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ADVANCED BIOFUEL PRODUCTION: ENGINEERING METABOLIC PATHWAYS FOR BUTANOL AND PROPANE BIOSYNTHESIS

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Paper IV G. Strijkstra, D. Das, P. Kallio, A. Pásztor, M. Twachtmann, L. Holtmann, E. N. G. Marsh, P. R. Jones and G. T. Hanke: **The contribution of interaction between ferredoxin and aldehyde deformylating oxygenase to the supply of electrons for alkane biosynthesis** (manuscript)

Abstract

Greenhouse gases emitted from energy production and transportation are dramatically changing the climate of Planet Earth. As a consequence, global warming is affecting the living conditions of numerous plant and animal species, including ours. Thus the development of sustainable and renewable liquid fuels is an essential global challenge in order to combat the climate change.

In the past decades many technologies have been developed as alternatives to currently used petroleum fuels, such as bioethanol and biodiesel. However, even with gradually increasing production, the market penetration of these first generation biofuels is still relatively small compared to fossil fuels. Researchers have long ago realized that there is a need for advanced biofuels with improved physical and chemical properties compared to bioethanol and with biomass raw materials not competing with food production. Several target molecules have been identified as potential fuel candidates, such as alkanes, fatty acids, long carbon-chain alcohols and isoprenoids.

The current study focuses on the biosynthesis of butanol and propane as possible biofuels. The scope of this research was to investigate novel heterologous metabolic pathways and to identify bottlenecks for alcohol and alkane generation using *Escherichia coli* as a model host microorganism. The first theme of the work studied the pathways generating butyraldehyde, the common denominator for butanol and propane biosynthesis. Two ways of generating butyraldehyde were described, one via the bacterial fatty acid elongation machinery and the other via partial overexpression of the acetone-butanol-ethanol fermentation pathway found in *Clostridium acetobutylicum*. The second theme of the experimental work studied the reduction of butyraldehyde to butanol catalysed by various bacterial aldehyde-reductase enzymes, whereas the final part of the work investigated the *in vivo* kinetics of the cyanobacterial aldehyde deformylating oxygenase (ADO) for the generation of hydrocarbons.

The results showed that the novel butanol pathway, based on fatty acid biosynthesis consisting of an acyl-ACP thioesterase and a carboxylic acid reductase, is tolerant to oxygen, thus being an efficient alternative to the previous *Clostridial* pathways. It was also shown that butanol can be produced from acetyl-CoA using acetoacetyl CoA synthase (NphT7) or acetyl-CoA acetyltransferase (AtoB) enzymes. The study also demonstrated, for the first time, that bacterial biosynthesis of propane is possible. The efficiency of the system is clearly limited by the poor kinetic properties of the ADO enzyme, and for proper function *in vivo*, the catalytic machinery requires a coupled electron relay system.

Tiivistelmä

Energiatuotannon ja liikenteen aiheuttamat kasvihuonekaasupäästöt muuttavat jatkuvasti maapallon ilmastoa, mikä vaikuttaa kaikkiin maapallolla eläviin lajeihin, myös ihmisiin. Uusiutuvien, ympäristöystävällisten polttoaineiden kehittäminen onkin yksi keskeisimmistä haasteista taistelussa ilmaston lämpenemistä vastaan.

Viimeisten vuosikymmenten aikana on kehitetty useita teknologioita uusiutuvien polttoaineiden valmistamiseksi fossiilisten polttoaineiden rinnalle. Esimerkiksi bioetanolin ja biodieselin osuus käytetyistä polttoaineista on ollut jatkuvassa kasvussa, joskin niiden markkinaosuus on edelleen vain murto-osa verrattuna uusiutumattomiin polttoaineisiin. Tutkijat ovat yhtä mieltä siitä, että muutokseen vaaditaan uusia vaihtoehtoisia biopolttoaineita, joiden fysikaaliset ja kemialliset ominaisuudet ovat nykyisiä paremmat ja joiden valmistamiseen käytettävä biomassa ei kilpaile ruuantuotannon kanssa. Mahdollisia uusiutuvista lähteistä saatavia polttoaineita ovat esimerkiksi alkaanit, rasvahapot, pitkäketjuiset alkoholit ja isoprenoidit.

Väitöskirjatyö keskittyy kahden polttoaineen, butanolin ja propaanin, valmistamiseen mikrobien avulla. Tavoitteena oli rakentaa keinotekoisia biosynteesireittejä polttoaineiden tuottamiseksi *Escherichia coli* –bakteerissa, sekä tutkia systeemien toimintaa ja niiden keskeisiä rajoittavia tekijöitä. Työn ensimmäinen vaihe keskittyi biosynteesireiteille yhteisen välituotteen, butyraldehydin, tuottamiseen. Kahdessa rinnakkain suunnitellussa systeemissä butyraldehydiä valmistettiin joko bakteerin omaa rasvahappobiosynteesikoneistoa apuna käyttäen, tai *Clostridium acetobutylicum* –bakteerin asetoni-butanoli-etanoli –käymistiestä muokatun biosynteesireitin kautta. Työn toisessa osa-alueessa tutkittiin butyraldehydin muuntamista butanoliksi solun sisällä aldehydi reduktaasi –ensyymien avulla. Kolmas osio käsitteli vaihtoehtoisen lopputuotteen, propaanin, biosynteesiä ja viimeistä reaktiota katalysoivan ADO-entsyymin (aldehydia deformyloiva oxygenaasi) toimintaa.

Työ osoitti, että rasvahappoaineenvaihdunnan välituotteisiin perustuva keinotekoinen butanolin tuottosysteemi ei ole herkkä hapelle ja tarjoaa näin ollen vaihtoehdon anaerobisille *Clostridium* –biosynteesireiteille. Lisäksi tulokset osoittivat, että butanolia voidaan tuottaa *E. coli* –bakteerissa myös asetyyli-CoA –lähtöaineesta, käyttäen hyväksi asetoasyyli CoA syntaasi - (NphT7) ja asetyyli CoA asetyylitrasferaasi (AtoB) –entsyymeitä. Työn keskeisimmät tulokset liittyivät propaanin biosynteesiin: Tutkimus todisti ensimmäistä kertaa, että propaanin tuottaminen bakteerisoluissa on mahdollista. Keskeisiä tuottotehoa rajoittavia tekijöitä olivat ADO-entsyymin kineettiset ominaisuudet, sekä entsyymin toiminnalle välttämättömän pelkistyssysteemin toiminta.

Abbreviations

ABE	acetone-butanol-ethanol fermentation
ACP	acyl carrier protein
ADH	alcohol dehydrogenase enzyme
ADO	aldehyde deformylating oxygenase enzyme
CoA	coenzyme A
DNA	deoxyribonucleic acid
FAME	fatty acid methyl ester
FAS II	fatty acid synthase complex type II
Fdx	ferredoxin enzyme
Fpr	ferredoxin-oxidoreductase enzyme
FFA	free fatty acid
GC	gas chromatography
GMO	genetically modified organism
IPCC	Intergovernmental Panel on Climate Change
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	lysogeny broth
LCA	life cycle assessment
LPG	liquefied petroleum gas
MS	mass spectrometry
OD	optical density
PHB	polyhydroxybutyrate
TB	terrific broth
TE	thioesterase enzyme

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1. Background

1.1. Renewable biofuels today

1.1.1. Developing a sustainable fuel economy

With its growing demand for energy, mankind has changed the living conditions on Earth. By combusting fossil fuels, the global atmospheric carbon-dioxide levels continue to rise and consequently our planet's climate started to react more and more severely. The waste product of fossil energy production, carbon dioxide, contributes the most to the greenhouse effect and global warming, as it is generated in such high quantities. Although other gases, like methane (CH_4), nitrous oxide (N_2O) and fluorinated gases have much higher radiative efficiency – implying they are more effective in reflecting solar heat back to the surface (Figure 1), they are present in significantly lower concentrations, compared to carbon dioxide.

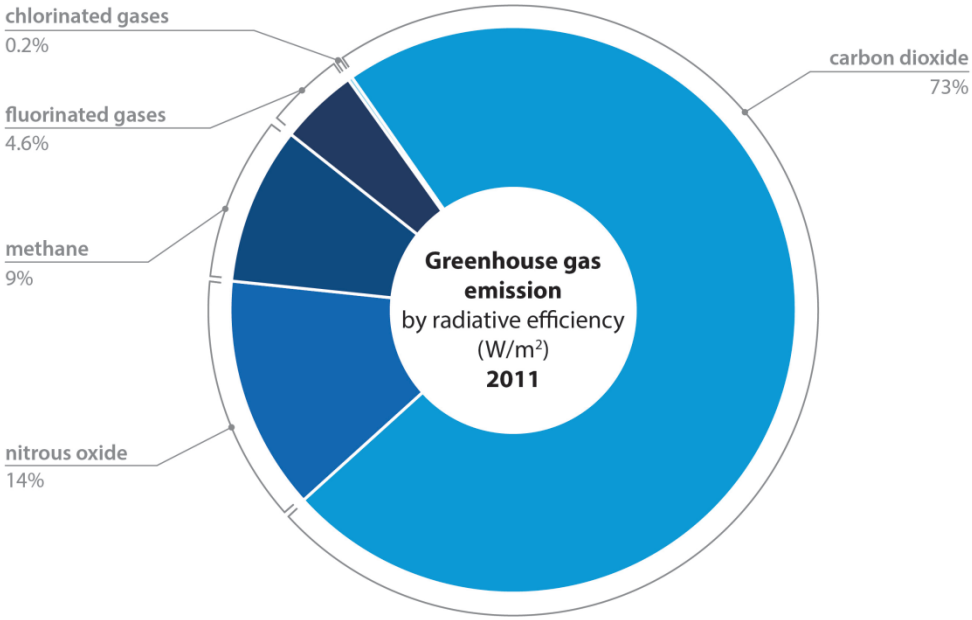

Figure 1 – Proportion of greenhouse gases produced by human activity in 2011, published by the Intergovernmental Panel on Climate Change (IPCC). The well-mixed, global atmospheric concentrations of greenhouses gases were normalized according to their radiative efficiency. The radiative efficiency is used in climate science to compare greenhouse gases based on their ability to absorb infrared radiation. The unit of W per m^2 surface has been used in this figure and the given percentage values represent data from four different measurement networks. Figure representing raw data from IPCC report (Hartmann et al., 2013).

Combustion of fossil fuels, including petroleum oil, coal and natural gas is the main source of atmospheric carbon dioxide emission (Hartmann et al., 2013). Fertilizers are the primary source of the second most effective greenhouse gas, nitrous oxide. Methane arises from shale gas production, agriculture and lately from thawing

permafrost lands near the Arctic Circle. Fluorinated and chlorinated gases are emitted from other industrial sources. Due to the emission of these greenhouse gases, the increase of average surface temperature, melting polar icecaps, rising sea levels, erosion of marine coastlines and the increased frequency of severe weather conditions have been reported. Climate change is negatively influencing the living environment of many species all around the globe (Kerr, 2007).

