdoctoral researcher in the Bioprocess Engineering group of Wageningen University.



List of publications

Hesselman, M. C., Odoni, D. I., Ryback, B. M., de Groot, S., van Heck, R. G. A., Keijsers, J., Kolkman, P., Nieuwenhuijse, D., **van Nuland Y. M.**, Sebus, E., Spee, R., de Vries, H., Wapenaar, M. T., Ingham, C. J., Schroën, K., dos Santos, V. A. P., Spaans, S. K., Hugenholtz, F., van Passel, M. W. J., 2012. A Multi-Platform Flow Device for Microbial (Co-) Cultivation and Microscopic Analysis. *PLoS One* **7**, 1–8.

Ryback, B. M., Odoni, D. I., van Heck, R. G. A., **van Nuland, Y. M.**, Hesselman, M. C., dos Santos, V. A. P., van Passel, M. W. J., Hugenholtz, F., 2013. Design and analysis of a tunable synchronized oscillator. *Journal of Biological Engineering* **7**, 26.

van Nuland, Y. M., Eggink, G. & Weusthuis, R. A., 2016. Application of AlkBGT and AlkL from *Pseudomonas putida* GPo1 for selective alkyl ester ω -oxyfunctionalization in *Escherichia coli*. *Applied Environmental Microbiology*. **82**, 3801–3807.

van Nuland, Y. M., de Vogel, F. A., Eggink, G. & Weusthuis, R. A., 2017. Expansion of the ω -oxidation system AlkBGT of *Pseudomonas putida* GPo1 with AlkJ and AlkH results in exclusive mono-esterified dicarboxylic acid production in *E. coli*. *Microbial Biotechnology*. doi:10.1111/1751-7915.12607

Overview of completed training activities

Discipline specific activities

Annual meetings BE-Basic consortium (Noordwijkerhout, 2013, 2014, 2015, 2017)^{1,2}

BE-BIC workshop (Wageningen, 2013)

Masterclass biobased innovation (Wageningen, 2013)¹

Advanced course microbial physiology and fermentation technology (Delft, 2014)

NBC meeting Biotechnology by Dutch design (Ede, 2014)

KNVM microbial biotechnology meetings (Delft, 2014, 2015)

Eco-Bio conference (Rotterdam, 2016)²

Bio world congress on industrial biotechnology (San Diego, USA, 2016)²

General courses

VLAG PhD week (Baarlo, 2013)

Techniques for writing and presenting a scientific paper (Wageningen, 2014)

VLAG workshop carousel (Wageningen, 2014)

Voice matters – voice and presentation skills training (Wageningen, 2015)

Philosophy and ethics of food science and technology (Wageningen, 2015)

Scientific Writing (Wageningen, 2016)

Optional activities

Research proposal (Wageningen, 2013)

PhD trip to Brazil (2013)¹

PhD trip to Portugal (2014)¹

Metabolic engineering tools meetings (Wageningen, 2015, 2016, 2017)¹

Bioprocess engineering group meetings (Wageningen, 2015, 2016, 2017)¹

¹Oral presentation(s)

²Poster presentation(s)

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