THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

A study on fatty acid ethyl ester production in a *Saccharomyces cerevisiae* cell factory

Metabolic engineering practice towards a sustainable transportation fuel

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CHALMERS UNIVERSITY OF TECHNOLOGY

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Abstract

Currently there is an obvious demand for alternative transportation fuels to fulfill the requirements for the future transportation. Fatty acid ethyl esters (FAEEs) have chemical properties that are comparable with crude diesel or the currently available FAME biodiesels, but can be produced in a more sustainable way, based on microbial cell factories. During this PhD project, three different metabolic engineering strategies were applied to improve the production of FAEEs in *Saccharomyces cerevisiae*.

In the first strategy the native cytosolic pyruvate dehydrogenase bypass was improved together with regulation of the acetaldehyde branch point, at which carbon can be converted into ethanol or into acetate and further to acetyl-CoA. Therefore, genes *ADH2*, *ALD6* and acs_{SE}^{L641P} from *Salmonella enterica*, containing a point mutation to prevent acetylation in combination with a wax ester synthase (*ws2*) from *Marinobacter hydrocarbonoclasticus* were overexpressed on 2-micron plasmids which resulted in an 2.7 fold (p-value: 0.03) improved yield of 359 ± 128 µg FAEEs $gCDW^{-1}$.

A second metabolic engineering strategy was performed to re-channel the carbon flow via the pentose phosphate pathway and the heterologous phosphoketolase (PHK) pathway towards acetate and acetyl-CoA and yield NADPH, needed for fatty acid synthase, as a byproduct. The first reaction of the two step PHK pathway is catalyzed by xylulose-5-phosphate phosphoketolase (encoded by *xpkA* from *Aspergillus nidulans*) and the second step is catalyzed by acetate kinase (encoded by *ack* from *A. nidulans*) or phosphotransacetylase (encoded by *pta* from *Bacillus subtillis*). The combined expression of *xpkA* and *pta* on a 2-micron plasmids resulted in a FAEE yield of 105 ± 30 µg $gCDW^{-1}$ which was an 5.7 fold (p-value: 0.03) improvement. Additional expression of *xpkA* and *pta* in a strain with 6 integrated copies of *ws2* resulted in 4670 ± 379 µg $gCDW^{-1}$ (p-value: 0.02), a 1.6 fold higher FAEE yield than a reference, whereas the expression of *xpkA* and *ack* in the same background strain improved the yield to 5100 ± 509 µg FAEEs $gCDW^{-1}$ (p-value: 0.01), which was a 1.7 fold improvement.

The combined overexpression of *ws2*, *ADH2*, *ALD6*, acs_{SE}^{L641P}, $ACC1^{S1157A,S659A}$ (acetyl-CoA carboxylase with point mutations to prevent phosphorylation) and *ACB1*(acyl-CoA binding protein), which were stably integrated into the chromosome, was investigated together with the disruption of acyl-CoA competing pathways towards the formation of triacylglyceride (*dga1Δ lro1Δ)*, sterol esters (*are1Δ are2Δ*) and acetyl-CoA (*pox1Δ*) and resulted in a yield of 1072 ± 160 µg total FAEEs $gCDW^{-1}$ (titer of 4.4 ± 0.7 mg l^{-1}), which was a 4.1 fold improvement compared to sole *ws2* expression.

Transciptional analysis was performed on FAEE producing mutants of *S. cerevisiae* which pointed out the increased cellular stress that influenced cellular growth and the increased demand of cofactor NADPH which is therefore considered an engineering target for the future.

Keywords: fatty acid ethyl ester, metabolic engineering, *Saccharomyces cerevisiae*, phosphoketolase pathway, transcriptome analysis, physiological characterization, genome integration.

List of publications

This thesis is based on the work contained in the following publications:

I. **de Jong B.W.**, Siewers V. and Nielsen J. (2012) Systems biology of yeast: enabling technology for development of cell factories for production of advanced biofuels. Current Opinion in Biotechnology 23:624-630

II. **de Jong B.W.**, Shi S., Siewers V. and Nielsen J. (2014) Improved production of fatty acid ethyl esters in *Saccharomyces cerevisiae* through up-regulation of the ethanol degradation pathway and expression of the heterologous phosphoketolase pathway. Microbial Cell factories, 13, 39.

III. **de Jong B.W.**, Shi S., Valle-Rodríguez J.O., Siewers V. and Nielsen J. (2014) Metabolic pathway engineering for fatty acid ethyl ester production in *Saccharomyces cerevisiae* using stable chromosomal integration. Journal of Industrial Microbiology and Biotechnology, DOI 10.1007/s10295-014-1540-2

IV. **de Jong B.W.**, Siewers V. and Nielsen J. Physiological and transcriptional characterization of *Saccharomyces cerevisiae* engineered for production of fatty esters ethyl esters. submitted (2015)

Additional publication, not part of this thesis:

V. Thankaswamy-Kosolai S., **de Jong B.W**., Roldao A. and Nielsen J. BEDA - batch fermentation analysis tool. Manuscript in preparation

Contribution manuscript

I. Drafted and edited the paper.

II. Performed all experimental work, analyzed the data. Drafted and edited the paper.

III. Performed all experimental work, analyzed the data. Drafted and edited the paper.

IV. Performed all experimental work, analyzed the data. Drafted and edited the paper.

Additional publication, not part of the thesis:

V. Performed all experimental work, analyzed the data. Drafted part of the paper and contributed editing the paper.

Preface

This dissertation serves partial fulfillment of the requirements of a PhD degree at Chalmers University of Technology, Sweden. The research, performed during the PhD project, was funded by the Knut and Alice Wallenberg Foundation, Vetenskapsrådet, FORMAS, Ångpanneföreningens Forskningsstiftelse and the European Research Council (grant no. 247013). Personal funding has been received from Stiftelsen Futura, Chalmers Internationalization Fund, The Novo Nordic Foundation, Ångpanneföreningens Forskningsstiftelse and Chalmersska Forskningsfonden. If necessary, copyrights were received for my already published work. The work was carried out under supervision of Professor Jens Nielsen and Dr. Verena Siewers.

Bouke Wim de Jong
December 2014

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To whom I love

1. Introduction

1.1. Necessity of biofuels

Motorized transport is a social-economic necessity which consequently creates a gigantic market for liquid fuels. World leading institutions like 'The International Energy Agency (IEA, U.S.) and committees from the European Union (EU) frequently publish reports on changes and future developments in the transport fuel market [1-3]. Independent of the exact size of the future liquid fuel market, it is clear that transportation fuels take an increasing share of the total energy demand. It has been reported that the energy consumption by the transportation sector has increased by more than 20% to 65% of all fuel produced, during the last 35 years [2]. The diesel engine and the gas turbine are the driving forces for almost all transportation modes. Diesel fuel production is the largest in volume and provides marine engines (150 million metric tons per year, low quality) and large parts of road traffic (650 million metric tons per year, high quality); whereas gasoline plays a role for lighter road vehicles and kerosene is used for aviation (190 million metric tons per year, high quality). The increase in energy demand can be mainly attributed to road transport which consumes about 75% of total transport energy and is largely driven by diesel fuel [4]. Predictions, based on this increase in energy consumption for transport modes, foresee a further energy increase of 50% until 2030 and an 80% increase in energy consumption until 2050. Additionally it is believed that the traditional petroleum based fuel consumption will decrease with 50% in 2050 and be replaced by alternative fuels [1]. Concomitantly, the biofuel share is expected to increase 10-fold between 2010 and 2050 (>30 Exajoules) (Figure 1.1) [2, 5] and therefore, there is an obvious demand for alternative transportation fuels, like biodiesel.

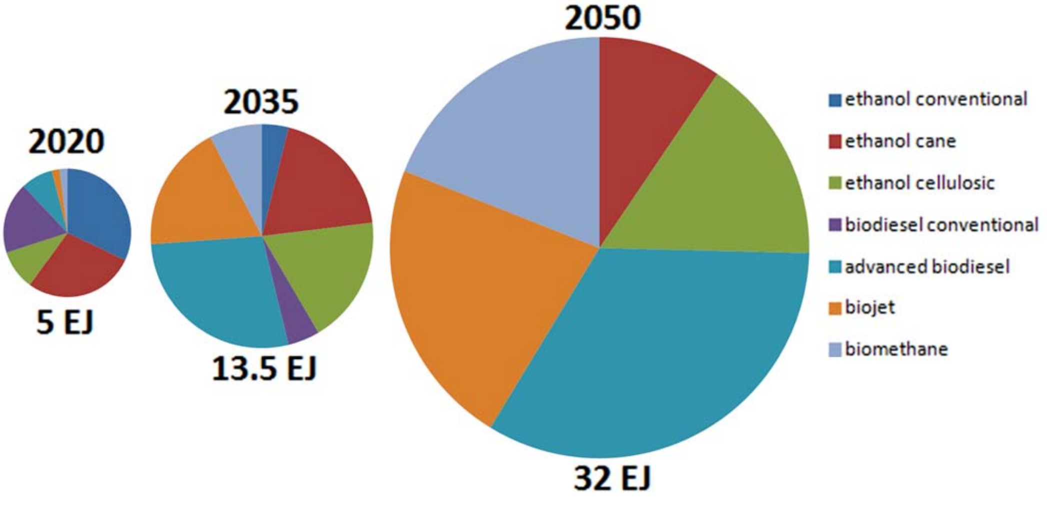

Figure 1.1: One assumption of the changing and growing biofuel market with the number above the chart representing the year and number underneath the total amount of energy in the biofuel market.

Nowadays, energy for transport is heavily leaning on the support from fossil fuels. However, the use of fossil fuels faces a number of challenges, among which the risk of depletion. Several predictions assumed that the so-called 'peak-oil' was reached around 2011. This means that the consumption of oil became larger than the production of it [5]. While discussions about the size of fossil fuel reserves and the necessity of alternatives remain active [6], it is broadly accepted, that an increased energy demand will result in fossil oil scarceness. This will consequently lead to higher energy friction, at the economic, political and environmental level and requires measures to be taken to provide a new and more sustainable source of energy. In parallel to new technological developments in the transport sector, biofuels and especially biodiesels, which has the largest energy density per volume and per weight [1, 2], will play an important role in fossil fuel replacement and therewith part of a more sustainable future.

1.2. History and maturation of biodiesel and other biofuels

The concept of biofuels is as old as the invention of the car engine itself. The first cars ever built were made to function on biofuels, rather than fossil fuels. In such a way, the first internal combustion engine to be patented in 1826 was designed to run on a blend of ethanol and turpentine (derived from pine trees). Henry Ford expected his Ford T to run on ethanol and in 1900 Rudolph Diesel demonstrated his peanut oil powered diesel engine during the world exhibition in Paris. Despite the early attempts of liquid biofuel powered transport, the use of fossil fuels was widely implemented due to the sudden large crude oil availability, the low price, the high efficiency and practicality at this time. These advantages remain until today. It took until the 70's and 80's of the last century before the idea of biofuels was revisited. Among others, an important milestone here was the 'Clean Air Act' by the American Environmental Protection Agency (EPA) in 1970 which set the standard for emissions and fossil fuel additives. Also the 1973-1974 Arab oil embargo and the Iranian revolution (1978-1979) and the resulting oil-scarceness gave the renewed development of biofuels an impulse. The American endeavor for independence of foreign oils pushed the development of biofuels further with the Energy Policy Act (EPACT; 1992). Even today, the biofuel market is influenced by the political climate and the crude oil price.

However, optimizations of the diesel engine towards fossil fuels made vegetable oils inapplicable as an energy source due to their high viscosity. Already in 1937 the Belgium inventor G. Chavanne patented the idea of a transesterification method, in which vegetable oils and short alcohols were converted into fatty acid alkyl esters with glycerol as a byproduct (Figure 1.2). Fatty acid alkyl esters are a very attractive diesel fuel replacement. This transesterification reaction is at the base of the current generation of biodiesel, which is the trade name for fatty acid methyl esters (FAMEs). Currently the two major biofuels available on large scale are ethanol with ~130 billion liter a year in 2014, as a gasoline substitute, which is a natural product of yeast fermentation and FAMEs, with a market size of more than ~30 billion liter a year (in 2014) as a crude oil diesel replacer.

Besides governmentally organized projects and initiatives (like for example 'The Billion Tons Study'; US) for future exploration of the biofuel scope, several industrial initiatives, mostly originating from chemical and/or oil companies, have taken shape and are steadily investing into the development of other biofuels. These cooperations show the commercial interest in sustainable cell factories for fuel production and are an indication for the strong commercial trust in successful and fast advanced biofuel implementation in a short future. The political support to use current biofuels and to develop the next generation sustainable biofuels by academic and industrial researchers might be another motivation to position this generation biofuels on the market and to push forward to the next sustainable but also commercially attractive generation of biofuels. The development and production of isobutanol, an ethanol replacer with a higher energy density, is for example tackled by Butamax^{TM} (www.butamax.com; joint venture of BP and DuPont) and Gevo (www.gevo.com). Dupont is also developing the production of 1,3-propanediol. BP and Verenium Corporation (www.verenium.com) cooperate on second generation bioethanol and ExxonMobil entered a joint venture with Synthetic Genomics on the production of microalgae based biodiesel. Amyris Inc (www.amyris.com; large shareholder: Total) is paving the road for the application of isoprenoid derived products, i.e. farnesene (biodiesel), whereas LS9 is producing fatty acid derived products in *E. coli* but was recently acquired by Renewable Energy Group (REG; www.regi.com). Other initiatives, like Joules Unlimited (www.jouleunlimited.com) are using phototrophic microbes for energy conversion. There are many other companies like Codexis (www.codexis.com), Raizen (www.raizen.com), Solazyme (www.solazyme.com), Mascoma Corporation (www.mascoma.com), Synthetic Genomics (www.syntheticgenomics.som), Novozymes (www.novozymes.com), and Genencor (www.biosciences.dupont.com) that are exploring the biofuel market.