Transportation industry is one of the main contributors of carbon dioxide emission as the major consumer of liquid petroleum fuels, mostly oil (G.J. Olivier et al., 2013). The transportation sector had a 28% share of the total CO_2 emission in the United States in 2012, which was the second largest share after electricity generation (EPA, 2012). At the same time global oil prices are expected to rise in the upcoming decades due to decreasing reserves and increasing demand as well as more expensive mining and drilling methods (BP, 2014). Developed countries like the United States are dependent on oil imports for gasoline production (EIA, 2015). Thus the long-term rise of petroleum fuel prices and the environmental costs of air pollution are driving governments to invest in renewable fuels development (Conti et al., 2013). However due to policy uncertainties the rate of investment has decreased by 14% worldwide in 2013 compared to the previous year (McCrone et al., 2014). If advanced biofuels can be made more wide spread, they could supplement or completely replace fossil fuels without the need to compromise with energy content, physical parameters or even food supply.

In contrast to petroleum fuels, biofuels are *renewable* as they are synthetized from natural resources, which are reproduced within a short period of time. These resources are typically plant, bacterial or other biomass consisting of high energy molecules, which could be utilized as liquid fuels or raw materials of fuels (Peralta-Yahya et al., 2012). Compared to fossil energy sources, biofuels are carbon-neutral or have extremely low CO_2 output, thus not increasing the global atmospheric carbon dioxide levels when combusted (Zeman and Keith, 2008). However in order to evaluate the sustainability of a certain type of biofuel technology as a whole, it is important to make a manufacturing lifecycle assessment (LCA). LCA is risk management technique, which is used to evaluate the environmental impact of a given technology by calculating all the relevant material and energy inputs and emissions (EPA, 2006).

1.1.2. Current status of available biofuels

Renewable electric energy production is developing rapidly; available technologies can harness the power of wind, sunlight, water, geological energy and even tidal movements and renewables now account for 5.3% of the global electrical power output (BP, 2014). However, fossil fuels are used also for heating, transportation and as raw materials for the petrochemical industry, not only for electricity generation. Thus electricity alone cannot complement for the need of fuels in our current society. However, this might change in the future, as electrical vehicles charged by renewable

solar power are becoming more prominent, led by companies like Tesla Motors, Inc. Until the phasing out of the more than 1 billion petroleum propelled motor vehicles currently in use, fossil liquid fuels dedicated for transportation need to be replaced with biofuels (OICA, 2013; WardsAuto, 2014).

Currently the share of biofuels is still minor nonetheless; in 2013 the total annual production was 65.3 million tons (oil equivalent), while the crude oil production accounted for 4132.9 million tons within the same period (BP, 2014). Fossil fuels, including crude oil, coal and natural gas accounted for 86.7% of global energy consumption in 2013 (Figure 2). Moreover the global primary energy consumption is increasing by 2.5% every year, and it is predicted to increase by 56% until 2040, with oil as the leading type of fuel (Conti et al., 2013).

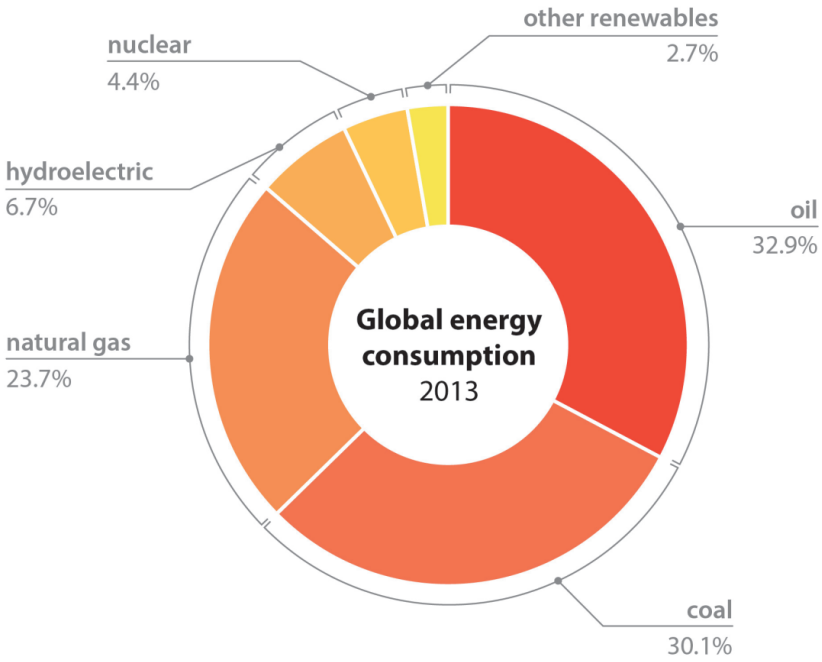

Figure 2 – Global energy consumption as a proportion of energy sources in 2013. The data includes all types of consumption, as well as power generation and transportation. In 2013 oil remained the primary source of global energy, while coal was the most rapidly growing fossil fuel with an annual 3.0% increase in consumption. Renewables contributed more to electrical power generation (5.3% global share) than to transportation, with wind energy generation growing the fastest by 21% (BP, 2014).

In the last 15 years crude oil prices started to increase sharply, while the global production is barely keeping the pace with the consumption. However, in the second half of 2014 oil prices started to fall to a 5-year low, due to temporal overproduction by Organization of the Petroleum Exporting Countries (OPEC) despite the global glut. The latest reserves-to-production ratios estimate 50 years for crude oil and about 100 years for coal, although these numbers are continuously re-evaluated, it is clear that the fossil fuel supply is finite (BP, 2014).

Biofuels on the other hand are independent from finite reserves, drilling and mining expenses and refining costs and even political insecurities of oil exporting countries. In 2013 the total renewable energy consumption accounted for 2.7% of global usage (BP, 2014). The largest producer was the North American region with almost 50% share (29.5 million tons, oil equivalent) of global biofuel generation (BP, 2014). Bioethanol is leading the global biofuel market, followed by biodiesel, with an opposite trend in Europe (Figure 3) (BP, 2014).

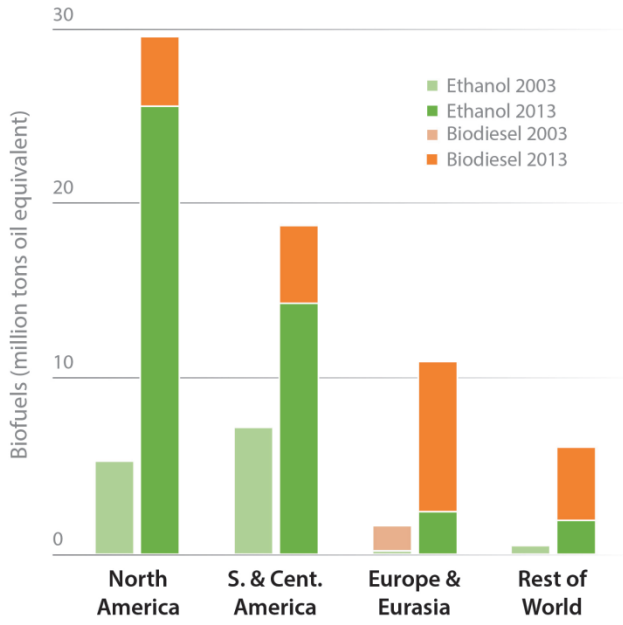

Figure 3 – Global liquid biofuel production by region comparing a 10 year period in million tons oil equivalent. Adapted from BP Statistical Report for World Energy 2014 dataset.

Despite of the increasing production (Figure 3), biofuels are still underrepresented compared to petroleum products and many biofuel technologies are still under development, thus the industry needs political incentives. According to ePURE, a European organization representing ethanol producers, biofuels could help the EU reduce its transport carbon footprint by 34 million tons per year (ePURE, 2015). However at the moment political uncertainties on Indirect Land Use Change (ILUC) are negatively influencing biofuel producers in Europe (Wrigh, 2015).

1.1.3. First generation renewable fuels

Based on the applied raw materials, renewables can be categorized as first generation and advanced biofuels. Advanced biofuels could be subdivided further to second, third and even fourth generation biofuels, based on feedstock utilization and/or chemical properties (Peralta-Yahya et al., 2012). First generation biofuels, including bioethanol and biodiesel, are probably the most well-known group of renewable

fuels, utilizing multiple applicable feedstocks. What the first generation biofuels share in common is that they are produced from plant biomass containing high concentrations of easily accessible carbohydrates or plant derived oils (Figure 4 – light grey block) (Peralta-Yahya et al., 2012; Wen et al., 2013). While serving as efficient substrates for biofuels, the main disadvantage of these raw materials is that they are competing with food and animal feed production.

The most widely used biofuel, ethanol, belongs to first generation fuels. Bioethanol is being produced from sugar cane (sucrose) in Brazil or from corn (starch) in the United States (Naik et al., 2010). The extracted and pre-treated carbohydrates are enzymatically converted to glucose and then used as a carbon source in bakers' yeast (*Saccharomyces cerevisiae*) fermentation to produce about 10% ethanol, which is thereafter separated from the fermentation broth, concentrated, purified and finally blended with gasoline in various proportions (Pimentel and Patzek, 2005). Compared to gasoline, the final product has considerably lower energy density per unit of volume (19.6 vs. 32 MJ/l), higher volatility and is miscible with water, which could lead to corrosion in traditional Otto-engines (Tornatore et al., 2011). Due to these poor physicochemical properties, ethanol can only be used as a pure fuel in specifically modified, flex-fuel combustion engines (Peralta-Yahya et al., 2012).

The second largest, but still first generation biofuel product is biodiesel. Unlike bioethanol, biodiesel does not have a defined chemical composition; it is a mixture of different carbon chain length alkyl esters. Biodiesel can be produced from a wide variety of vegetable oils, animal tallow or microbial lipids (Figure 4) (Fortman et al., 2008; Lu et al., 2008). In Europe mostly rapeseed oil, sunflower oil and used cooking oil is utilized, whereas in the United States the main source is soybean oil and peanut oil. In Eastern-Asia palm oil and coconut oil is harvested for their high crop yields for biodiesel production (Bharathiraja et al., 2014).

Traditional biodiesel consist of fatty acid methyl esters (FAME), not hydrocarbons, as a consequence it has slightly different physical and chemical properties compared to petroleum diesel (Naik et al., 2010). These include higher viscosity, low performance in cold climates and risk of engine corrosion due to water accumulation. Furthermore because biodiesel is mostly synthetized from food crops, it shares the disadvantage of ethanol, and many researchers argue that due to its production food prices are rising in developing countries, where farmers have switched to grow more profitable oil crops.

Currently there are two available technologies to produce alkyl esters (the main component of traditional biodiesel) from oils containing triacylglycerols: transesterification and pyrolysis. Transesterification is extensively studied and applied, whereas pyrolysis is more costly than the former and can yield undesirable by-products (Bharathiraja et al., 2014). Transesterification is the treatment of vegetable or other oils with alcohols in the presence of a catalyst to form FAME

(biodiesel) and glycerol as a by-product. The alcohol serves as an acyl acceptor, and it could be ethanol or methanol; however the latter is preferred in the industry for its cost effectiveness and broad availability (Bharathiraja et al., 2014; Kirrolia et al., 2013). The transesterification process could be chemically or enzymatically catalysed, with acid or alkali or by lipase enzymes respectively. Lipase has the advantage of reusability without toxic waste product accumulation.

1.1.4. Producing advanced biofuels from non-food biomass

Advanced biofuels, like first generation biofuels, are produced from various types of plant, animal or microbial biomass. The main difference in comparison to first generation biofuels is that advanced biofuels are made from non-food resources (Figure 4), like lignocellulose or other agricultural waste streams (Peralta-Yahya et al., 2012). However, there is a partial overlap between first generation and advanced biofuels, as ethanol and biodiesel can be produced from different feedstocks. Moreover, the category of advanced biofuels could also include other sustainable fuel types, like algae oil (Jones and Mayfield, 2012) or biohydrogen (Meher Kotay and Das, 2008); the latter is not yet available commercially.

Plant based, non-food biomass has a great potential for biofuel production. Typically 75% of plants dry matter consists of di- and polysaccharides, including sucrose, starch, cellulose and hemicellulose (Naik et al., 2010). This rich sugar content makes it possible to obtain advanced biofuels from lignocellulosic biomass by enzymatic hydrolysis and fermentation. The fermentation can produce both ethanol and other fuels with more complex chemical structures, e.g. farnesene (Figure 4) (George et al., 2015). Cellulosic ethanol production is discussed in Chapter 1.1.5. in more detail. Apart from plant-based polysaccharides, the whole plant biomass can be utilized for biofuel production by chemical synthesis, using Fischer–Tropsch gasification method (Kallio et al., 2014; Steele et al., 2012) or pyrolysis.

Furthermore non-edible oils from waste animal or plant resources can also be converted to diesel type fuels with hydrogenation with similar methods to petroleum oil refining (Neste, 2015). Tall oil, the by-product of chemical pulping of wood materials, can also be converted to renewable biodiesel using hydrotreatment and fractionating (UPM, 2015). Companies like Neste Oil (Finland) and UPM (Finland) are producing these advanced diesel fuels at commercial levels.

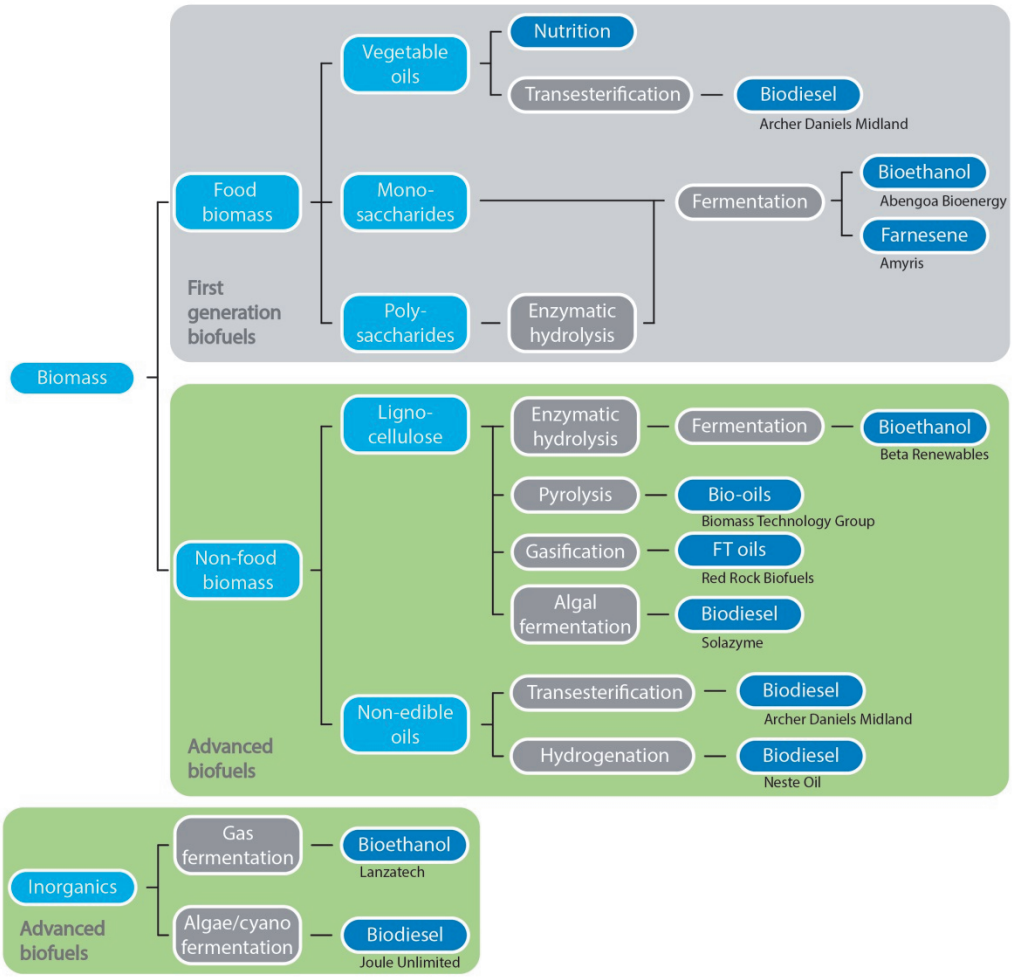

Figure 4 – Block representation of commercially available biofuel technologies to-date. The different biofuel technologies have been categorised by the available raw materials (light blue blocks), which are converted with a specified technology (dark grey block) to an end-product (dark blue). For each technology a producing company was given as an example (note that these corporations are not exclusive producers, furthermore some companies can use multiple feed stocks). First generation biofuel production technologies are typically utilising food biomass (light grey background); advanced biofuels are produced from inorganics or non-food biomass (green background). The figure is partially based on the work of Naik and co-workers (Naik et al., 2010) and press research on Biofuels Digest (Lane, 2015).

To overcome the technical limitations of ethanol as a fuel molecule and also to broaden the available raw materials for biofuel production, extensive research has been conducted in the field of advanced biofuels in the recent years. The development has been focused on longer-chain alcohols (Atsumi et al., 2008b; Jones and Woods, 1986; Lan and Liao, 2012; Shen et al., 2011), fatty acid based molecules (Blatti et al., 2012; Lu et al., 2008), isoprenoids (George et al., 2015) and alkanes (Rabinovitch-Deere et al., 2013). For more details see also Chapter 1.1.5. and Table 1.

1.1.5. Cells as biofuel factories

Microorganisms have been used for producing fermented food and drinks for human consumption for thousands of years. Nowadays, microbes are utilized for the production of a wide array of complex drug molecules currently in therapeutic use including antibiotics, cancer drugs and immunosuppressants (Chen and Nielsen, 2013; Nielsen et al., 2014). However this chapter only focuses on microbial hosts synthesizing potential biofuels and their precursors.

Microbes are also an integral part of modern biofuel production technologies as the primary catalysts in bioethanol fermentation (Agarwal, 2007; Pimentel and Patzek, 2005). In all cases the basic concept is that cells provided with suitable raw materials are engineered to produce the selected target metabolites or their precursors as part of the native metabolism. There are various different biosynthetic pathways and their synthetic variants that have been utilized for biofuel production purposes and in most of these (e.g. ethanol, biodiesel, terpenoids, butanol and propane) the common initial chemical building blocks are acetyl-CoA or pyruvate (Fischer et al., 2008; Rabinovitch-Deere et al., 2013).

Owing to relatively high growth rates, the depth of genetic and microbiological knowledge and available metabolic engineering tools, bacteria and yeast are used as cell factories for biofuel production (Alper and Stephanopoulos, 2009; Fischer et al., 2008). *Escherichia coli* (hereafter *E. coli*), a Gram-negative enterobacterium and *Saccharomyces cerevisiae*, the budding yeast are well-studied model organisms and have been used for biofuel synthesis from the early times (Clomburg and Gonzalez, 2010). Various metabolic pathways, including fermentation and respiration, have been developed for the production of different biofuels using these hosts (Atsumi et al., 2008b; Cho et al., 2015; Steen et al., 2008). The host, expressing such pathways, mostly utilize monosaccharides as raw materials. As a result of recent development in the field of photosynthesis, cyanobacteria and microalgae are being recognised as host organisms for biofuel generation (Savakis and Hellingwerf, 2015). Cyanobacteria are capable of utilizing CO_2 and light for growth and the biosynthesis of certain useful products (Machado and Atsumi, 2012). The utilization of inorganic carbon by photosynthesis would have a significant benefit over sugar-based carbon sources as being easily accessible and cheap (for more details on photosynthetic hosts see Chapter 1.1.6.).

One of the most prevalent biofuels, ethanol is produced from pyruvate (derived from glucose) via acetaldehyde, through decarboxylation and a consecutive reduction step. Traditionally the unicellular yeast, *Saccharomyces cerevisiae* is being used for ethanol fermentation (Fortman et al., 2008), however lately the Gram-negative bacteria, *Zymomonas mobilis* (Wirawan et al., 2012) and some recombinant *E. coli* strains have gained attention for their relatively high ethanol yields (Fischer et al., 2008). In the United States over 200 first generation bioethanol plants are using sugar based

technology, including producers, like Cargill, Inc. and Abengoa Bioenergy Co. (BBI, 2014).

Microbes, like filamentous fungi, can also be used indirectly for biofuel production. Species such as *Trichoderma reesei* and *Myceliopthora thermophila* are used for cellulose enzyme production (Ferreira et al., 2014; Singh, 2014). The breakdown of cellulose, derived from waste plant parts (straw, stems and leaves), other non-food plant biomass (wood chips, pulp and saw dust) or even non-food crops dedicated for ethanol production (switch grass, *Jatropha* and other kinds of grass), require various mechanical and chemical pre-treatment processes and an array of fungal enzymes. These include endocellulases, exocellulases, β-glucosidases, cellobiose dehydrogenase and polysaccharide monooxygenase (GH61) (Xu et al., 2009). The cellulase enzyme mixtures produced by these fungi species are currently commercially available from biotechnology companies like Novozymes, DSM and Dupont-Genencor (Lane, 2015).

After the hydrolysis of cellulose the C5 sugars (xylose, arabinose etc.) and glucose end-products can be used as a substrate for ethanol production. The cellulosic ethanol technology has recently passed commercialization by several companies, like Dong Energy (Inbicon A/S, Denmark), Beta Renewables (Crescentino, Italy), Dupont (Nevada, US), Abengoa (Hugoton, Kansas, US) and POET-DSM (Project Liberty, Iowa, US) (Lane, 2015).

Advanced biofuels with superior physicochemical properties to ethanol require novel metabolic pathways, extensive research in the genetic background of host microorganisms, molecular biology tools, optimized growth conditions, media utilization, end-product collection/separation and cultivation processes. Due to these complex obstacles only a handful of technologies have reached beyond laboratory scale production rates. For example outstanding progress has been made in increasing the titres of short chain alcohols (1-butanol, 2-propanol, isobutanol) and farnesene (a type of terpenoid), as described in Table 1. However some other potential biofuels, such as microbial free fatty acids and medium chain alcohols still remain in the early proof-of-concept stage. Based on the titres of farnesene, a diesel substitute developed by Amyris (Table 1), a viable biofuel production process would require titres above 100 g/l with substrate conversion yields at approximately 20% and cost-effective downstream processing techniques. Thus numerous technologies with lower titres still need many years of development.

Table 1 – Comparison of the highest reported advanced biofuel titres in various host organisms. The comparison is purely based on maximal product concentration reached in a biological process, not taking production time, rate or yield into account as such data was not available in most cases. The data represented in this table is partially based on (Rabinovitch-Deere et al., 2013) and (Lane, 2015).