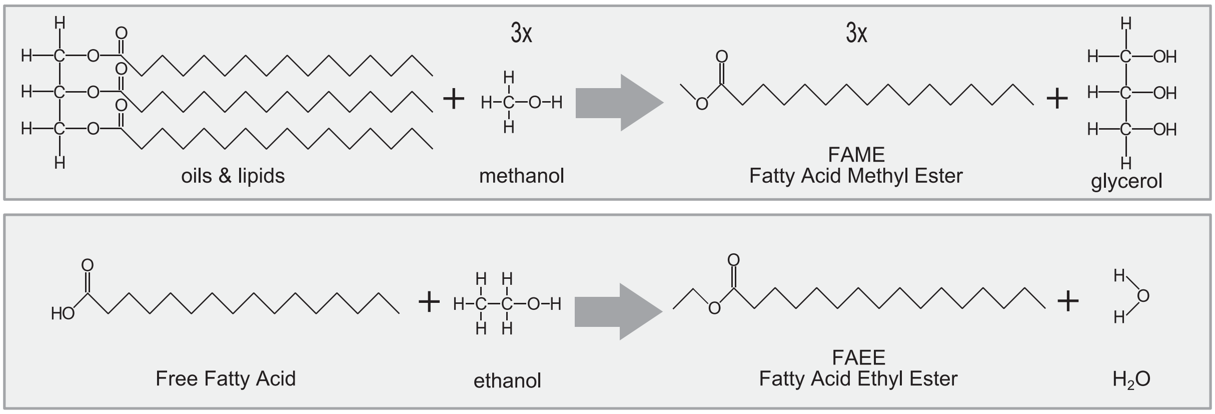

Figure 1.2: Chemical reaction of FAMEs from oils and lipids and biochemical conversion of FAEEs from free fatty acids.

1.3. *Advantage of FAEEs*

Novel fuel molecules (advanced biofuels) should possess superior fuel and operational properties to compete with the current fossil fuel market. Both major currently available biofuels, ethanol and biodiesel, however, suffer from major drawbacks. The short chained ethanol for example has lower molecular properties for combustion purposes than crude oil gasoline and can therefore only be blended into this fuel. Therefore it is, among others, important to design new sustainable fuels and extend the variety of fuels with fuel molecules with improved physical properties to achieve a high energy density, a low octane number and a suitable viscosity. Candidates for advanced biofuels are beyond others longer chain alcohols (1-propanol, 1-butanol, isobutanol, 2-methyl-1-butanol, isopropanol etc.), biodiesels (fatty acid ethyl esters (FAEEs) or (isoprenoic-) farnesane) and alkanes or alkenes. Furthermore it is important to have a reliable, storable, flexible, safe and clean fuel that can compete economically with fossil fuels. It is important to note that each transportation node requires a fuel with specific properties. Additionally, there are fine-tuned differences required within one transportation node due to, for example geographical location. A general rule of thumb is that the faster the transportation the higher the quality of the fuel should be.

Next to the chemical and physical properties of the fuel molecule, the reliability of the substrate source is also important. The current process of FAME production requires a large supply of lipids. These lipids are mainly harvested from rapeseed and soybean plants but the vegetable oils can also be obtained among others from palm, coconut, peanut and sunflower. However, the above mentioned substrate sources do not yield sufficient amounts of vegetable oil per (agricultural) area and are therefore not able to supply the large amount of lipids necessary for bulk FAME production. The second metabolite, methanol, is currently obtained from fossil oil resources and is as such a less sustainable substrate. Another disadvantage is the large amount of glycerol being produced during the formation of FAMEs. An attractive alternative is the production of fatty acid ethyl esters (FAEEs), an equally well suitable biodiesel. FAEEs can be produced by microbial cell factories, which use sugar as a source of energy. Sugars can be grown and harvested more efficiently than lipids and are currently also used for several other products using cell factories as a production platform. As a substrate source, sugars are therefore more flexible, storable and reliable than lipids. During microbial production, the precursors, acyl-CoA (activated form of free fatty acids (FFA)) and ethanol are converted by a transesterification reaction, catalyzed by a wax ester synthase into FAEE and water [7]. No byproduct is formed and therefore the theoretical carbon yield of the transesterification reaction is 100% (Figure 1.2), which is around 5% higher than the yield from the classical transesterification reaction converting vegetable oils and methanol into FAME and glycerol. The byproduct, glycerol, is responsible for the lower yield during the classical chemical reaction.

1.4. *The microbe – choosing between fat, smart and robust*

Already for centuries, humans have been selecting microbes with a desired phenotypical traits and used them for different, industrial, processes. One of the first examples of the cell factory concept was the production of penicillin by *Penicillium chrysogenum*. During the Second World War there was a high demand for antibiotics for wounded soldiers. The *P. chrysogenum* cell factory was suitable of producing penicillin and could therefore 'save many lives'. This would not have been possible by chemical synthesis of the antibiotic, which was only established in 1957. Another important milestone was the production of the first 'heterologous' protein, human insulin for diabetic patients, produced in a recombinant *E. coli* cell factory in the early eighties [8]. Before, animal insulin was extracted from mostly pig pancreas, which was difficult to supply and had different activity due to the fact that it was animal insulin. A third microbe with large industrial impact is the yeast *Saccharomyces cerevisiae*, which has been optimized for the production of ethanol, resulting in yields close to the theoretical yield. *S. cerevisiae* is furthermore very robust against several industrial stresses and has been (genetically) engineered for many other production advantages. Nowadays, some of the most widely used, heterologous cell factories include *E. coli*, *S. cerevisiae*, *Bacillus subtilis*, and *Streptomyces coelicolor*, but many other hosts exist for different purposes. Overall, a broad range of (microbial, mammalian and insect) cell factories have specific physiological traits and have therefore been incorporated to the cell factory family [9, 10]. From a general industrial point of view, algae, fungi, psychrophilic bacteria, mosses and others are gaining in interest [11-14].

For microbial production of FAEEs, the question what microbial host to use is a relevant one. In general, the product, the metabolic conversion and the substrates affect the choice for a host. For the direct, cellular conversion of sugar into FAEEs several factors are important. First of all, FAEEs are supposed to become a bulk product. Therefore the organism of choice should be able to handle large scale and long term cultivation conditions in low-cost media. Therefore the host has to be genetically stable while expressing heterologous pathways. Additionally, the conversion of glucose into FAEEs requires that most of the consumed carbon is used for the production of the long fatty acid chain and the minor part being converted into ethanol. An organism with the desired metabolic pathway or that allows introduction of the pathway is required. One possibility is an oleaginous organism, which produces large amounts of lipids. Examples of oleaginous organisms which were reported to accumulate lipids up to 65–75% of their cell dry weight (CDW) are *Botryococcus braunii*, *Gordonia sp.*, *Humicola lanuginose* or *Lipomyces starkeyi* [15-19]. Microbial hosts, for industrial purposes, should in general be genetically manipulated to improve product yield, titer and productivity, and additionally have to introduce the FAEE pathway. It is therefore inevitable to have (good) genetic tools available to manipulate the host. Unfortunately, above listed organism do not fulfill these requirements.

The oleaginous yeast *Yarrowia lipolytica* stores lipids up to 36% of CDW and is used as industrial workhorse and could therefore be an attractive host for microbial FAEEs production [20-22]. Additionally, its genome has been sequenced [23] because of increased industrial interest for e.g. citric acid production, protein production, intracellular lipids and bioremediation [24, 25]. However, there is a necessity to metabolically engineer *Y. lipolytica* for the production of FAEEs because it naturally contains no wax ester synthase and is not able to produce ethanol, one of the FAEE precursors during direct *in vivo* conversion from sugars. Genetic modifications, however, are not straight forward in *Y. lipolytica* [26, 27]. Because of this lack of a decent genetic tool-box no direct conversion from sugars into FAEEs has been reported in *Y. lipolytica* so far. The realization of oleaginous hosts for the production of FAEEs is unsuitable within a close future because of the missing valuable engineering knowledge of these organisms.

In contrast, *S. cerevisiae* and *E. coli* were not only used as microbial platforms for cell factories for many years but also as model organisms for fundamental research. For both organisms, the availability of an established genetic toolbox, large physiological experience, accessibility to metabolic engineering and growth on simple inexpensive medium with minimal additions increased their applicability as industrial host [7, 28-31]. Due to the fact that *E. coli* is a small and therefore fast growing organism, due to its lack of compartmentalization and due to its easily accessible DNA, it would be very suitable for engineering the FAEEs pathways in this microbial host. The first examples of FAEEs production in a heterologous organism were therefore also from *E. coli* research [32-34]. However, *E. coli* has the disadvantage of being susceptible to attack by bacteriophages and therefore its performance in large scale and long term cultivations in low-cost media is suboptimal.

The yeast *S. cerevisiae* is not only widely accessible for metabolic engineering purposes, but also has a long history of being used in industrial large scale fermentations and therefore represents an ideal candidate as a FAEEs producing platform. *S. cerevisiae* is a natural ethanol producing microbe, however, only 10% of its CDW consists of lipids [35]. Therefore, improving the availability of acyl-CoA for the formation of FAEEs is considered to be the main engineering challenge in *S. cerevisiae*. The combined knowledge of bioprocess engineering and metabolic engineering together with the availability of a large toolbox provides an excellent starting position for the creation of an economically competitive production platform for the biodiesel FAEE.

1.5. The microbial cell factory process

The successful implementation of any microbe in an industrial setting should address three major points: the feedstock, the bioconversion process and the product purification. Only optimization of all three fields will result in a successful, economically feasible and competitive process. A centralized position in the interplay is reserved to the microbe, which has to interact with its substrate and convert it into the product, and which dictates the downstream processing, and is therefore attracting most engineering attention (Figure 1.3).

1.5.1. Feedstock

In general, the most costly part of the cell factory process is the substrate provided to the microbes. Therefore, the economical success of microbial cell factories for bulk products is dependent on the ability to consume cheap substrates from cellulosic biomass. While microbes can consume most exotic substrates, *S. cerevisiae* prefers to consume energy rich glucose. Additionally most microbes cannot or have difficulties consuming sugar mixtures (C5 and C6) but recent engineering of yeast *S. cerevisiae* did not only enable successive consumption of both sugars, but also enabled the co-catabolism of these sugars [36, 37]. However, for bulk products like FAEEs, pure glucose is too expensive and consequently cheaper feedstocks such as sugarcane (Brazil), corn (Northern America) or sugar beets (Europe) are used. As *S. cerevisiae* cannot consume large polysaccharides, these need to be depolymerized mostly by cellulases and hemicellulases into monosaccharides before cellular uptake and conversion [38, 39]. On a longer term, microbial cell factories are meant to consume substrates (from specially designed crops) that are non/less-competitive with food consumption and in a long future photoautotrophic mechanism might be preferred [40]. There are large engineering efforts to create a better interaction between the release of sugar mixtures from plant materials and the microbes consuming them. Main efforts focus on fast and efficient consumption of multiple sugars by microbes, enzyme technology for lignocellulosic degradation, design of increased cellular robustness to stand the high impurities in the lignocellulosic feedstocks and design for fast growing, non food-competitive, high sugar containing plants [41, 42].

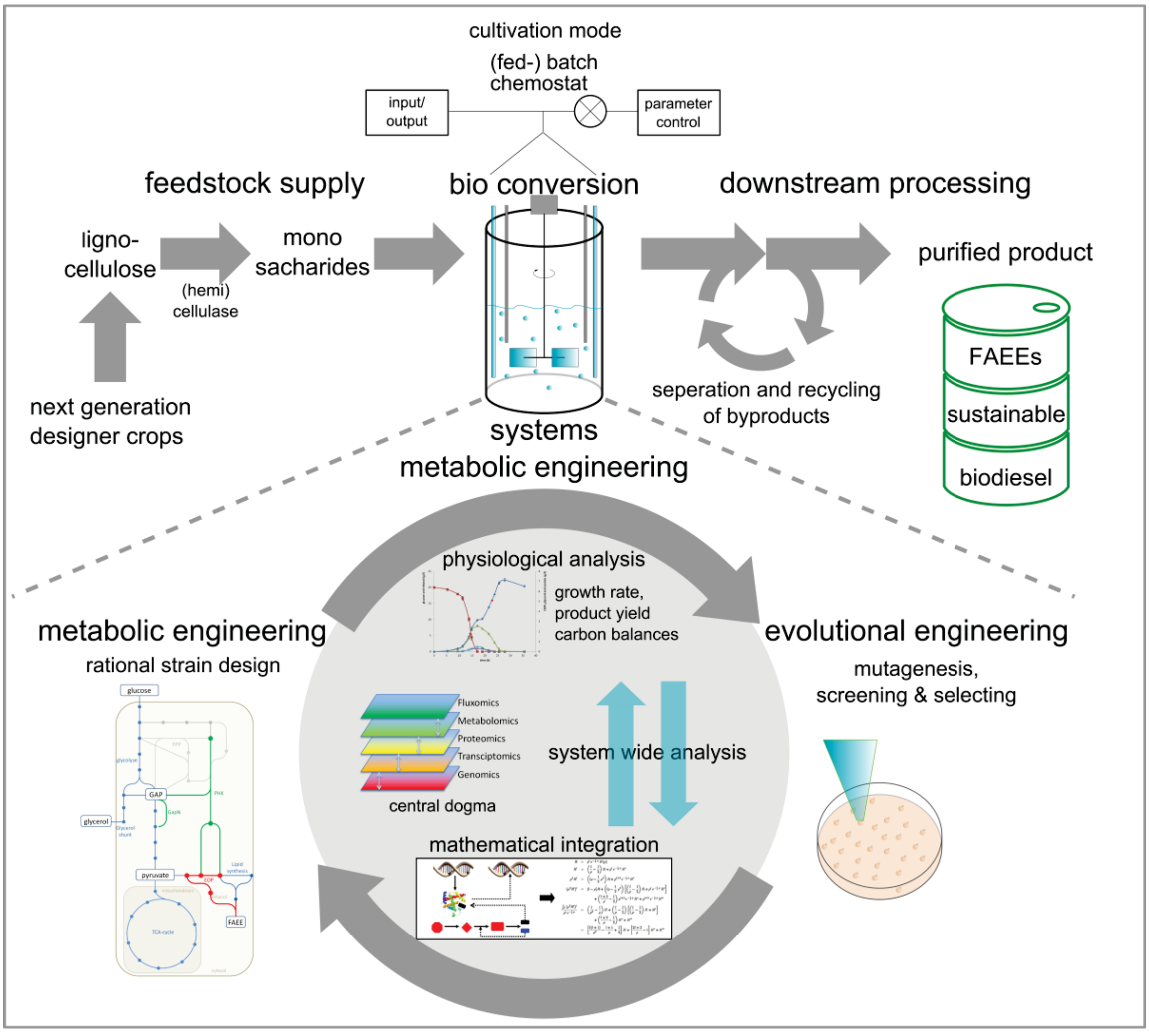
cultivation mode
(fed-) batch
chemostat
input/
output
parameter
control
feedstock supply
bio conversion
downstream processing
ligno-
cellulose
(hemi)
cellulase
mono
sacharides
next generation
designer crops
purified product
FAEEs
sustainable
biodiesel
seperation and recycling
of byproducts
systems
metabolic engineering
physiological analysis
growth rate,
product yield
carbon balances
metabolic engineering
rational strain design
evolutional engineering
mutagenesis,
screening & selecting
Fluxomics
Metabolomics
Proteomics
Transciptomics
Genomics
central dogma
system wide analysis
mathematical integration
glucose
GAP
glycerol
pyruvate
FAEE
TCA cycle

Figure 1.3: The connection between the different fields of expertise that come together for the construction of cell factories.