Biofuel	Species	Titre [g/l]	Comments	Reference
2-propanol	*E. coli*	143		(Inokuma et al., 2010)
1-butanol	*C. acetobutylicum* JB200	113.3	fed-batch ABE	(Xue et al., 2012)
farnesene	*S. cerevisiae*	104.3	Amyris Inc.	(Amyris, 2010)
isobutanol	*E. coli*	50		(Baez et al., 2011)
isoprene	*Y. lipolytica*	40	Danisco Inc.	(Calabria et al., 2012)
1-butanol	*E. coli*	30		(Shen et al., 2011)
3-methyl-1-butanol	*E. coli*	9.5		(Connor et al., 2010)
free fatty acids	*E. coli*	7		(Dellomonaco et al., 2011)
2-methyl-1-butanol	*E. coli*	1.25		(Cann and Liao, 2008)
farnesene	*E. coli*	1.1		(Zhu et al., 2014)
bisabolane	*S. cerevisiae*	0.994		(Peralta-Yahya et al., 2012)
limonene	*E. coli*	0.4		(Alonso-Gutierrez et al., 2013)
1-hexanol	*E. coli*	0.21		(Dellomonaco et al., 2011)
1-octanol	*E. coli*	0.1		(Dellomonaco et al., 2011)
limonene	*S. sp.* PCC 7002	0.004		(Davies et al., 2014)

As described in Table 1, the highest biofuel titres have been achieved by non-phototrophic bacteria or yeast. However, carbohydrate utilization via heterotrophic metabolism could be also a major drawback for sustainability. A large portion of the sugars utilized by heterotrophic host organisms are originated from non-sustainable, potential food resources, mostly plant biomass originated from corn and sugarcane. Thus, by replacing the heterotrophic metabolism with a host that is capable of utilizing solar energy and inorganic carbon sources, especially carbon dioxide, would make the biofuel production commercially more feasible and competitive and also provide a solution to avoid increasing greenhouse gas concentrations.

1.1.6. Photosynthetic hosts, a sustainable concept for biofuel production

On the surface of Earth approx. 200W/m^2 solar energy is available throughout the year, which of 29.2% can be harvested by photosynthesis, accounting for 1.85x10^6 KJ/m^2/year (Lan and Liao, 2011). This is a significant renewable and sustainable energy source, which could be used not only for food production, but also for the synthesis of biofuels. It has been estimated that with a theoretical maximum photosynthetic efficiency (not accounting for cell growth and maintenance), 16 kg of butanol fuel could be produced per m^2 per year by engineered photosynthetic organisms (Lan and Liao, 2011).

In comparison, heterotrophic bacteria use organic carbon sources derived from plant photosynthesis, which increases the overall number of the metabolic steps to generate the fuel molecules. In addition the solar energy conversion efficiency is much higher for cyanobacteria than for higher plants (3-9% vs. 0.25%), e.g. corn, a major source for bioethanol (Ducat et al., 2012; Pimentel and Patzek, 2005). From this perspective, photosynthetic microorganisms have a great biotechnological potential for delivering bio-products and biofuels (Machado and Atsumi, 2012).

The research on photoautotrophic organisms has received increased attention over the past years. Molecular genetic tools including genome sequences are available for the modification of cyanobacteria and microalgae. This has led to the production of a large variety of compounds, including ethanol, lactic acid and 2,3-butanediol in lab-scale (Savakis and Hellingwerf, 2015). Although the numbers of engineered, synthetic pathways in cyanobacteria are increasing rapidly, many challenges are needed to be solved in order to develop successful host organisms. For example if a compound is synthesized intracellularly, it needs to be excreted from the cytoplasm during cultivation; in cyanobacteria still little is known of these transportation processes (Niederholtmeyer et al., 2010). Many biofuel producing pathways are using anaerobic fermentative metabolism to generate reduced compounds. In order to overcome the potential incompatibility of oxygen sensitive enzymes with the process of oxygenic photosynthesis, metabolic pathway engineering (Lan et al., 2013) or spatial compartmentalization of the heterologous enzymes can be used (Agapakis et al., 2012). In cyanobacteria the decarboxylation reactions can be manipulated to improve driving forces towards the compound of interest, for example by overexpressing 2-acetolactate decarboxylase (*alsD*) for 2,3-butanediol synthesis (Oliver et al., 2013). In addition end-product toxicity at higher titres can be detrimental to growth, thus a number of studies have investigated the tolerance to potential biofuel compounds (Anfelt et al., 2013; Dienst et al., 2014). In addition more development is required for large-scale cultivation processes in photobioreactors, to solve issues like open-pond contamination, CO_2 distribution and evaporation. Despite the on-going fundamental research, there are only a few companies developing pilot-scale photoautotrophic production systems (e.g. Joule Unlimited Inc., US and Cellana Inc., US) and currently no corporations are at commercial scale.

1.2. Basic research towards advanced biofuels

1.2.1. Need for advanced biofuels

As discussed in Chapter 1.1. there is a need to overcome the limitations of first generation biofuels. Currently bioethanol and biodiesel accounts approximately 90% of the biofuel market (Agarwal, 2007; Rabinovitch-Deere et al., 2013), with the worldwide share of 57% of bioethanol and 43% of biodiesel (BP, 2014). At present the main feed stock for bioethanol and biodiesel production is food biomass (Figure 4) (Agarwal, 2007; Liang et al., 2012). As an example, already in 2006 bioethanol production in the United States consumed 11% of the corn harvest alone (Agarwal,

2007), thus it is not sustainable in the long term. Furthermore due to its less than optimal chemical and physical properties (Chapter 1.1.3.) ethanol is mostly used as a fuel additive to gasoline in vehicles equipped with regular Otto-engines (G.J. Olivier et al., 2013).

Currently extensive fundamental and applied research is being carried out to introduce competitive alternatives to bioethanol (Cho et al., 2015; George et al., 2015; Rabinovitch-Deere et al., 2013). In addition to lignocellulose based bioethanol (see Chapter 1.1.5.) already a few alternatives of advanced biofuels are available (Table 1) at demonstration and commercial scale (Lane, 2015), including biobutanol (Butamax Joint Venture, US and Gevo Inc., US), algae oil (Solazyme, US) and farnesene (Amyris, US). To increase the share of advanced biofuels on the market, more governmental support and collaboration between academia and biotech industry is needed.

1.2.2. Early stage, non-photosynthetic biofuel research

Besides lignocellulose-based bioethanol many other advanced biofuels are currently being developed and evaluated by academia and industry. These include medium and long chain alcohols (e.g. butanol, hexanol), fatty acids and their derivatives (fatty acid alkyl esters), alkanes (including propane) and alkenes and isoprenoid based biofuels (Fortman et al., 2008; Rabinovitch-Deere et al., 2013) from non-food plant resources.

n-butanol, a key metabolite under focus in this Thesis, is one of the first products of modern day biotechnology; it has been commercially produced by the solventogenic bacteria *Clostridium acetobutylicum* in the acetone-butanol-ethanol (ABE) fermentation up until the and 1960's (Jones and Woods, 1986). Recently the need for renewable chemicals and fuels has resurrected the interest towards butanol research. Butanol offers improved physical characteristics for internal combustion engines, with an energy density (29.2 MJ/litre) comparable to gasoline, lower volatility than ethanol and low hygroscopicity (Tornatore et al., 2011). However, n-butanol has not so far reached commercial scale production as a renewable replacement of gasoline. Scientists have managed to get a good understanding of the ABE fermentation and with the help of metabolic engineering, different *Clostridia* and *E. coli* species have been engineered for n-butanol production (Fischer et al., 2008). Butanol has been produced via the coenzyme A (CoA) dependent pathway, the keto acid pathway or the acyl carrier protein (ACP) pathway. The CoA-dependent pathway from *Clostridium acetobutylicum* was expressed in *E. coli* to overcome the slow growth, the difficult cultivation characteristics and the mixed product formation of *Clostridia*. After several rounds of strain engineering and the deletion of competing fermentation pathways of the host, Shen and co-workers achieved 30 g/l butanol production in bioreactors (Shen et al., 2011). The ACP-pathway is utilized by genetically engineered *E. coli*, described in **Paper I**.

Another advanced biofuel candidate discussed in this Thesis is propane, the bulk component of liquefied petroleum gas (LPG). It is a widely used hydrocarbon fuel in automobiles, forklifts, gas burners and heaters. Although propane is not directly compatible with traditional Otto engines, many manufacturers are producing LPG-only or bi-fuel engines and Otto engines can be retro-fitted with third-party accessories for LPG utilization. Propane is currently produced as a by-product of cracking in petroleum refining or natural gas production, thus it is not a renewable energy source nowadays. Propane could be also generated from biomass containing triacylglycerols, as a by-product of NExBTL advanced biodiesel manufacturing, and soon it will be commercially available (Neste, 2015). Prior to this study, propane has not been generated by any microbial system; a novel method for the biosynthesis of renewable propane from glucose by the utilization of fatty acid biosynthesis has been described in **Paper II**.

1.2.3. Metabolic engineering for photosynthetic biofuel production

In the field of cyanobacteria research most of the metabolic engineering has been conducted with *Synechocystis sp.* PCC 6803 (hereafter *Synechocystis*) or *Synechococcus elongatus sp.* PCC 7492 (Savakis and Hellingwerf, 2015). Scientists have been able to introduce new pathways and use existing molecular genetics knowledge to produce compounds for fuel applications. The list of potential fuel products already include ethylene (Ungerer et al., 2012), ethanol (Gao et al., 2012), butanol (Lan and Liao, 2012), lactate (Angermayr et al., 2014), free fatty acids (Kaiser et al., 2013), fatty acid derived alcohols (Yao et al., 2014) and terpenoids (Lindberg et al., 2010). The highest reported ethanol production so far in cyanobacteria was 5.5 g/l in 26 days that is still far below the capabilities of *Saccharomyces cerevisiae* (Savakis and Hellingwerf, 2015).

In order to produce fuel compounds in cyanobacteria the photon conversion efficiency needs to be improved, which would allow more effective utilization of sunlight (Badger and Price, 2003; Ducat et al., 2012). Furthermore pathways are needed to be optimized for host metabolism for increased flux. An example for this approach is the modification of heterologous biofuel pathways from *Clostridia*, by excluding oxygen sensitive bioconversion and introducing oxygen-tolerant enzymes (Lan et al., 2013). Alternatively the separation of oxygen sensitive biological processes could take place also spatially, by targeting nitrogen-fixing heterocysts (Ihara et al., 2013; Savakis and Hellingwerf, 2015).

Metabolic engineering tools are required not only to introduce new target pathways in cyanobacteria, but to radically modify host metabolism. For example by inactivating glycogen and polyhydroxybutyrate (PHB) storage metabolisms in cyanobacteria (Panda et al., 2006), the excess carbon could be directed towards product formation. It has been shown that the inactivation of PHB accumulation increases the yields of 3-hydroxybutyrate, heptadecane and heptadecene (Savakis and Hellingwerf, 2015; Wang et al., 2013), while the inactivation of glycogen was successful under nitrogen

depleted conditions (Li et al., 2014). In cyanobacteria the modification of decarboxylation and phosphoester bond cleavage reactions can lead to the diversion of driving forces and improved product formation (Savakis and Hellingwerf, 2015). As an example for modified decarboxylation, for the production 2,3-butanediol an oxygen insensitive pathway has been designed, coupled with irreversible enzymatic steps (Oliver et al., 2013). In another study the overexpression of pyruvate decarboxylase and alcohol dehydrogenase resulted in enhanced ethanol production in *Synechococcus elongatus* PCC7942 (Deng and Coleman, 1999). Carbon flux was directed for D-mannitol production in *Synechococcus sp.* PCC 7002 using mannitol-1-phosphate dehydrogenase and mannitol-1-phosphatase (Jacobsen and Frigaard, 2014). It has been suggested that CO_2 fixing efficiency can be increased, by modifying the Calvin-Benson cycle in *Synechococcus elongatus* PCC7942 (Atsumi et al., 2009).