1.5.2. *Downstream processing*

The fermentation broth is a mixture of water, yeast, salts, the product and other metabolites from which the target molecules have to be separated and purified. Dependent on the chemical properties of the compounds of interest and the composition of the fermentation broth the separation can be challenging and costly. Especially energy consumption can add up to the total costs [43]. Therefore it is essential to design a downstream process with minimal product loss that results in the desired purity of the product, at lowest cost possible. FAEE molecules are hydrophobic therefore create separate layer when secreted by the cell and can therefore be separated and purified relatively easily [40]. More information on the economics of down-stream processing can be found in [43].

1.5.3. Bioconversion process

The microbial conversion of substrate to product is the core of the cell factory process and due to its high impact, large engineering efforts are performed in this area. The harvest of a product yield as close to the theoretical yield as possible is important for economical feasibility and additionally, ‘time is money’ and therefore the product should be converted in a minimum amount of time. Essential parameters for an economically feasible process are the product yield, the titer and the production rate [44].

1.6. Microbial design

1.6.1. Engineering the metabolism

Traditionally, microbial cell factories were improved by mutagenesis and screening for specific properties of microbes, like high production rates, yields and titers. From the 1980s onwards the development of cell factories brought its first engineering successes by targeted and rational strain design. Nascent recombinant DNA technologies supported the first cell factory successes, as an example the production of the earlier mentioned recombinant insulin. However, strain engineering for biotechnological purposes has a fundamental different objective than the objective of the cell. Often the engineering purpose acts against the cells nature, for example in the case of shifting the metabolism entirely towards the product and minimizing byproduct formation. Knowledge of targeted genetic modifications, computational modulations, and cell physiology is a powerful combination, which led to the development of the field of metabolic engineering, originally coined by Bailey and Stephanopoulos [45-48]. The field of metabolic engineering, which deeply penetrated the area of microbial cell factories and became a widespread platform for the production of molecules, has been discussed and reviewed extensively and has also been defined differently [49]. Here the definition given in the metabolic engineering textbook by Stephanopoulos, Aristidou and Nielsen was followed.

Metabolic engineering
‘*The directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology.*’[35]

The performance of many different cell factories, including the FAEEs production in *S. cerevisiae*, was improved by regulation of the expression of endogenous and heterologous genes and specific gene knockouts. However, without considering the whole cellular system the microbial cell factory is prone to suboptimal performance and therefore it became clear that system wide behavior of the cell is an important factor for the consideration of metabolic engineering targets.

1.6.2. System wide perspectives

The field of metabolic engineering, and therefore the creation of new phenotypes, is driven forward by understanding complex cellular systems. The system wide perspective provided by systems biology, which shed light into hidden cellular properties, has several advantages compared to traditional molecular biology tools in which only few metabolic engineering features could be evaluated at the same time. Systems biology has therefore changed and advanced the analysis of cellular phenotypes for industrial biotechnology and metabolic engineering.

Omic technologies are the driving power for analyzing large numbers of cellular components for industrial cell factories. Transcription analysis is the most commonly used technique in metabolic engineering and demonstrated its value as it screens genome wide messenger RNA levels which allows integration with genome wide metabolic models [50-55]. The first system wide analysis at the transcriptome level of a FAEE cell factory was performed during this work. However, messenger RNA levels and protein levels do not necessary show a direct correlation [56] and therefore the mass spectrometry based proteomics enables the monitoring of large parts of the cellular proteome [57, 58]. The use of mass spectrometry also enables a relative high-throughput measure of metabolic pathway intermediates, which is referred to as metabolomics. However, challenges related to sampling and extraction [59] as well as to obtaining quantitative information about the metabolite levels remain [60]. The different omic techniques can reveal essential parts within a specific cellular layer and especially metabolic fluxes represent the cellular phenotype as integrated output of all cellular components, such as genes, transcripts, proteins and metabolites [61-63].

An import milestone for recombinant DNA techniques was complete genome sequencing of organisms. Genome sequencing enables the possibility of reversed engineering which in turn facilitated the identification of driving mutations behind adaptively evolved strains [64, 65]. The more recent genome wide RNA sequencing improved expressing profiling of organisms and might provide more information for the integration of metabolic networks with regulatory networks or cellular stress and will further optimize the understanding of cell factory engineering [66]. The use of additional omics technologies for detailed phenotypic analysis is still important in order to identify driving mutations, despite the existing possibility to identify genomic mutations [65], in particular because adaptive evolution often results in appearance of many silent mutations that can be difficult to filter out. The combination of different omics technologies has been found to be very worthwhile to understand how gene deletions or gene overexpressions lead to a certain phenotype. This has been demonstrated to be valuable for gaining insight into complex regulatory pathways involved in controlling metabolism. Investigation of the yeast protein kinase Snf1 [67], the identification of transcription factors determining metabolic fluxes in yeast and *E. coli* [68, 69] and the interaction between Snf1 and protein kinase Tor1 in yeast [70] have been good examples for this. As it will be discussed later on, for example Snf1-dependent phosphorylation attenuates the activity of acetyl-CoA carboxylase and therefore plays an important role for the production of FAEEs in *S. cerevisiae*.

Since the foundation of the field of metabolic engineering, mathematical modeling has been an integral part of it, but especially with the advancement of genome scale metabolic models the models have been directly coupled with identification of metabolic engineering targets and also allow the evaluation of the global physiology of a cell. The mathematical modeling of metabolic networks [55, 71-74] and the development of online tools [62, 63] has been reviewed extensively.

The rather new field of synthetic biology might enable the creation of novel regulatory mechanism for metabolic pathways and therewith maximizing the metabolic flux and the strain development [75]. The ability to turn on and off cellular metabolism, adjusting it to its environment and dynamically control its (heterologous) pathways will push the creation of flexible cell factories forward [76, 77]. Rapid development of a technological platform for microbial design for cell factories will enhance by integration of the field of metabolic engineering, systems biology screening and synthetic biology tools, also described as systems metabolic engineering [31].

1.7. FAEE producing cell factories

The early production of FAEEs in microbial hosts was established due to the appliance of above briefly described metabolic engineering strategies. These first metabolic engineering projects were performed in *Escherichia coli* [32-34] and efforts on combining strategies resulted in a highly engineered *E. coli* strain with cytosolic expression of thioesterase ’TesA, overexpression of acyl-CoA ligase, inhibition of β-oxidation, introduction of the alcohol synthesis pathway from *Zymomonas mobilis*, and high expression of a heterologous wax ester synthase (*atfA*), which led to production of 674 mg FAEEs/l during cultivation on glucose [78].

For several reasons, which were discussed earlier, researchers switched to the production of FAEEs in the yeast *S. cerevisiae* and therefore some of the engineering history is briefly described here. The disruption of acyl-CoA competing pathways (see chapter 3 for details), in combination with the over expression of the heterologous *ws2* from *Marinobacter hydrocarbonoclasticus* resulted in a yeast strain with the inability to synthesize storage lipids and an FAEE production of 17.2 mg/l [32, 79, 80]. Another research combined expression of Acc1^{S1157A,S659A}, an acetyl-CoA carboxylase variant with partly abolished posttranslational regulation, and *ws2* resulted in an FAEE production of 15.8 mg/l [81]. Furthermore, overexpression of native *ACC1*, *FAS1* and *FAS2* and the deletion of the β-oxidation pathway in combination with expression of a wax ester synthase from *Acinetobacter baylyi* (AtfA) resulted in an FAEE production of 5.44 mg/l [82]. Following up on the previous work, an abolished β-oxidation pathway, disruption of acyl-CoA synthetase gene *FAA2* and acyl-CoA transporter gene *PXA2* as well as elimination of acyl-CoA binding protein Acb1 in combination with expression of codon optimized *atfA* under nitrogen limitation cultivation conditions resulted in a FAEE titer of 13.9 mg/l [83]. One study focused on increased integrated copy numbers of the wax ester synthase, which were randomly integrated into the genome, and resulted in a total of six copies of *ws2* and a FAEE titer of 34 mg l^{-1} [84]. The brief description of metabolic engineering strategies on the previous performed work indicates the interest to establish a FAEEs producing platform in *S. cerevisiae*.

1.8. Outline of thesis

This work shows the essence of microbial cell factory design with a focus on the central part of engineering the microbe, *S. cerevisiae*, for the establishment of fatty acid ethyl ester production. During the introduction (Chapter 1) argumentation and reasoning for the concept of the microbial cell factory in a *S. cerevisiae* host for the production of fatty acid ethyl esters was given. The following chapters describe the research performed during this study. The different chapters are organized into different categories that play an important role for the development of a FAEE cell factory.

Chapter 2 **'metabolic pathway engineering'** describes the necessity of several techniques which are required for the construction of long and complicated pathways. The development of the molecular biology techniques is necessary to reduce the time and therewith the investment involved for constructing new cell factories. During the time of this study several different techniques were used for the construction of the strains described in chapter 2. However, not all microbial techniques were included in the publications (**paper II** and **paper III**) and therefore metabolic pathway engineering is reviewed in a broader perspective, here.

Chapter 3, **'yeast metabolism and fatty acid ethyl esters'** describes the core of *S. cerevisiae's* metabolism and the pathway engineering strategies for the development of an industrial microbial system which is able to produce the heterologous product, FAEEs. First of all, wax ester synthase is described as the key-enzyme for the conversion of fatty acyl-CoA and ethanol into FAEEs. Several endogenous as well as heterologous genes were up-regulated to improve the supply of metabolite intermediates ethanol and acyl-CoA. Additional, a heterologous pentose phosphate pathway was introduced into the host cell which rechanneled the carbon flow through the pentose phosphate pathway, producing NADPH as a byproduct. Finally, different metabolic engineering strategies were combined and stably integrated into the yeast chromosome. The results presented in this chapter find their origin mostly in **paper II** and **paper III**.

Chapter 4, '**phenotypic characterization**' describes the techniques involved to understand the metabolic changes in a constructed cell factory. First of all, phenotypes, physiological behavior, and the important role of systems wide 'omic' analyzing methods were elucidated. The results presented here demonstrate the importance of systems wide transcriptional regulation for the production of FAEEs in *S. cerevisiae*. The correct interpretation of the combination of the analytical results leads to new rounds of strain engineering and strain improvement. The results presented here find their origin mostly in **paper IV**.

The last chapter, '**conclusion and future perspective**' explains the lessons learned during the performed studies and looks towards the scientific challenges that lie ahead for paving the ideal road for the implementation of the FAEE cell factory.

2. Metabolic Pathway Engineering

2.1. Background

To construct microbial cell factories one of the most important elements is the engineering of the metabolic pathways. Well engineered cell factories have significant advantages in physiological characteristics and therefore reach for example better yields, titers or robustness. The ease to apply genetic modifications in *S. cerevisiae* is essential for industrial competitiveness of these yeast cell factories. Heterologous genes can be inserted, endogenous genes or promoters can be deleted/replaced, the compartmentalization of the encoded proteins can be changed and enzymes can be switched on or off for regulation purposes. Typically, a combination of several of these strategies forms the core of *de novo* pathway engineering [85]. Where traditionally the modification of one gene was targeted within the field of genetic engineering, the field of metabolic engineering approaches the overall efficiency of the metabolic pathway in connection to the fitness of the microbial cell factory. Recent developments in the field tilt towards an engineering approach with several parallel modifications of an increased amount of sensitively regulated genes, which could be adapted fast towards new understanding of the microbial metabolism [77, 86]. Therefore and due to the fact that pathway engineering is a highly costly and time consuming workmanship, there is a parallel demand for the development of fast, cheap, reliable, robust and high-throughput metabolic pathway engineering techniques. In this chapter, the development of pathway construction is discussed to give a view on the challenges, drawbacks and hurdles to be dealt with.

2.2. Pathway engineering's core architecture

The fundament of gene expression needs to be well understood in a detailed way before more complex multi-gene modifications can be performed. In *S. cerevisiae* it is possible to express genes (or open reading frames (ORFs)) from plasmids as well as from genomic DNA. In both cases, when expressed, it is recommended that the functional gene sequence is preceded by a promoter and an optional Kozak sequence [87] to increase transcription and followed by a terminator. The DNA sequence starts with the ATG start codon whereas it ends with a terminating stop codon (TAA, TAG or TGA). The gene of interest (GOI) forms the core of the construct but can be accompanied by different, exchangeable promoters and terminators. Especially the promoter site is important for the regulation of the gene expression and therefore of special interest in metabolic engineering.

In general, for *S. cerevisiae*, two different types of promoters can be distinguished; constitutive and regulatory promoters. The choice of the promoter is essential for achieving a desired expression level of the gene, which can differ by four orders of magnitude [85]. The strongest and therefore most commonly used constitutive promoters are among others *pPGK1*, *pADH1* and *pTEF1*, which have been used during this work **(paper II, III and IV)** for the overexpression of the genes *ws2*, acs_{SE}^{L641P}, *ACB1* (all preceded by *pTEF*) and *ALD6*, $ACC1^{ser1157ala,ser659ala}$ (both preceded by *pPGK1*). These strong promoters were used to demonstrate an increased yield of FAEEs and product intermediates and were therefore important. However, in the future it will be necessary to fine-tune the individual gene expressions of the overall pathway, in which weaker constitutive promoters and regulatory promoters will become more meaningful. Regulatory promoters are commonly induced by specific signals, but as well by environmental changes. For example *pHXT7* (**paper II**) or *pADH2* [88] are glucose repressed and will be useful in industrial fermentative applications to induce genes in pathways needed once glucose is depleted. A truncated version of the promoter *HXT7*, however prevents glucose repression and is therefore active during glucose consumption [89]. To improve the production of FAEEs this truncated version of *HXT7* was used for the expression of gene *ADH2* in **paper III and paper IV**. During industrial processes the costs of induction of such promoters might be a limiting factor and therefore it is important to search for promoters which are regulated by cheap inducers [77]. In general it should be considered that the application of strong promoters might lead to high yields and titers, but that after evaluation and fine-tuning of the metabolic pathway the adaption towards regulatory or weaker promoters for individual genes, part of the overall pathway, might lead to an increased productivity. In addition, it should be kept in mind that unnecessary overexpression of genes and plasmids might also cause stress, by drain of co-factors or other metabolites, by accumulation of (toxic) intermediates, by negative feedback loops or by the development of instability for the cell. However, it seems unlikely that FAEEs are toxic to *S. cerevisiae* at the concentrations produced here (see next chapter). There was no negative effect on cell growth when 1 g/l of myristic acid ethyl esters were added to the medium (data not shown). In **paper II**, two assembled pathways (resulting in plasmids larger than 15 kb) were expressed on 2-micron plasmids resulting in a high copy numbers and therefore high expression. Although it is well known that expression of pathway enzymes from self-replicating plasmids might cause a metabolic burden on the host cell and that both plasmid size and promoter strengths influence the stability of the plasmid and therefore the gene expression when propagated in *S. cerevisiae* [39-41] most previous work on modifications for FAEE production in yeast were based on plasmids. The successive segregational and structural instability makes these strains non-suitable for industrial fermentations [90-92]. Strains that were constructed based on plasmids (in **paper II**) indeed showed a high clonal variation (see chapter 3).

Fine-tuning and balancing of the expression levels of individual genes within a larger metabolic pathway is essential to reach optimal functionality of metabolic pathways, and therefore there is extensive research to create an enlarged promoter library for different inducible promoters as well as for stronger and weaker constitutive promoters. New promoter properties can for example be achieved by combining regulatory elements derived from different promoters and new promoter-libraries are created by error-prone PCR from existing yeast promoters [85, 93, 94].

Another difficulty is the fact that DNA sequences coding for functional proteins in an original organism might not lead to functional expressions in the *S. cerevisiae* host. Therefore, codon optimization is essential for the functionality of heterologous genes in *S. cerevisiae*. Understanding codon-optimization will dramatically improve predictability of expression of

ORFs but it should be kept in mind that other parameters, like the choice of promoter region also controls gene regulation [95-100]. All in all, understanding what influences the expression levels of (heterologous) genes, will improve the final outcome of metabolic engineered pathways including multiple modifications.

The proper termination of transcription is required for the stability of mRNA and is therefore also important for the expression level. However, the choice of a terminator is less sensitive towards the final expression level of a gene compared to the choice of promoter. The terminators applied in **paper II, III and IV**, *tADH1*, *tCYC1* and *tTEF1*, belong to the most commonly used terminators in *S. cerevisiae* [85].

In general there are two major expression platforms for expressing genes and pathways in yeast. A very common way is plasmid based expression. Overexpression of genes typically relies on high-copy yeast episomal plasmids (YEps) which come with the risk of an energy burden by transcription and translation costs and a metabolic burden due to drain of nucleotides and amino acids. Secondly, expression of genes after integration into the host genome constitutes a more stable option for a cell factory with consequently several advantages on plasmid based expression of genes [90, 101, 102]. Plasmids, however, play an important role for the assembly of metabolic pathways, which involve one or more promoter-gene terminator bricks.

Results in **paper III** demonstrated that chromosomal integration of the metabolic engineering strategy into the chromosomes of *S. cerevisiae* resulted in stable expression and it was therefore possible to quantitatively evaluate complex engineering strategies involving several different genetic modifications. This combined overexpression of the six genes on two 2μ plasmids with a total size of 15 kb and 18 kb however, decreased the yield of FAEE in *S. cerevisiae* (unpublished results) and was therefore due the instability of plasmid expression not possible. The study also demonstrated much reduced clonal variation of the integrated strains in comparison to the plasmid-based strains used in previous studies. A very good starting point for further engineering was established due to the success of chromosomal integration e.g. evaluation of the effect of increased expression of the wax ester synthase, which is likely to be limiting production the engineered strains.

2.2.1. Selection markers

While introducing genetic traits into host cells, there is an interest to select on the cells expressing correct insertions, which is performed by the use of marker genes. Different selection markers have been developed for this reason, which includes prototrophic markers (require the availability of an auxotrophic host strain carrying a nonfunctional version of the respective gene; ex.: *URA3, HIS3* or *LEU2*), markers conferring drug resistance, autoselection markers (marker gene is essential for cell viability) and counter selectable markers (selecting for absence of marker gene). An extensive review on selection markers was reviewed by Siewers [103]. Despite the variety of selection markers available in *S. cerevisiae*, mostly only few selection markers are used [103]. During this work, marker genes *URA3* and *HIS3* were used (**paper II and III**). A reason for the limited use of the full variety of selection markers is the availability of background strains that allow the use of different auxotrophic markers. To perform a second round of metabolic modifications (with the same selection marker) on the host cell, the selection marker needs to be recycled. The marker recycling is mainly done in three different ways: direct repeats [104-106] which are looped out by homologous recombination, site specific recombinases in which the *Cre/loxP* system from bacteriophage P1 is most commonly used [107, 108] or bridging oligonucleotides, which is a scar-free approach working with overlapping oligonucleotides, carrying up- and downstream sequences of the marker cassette and homologous recombination [109]. As an example: marker gene *URA3* is able to convert 5-fluoroorotic acid (5FOA) into a toxic compound (5-fluorouracil) and therefore selects on cells without gene URA3 [110]. The recent development for easier recyclable selection markers in *S. cerevisiae*, performed by Jensen et al. (2013) [90] and Kuijpers et al. (2014) [111] indicates the industrial demand for good selection pressure.

2.2.2. Limitation of large pathway assembly

The necessity for each gene in a sequential pathway to individually be regulated by a unique promoter and a terminator creates a major drawback for the integration of large multigene pathways and increases the risk of recombinations between the constructs. A solution to this problem was i.a. given by the development of scattered integration sites along the chromosomes [112], and in addition to this, the development of polycistronic (several genes under the control of one promoter) expression of genes is most interesting (reviewed by David et al., 2014 [113]). However, disadvantages might be, respectively, the limitation of the amount of promoters and terminators for each integration site and the lack of fine-tuning for individual gene expression in the latter case.

2.3. From gene to pathway

Increased availability of genome sequence data and improved metabolic models, which lead to identification of multiple metabolic engineering targets, shifted the limitation of cell factory design towards strain construction [114-116]. Consequently, efforts to improve pathway engineering have been on the rise. Pathway expression, as described earlier, is done on two different platforms, plasmids or chromosomes, where the latter is the preferred method of pathway expression due to above mentioned limitation of plasmid based gene expression. Integration of metabolic pathways into the yeast chromosome has traditionally been performed via cloning into integrative plasmids, which are subsequent linearized before transformed into the yeast, followed by the insertion into the chromosome. Therefore, plasmids take an essential function for pathway assembly. Traditionally, cloning was dependent on restriction-ligation methods; however, the demand for high-throughput pathway assembly methods led to the development of new pathway construction methods. Several methods for pathway assembly are widely used (Gibson cloning [117], SLIC [118] or InFusion [119]), however, here only a couple of recent examples of pathway assembly methods are described.

2.3.1. Uracil-Specific Excision Reagent cloning

Uracil-Specific Excision Reagent (USER) cloning was developed for fast, high-throughput and stable cloning. The development of the method started in the early 1990s with the uracil excision based cloning (ligase free Uracil DNA Glycosylase (UDG) cloning) which represented an alternative to the disadvantages of restriction-ligation cloning [120-123]. The principle of the method was based on PCR amplification of inserts and vector that were constructed with overlapping primers. The primers contained at least four deoxyuridines (dU) which, after treatment with UDG, resulted in 3' single stranded overhangs of the insert, complementary to the single stranded overhangs of the vector. Cross-annealing of vector and insert resulted in successful scar-less cloning [122, 123] but had several limitations preventing it for wide usage [122-124]. Therefore, improvements resulted in a vector and insert with single stranded overhangs with specifically designed nucleotide composition preventing (self-) complementation and resulting in flexible lengths of the overhang permitting the assembly of multiple inserts into the vector. An important milestone for the development of the method was the insertion of a small restriction cassette which enabled, first linearization of the vector using AsiSI restriction enzyme and secondly the creation of specific overhangs by nicking the sequence with Nb.BsmI. An advantage of the use of these two restriction endonucleases prevents the plasmid for proof-reading mistakes that might occur during PCR amplification. The PCR fragments are amplified with *Taq* DNA polymerase or *Pfu* DNA polymerase, which are both able to amplify the dU residue. Recently a (point-) mutated version of PfuTurbo Cx hotstart was created with even slightly better performance (Pfu X7 DNA polymerase). The 3' end single stranded extensions of the PCR products are generated by removing the dU residue by the USER TM enzyme. The deoxyuridine-excision reagent (USER TM enzyme) is a mix of UDG and DNA glycosylase-lyase endonuclease VIII which is able to break the phosphodiester backbone at the 3' and 5' site of the dU [121, 124, 125].

2.3.2. Circular Polymerase Extension Cloning

Circular Polymerase Extension Cloning (CPEC): Restriction digestion, ligation or single stranded overhangs are necessary in several different cloning techniques but also cause difficulties while performing the cloning. To circumvent these techniques, CPEC was developed, which only requires the use of a high fidelity DNA polymerase during the one step simultaneous *in vitro* assembling of multiple inserts into any vector [95, 126]. CPEC solely relies on the mechanism of homologous overlapping sequences (20-25 bp) at the end of the linearized inserts and the linearized vector which form a circular insert(s)-vector product. A limitation of CPEC is the compulsory use of high fidelity proofreading DNA polymerases, which could be problematic, especially with sequences with repeats or GC-rich parts.

Independent of which cloning technique is used, it is important to gain a high efficiency in cloning and therefore reduce false positives. One of the possible reasons to lose efficiency is the reannealing and consequently recirculization of the vectors. While using CPEC, it has been demonstrated that treatment with methylation sensitive *DpnI* restriction enzyme resulted in more efficient cloning [95, 127]. This enzyme digests all methylated plasmids produced by a bacterial host and therefore automatically selects for linearized products. This technique can generally be applied for similar cloning techniques and has already shown its usefulness for USER-cloning in the Nielsen lab.

2.3.3. Ligase Cycling Reaction

Ligase cycling reaction: The development of the ligase cycling reaction (LCR) method for plasmid construction pioneered with the detection of single-nucleotide polymorphisms in genomic template DNA with a high specificity [128-130]. Single stranded oligonucleotides are designed, complementary to the ends of two DNA parts to assemble these DNA sequences. The LCR-process is initiated when the bridging oligonucleotides anneal to the two complementary DNA parts after a denaturation step at high temperature. A thermostable ligase then joins the DNA backbones via a phosphodiester bond in a scar-less manner. This denaturation-annealing-ligating reaction of a single strand DNA serves as a template for the double stranded DNA string and by applying different temperature cycles, many DNA parts can be assembled into complex DNA constructs. Initially only a 40% success rate was achieved with this method [128] whereas later improvement of ligase, PCR and primer conditions resulted in a success rate of 60-100% [86]. This is comparable to the process of homologous recombination in yeast (see section below), however constituting a faster option [86].

2.3.4. Homologous Recombination

Homologous recombination is a process functional in *S. cerevisiae* to secure the repair of a DNA sequence after double strand breakage. Due to the efficiency and simple working procedure the use of *in vivo* homologous recombination in *S. cerevisiae* it was demonstrated for gene cloning, plasmid construction and library creation since the late seventies of the last century [131-140] and was recently investigated by several groups for its potential for pathway engineering on a lab scale as well as for high throughput strain construction. Shoa et al. (2009) pioneered herein with the construction of three distinct functional pathways: a D-xylose utilization pathway (9 kb, 3 genes), a zeaxanthin biosynthesis pathway (11 kb, 5 genes) and the combination of both pathways (19 kb, 8 genes). All efforts resulted in assembly efficiencies of 70-100% on both, plasmids and chromosome [141]. This work was followed by others, for example by assembling nine different constructs that reached up to a total size of 21 kb and 95% correct assembly [111]. Despite the pathway assembly successes reached, incorrect and imprecise genome integration remained a problem for strain engineering programs. Low cloning efficiency was reached by occurrence of false positive clones, caused by re-circularization of plasmid backbones containing all requirements for selection and propagation [139, 140]. Additionally, the lengths of the homologous regions and the number and size of the assembled fragments were considered to be main factors for incorrect assembly of pathways [141-143]. Recent method development introduced the meganuclease I-SceI, which causes a double strand breakage at a specific recognition site within the targeted chromosomal locus [144, 145]. This breakage facilitated the integration of the assembled pathway and led to an radical efficiency improvement from 5% to 95% while assembling a ten-fragment 22 kb construct [146].

Comparative studies have been performed to find out which method is most trustworthy for high-throughput cloning and integration [86, 95]. Therefore metabolic pathways with different sizes and amounts of inserts have been tested for efficiency. A clear conclusion was that homologous recombination and LCR results in a higher efficiency while comparing the correct assembly of 12 DNA parts than CPEC, Gibson cloning or others, independently of the amount or size of fragments and the final size of the metabolic pathway (Gibson isothermal assembly and CPEC showed a clear efficiency drop with 6 DNA parts or more) [86]. However, pathway construction and assembly is highly context dependent and might need different conditions in different situations. Additionally, not only final cloning efficiency is a relevant parameter, but also the efficiency of individual steps, which might differ from method to method.

2.3.5. Different methods for chromosomal integration

Once metabolic pathways have been assembled, the produced genetic fragments need to be transformed into the host and targeted towards the chromosomal positions of interest if stable expression is desired. The constructed fragments are therefore flanked by an up- and downstream recognition sequence [147]. A single or multicopy integration site can be selected for this purpose. As an example, in order to increase the copy number of the gene *ws2*, integrated into the *S. cerevisiae* chromosome, an integration cassette including the usage of a weak promoter and antibiotic selection was used for multiple rounds of transformation and strain evolution based on elevated antibiotic resistance [148-150]. The strain CB2I20, used in this research (**paper II and paper IV**), was evolved with stationary increased copy numbers of the *ws2* gene to a final copy number of 6 [84]. Awareness about the importance of stable integration led to a recent research investigating 14 integration sites situated in between essential genes on chromosomes X, XI and XII which reduced the risk of intra-chromosomal recombinations [112]. The beta-galactosidase activity was measured during this comparison study for strains with the *lacZ* cassette integrated at the 14 integration sites [112] and the sites emerging were used for integration of the FAEEs producing pathway in **paper III** (see next chapter).

2.4. Pathway assembly and expression experience during this work

During this work several yeast strains have been constructed. At the starting point of the project, restriction-ligation cloning was still common practice (in our lab), and therefore I could follow the pathway engineering development from small towards large genetic fragments. At the same pace, different cloning strategies were established and therefore first-hand experience demonstrated the difference between practical and theoretical cloning efficiency. During the same period a growing realization for the necessity of integration of genes and pathways into the genome was established.