More genome sequences become available and the variety of genetic tools are increasing, tightly regulated expression systems and new strain selection systems are researched; these improvements lead to a better understanding of cyanobacteria as biofuel production hosts. Several challenges however remain open. These include reaching the industrial scale production, integration of large-scale photobioreactors with downstream processing functions and also the genetic instability of cyanobacteria should be carefully addressed (also see Chapter 1.1.6. for more details).

1.3. Industrial host organisms for biofuel production

Selecting a suitable host organism is one of the first steps towards developing advanced biofuels (Alper and Stephanopoulos, 2009). There are two different models of industrial host construction: (1) choosing native organisms and consortia or (2) selecting a recombinant, well established host. Both solutions have their benefits and disadvantages.

The native host can possess a complete pathway for the production of a target molecule; it usually has a better ability to degrade substrates, like carbohydrates thus resisting substrate (e.g. carbon catabolite repression) and product inhibition and tolerates high concentrations of toxic end products. On the other hand, molecular biology tools are underdeveloped for newly isolated, native strains and they also usually need a highly specific (non-industrial) cultivation environment (Alper and Stephanopoulos, 2009). In contrast, the recombinant industrial strains have a long track record for produced molecules and well-established genetic tools. Strains like *E. coli* and *S. cerevisiae* can be engineered for improved metabolism using the approach of global transcription machinery engineering.

Many natural catabolic activities occur simultaneously and involve large bacterial consortia, consisting of numerous co-existing strains instead of isolated single organisms, which degrade complex polysaccharides (from lignocellulose) and other substrates via highly specialized metabolic routes. If multiple parallel metabolic

pathways are required for complex biomass conversion, the application of microbial consortia could be economical; although genetic manipulation and optimization of a consortia could require great scientific effort (Alper and Stephanopoulos, 2009).

With the powerful tools of metabolic engineering and synthetic biology, heterologous pathways can be created, using genes from native microorganisms. However, legal and ethical regulations related to the use of transgenic genetically modified organisms (GMO) may restrict or direct the use of heterologous expression systems even in the field of biofuel research, which will clearly be a challenge for future policy makers.

1.4. From basic research towards applications: Long term goals in biofuel production

Universities and state research institutes perform dedicated multi-disciplinary basic research in the field of biofuels. Many enzymes and metabolic pathways for biofuel production have been discovered and characterized by state funded R&D projects. As an example, the microbial production of farnesene (a C15 isoprenoid molecule) with chemical properties to replace diesel as a transportation fuel, has been recently explored by academia and commercialized by Amyris, US (George et al., 2015).

Despite the number of potential fuel molecules, which have been discovered in the recent years, low titres and production rates hinder the commercial introduction of cheap advanced biofuels (Hollinshead et al., 2014). Fermentation engineering should adopt new tools, such as proteomics, flux balance analysis and molecular biology to overcome these hurdles (Hollinshead et al., 2014). If these goals are met, along with climate policy changes, then many technologies which are currently at demonstration scale or early commercial scale (Figure 4), have the potential to overcome initial technical hurdles and could become cost-competitive with fossil fuels by 2030 (Fiorese, 2012).

2. Aims of the Thesis

This thesis focuses on the biosynthesis of two different types of biofuels, butanol and propane. Suitable heterologous metabolic pathways were designed and investigated using *E. coli* as a model host organism. Performance of the different pathways and the *in vivo* characteristics of the involved biocatalysts were explored under various genetic contexts and physical culture conditions. The key enzymes under focus included acyl-ACP thioesterase, carboxylic acid reductase and alcohol dehydrogenase, which catalyse the butanol synthesis, and ferredoxin, NADPH:ferredoxin/flavodoxin-oxidoreductase and aldehyde deformylating oxygenase, which are responsible for propane synthesis.

The aims of research were:

(1) To generate a novel method for renewable alkane production with the goal to show that microbial propane biosynthesis is possible
(2) To find efficient, oxygen tolerant alternatives of currently available *Clostridial* butanol pathway
(3) To develop biochemical tools and analytical methods to evaluate the performance of the pathways and functions of the individual enzymatic steps
(4) To identify and analyse the most significant limiting factors restricting metabolic flux towards the end-products

Both pathways were designed and engineered to be transformable to phototrophic bacteria with the future aim to replace the glucose carbon source with carbon dioxide, water and sunlight.

3. Materials and methods

3.1. General description

A brief overview of the molecular biology tools, biochemical reactions and analytical methods and materials used in this Thesis work is presented here. A more detailed description can be found in original **Papers I-IV**.

3.1. Applied molecular biology methods and pathway construction

Synthetic gene fragments were purchased from GenScript (USA) and delivered in pUC57 Amp^R plasmids with custom restriction sites. Oligonucleotides for PCR and sequencing reactions were purchased from Eurofins MWG (Germany). Restriction enzymes, antarctic phosphatase, ligase and DNA polymerase enzymes were acquired from New England Biolabs (USA) or Thermo-Fisher Scientific (Finland).

E. coli DH5α was used to propagate all plasmids and *E. coli* BL21 (DE3) was used for enzyme expression and alcohol/alkane/fatty acid production studies. Standard molecular biology methods were used to clone synthetic DNA fragments to plasmid vectors. Plasmids were transformed to *E. coli* competent cells using heat shock transformation. Gene knockouts of *yqhD* and *ahr* were created by Red recombinase incision and FLP mediated excision of a kanamycin resistance cassette (Datsenko and Wanner, 2000).

3.2. Bacterial growth conditions and biofuel production

Metabolite production experiments were initiated either from LB (lysogeny broth) agar plates or LB liquid media incubated over-night, supplemented by specific antibiotics. Main cultures of TB or M9 media (recipes in **Paper I**) were inoculated from over-night precultures (1% v/v), cultivated in 250 ml baffled Erlenmeyer flask or 160 ml serum bottle and induced with 0.5 mM IPTG at OD_{600} = 0.5-0.7 for butanol production. A similar method was used for propane biosynthesis with an additional incubation period in gas-tight 2 ml GC vials.

3.3. Metabolite detection and analysis

A GC-MS was used to analyse alcohol and fatty acid samples from purified culture supernatants, mixed with acetone (**Paper I**). The signal abundance of the compounds was compared to a freshly prepared, linear calibration curve of known amounts of the analysed alcohol and fatty acid. Volatile alkanes (propane and heptane) were quantified using a similar GC-MS method with the exception of that samples were injected as gases from the headspace of sealed GC vials described in **Paper II**.

3.4. Stoichiometric analysis

A modified stoichiometric *E. coli* model iAF1260 (Feist et al., 2007) was used for the ACP-dependent butanol pathway (strain TPC4) by inserting the necessary catabolic reactions and the active transport constraint of butanol through the cytoplasmic

membrane (**Paper I**). The stoichiometric fluxes were calculated based on the model and experimental data obtained from cultivation in M9 minimal media. The applied approach was similar to a previously described method, called dynamic flux balance analysis (Varma and Palsson, 1994).

4. Overview of the results

4.1. Introduction

This section summarizes the scientific findings of the experiments described in the original research **Papers I-IV**. The experimental work was carried out in close co-operation within our research group (Bioenergy Group, Molecular Plant Biology Unit, Department of Biochemistry, University of Turku, Finland) and our collaborators in London (Department of Life Sciences, Imperial College London, UK), Manchester (BBSRC/EPSRC Centre for Synthetic Biology of Fine and Specialty Chemicals, Manchester Institute of Biotechnology, University of Manchester, UK) and Osnabrück (Pflanzenphysiologie, University of Osnabrück, Germany).

4.2. Butyraldehyde, the common denominator for the biosynthesis of butanol and propane

4.2.1. Butyraldehyde as an intracellular precursor

Butyraldehyde [butanal; $CH_3(CH_2)_2CHO$] is a flammable and toxic organic compound with a distinct, acrid smell. Under standard conditions it is a colourless liquid with limited solubility in water; it is highly miscible with organic solvents, such as ethanol, acetone, ether, and toluene. It is produced in large quantities from propylene, carbon monoxide and hydrogen and mostly used as an intermediate for manufacturing synthetic resins, rubbers, pharmaceuticals and other commodity chemicals. It is also used to produce industrial solvents, like n-butanol and 2-ethyl hexanol (DowChemicals, 2009).

Butyraldehyde is not present in nature in high quantities, but it can be formed in certain biochemical reactions. Butyraldehyde, like many other aldehydes, could disrupt cellular processes in prokaryotes and eukaryotes by reacting with amino and thiol groups of certain enzymes (Pérez et al., 2008). As a result, many species have evolved biological systems to neutralize the toxic effects of aldehydes. In bacteria, the so called Group III metal-containing alcohol dehydrogenases are mostly involved in reducing potentially harmful aldehydes to alcohols (Elleuche and Antranikian, 2013).