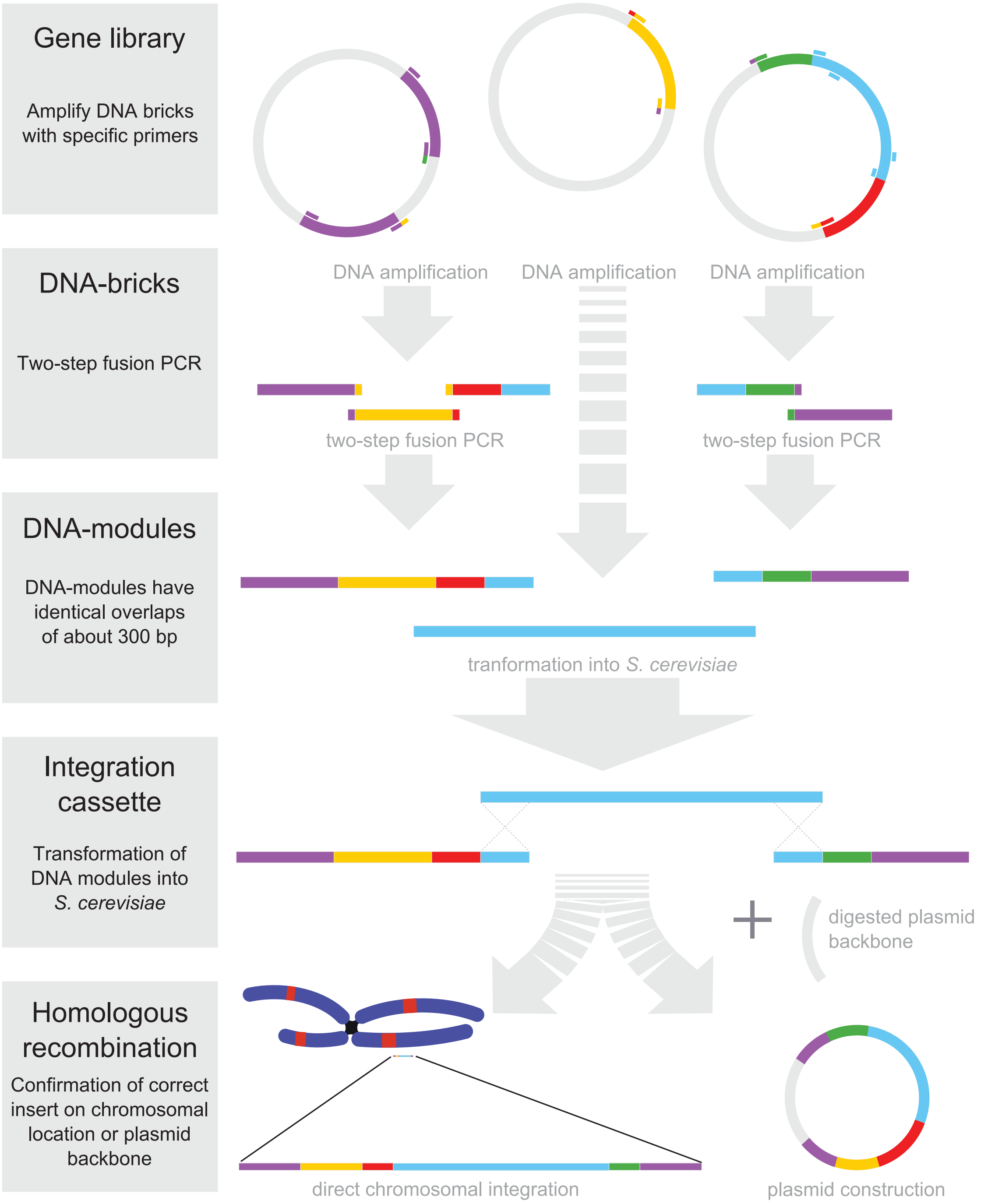
Gene library
Amplify DNA bricks with specific primers
DNA-bricks
Two-step fusion PCR
DNA-modules
DNA-modules have identical overlaps of about 300 bp
Integration cassette
Transformation of DNA modules into S. cerevisiae
Homologous recombination
Confirmation of correct insert on chromosomal location or plasmid backbone
DNA amplification
DNA amplification
DNA amplification
two-step fusion PCR
two-step fusion PCR
tranformation into S. cerevisiae
digested plasmid backbone
direct chromosomal integration
plasmid construction

Figure 2.1: Pathway assembly pipeline for linear chromosomal integration or plasmid construction [151].

While the yeast strains described in **paper II** expressed the genetic modifications from a plasmid based platform, in **paper III** all modifications were integrated into the yeast chromosomes. In agreement with the comparative studies that have been performed between different pathway assembly methods, homologous recombination has been most successful and was therefore used for the pathway construction pipeline is outlined as shown in figure 2.1. Different than in some companies (definitely true for Amyris Inc), pathway construction in academia is, for several reasons, performed in more low throughput measures. Therefore most important argument for the application of methods is high efficiency but of course economics or speed also have to be considered.

Here, three different pathways (described in **paper III**) were constructed, assembled and integrated into the chromosomes of two different background strains, CEN.PK113-11C and JV04. The first pathway contained the gene *ws2* and the strains carrying this integration cassette were used as a reference to the other strains. The next strains contained a pathway with genes *ADH2*, *ALD6* and acs_{SE}^{L641P} in addition to the *ws2* gene, while the last strains contained genes $ACC1^{S1157A,S659A}$ and *ACB1* in addition to the previously named genes. The yeast strains that were constructed with help of linear chromosomal integration cassettes, carrying above described genes. All strains are listed in table 2.1 and all integration cassettes are shown in figure 2.2. Integration cassettes 1, 2 and 3 had a total length of respectively 4.915 bp, 12.033 bp and 11.614 bp and where integrated into background strains chromosomal position XI-3 (cassette 1 and 2) and XI-5 (cassette 3) of CEN.PK113-11C and JV04 with an efficiency of respectively 84%, 58% and 63%, 56% and 80%, 8%.

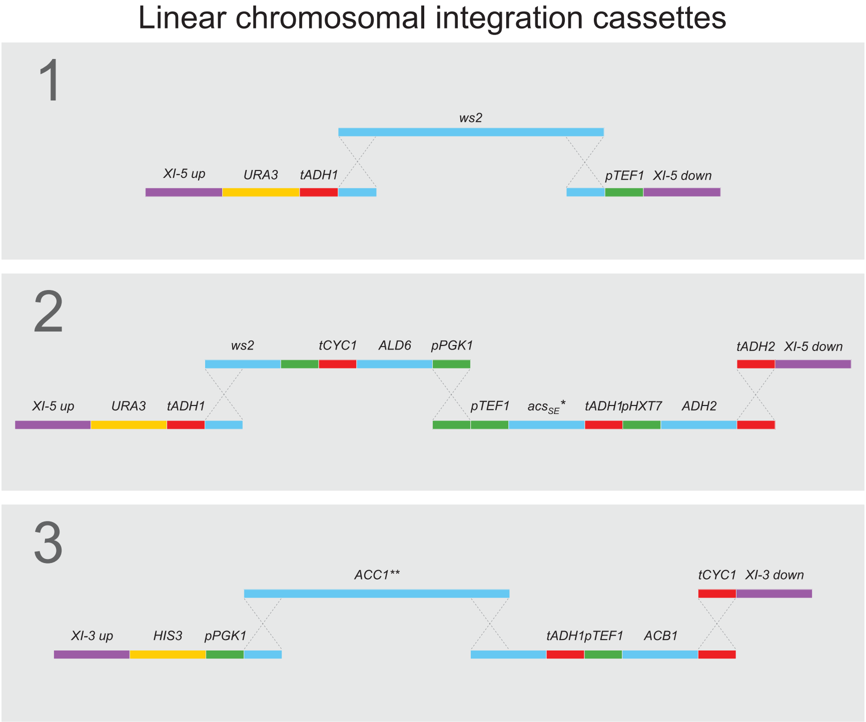

Figure 2.2: Linear chromosomal integration cassettes assembled during this work [151].

Figure 2.1 shows the pathway assembly and integration pipeline which was used here. To construct the linear chromosomal integration cassettes, it was first necessary to amplify DNA bricks from a given set of plasmids by polymerase chain reaction (PCR). The primers used, were specifically designed with overhangs matching the end of the previous or next brick, respectively. Subsequently, a two-step fusion PCR [152] was performed to create the linear chromosomal integration modules if necessary. The different DNA modules formed the chromosomal integration cassettes (which included genome recognition sequences) and were transformed into *S. cerevisiae*, where they integrated into the chromosome by homologous recombination. Alternatively the recognition sites were homolog to the ends of a digested plasmid backbone and the constructed plasmids were purified and expressed in *E. coli* to reproduce (figure 2.1).

Table 2.1: List of strains used in this study (**paper III**)

Strain	Genotype or relevant characteristics[†]	Engineered pathways*	Source
CEN.PK 113-11C	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1*	-	P. Kötter
BdJ10	CEN.PK113-11C *ws2*	WS	paper III
BdJ11	CEN.PK113-11C *ws2 ADH2*↑ *ALD6*↑ *acs*$_{se}^{L641P}$	WS + EDP	paper III
BdJ12	CEN.PK113-11C *ws2 ADH2*↑ *ALD6*↑ *acs*$_{se}^{L641P}$ *ACC1*^{S659A,S1157A} *ACB1*↑	WS + EDP + FASP	paper III
JV04	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1 are1Δ dga1Δ are2Δ lro1Δ pox1Δ*	-	paper III
BdJ13	JV04 *ws2*	WS	paper III
BdJ14	JV04 *ws2 ADH2*↑ *ALD6*↑ *acs*$_{se}^{L641P}$	WS + EDP	paper III
BdJ15	JV04 *ws2 ADH2*↑ *ALD6*↑ *acs*$_{se}^{L641P}$ *ACC1*^{S659A,S1157A} *ACB1*↑	WS + EDP + FASP	paper III

[†] native overexpressed genes are market with ↑, *Engineered pathways are defined as followed: WS: wax ester synthesis, EDP: ethanol degradation pathway, FASP: fatty acid synthesis pathway.

3. Yeast metabolism and fatty acid ethyl esters production

S. cerevisiae is known as an industrial robust organism very suitable as a cell factory for production of bulk products. Since ancient times the organism has already been used for the fabrication of different products. For example, the production of bread was made possible due to the excretion of CO_2 by yeast and beer and wine are bringing joy to people due to the alcoholic fermentation capacity of yeast. Since the 1980th, *S. cerevisiae* metabolism was adapted for maximized ethanol production followed by the development of metabolic engineering of the yeast to become a versatile production host for e.g. FAEEs. Therefore, in current yeast cell factories CO_2 has mostly been considered to be a byproduct (and a loss in carbon). The above examples do not only demonstrate the metabolic engineering ability, but foremost the multi-functionality of the *S. cerevisiae* metabolism. This chapter, which has a focus on the production pathway of FAEEs in yeast, will therefore start with a short overview of the general metabolism of *S. cerevisiae* before deepening into the possibilities and its experimental outcomes of turning the alcohol producer into a FAEE cell factory.

3.1. Central carbon metabolism in yeast

S. cerevisiae consumes its substrates (foremost glucose), which function often as carbon as well as energy source and converts them through a fueling cascade of highly conserved and therefore finely tuned and tightly regulated chemical reactions towards the central metabolite precursor, pyruvate. The combined catalytic reactions of **glycolysis** and the **pentose phosphate (PP) pathway**, leading towards pyruvate are called the central carbon metabolism (figure 3.1). Whether, pyruvate is then further catabolized through the **tricarboxylic acid cycle** (TCA or Krebs cycle), for Gibbs free energy provision in the form of ATP or GTP, or converted into one of the 12 central metabolic precursors which enable the formation of yeast biomass by e.g. anabolic formation of amino acids, nucleotides or different lipids which form cellular membranes and are important for cellular functionality and energy storage. Formation of biomass is mostly coupled to a reduction of power and therefore an important function of the PP pathway is the production of reducing equivalents (mostly NADPH) which are required in biosynthetic reactions. Also, the individual defined metabolic pathways are not merely self-sufficient, but are connected by shared cofactors (ATP/ADP, NAD/NADH, NADP/NADPH and equivalents), metabolites and compartmentalization which results in a tight cellular regulatory effect.

Because of the important role that the central carbon metabolism plays for the total yeast metabolism, the individual pathways are reviewed more in detail: **Glycolysis** describes the total of biochemical reactions in which glucose is broken down via the so called Embden-Meyerhof-Parnas (EMP) pathway towards pyruvate. The EMP pathway can be described with the overall reaction:

$$\text{glucose} + 2\ \text{ADP} + 2\ \text{P}_i + 2\ \text{NAD}^+ \rightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 2\ \text{NADH} + 2\ \text{H}_2\text{O} + 2\ \text{H}^+$$

(reaction 3.1)

Also the **pentose phosphate (PP) pathway,** consisting of an oxidative part and an anabolic function is involved in converting carbon from glucose into pyruvate. The overall reactions, divided into an anaplerotic and an oxidative function are described as follows:

Anaplerotic PP function:

$$5\ \text{glucose-6-P} + 5\ \text{ATP} \rightarrow 6\ \text{ribose-5-P} + 5\ \text{ADP} + 4\ H_2O + 4\ P_i \qquad \text{(reaction 3.2)}$$

Oxidative PP function:

$$\text{Glucose-6-P} + 12\ \text{NADP}^+ + 7\ H_2O \rightarrow 12\ \text{NADPH} + 12\ H^+ + 6\ CO_2 + P_i \qquad \text{(reaction 3.3)}$$

However, while the description of above pathways is very brief, they can be reviewed more in detail in biochemistry or metabolic engineering textbooks [35]. The relative flux through the two pathways is dependent on the requirements of Gibbs free energy, reducing power in the form of NADH and NADPH and the resulting precursor metabolites. For example, the requirement of NADPH for the synthesis of large amounts of lipids might influence the distribution of the carbon flux through EMP- or PP pathways.