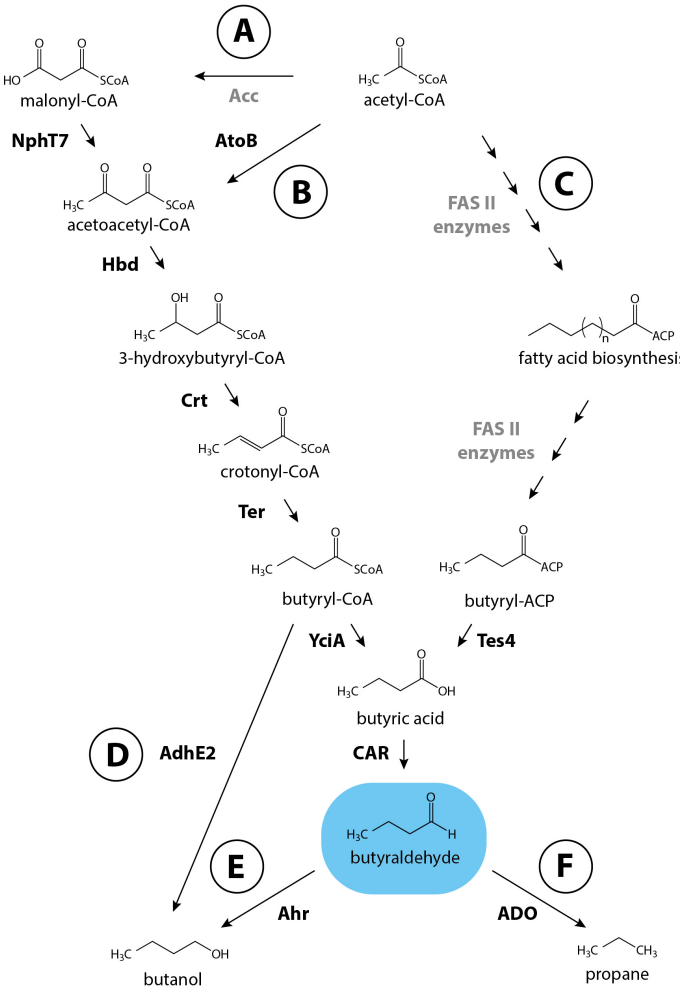

Figure 5 – Schematic representation of various heterologous pathways producing butyraldehyde, the common precursor for n-butanol and propane biosynthesis. All pathways are initiated from acetyl-CoA; enzymatic steps coloured in grey represent native biochemical routes (Acc and FAS II refers to acetyl-CoA carboxylase and fatty acid synthase type II respectively). **Route A** converts native malonyl-CoA to butyraldehyde using enzymes NphT7 (acetoacetyl CoA synthase), Hbd (3-hydroxybutyryl-CoA dehydrogenase), Crt (3-hydroxybutyryl-CoA dehydratase), Ter (trans-2-enoyl-CoA reductase), YciA (acyl-CoA thioester hydrolase) and CAR (carboxylic acid reductase). **Route B** utilizes the same enzymes as Route A to convert acetyl-CoA to butyraldehyde, except NphT7, which is replaced with AtoB (acetyl-CoA acetyltransferase). **Route C** diverts endogenous fatty acid biosynthesis to convert butyryl-ACP to butyraldehyde, using enzymes Tes4 (acyl-ACP thioesterase) and CAR. **Route D** is an exceptional pathway for n-butanol production, as no free butyraldehyde intermediate is released; butyryl-CoA is reduced in a two-step reaction to n-butanol by AdhE2 (aldehyde-alcohol dehydrogenase). The combination of Route B and Route D is found in *Clostridia*, as part of ABE fermentation. **Route E** is a one-step pathway for reducing butyraldehyde intermediate to n-butanol, either by endogenous or over-expressed alcohol dehydrogenases. **Route F** converts butyraldehyde (and other potentially present aldehydes) to propane (or longer chain-length alkanes) with the help of various over-expressed *cyanobacterial* ADO (aldehyde deformylating oxygenase) enzymes. Other abbreviations used: Acc (acetyl-CoA carboxylase), FAB (fatty acid biosynthesis).

Generally, aldehydes may serve as precursors for alcohol biosynthesis by reduction of the formyl group into primary hydroxyl, or for generation of n-1 alkanes by deformylation. In this work, as illustrated in Figure 5, butyraldehyde was the intermediate for the biosynthesis of n-butanol (**Papers I and III**) and propane (**Papers II and III**).

4.2.2. Generating butyraldehyde via the ACP route

One way to biosynthesize butyraldehyde is intercepting the bacterial fatty acid machinery. This route utilizes a heterologously expressed acyl-ACP thioesterase (TE), which can hydrolyse the fatty acyl-ACP bond and terminate the fatty acid elongation process (Figure 5, Route C). Thus the route generates a range of free fatty acids (FFA), including butyric acid, the precursor for butyraldehyde biosynthesis.

TesA thioesterase I, an enzyme expressed in cytosol was amongst the first acyl-ACP thioesterases discovered (Cho and Cronan, 1995). Since then a large group of acyl-ACP thioesterases from bacteria and plants with similar activity to TesA have been experimentally characterized (Jing et al., 2011). Jing and co-workers have selected 31 thioesterases and determined the *in vivo* substrate specificity of these enzymes in *E. coli* K-27, a strain lacking FadD (fatty acyl-CoA synthase). Many of these thioesterases have broad-range substrate specificity and are capable of acting on short and medium carbon chain length acyl-ACPs and producing the corresponding fatty acids. The analysis of the product composition of the bacterial acyl-ACP TE from *Bacteroides fragilis* showed that it was capable of producing 4:0, 6:0, 12:1 and 14:1 fatty acids; *Marvinbryantia formatexigens* had specificity for 4:0, 6:0 and 8:0 products; whereas *Lactobacillus brevis* was mostly generating 8:0 and minor amounts of 4:0 and 6:0 fatty acids (Jing et al., 2011). The same study reported that although the product composition of these selected TEs tends to concentrate in short chain fatty acids, there is also a high variation of the total produced FFA content. Taking the FFA composition and the total fatty acid content into account, the genes encoding acyl-ACP thioesterases (E.C. 3.1.2.14) from *B. fragilis*, *M. formatexigens* and *L. brevis* were selected as the most potent candidates for butyric acid production in *E. coli* BL21 (DE3) (**Paper I**).

The three selected acyl-ACP thioesterases were assembled in a previously described plasmid backbone, developed in our lab (Akhtar et al., 2013) containing synthetized genes encoding Sfp (maturation factor phosphopantetheinyl transferase from *Bacillus subtilis*), and CAR (carboxylic acid reductase from *Mycobacterium marinum*). The resulting pathways (Figure 5) were named TPC4-6, with the numbers indicating the acyl-ACP thioesterase homolog in question. The performance of the assembled pathways expressing the different acyl-ACP thioesterases were analysed and compared in regards to butyraldehyde production by measuring n-butanol production, as butyraldehyde is endogenously reduced to n-butanol in *E. coli* (**Paper I**; Section 4.3 in this thesis).

The effect of culture aeration (oxygen) on n-butanol productivity was evaluated in **Paper I**. Two different aeration conditions (micro aerobic and aerobic) were chosen based on earlier studies (Shen et al., 2011) and preliminary experiments. Aerobic conditions had a clear positive effect on butyraldehyde and consequently n-butanol production of the constructed pathways; TPC4 strain was most affected and the enhanced aeration increased the production over 10 fold in TB medium (Figure 2C and 3 published in **Paper I**). To verify these results and the involvement of TEs, the effect of increased atmospheric oxygen concentration was tested directly on Tes4 acyl-ACP thioesterase *in vivo*, using a plasmid that was lacking CAR and Sfp. When the oxygen concentration increased from 21 to 80% (v/v), the butyric acid formation nearly doubled (Figure 6).

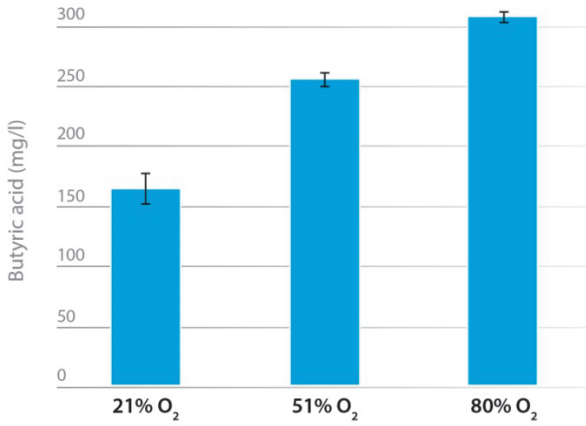

Figure 6 – The effect of oxygen concentration on butyric acid production of the strain expressing Tes4 acyl-ACP TE. Butyrate production was evaluated at 21, 51 and 80% v/v oxygen concentrations in 2 ml gas-tight GC vials with 3h incubation time in an identical manner as propane production experiments were carried out. Samples were collected; butyrate was extracted from the supernatant and analysed by GC-MS using the method described in **Paper II**. Error bars represent standard deviation (n = 4).

Oxygen tolerance is the largest advantage of ACP-dependent pathway for generating butyraldehyde intermediate, as it allows the utilization of oxygenic photosynthetic organisms as production hosts. However, the ACP route is relying on the bacterial fatty acid synthesis and lipid generation. Because of the stringent regulation of native fatty acid biosynthesis, the productivity of any biofuel pathway relying on fatty acid synthase complex (FAS II) would be limited. One possibility to overcome these limitations would be the application of fatty acid overproducing strains (Lu et al., 2008), such *E. coli* strains have achieved titres up 7 g/l of free fatty acids (Janssen and Steinbuchel, 2014). Alternatively, the fatty acid biosynthesis could be by-passed by using the *Clostridial* CoA pathway for butyraldehyde generation.

4.2.3. Biosynthesis of butyraldehyde via the CoA pathway

To overcome the limitations associated with the dependence on fatty acid biosynthesis, alternative butyraldehyde production pathways were investigated. The goal was to generate butyraldehyde directly from an upstream metabolic building block, acetyl-CoA, by partially overexpressing the ABE fermentation pathway from *Clostridium acetobutylicum*. In the recent years the *Clostridial* CoA pathway for the generation of n-butanol has been well studied and expressed in *E. coli* (Atsumi et al., 2008a); by optimizing NADH co-factor utilization, approx. 30 g/l n-butanol titre has been achieved in fed-batch fermentation (Shen et al., 2011).

In this Thesis work several variations of the *Clostridial* CoA-dependent pathway were assembled (**Paper III**). The NphT7 pathway (Figure 5, Route A) initiates from malonyl-CoA generated by native metabolism, whereas the AtoB pathway (Figure 5, Route B) initiates with a condensation step of two acetyl-CoA molecules. Both of these pathways share the subsequent reduction steps of acetoacetyl-CoA catalysed by Hbd (3-hydroxybutyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824), Crt (3-hydroxybutyryl-CoA dehydratase from *C. acetobutylicum* ATCC 824) and Ter (trans-2-enoyl-CoA reductase from *Treponema denticola* ATCC 35405), leading to butyryl-CoA. Butyraldehyde can be produced from butyryl-CoA via two possible biochemical routes, either by the two-step reaction of YciA from *Haemophilus influenza* and CAR or the bi-functional aldehyde-alcohol dehydrogenase AdhE2 from *C. acetobutylicum* ATCC 824 (Figure 5, Route D).

Although the aldehyde-alcohol dehydrogenase AdhE2 has been demonstrated to be able of generating high titres of n-butanol, free butyraldehyde is not released during the catalysed reduction step (Shen et al., 2011). This finding has been verified in **Paper III**. This is consequently limiting the applicability of AdhE2 for the production of aldehyde-derived biofuels. To overcome this limitation, the AdhE2 route was by-passed by using an acyl-CoA thioesterase, YciA. This enzyme effectively regulates the intracellular levels of acyl-CoA and fatty acids, acting on a wide range of acyl-CoA compounds (Willis et al., 2008). By co-expressing YciA with CAR and Sfp, free butyraldehyde was produced, which was again detected as n-butanol (**Paper III**).

4.3. Production of n-butanol from butyraldehyde

4.3.1. Removal of potentially toxic aldehydes by alcohol dehydrogenases

Alcohol dehydrogenases (ADH) or aldehyde reductases (EC 1.1.1.1 and EC 1.1.1.2) are enzymes catalysing the inter-conversion of alcohols and aldehydes or ketones, as demonstrated by the conversion of butyraldehyde into n-butanol as part of this work (**Paper I-III**). These enzymes are present in all taxonomical kingdoms, also in several mammalian tissues and also in microorganisms growing in extreme environmental conditions. Based on the specific co-factors, ADHs can be grouped into (1) NADP-dependent enzymes, (2) pyrroloquinolinequinone-, haem-group and F_{420}-dependent ADHs and (3) flavin adenine dinucleotide-dependent isozymes. From metabolic

engineering perspective NADP-dependent alcohol dehydrogenases are the most important ones, used in numerous heterologous pathways and they can be categorized into three groups: (1) zinc-containing, medium/long chain ADHs, (2) metal-free, short chain ADHs and (3) the metal-containing ADHs (Elleuche and Antranikian, 2013). Alcohol dehydrogenases play an important biochemical role in microorganisms and they are also targets of applied biotechnology research for biofuel production.