S. cerevisiae is able to grow under aerobic as well as anaerobic conditions and can use multiple substrate sources, like glucose (C6), glycerol (C3) or ethanol (C2). During aerobic growth, the complete oxidation of pyruvate is performed inside the mitochondrion. The pyruvate dehydrogenase complex (PDC) catalyses the reaction in which pyruvate is converted to acetyl-CoA, which subsequently enters the TCA cycle in which it is reduced to CO_2, H_2O, $FADH_2$ and NADH. The **electron transport chain** pumps protons (from reduced electron carriers generated by the TCA cycle) out of the inner mitochondrial membrane, creating a proton gradient over the membrane which is used by the ATP synthetase for the generation of ATP. This so called **oxidative phosphorylation** has a theoretical P/O ration of 2 in *S. cerevisiae* which lacks the system 1 electron in the transport chain for reduction of NADH and 1 for reduction of FADH. However, the operational P/O ratio is only about 1. To prevent replenishing of the TCA cycle metabolites (i.a. precursors needed for amino acids formation), the **glyoxylate shortcut** fulfills an essential anaplerotic role and in addition, the pathway is indispensible for cellular growth on C2 and C3 carbon sources (mostly ethanol, glycerol or acetate).

In the yeast *S. cerevisiae*, the main **fermentative pathway** leads to ethanol (and small amounts of acetate and succinate) and is therefore also called alcohol fermentation. In *S. cerevisiae*, formation and consumption of NAD^+ cofactor is balanced for sole formation of ethanol from glucose, however because of the production of metabolic building blocks for biomass formation, a net production of NADH occurs. To maintain the redox balance, glycerol is excreted and in consequence NADH is converted into NAD^+.

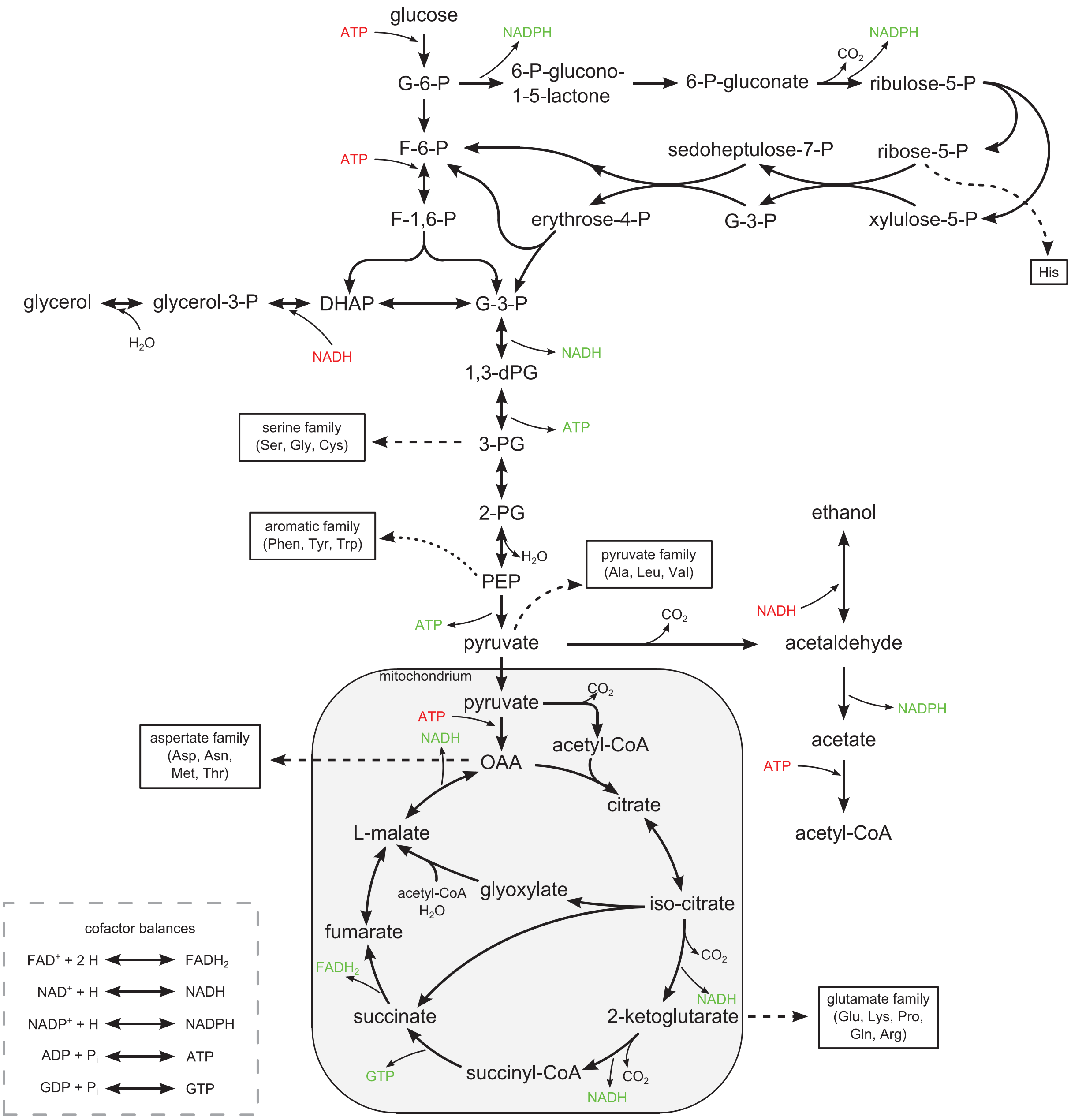
glucose
ATP
G-6-P
NADPH
6-P-glucono-1-5-lactone
6-P-gluconate
CO$_2$
NADPH
ribulose-5-P
F-6-P
ATP
sedoheptulose-7-P
ribose-5-P
F-1,6-P
erythrose-4-P
G-3-P
xylulose-5-P
His
glycerol
glycerol-3-P
DHAP
G-3-P
H$_2$O
NADH
NADH
1,3-dPG
ATP
serine family (Ser, Gly, Cys)
3-PG
2-PG
ethanol
aromatic family (Phen, Tyr, Trp)
H$_2$O
pyruvate family (Ala, Leu, Val)
PEP
NADH
CO$_2$
ATP
pyruvate
acetaldehyde
mitochondrium
pyruvate
CO$_2$
NADPH
ATP
acetyl-CoA
acetate
NADH
aspertate family (Asp, Asn, Met, Thr)
OAA
ATP
citrate
acetyl-CoA
L-malate
glyoxylate
acetyl-CoA
H$_2$O
iso-citrate
fumarate
CO$_2$
FADH$_2$
NADH
cofactor balances
FAD$^+$ + 2 H ↔ FADH$_2$
NAD$^+$ + H ↔ NADH
NADP$^+$ + H ↔ NADPH
ADP + P$_i$ ↔ ATP
GDP + P$_i$ ↔ GTP
succinate
2-ketoglutarate
glutamate family (Glu, Lys, Pro, Gln, Arg)
GTP
succinyl-CoA
CO$_2$
NADH

Figure 3.1: Central carbon metabolism with MEP, PP pathway, TCA cycle and amino acid groups.

3.2. *Wax ester production*

Wild type *S. cerevisiae* lacks a suitable enzyme to perform a transesterification reaction to produce fatty acid ethyl esters (fatty acyl esters) from acyl-CoA and ethanol for the production of fuels (figure 1.2). Fortunately other organisms, mostly originating from the species of plants, mammals and bacteria, naturally express wax ester synthases and are therefore able to convert alcohols and fatty acids to acyl esters [153]. Wax esters are naturally formed for energy storage purposes, specific membrane functions or, in the case of plants, as a protective layer on top of the leaves. From an industrial perspective, wax ester molecules have been harvested for cosmetics, food additives and industrial lubricants. The energy storage ability, however, was not industrially explored until FAEEs became important for the biofuel industry as a biodiesel substitute.

Heterologous enzymes, like wax ester synthase in *S. cerevisiae*, are dependent on several factors i.a. promoter strength and transcriptional regulation, for successful and strong expression. Functionality of enzymes in their original hosts does not automatically result in functional enzyme expression in the cell factory host. Functionality of heterologous genes being expressed can result in large phenotypical differences to its endogenous host (see chapter 2) [80]. For example, the expression of jojoba embryo ester synthase did not show activity while expressed in *S. cerevisiae* [154]. Another wax ester synthase from bacteria did not only function as those but also as acyl-CoA:diacylglycerol acyltransferase (DGAT) and might therefore result in sub-optimal conversion into FAEEs [155].

One of the first functional wax esters synthases expressed in *S. cerevisiae* was a WS/DGAT from *A. calcoaceticus* ADP1, which not only produced FAEEs but also accumulated TAGs and fatty acid isoamyl esters [32]. The highly unspecific acyltransferase activity of WS/DGAT demonstrated that the specificity of the enzyme towards its substrate and products is essential for a successful functioning of the enzyme to avoid unwanted side effects. Wax ester synthases originating from different bacteria were expressed and compared for their enzyme activity and affinity for different alcohol chain lengths in an *E. coli* cell factory, which was an obvious choice, because of its bacterial origin [33, 34, 78, 83, 156-160]. The *atfA* originating from *A. baylyi* and the wax ester synthases from, *Marinobacter hydrocarbonoclasticus*, *Rhodococcus opacus*, *Mus musculus* and *Psychrobacter arcticus* were characterized in a *S. cerevisiae* host [80]. All enzymes catalyzed a broad range of alcohol chain-lengths, but, preferred 1-dodecanol and 1-tetradecanol. However, in *S. cerevisiae*, the wax ester synthase (*ws2*) from *M. hydrocarbonoclasticus* did not only show the highest activity over the whole range of tested alcohol lengths, but also resulted in the largest production of FAEEs [80, 161]. The expression of *ws2* resulted in a 1.3-fold larger FAEEs production than expression of the second best wax ester synthase (*atfA)* from *A. baylyi* [80]. It was shown that there were differences in functional expression of *atfA*, depending on which microbial host, *E. coli* or yeast was used [78, 154, 156, 162, 163].

In this work, wax ester synthase (*ws2*) from *M. hydrocarbonoclasticus* was used to result in an optimum yield of FAEEs (**paper II, paper III and paper IV**) [151, 164]. However, yeast naturally provides ethanol (a two-carbon molecule) and saturated as well as unsaturated acyl-CoAs of mostly 16 and 18 carbons. Keeping the alcohol preferences of *ws2* in mind, the enzyme activity during ethanol consumption is only 16% compared to the enzyme activity during 1-tetradecanol consumption [80]. Therefore it is clear that the wax ester synthase requires enzyme engineering to adapt to the available substrates and microbial host environment. It will remain challenging to find a wax ester synthases which perfectly fit towards a *S. cerevisiae* host. Also the comparison of enzymes from different origins could contribute to understanding essential enzyme (regulatory) mechanisms and therefore it is important to enlarge the wax ester enzyme library from different hosts. A library of wax ester synthase could also point out differences in substrate and product preferences. Enzyme engineering was shown to result in more than 1000 fold improvements of enzyme activity in industry and therefore will also play an important role for the adaptation of the wax ester synthase in a heterologous environment.

3.3. Strategy one: Ethanol degradation pathway

3.3.1. Central precursor acetyl-CoA

One of the main challenges for the production of FAEEs in *S. cerevisiae* is the improvement of the production of cytosolic acyl-CoA. The main precursor for the fatty acid biosynthesis is acetyl coenzyme A (acetyl-CoA) and therefore the provision of acetyl-CoA is important for the improvement of the amount of acyl-CoA and subsequently the amount of FAEEs.

Acetyl-CoA plays a central role in several cellular pathways and compartments, and is involved in regulatory mechanisms [165, 166]. Acetyl-CoA is found in the cytosol, the mitochondria, the peroxisome and the nucleus, but is not able to cross the organelle membranes. Acetyl-CoA also functions as a gateway for metabolic routes to many other biotechnologically valuable compounds, like polyhydroxybutyrates (PHB), isoprenoids, alkanes/olefins, waxes, polyketides, polyphenols or fatty alcohols and provision of the metabolite is therefore valuable as a platform stain. Due to the fact that the products needs to be excreted from the cells, there consists an interest to increase the production of acetyl-CoA in the cytosol [166, 167].

To ensure large amounts of cytosolic acetyl-CoA in *S. cerevisiae* and therefore an increase of the carbon flux towards cytosolic acetyl-CoA synthesis, the cytoplasmic pyruvate dehydrogenase (PDH) bypass, was metabolically engineered. The cytoplasmic PDH bypass converts pyruvate towards acetyl-CoA via acetaldehyde and acetate. The conversions involve the enzymatic activities of, respectively, pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase. Additionally, the major byproduct, ethanol, is formed from acetaldehyde by alcohol dehydrogenase. However, the reaction is reversible and Adh2 converts ethanol into acetaldehyde and was therefore also target of the metabolic engineering strategy which was termed the ethanol degradation (EDP) pathway (see figure 3.2). This subchapter will discuss the reaction of the ethanol degradation pathway more in detail and will show the experimental outcome of engineering this pathway.

3.3.2. Acetyl-CoA synthetase

The conversion of acetate into acetyl-CoA is catalyzed by tightly regulated isoenzymes encoded by the genes *ACS1* and *ACS2* [168, 169] which are both cytosolically expressed [170-172]. It was shown that *S. cerevisiae* was not viable without *ACS1* and *ACS2* during growth on glucose and that both enzymes are subject to substrate regulation [168, 173]. On a transcriptional level, *ACS1* is subject of glucose (and ethanol to a lesser extend) repression whereas absence of *ACS2* prevented complete glucose repression [169, 173].

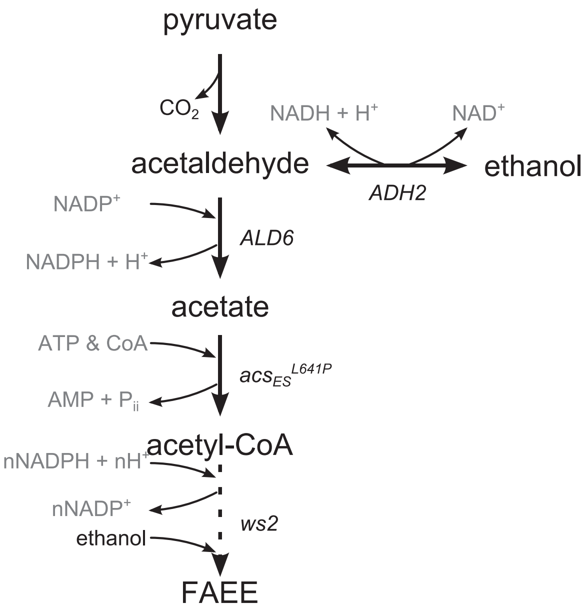

Figure 3.2: Ethanol degradation pathway

As indicated before, Acs is subject to posttranslational regulation by acetylation in bacteria. However, it was reported that substituting proline for leucine at position 641 on *Acs* of *Salmonella enterica* prevents the acetylation of Acs and hereby keeps it in its active state [174]. Over-expression of heterologous $\text{acs}_{\text{SE}}^{\text{L641P}}$ in *S. cerevisiae* resulted in a three times improvement of the enzyme activity compared to wild type *ACS1* overexpression and in a 2 times improvement in overexpression of the non-modified acs_{SE} [175]. The improvement of enzyme activity indicated that the amino acid sequences around the acetylation site between *Salmonella* and yeast are well conserved and that posttranslational regulation of acetyl-CoA synthetase in yeast and *Salmonella* have similarities, although, similar regulation in yeast has not been identified yet [175]. Therefore, the heterologous acetyl-CoA synthetase variant, carrying an amino acid substitution, preventing inactivation by acetylation, from *S. enterica* encoded by $\text{acs}_{\text{SE}}^{\text{L641P}}$ was overexpressed in **paper II** and **paper III** [164]. The overall reaction from acetate to acetyl-CoA can be described by the following reaction:

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{AMP} + \text{PP}_i \qquad \text{(reaction 3.4)}$$

The conversion of ATP to AMP and diphosphate is a very unfavorable byproduct of the reaction and energetically equivalent to the conversion of two ATP molecules to two ADP and two phosphate molecules. It was estimated that such an energy expenditure is not compatible for process economics [176]. Recently, attempts have been targeting the circumvention this energy loss by the implementation of bacterial genes encoding acetylating acetaldehyde dehydrogenase (A-ALD) and pyruvate-formate lyase (PFL) in *acs1Δ, acs2Δ S. cerevisiae* strains [177]. Expression of *ACS1* and *ACS2* was successfully replaced by the heterologous pathways, however, both strategies do not directly fit within the FAEE producing yeast cell factory, because of the circumvention of NADPH production catalyzed by *ALD6* (A-ALD) and the circumvention of ethanol production (needed as FAEE substrate) by the PFL pathway.

3.3.3. Acetaldehyde dehydrogenase

The enzyme family of acetaldehyde dehydrogenases catalyzes the conversion of acetaldehyde to acetate. In the *S. cerevisiae* genome there are five genes known to encode the acetaldehyde dehydrogenase (*ALD2*, *ALD3*, *ALD4*, *ALD5* and *ALD6*) [178]. The most relevant gene is *ALD6* which is constitutively expressed in the cytosol, but is relocated to the mitochondrial outer surface upon oxidative stress. *ALD6* encodes a Mg^{2+} dependent enzyme which uses $NADP^+$ as cofactor [179, 180]. The full reaction catalyzed by acetaldehyde dehydrogenase looks as follows:

$$\text{Acetaldehyde} + NADP^+ + H_2O \rightarrow \text{acetate} + NADPH + 2\,H^+ \qquad \text{(reaction 3.5)}$$

Not only *ALD6*, but also *ALD2* and *ALD3* are cytosolically expressed, however, Ald2 and Ald3 use NAD^+ as a cofactor. Both enzymes are induced as response to ethanol or by stress but are repressed by glucose. The two K^+ dependent acetaldehyde dehydrogenases, *Ald4* and *Ald5* are expressed in the mitochondrial compartment [181]. Ald5 is constitutively expressed whereas Ald4 is repressed by glucose.

Next to production of NADPH in the oxidative part of the PP pathway is the conversion of acetaldehyde to acetate catalyzed by Ald6, the main reaction in wild type *S. cerevisiae* for producing the cofactor NADPH. (see figure 3.2). Grabowska et al. demonstrated this by constructing a *S. cerevisiae* mutant with deletions in *ZWF1* (encoding glucose-6-phosphate dehydrogenase) and *ALD6* (encoding cytosolic aldehyde dehydrogenase), which was not viable on glucose [35]. NADPH plays an important role for building the acyl-CoA chain and therefore it is essential to look into metabolic pathways producing this compound. An additional source of NADPH was identified during growth on lactate, namely cytosolic isocitrate dehydrogenase (Idp2) [182]. During this study glucose was used as a carbon source and the endogenous *ALD6* was overexpressed to gain a larger cytosolic carbon flow towards acetyl-CoA in combination with NADPH production. Overexpression of cytosolic Ald2 and/or Ald3 might belong to the future engineering options, however, the effect of the modification needs to be carefully evaluated, due to competitive interest with NADPH producing Ald6.

3.3.4. Alcohol dehydrogenase

During growth of *S. cerevisiae* on high concentrations of glucose, most glycolytic carbon is directed towards the production of ethanol from acetaldehyde, catalyzed by the tightly regulated alcohol dehydrogenase enzyme family. The production of ethanol is a direct effect of balancing the NAD^+/NADH equilibrium. During growth with excess glucose, the TCA cycle and oxidative phosphorylation are repressed and ethanol functions as carbon sink. During the so called Crabtree effect [183, 184] there is also increased production of CO_2, acetate, pyruvic acid and glycerol to maintain the redox balance [185, 186]. The reversible reaction from acetaldehyde to ethanol is catalyzed by alcohol dehydrogenase an enzyme family encoded by *ADH1*, *ADH2*, *ADH3*, *ADH4* and *ADH5*. Adh1, Adh3, Adh4 and Adh5 reduce acetaldehyde to ethanol during glucose fermentation while Adh2 catalyzes the reverse reaction of oxidizing ethanol to acetaldehyde [88, 187-190]. The reaction is described as followed:

$$\text{Acetaldehyde} + \text{NADH} + \text{H}^+ \leftrightarrow \text{ethanol} + \text{NAD}^+ \qquad \text{(reaction 3.6)}$$

Acetaldehyde forms the branch point between carbon flux directed towards ethanol and directed towards cytosolic acetyl-CoA. Both ethanol and acyl-CoA are precursors for the production of FAEEs and therefore precise regulation of this branch point will be required to distribute the fluxes correctly without disturbance of the cellular redox balance. Complete deletion of the alcohol dehydrogenase enzyme family, resulting in depletion of ethanol is therefore not favored.

The expression of the major alcohol dehydrogenase isoenzyme, *ADH1*, is required for the reduction of acetaldehyde to ethanol, which is the last step of the glycolytic pathway [191]. Although, *ADH1* and *ADH2* share about 90% sequence similarity, Adh2 differs in metabolic directionality due to differencse in substrate affinity and also shows a ten-fold lower K_m value for ethanol than the other alcohol dehydrogenases [88, 192]. Therefore, when *S. cerevisiaes* metabolism changes to ethanol consumption after glucose depletion Adh2 is responsible for catalyzing the conversion of ethanol to acetaldehyde. The *ADH2* promotor contains two cis-acting elements (UAS1 and UAS2/CSRE) necessary for maximal *ADH2* expression [193, 194]. In absence of a fermentable carbon source, these sites are bound by transcriptional activators Adr1 and Cat8 [193, 195]. The presence of glucose down-regulates the transcriptional factors and consequently *ADH2* is repressed several hundred-fold [193, 196]. *ADH3* expresses an enzyme with 80% sequence similarity to *ADH1* and *ADH2*, but it is located inside the mitochondrial matrix and is repressed in presence of glucose [189]. It was suggested that Adh3 was involved in the mitochondrial ethanol-acetaldehyde redox shuttle because of the demonstrated growth reduction of an *adh3Δ* mutant. Despite the fact that *ADH4* encodes an enzyme, which is less related to the other alcohol dehydrogenase sequences, expression of the enzyme seems essential in case of malfunction of the major isoenzyme Adh1 [190]. All alcohol dehydrogenases are zinc dependent.

3.3.5. Experimental outcome

Summarized, the ethanol degradation pathway (figure 3.2) was employed to re-channel carbon flow towards the synthesis of acetyl-CoA (**paper II**). Therefore, *ADH2* and *ALD6* encoding, respectively, alcohol dehydrogenase and acetaldehyde dehydrogenase were overexpressed together with the heterologous gene acs_{SE}^{L641P} encoding acetyl-CoA synthetase and *ws2* from *M. hydrocarbonoclasticus* encoding wax ester synthase. The genes were under control of strong constitutive promoters (respectively, *pHXT7*, *pPGK1*, *pTEF1* and *pTEF1*) and expressed from 2-micron plasmids [197]. However, expression of the four genes on a 2-micron plasmid resulted in a plasmid size of 15.087 bp. Plasmid size as well as promoter strengths influence the stability of a plasmid and therefore the gene expression when

Table 3.1: List of plasmids used in metabolic engineering strategy 1 and 2.

Plasmid name	Genes	Marker gene	Source
pIYC04	---	*HIS3*	[166]
pBdJ01	*ws2*	*HIS3*	this study
pICY09	*ALD6*, *ADH2*, acs_{SE}^{L641P}	*HIS3*	[167]
pBdJ02	*ws2*, *ALD6*, *ADH2*, acs_{SE}^{L641P}	*HIS3*	this study
pSP-GM2	---	*URA3*	-
pSPB2N	*ws2*	*URA3*	[84]
pMPa	*xpKA*, *ack*	*URA3*	[198]]
pMPp	*xpKA*, *pta*	*URA3*	this study

propagated in *S. cerevisiae* [199-201]. Therefore the four genes were also distributed over two different plasmids resulting in a smaller size of the individual plasmids (8.872 bp and 12.875 bp). The strains and plasmids used for this strategy are listed in tables 3.1 and 3.2.

The yield of FAEEs in four different strains (figure 3.3) was measured. It could be observed that the introduction of a wax ester synthase resulted in a FAEE yield of 133 ± 113 µg gCDW^{-1}. Up-regulation of *ALD6*, *ADH2* and acs_{SE}^{L641P}, expressed from the same plasmid, led to a yield of 408 ± 270 µg gCDW^{-1}, a 3-fold improvement compared with sole wax ester expression (p-value: 0.08). This strain showed a large variation of the FAEEs yield in the different clones measured, which might be caused by variations in copy number of the relatively large plasmid. Therefore, a similar strain was constructed, reducing the plasmid size by expressing the genes on two different plasmids. This strain, in which *ws2* and the three genes *ALD6*, *ADH2* and acs_{SE}^{L641P} were expressed from separate plasmids resulted in a FAEEs yield of 359 ± 128 µg gCDW^{-1}. This modified strain showed a 2.7 fold improvement compared to sole expression of the wax ester (p-value: 0.03) and also showed a lower clonal variation than the strain expressing one large plasmid with all genes.

Table 3.2: List of strains used in metabolic engineering strategy 1 and 2.

Name	Genetic background	*HIS3* based plasmid	*URA3* based plasmid
		Reference strain	
BdJref	CEN.PK 113-11C	pIYC04 (---)	pSP-GM2 (---)
		Phosphoketolase strains	
BdJ01	CEN.PK 113-11C	pBdJ01 (*ws2*)	pSP-GM2 (---)
BdJ02	CEN.PK 113-11C	pBdJ01 (*ws2*)	pMPa (*xpkA*, *acK*)
BdJ03	CEN.PK 113-11C	pBdJ01 (*ws2*)	pMPp (*xpkA*, *pta*)
BdJ04	*CB2I20*	---	pSP-GM2 (---)
BdJ05	*CB2I20*	---	pMPa (*xpkA*, *acK*)
BdJ06	*CB2I20*	---	pMPp (*xpkA*, *pta*)
		Ethanol degradation strains	
BdJ07	CEN.PK 113-11C	pIYC04 (---)	pSPB2N (*ws2*)
BdJ08	CEN.PK 113-11C	pBdJ02 (*ws2*, *ALD6*, *ADH2*, acs_{SE}^{L641P})	pSP-GM2 (---)
BdJ09	CEN.PK 113-11C	pIYC09 (*ALD6*, *ADH2*, acs_{SE}^{L641P})	pSPB2N (*ws2*)

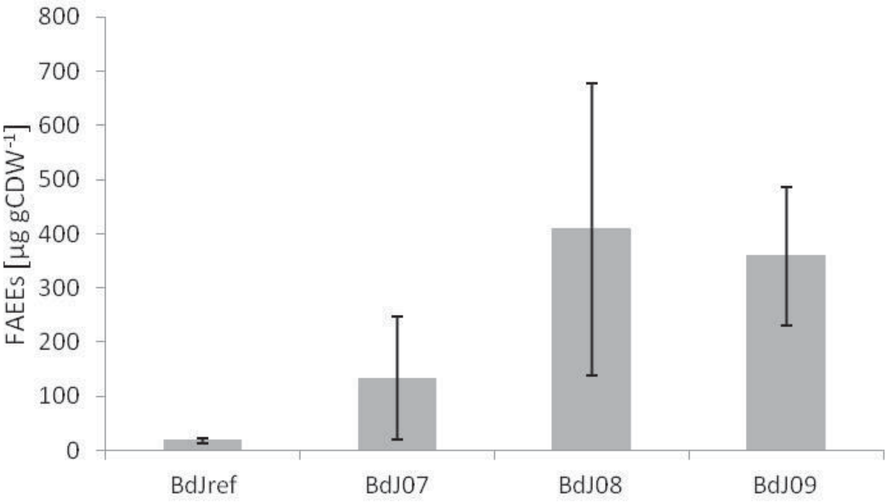

Figure 3.3: FAEE yield (µg gCDW^{-1}) of investigated *S. cerevisiae* strains expressing the ethanol degradation pathway. Biological triplicates of the strains were investigated. The experiment was performed twice and standard deviations are indicated.

The ethanol degradation strategy has been applied for different engineering targets before. Chen et al. investigated the physiological effect of some major enzymes relevant for acetyl-CoA metabolism on different carbon sources in *S. cerevisiae* [166]. The enzyme variant encoded by acs_{SE}^{L641P} was previously overexpressed together with *ALD6*, mainly responsible for the metabolic reaction to form acetate from acetaldehyde, in *S. cerevisiae* for high level production of amorphadiene [175]. This successful strategy had been improved by additional overexpression of *ADH2* and *ERG10* for the production of α-santalene which resulted in a 1.75 times higher production than the reference strain and a 25% increase in titer compared to the strategy introduced by Shiba et al. [175]. The pull-push strategy, pushing the carbon flow down to acetyl-CoA by overexpression of *ADH2*, *ALD6* and acs_{SE}^{L641P} and pulling it towards the product by overexpression of *ERG10* was also applied in the production of polyhydroxybutyrate (PHB) in *S. cerevisiae* [202]. A 16-fold improvement of PHB production was detected compared to the reference strain. In this study, the wax ester synthase was used to pull the carbon flow towards production of FAEEs. Comparable to the previously performed studies described, we here found the improvement of product formation to be 3-fold compared with the reference strain. This lower fold improvement might be caused due to regulation of downstream pathways leading to the formation of FAEEs.