As a by-product of aerobic respiration, reactive oxygen species are formed within prokaryotic and eukaryotic cells. These oxides and radicals can oxidize membrane lipids in cell walls, which leads to the formation of lipid peroxides (Pérez et al., 2008). The cellular degradation of these lipid peroxides could result in the formation of toxic, short-chain aldehydes, including butyraldehyde. Metal-containing ADHs, like YqhD have been shown to have affinity towards aldehyde substrates, being capable of reducing these substances to alcohols. The bi-functional aldehyde-alcohol dehydrogenase AdhE2, described in Section 4.2.3., has high affinity towards butyryl-CoA and butyraldehyde and is capable of producing high titres of n-butanol. The effect of over-expression and deletion of selected alcohol dehydrogenases has been studied in the research **Papers I-III**, in regards to n-butanol (Section 4.3.2.) and propane (Section 4.4) production.

4.3.2. Overexpression of alcohol dehydrogenase improves butanol titre

Butyraldehyde can be converted into n-butanol by enzymatic reduction. This conversion is occurring endogenously in *E. coli*, as it has six different alcohol dehydrogenases present in its genome (*adhE*, *adhP*, *eutG*, *yiaY*, *yqhD*, and *yjgB*, the latter reclassified as *ahr*) (Atsumi et al., 2010). These endogenous ADHs were responsible for the formation of n-butanol in the first-generation strains expressing the TPC pathways producing butyraldehyde (**Paper I**).

Previous studies have showed that the native conversion of aldehydes to alcohols can be limited in the microbial host organism (Rodriguez and Atsumi, 2012). This was suggesting that the over-expression of aldehyde reductases with specific activity towards short chain substrates could improve the metabolic flux towards n-butanol production. In **Papers I and III**, two NADP-dependent ADHs, Ahr from *E. coli* and the aldehyde reductase homolog encoded by *slr1192* from *Synechocystis* were evaluated for improved n-butanol production. The over-expression of Ahr increased the n-butanol productivity of TPC4 and TPC5 strains (Figure 7), demonstrating that the endogenous flux towards n-butanol production can be further enhanced. The most significant change was observed within the TPC4 strain, the productivity increased from 3.6 to 9.9 mg/l/h in aerobic shake flask cultures. The ADH encoded by *slr1192* however did not affect n-butanol production, possibly due to different substrate specificity. Based on these findings, Ahr was overexpressed in AtoB-TPC7 and NphT7-TPC7 strains as well. An increased n-butanol production was observed in strains with

either of the pathways, n-butanol titres doubled for AtoB-TPC7 strain to 160 mg/l, and moderately increased for NphT7-TPC7 strain (Figure 7).

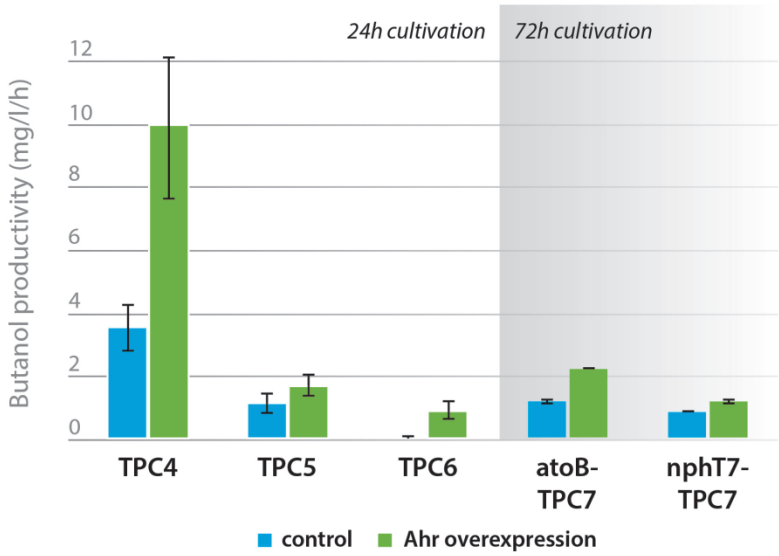

Figure 7 – The effect of Ahr overexpression on n-butanol productivity. Strains TPC4-6 produce n-butanol using the ACP-dependent route, described in more detail in Section 4.2.2. in a 24h cultivation. Strains atoB-TPC7 and nphT7-TPC7 produce n-butanol using the CoA-dependent route described in more details in Section 4.2.3. in a 72h cultivation. The productivity is given in mg n-butanol produced per litre of culture per hour. As the ACP and CoA-dependent pathways were investigated under differing environmental conditions, the direct comparison between these strains should be addressed with reservations (see **Paper I and III** for more details).

4.4. Biosynthesis of propane via the butyraldehyde route

4.4.1. Alkane biosynthesis in cyanobacteria; aldehyde deformylating oxygenase

Alkanes are found in nature mainly as components of cuticular wax in plants. It was discovered that cyanobacteria are also capable of synthesizing alkanes, although the physiological function of these C13-17 carbon long hydrocarbons (Schirmer et al., 2010) is not yet fully understood. The key enzyme responsible for cyanobacterial hydrocarbon production is the soluble, di-iron non-heme enzyme called aldehyde deformylating oxygenase (ADO; earlier called *cyanobacterial* aldehyde decarbonylase) (Schirmer et al., 2010). ADO has enabled scientists to research metabolic pathways for the generation of engine-ready alkane biofuels. ADO catalyses the key biochemical reaction step in the production of propane and heptane in the current study (**Papers II-IV**).

At the time of the discovery of ADO, it was believed that the catalytic conversion of the aldehyde molecule releases carbon monoxide. This hypothesis was proved to be false, when the one of the first *in vitro* characterization of the enzyme revealed that the by-product of the reaction was formate (Warui et al., 2011). As the formate by-

product has one additional oxygen atom compared to carbon monoxide, this implied that oxygen plays a major role in the reaction. Accordingly, biochemical studies revealed that the reaction requires oxygen as a co-substrate (Li et al., 2012; Li et al., 2011). This was further corroborated by showing that the incorporated oxygen atom in formate derives from molecular oxygen and not from water. The role of oxygen in the catalytic reaction has been further linked with the catalytic mechanism, in which the carbon-carbon bond is cleaved via an oxygen radical intermediate (Warui et al., 2011). During the redox oxygenation process NADPH, ferredoxin and ferredoxin-oxidoreductase provide four electrons for the complete reduction of molecular oxygen (Andre et al., 2013; Li et al., 2012).

4.4.2. Engineered ADO-based hydrocarbon pathways

So far various metabolic pathways have been constructed to heterologously express ADO *in vivo* and volatile short carbon chain (C3 and C7; **Papers II-IV**) and medium chain (C9-C17; (Choi and Lee, 2013; Harger et al., 2012; Howard et al., 2013; Schirmer et al., 2010)) alkanes have been produced using *E. coli* as a model host organism. In this Thesis the emphasis was placed on the development of novel propane and heptane production pathways. Based on the research conducted with acyl-ACP thioesterases (**Paper I**), the TPC4 pathway (capable of producing the butyraldehyde intermediate) was co-expressed with ADO and a ferredoxin reducing partner for the production of short chain alkanes. The pathway was designed to be tolerant to oxygen, allowing future implementation in photosynthetic host organisms.

The experiments have shown that the pathway is functional, however the production of propane ceased within an hour during small-scale, gas-tight incubation. In order to understand the limiting factors of the metabolism, further investigation was performed on the effects of oxygen (as a known co-substrate of ADO), reaction redox balance, flux limiting bypass pathways and enzyme engineering concepts towards improved substrate specificity and redox partner binding.

The production of propane was conducted is sealed GC vials, where no gas exchange was allowed with the external atmosphere. When the oxygen concentration inside the vials was gradually elevated to 80% (v/v), the propane production nearly doubled (Figure 2C published in **Paper II**), as expected from preceding experiments on n-butanol biosynthesis. When butyric acid was fed directly to the culture medium, using a strain lacking the acyl-ACP thioesterase, increased propane production was observed. This demonstrated that the fatty-acid synthesis derived pre-cursor availability of the alkane pathway was limited (Figure 6).

As described in Section 4.3.2. *E. coli* has the potential of removing toxic aldehydes from the cell metabolism, hence limiting the flux of the heterologous alkane pathway, which was indicated by the accumulation of n-butanol in the culture medium during propane production. To overcome these limitations, in **Paper II** two important aldehyde reductase genes, *ahr* and *yqhD* were deleted. The resulting mutants showed

enhanced propane generation (Figure 2E and 3A published in **Paper II**) and concurrently decreased n-butanol output flux.

4.4.3. ADO reduction: Role of Fdx and Fpr

The conversion of aldehydes into alkanes requires four electrons for every alkane produced. This electron supply chain has been reconstituted previously in various other *in vitro* pathways (Li et al., 2012; Schirmer et al., 2010). The *in vivo* electron supply of the reaction and the interaction between ADO, ferredoxin and Fpr (ferredoxin-oxidoreductase) were investigated for the first time in **Paper II**. The PetF ferredoxin from *Synechocystis* was over-expressed, instead of relying solely on native reducing systems of *E. coli*, in order to provide adequate electron supply to the alkane pathway. In **Paper III** it is shown that the co-expression of PetF ferredoxin with ADO increases the efficiency of the electron supply chain, independent of the metabolic route used for alkane production or the variant of the ADO enzyme. PetF nearly doubled the propane titre of the Route B (with atoB), while it moderately increased the production of Route A (with NphT7) (Figure 5).

To further investigate the reduction mechanism of aldehydes to alkanes, the enzymatic binding effect of ADO and Fdx was evaluated *in vitro* and *in vivo* in **Paper IV**. Our collaborators from University of Osnabrück (Germany) created different mutants of the ADO enzyme from *Nostoc punctiforme* NP73102. The mutants showed increased binding affinity towards ferredoxin *in vitro*, which was hypothesized to boost enzyme activity for alkane production *in vivo*. The pathway used in this study for *in vivo* experiments was modified from the heptane producing alkane pathway (described in **Paper II**), by removing the acyl-ACP thioesterase, thus octanoic acid was directly fed to the culture medium to generate the alkane. The *in vivo* kinetic experiments in *E. coli* demonstrated that without over-expression of Fdx and Fpr (Figure 8), the heptane production rates drastically decreased, however no significant heptane production increment was observed in the ADO mutant strains in comparison to the wild type.

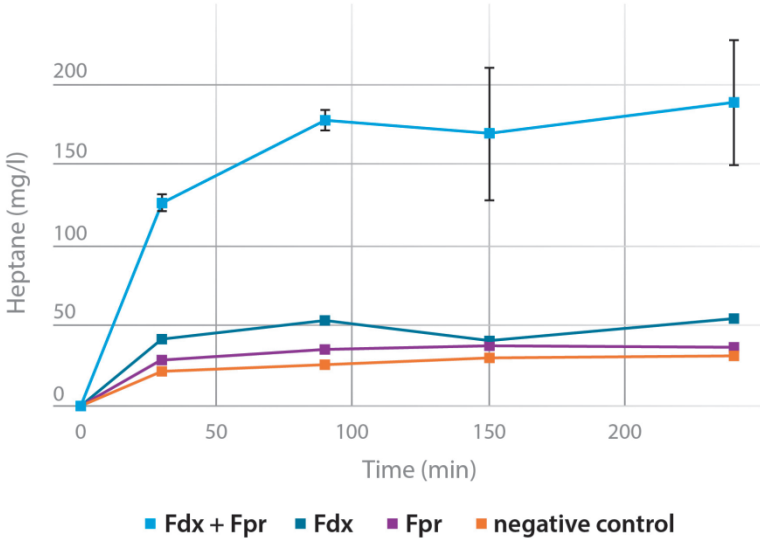

Figure 8 – The effect of Fdx and Fpr overexpression on heptane production in *E. coli*. Heptane was produced *in vivo* using octanoic acid feeding to *E. coli* BL21 (DE3) ΔAhr ΔYqhD strains harbouring plasmids expressing enzymes Sfp, CAR and ADO and optionally Fdx and Fpr. The strain was lacking the acyl-ACP thioesterase. Figure adapted from **Paper IV**.

To ensure sufficient *in vivo* reduction of ferredoxin, a ferredoxin-oxidoreductase (Fpr) was also introduced into the alkane production system based on the ACP route (Figure 5, Route C). The Fpr overexpression resulted in a moderate increase of propane production under atmospheric oxygen levels. Interestingly, when the oxygen concentration of the reaction was raised to 80% (v/v), a 5-8 fold increase of propane titre was observed (**Paper II**).

4.4.4. The kinetics of ADO *in vitro* and *in vivo*

An *in vitro* enzyme kinetics study of ADO (*Prochlorococcus marinus* strain MIT9313) showed that the enzyme follows the Michaelis-Menten reaction model and displays lower catalytic turnover towards short carbon chain aldehydes (Khara et al., 2013). The poor k_{cat} (0.0031 ± 0.0001 1/min) and high K_m (10.1 ± 0.9 mM) values for the conversion of butyraldehyde indicated that the wild-type activity of ADO towards this substrate is extremely low. The limiting activity of ADO was also verified separately *in vivo* (**Paper II**); moreover an experiment revealed that besides propane, longer carbon chain alkanes like heptane, were also produced, along with alcohol by-products (Figure 5 published in **Paper II**). To improve the enzyme's activity towards shorter carbon chain length products, multiple mutated enzymes were created and screened for to introduce a steric block, and prevent the binding of potential long-chain inhibitors (palmitic acid) and longer chain aldehydes. The resulting ADO_{A134F} mutant generated enhanced levels of short chain alkanes *in vitro* (Khara et al., 2013).

Benefiting from the work of Khara *et al.*, the ADO_{A134F} mutant strain was utilized to increase the substrate specificity of the enzyme towards short chain aldehydes (**Paper III**). The ADO_{A134F} was co-expressed with Fdx within the *nphT7* (Figure 5, Route A) and *atoB* (Figure 5, Route B) pathways to study the *in vivo* kinetics of propane generation via the CoA route. As expected from the preceding *in vitro* data (Khara et al., 2013), the structure-engineered ADO enzyme nearly doubled the propane titre within both heterologous pathways *in vivo*, compared to wild-type ADO (Figure 7 published in **Paper III**).

5. Discussion

5.1. Using photosynthesis for advanced biofuel production

Although the production and use of first generation bioethanol and biodiesel are gradually increasing, there is an urgent need for advanced biofuels, which derive from non-food biomass. The replacement of the current plant-based biofuel technologies with more sustainable and technologically improved alternatives is necessary to preserve arable land and fresh water for food production. To achieve these goals, two possible strategies could be followed. One solution would be to utilize non-photosynthetic bioconversion to transform waste materials from food and agricultural industry (e.g. lipids or lignocellulosic biomass) to advanced biofuels. Another obvious strategy is the application of photosynthetic microorganisms, which could directly use solar energy to convert CO_2 and water into biomass or potentially useful end-products.

Photosynthetic cyanobacteria and microalgae are appealing candidates for alternative renewable fuel production. These light-harvesting microorganisms have relatively fast growth rates, high (theoretical) photosynthetic efficiency and good capabilities for propagating in salty sea waters compared to land-based crops. Many heterologous pathways have already been introduced to cyanobacteria for the production of ethylene, ethanol, n-butanol, 2,3-butanediol, lactate, 3-hydroxybutyrate, isobutanol and many other potential fuel compounds (Savakis and Hellingwerf, 2015). On the other hand, microalgae have a high natural lipid content, which could reach up to 80% of their dry biomass, exceeding many currently cultivated oil plants (Kirrolia et al., 2013). These lipids could be efficiently utilized for advanced biodiesel and renewable jet fuel production.

To use photosynthetic microorganisms as biofuel factories, a wide range of criteria has to be considered. These include fundamental know-how and general understanding of the host organism metabolism, molecular biology tools for metabolic pathway engineering, optimal growth conditions and bioreactor design as well as oxygen tolerant enzymes. The engineering and the assembly of biofuel pathways in cyanobacteria is relatively complicated, and is usually preceded by extensive *in vitro* studies and evaluation in simpler model systems *in vivo*.

In this Thesis, the engineered pathways for n-butanol and propane biosynthesis and specific, oxygen tolerant (photosynthesis compatible) enzymes have been investigated. n-Butanol, a short chain-length alcohol, possesses better technical properties and higher energy content than bioethanol. Propane, a gaseous hydrocarbon fuel used in gas engines, heaters and refrigerators is traditionally obtained from natural gas and petroleum refining. Both of these compounds are excellent candidates for advanced biofuels. The pathways have been evaluated in *E. coli* BL21 (DE3), a model host organism which is capable of faster growth compared to

cyanobacteria and allows convenient assembly of a large variety of metabolic routes (Figure 5). Although *E. coli* is a heterotrophic host organism, the expressed pathways were designed in a way that takes the biochemistry of phototrophs into account. For example the propane biosynthesis was achieved by enzymes (particularly ADO and PetF), which originate from cyanobacteria, and use ATP and NADPH as catalytic co-factors.

5.2. Pathways for n-butanol and propane biosynthesis

This work focused on butyraldehyde generation as a key intermediate for the microbial production of alternative biofuels, n-butanol and propane. These metabolic pathways can be categorized mainly into two different groups. The pathways of the first group are ACP-dependent and use native bacterial fatty acid biosynthesis as a precursor (**Papers I and II**). The pathways of the second group are reliant on CoA and originate from *Clostridial* ABE fermentation (**Paper III**). All of these pathways were shown to produce butyraldehyde, which was endogenously reduced in *E. coli* to n-butanol, to eliminate the toxic effect of the aldehyde intermediate.

The ACP-dependent pathways enabled a novel oxygen tolerant n-butanol synthesis, based on bacterial fatty acid production and the utilization of different heterologous acyl-ACP thioesterases. Cultivation conditions, in regard to oxygen sensitivity and culture media, as well as the overexpression of aldehyde reductases were investigated within these ACP-dependent pathways. One of the engineered pathways, harbouring an acyl-ACP thioesterase from *Bacteroides fragilis* (TPC4 strain in **Paper I**), showed n-butanol productivity comparable with that of a previously described high yielding *Clostridium*-based reference pathway (**Paper I**). However, the ACP-based pathways have limited carbon flux restricted by the native fatty acid metabolism of *E. coli*. To overcome this restriction, a CoA-dependent production route was investigated, using enzymes mostly found in the *Clostridial* ABE fermentation. These enzymes were co-expressed with acyl-CoA thioesterase, YciA from *Haemophilus influenza* to produce butyraldehyde and eventually n-butanol (**Paper III**).

After verifying butyraldehyde production, both the ACP and the CoA dependent pathways were coupled with a unique enzyme, the aldehyde deformylating oxygenase (ADO) to produce propane from glucose, for the first time *in vivo* (**Paper II**). The key biochemical factors limiting the biosynthesis of short-chain alkanes were identified and the reaction's dependence on oxygen, the effect of increased electron supply and competing endogenous metabolism were investigated. For the first time, the entire redox cycle of ADO, ferredoxin (PetF) and ferredoxin-oxidoreductase (Fpr) was reconstructed and studied *in vivo* for alkane synthesis. Furthermore the effect of altered enzyme-enzyme binding affinities between ADO and Fdx were explored *in vitro* and *in vivo* in *E. coli* (**Paper IV**).

5.3. Future perspectives

In order to reach sustainable and renewable systems for biofuel production it is imperative to explore, evaluate and compare different biosynthetic platforms and strategies. Only this way it is possible to eventually find and optimize systems which would be sufficiently efficient and up-scalable for commercial use. Future modification of the presented n-butanol and propane pathways should focus on improving the metabolic flux through acetyl-CoA and fatty acid biosynthesis, and at the same time on eliminating competing metabolism of the host organism to increase production titres.

To improve the total fatty acid yields, various genetic modifications can be implemented, for example the overexpression of certain thioesterases, deletion of *fadD* and *fadL* genes, to eliminate fatty acid degradation (Liu et al., 2012; Lu et al., 2008) or by carefully tuning the regulation of fatty acid biosynthesis (Janssen and Steinbuchel, 2014). The enzymatic reduction of butyric acid to butyraldehyde could also be a limiting step, as the enzyme carboxylic acid reductase (CAR) responsible for this step has a low affinity towards shorter chain substrates (Akhtar et al., 2013). In this Thesis work it has been found that under aerobic conditions the reduction of butyraldehyde to n-butanol could also be limiting; consequently the overexpression of multiple specific aldehyde reductases could enhance n-butanol productivity in the host organism. It was also shown that throughout the production phase the majority of carbon was not used for n-butanol biosynthesis, instead acetic acid was formed. Transcriptional down-regulation of such by-pass pathways or traditional gene knock-out methods could focus the metabolic flux towards n-butanol biosynthesis (Na et al., 2013).

Kinetic studies (published in **Paper II**) of the key enzyme catalysing the last step in hydrocarbon biosynthesis (ADO) have revealed the poor catalytic efficiency of the enzyme. Protein engineering of ADO towards lower carbon chain substrate specificity as well as its replacement with a different variant from another cyanobacterial host could increase propane productivity. Alternative alkane producing enzymes, such as CER1 (Bourdenx et al., 2011) should also be explored and compared to ADO.

6. Concluding remarks

In conclusion, a series of alternative heterologous biosynthetic pathways were designed, constructed and evaluated in *E. coli* host for n-butanol and propane production. These proof-of-concept pathways were functional and allowed the detailed characterization of the individual biosynthetic steps and associated metabolic constraints, despite the fact that the overall yields still remained far from any commercial realization. The information obtained in this Thesis work lays the basis for further engineering of the pathways to optimize the production, and it was shown that:

- The n-butanol pathway, employing a specific acyl-ACP thioesterase, phosphopantetheinyl transferase, carboxylic acid reductase and aldehyde reductase, is tolerant to oxygen, which is a significant improvement compared to previously published anaerobic metabolic routes.
- n-Butanol can be also produced from acetyl-CoA with steps initiated by NphT7 acetoacetyl CoA synthase or AtoB acetyl-CoA acetyl transferase, which releases metabolic pressure from fatty acid synthesis, by not using ACP compounds.
- Renewable propane can be biosynthesized *in vivo*, using cyanobacterial aldehyde deformylating oxygenase for converting the butyraldehyde precursor to propane, as demonstrated for the first time.
- An effective *in vivo* electron transport chain, employing PetF ferredoxin and Fpr ferredoxin-oxidoreductase is necessary for the production of propane.
- New analytical methods could be developed for monitoring batch-scale *in vivo* production of volatile hydrocarbons.

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