3.4. Strategy two: Heterologous phosphoketolase pathway

The expression of heterologous pathways in *S. cerevisiae* could establish (a chain of) new metabolic reactions or improve endogenous reactions and is therefore of interest to metabolic engineering. For the improvement of fatty acid derived products in *S. cerevisiae*, several heterologous pathways have been implemented including pyruvate formate lyase (PFL) or acetylating aldehyde dehydrogenase (A-ALD) [177, 203, 204]. A metabolic engineering landmark was recently reached with the implementation of a pyruvate dehydrogenase complex from *Enterococcus faecalis*, which circumvented energy loss of cytosolic acetyl-

CoA synthase [176]. In strategy two of my thesis project (**paper II**) the phosphoketolase (PHK) pathway was investigated for the production of FAEEs.

The redox co-factor NADPH is required for formation of acyl-CoAs, which typically has 7 rounds of reaction cycles including two reduction steps, each requiring NADPH [205]. The most important NADPH source in *S. cerevisiae* is assigned to the oxidative part of the pentose phosphate pathway, and therefore, a metabolic pathway leading towards the synthesis of acetyl-CoA by rechanneling carbon flow via the PPP was investigated. The phosphoketolase pathway was described previously as potential alternative carbon route for different industrially relevant metabolites due to the increase of acetyl-CoA production with NADPH as co-product [198, 206, 207]. The carbon flow leading towards the PHK pathway starts with the conversion of glucose to glucose-6-phosphate in the EMP pathway followed by conversion of glucose-6-phosphate in three reactions into ribulose-5-phosphate in the oxidative part of the pentose phosphate pathway. Byproducts during these reactions are two moles of NADPH and one mole of CO_2 per mole of glucose. The next metabolite in the pathway, xylulose-5-phosphate, represents the precursor of the PHK pathway as it can be converted to acetyl-phosphate and glyceraldehyde-3-phosphate.

$$\text{xylulose-5-P} + P_i \rightarrow \text{acetyl-P} + \text{GAP} \qquad \text{(reaction 3.7)}$$

This reaction is catalyzed by xylulose-5-phosphate phosphoketolase encoded by *xpkA* in *Aspergillus nidulans*, but the gene is also expressed in other organisms [208]. During the second step of the PHK pathway, acetyl phosphate can either directly be converted into acetyl-CoA or indirectly with acetate as intermediate. Direct conversion of acetyl phosphate into acetyl-CoA in *Bacillus subtilis* is catalyzed by phosphotransacetylase encoded by the gene *pta* [209].

$$\text{acetyl-P} + \text{CoA} \rightarrow \text{acetyl-CoA} + P_i \qquad \text{(reaction 3.8)}$$

The formation of acetate and ATP from acetyl phosphate is performed by acetate kinase, encoded by the gene *ack* in *A. nidulans* [210, 211]. The PHK pathway is shown in Figure 3.4.

$$\text{acetyl-P} + \text{ADP} + P_i \rightarrow \text{acetate} + \text{ATP} \qquad \text{(reaction 3.9)}$$

The PHK pathway was first reported in heterofermentative and facultative homofermentative lactic acid bacteria, in bifidobacteria and sporadic in other microorganism, like xylose fermenting yeasts [212]. It has been demonstrated that *S. cerevisiae* has the ability to functionally express phosphotransacetylase from *B. subtilis* as well as phosphoketolase and acetate kinase from *A. nidulans* [198, 213]. Although, the above mentioned microbial sources were used in these studies, several other microbial sources for enzymes of the different reactions are known and were also tested in a *S. cerevisiae* host [208].The fact that more NADPH is formed when glucose is metabolized via the PP pathway instead of the EMP pathway makes the combination of the PP pathway and the PHK pathway an interesting alternative for production of FAEEs. The demand of NADPH for lipid synthesis might create the driving force for carbon to flow through the PHK pathway. It was demonstrated that glucose catabolism through the PHK pathway would improve the net NADPH supply for FAEE production.

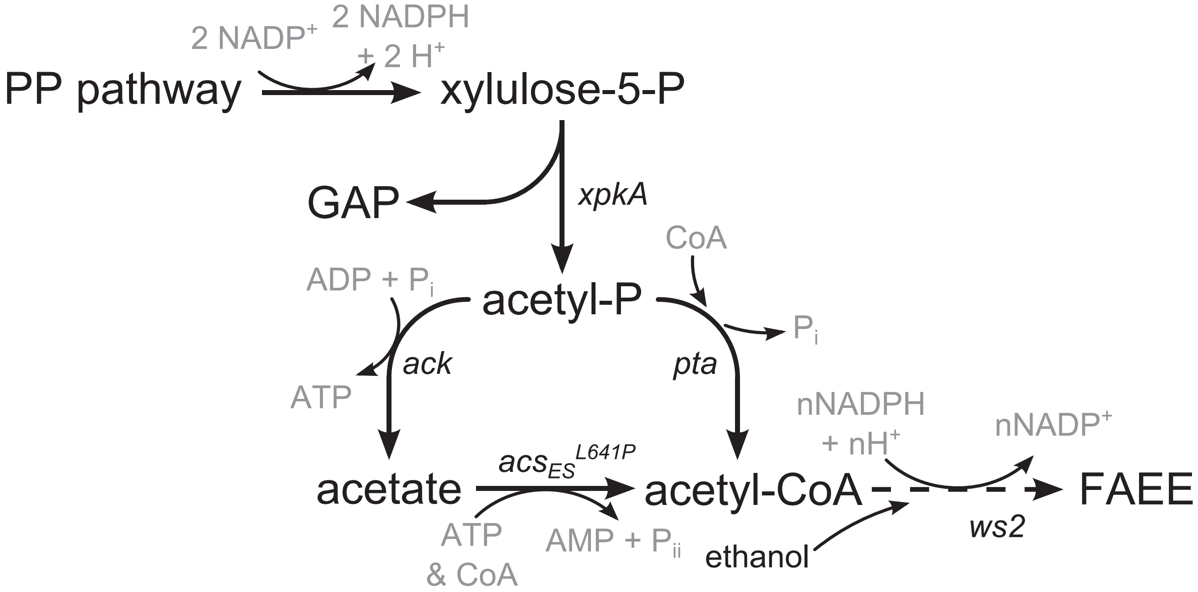

Figure 3.4: Overview of the phosphoketolase pathway. *xpkA* and *ack* are originally from *A. nidulans* while *pta* is from *B. subtilis*.

3.4.1. Experimental outcome:

During this work (**paper II**) several strains were constructed to test the expression of the PHK pathway genes in *S. cerevisiae* for the production of FAEEs (the strains are listed in table 3.2). Therefore, two genes, *xpkA* and *ack*, both descending from *A. nidulans*, were expressed to catalyze, respectively, the conversion of xylulose-5-phosphate to acetyl phosphate and glyceraldehyde-3-phosphate and acetyl phosphate to acetate with the gain of one ATP. A second strain was constructed, in which the gene *pta* from *B. subtilis* replaced *ack*. Pta converts acetyl phosphate directly to acetyl-CoA. Both strains were compared with a strain containing solely the *ws2* gene and to a reference strain, containing the empty reference plasmids. Figure 3.5 shows the quantification of total FAEEs of the described *S. cerevisiae* strains. The expression of *xpkA*, *ack* and *ws2* resulted in a FAEE yield of 28 ± 3.5 µg gCDW^{-1} which is 1.5 times higher than for the strain only expressing *ws2*. However, the difference between the two strains showed insufficient statistical significance (p-value: 0.10). The expression of *xpkA*, *pta* and *ws2* led to a FAEE yield of 105 ± 30 µg gCDW^{-1} which was 5.7 fold higher than the strain with only *ws2* expression (p-value: 0.03) and a 3.7 times higher FAEE yield than the strain expressing *xpkA*, *ack* and *ws2* (p-value: 0.05). However, due to the relatively low yield of the strains described above and because of presumed fluctuating plasmid stability during repetitive experiments, the PHK pathway was also expressed in strain CB2I20, a strain with multiple chromosomal *ws2* integrations [84]. These strains are listed in table 3.2. It was also hypothesized that multiple expression of the *ws2* gene might contribute to a stronger carbon-pull through the PHK pathway. Expression of *xpkA* and *pta* in strain CB2I20 resulted in 4670 ± 379 µg gCDW^{-1} (p-value: 0.02), a 1.6 fold higher yield of FAEEs compared to the reference strain CB2I20, whereas the expression of *xpkA* and *ack* in CB2I20 improved the final yield of FAEEs to 5100 ± 509 µg $gCDW^{-1}$ (p-value: 0.01), which is a 1.7 times improvement compared to the reference strain (figure 3.6). It could also be observed that the production levels of FAEEs in strains BdJ04-BdJ06 were stable and reproducible.

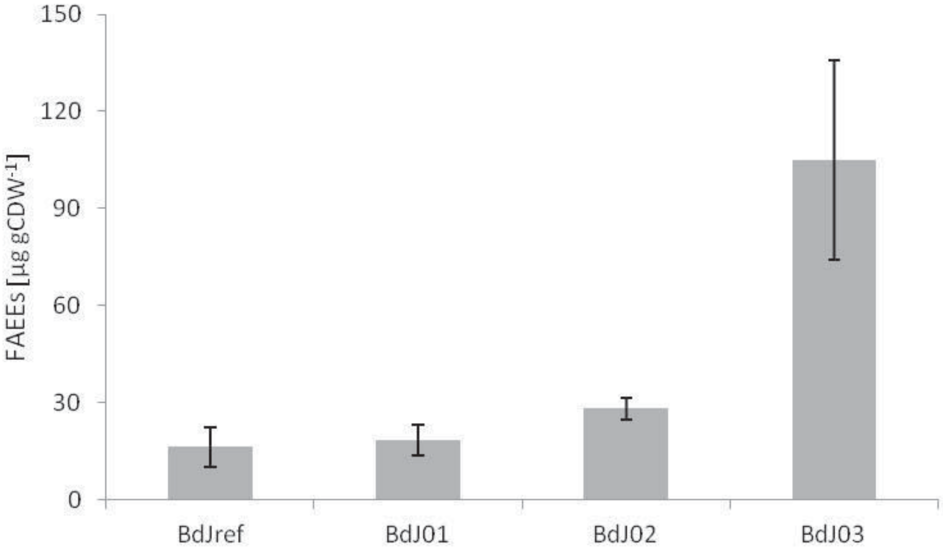

Figure 3.5: FAEE yield (µg $gCDW^{-1}$) of investigated *S. cerevisiae* strains expressing a heterologous PHK pathway. Biological triplicates of the strains were investigated. The standard deviations are indicated.

In this study, it was found that expression of the phosphoketolase pathway in *S. cerevisiae* could re-channel carbon flux through the oxidative part of the PP pathway, via the PHK pathway, towards the precursors acetyl-CoA and malonyl-CoA for synthesis of acyl-CoA. As a consequence, two molecules of the NADPH are being produced for each sugar molecule passing through the PP pathway. As was demonstrated earlier by Papini et al. the expression of genes *xpkA* and *ack* showed a functional carbon flux through the PHK pathway and as shown in this study the expression of genes *xpkA*, *ack* and *ws2* resulted in a yield of FAEEs 50% higher than in a strain not expressing the PHK pathway [198]. It was also shown that the expression of *xpkA* and *ack* resulted in an improved polyhydroxybutyrate (PHB) producing strain [207]. Papini et al. could not demonstrate the functionality of the PHK pathway using phosphotransacetylase gene *pta* (instead of *ack*), which would result in a direct conversion of acetyl phosphate to acetyl-CoA. However, in their study they did not insert a pull of acetyl-CoA for a specific product, whereas our result clearly shows that expression of this enzyme combination results in improved FAEEs production (minimal 60% improvement), which strongly indicates that the enzymes are active.

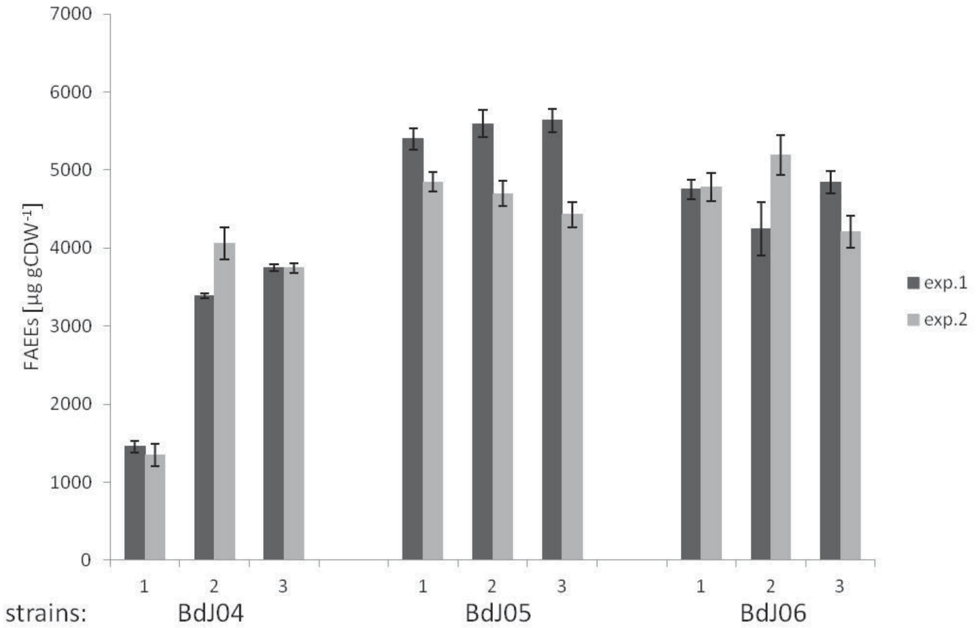

Figure 3.6: FAEE yield (µg gCDW^{-1}) of investigated *S. cerevisiae* strains containing multiple *ws2* chromosomal integrations and expressing a heterologous PHK pathway. Triplicate clones of each strain were investigated (described by 1, 2 and 3). The experiment was performed twice (light and dark columns) and standard deviations are indicated.

3.5. Strategy three: Integration of metabolic engineering strategies

Many different metabolic engineering strategies have been employed to improve *S. cerevisiae* cell factories for fatty acid derived products. However, the combined effect has never been investigated for the production of FAEEs. An important engineering target is shaped by the two reactions, acetyl-CoA carboxylase and fatty acid synthase, which convert acetyl-CoA into acyl-CoA chains. In **paper III**, a combination of multiple successful FAEE engineering strategies was chosen to be stably (over)expressed in *S. cerevisiae*.

3.5.1. Acetyl-CoA carboxylase

In yeast, *S. cerevisiae*, malonyl-CoA is formed from acetyl-CoA by acetyl-CoA carboxylase [81, 151, 214] and together the two molecules form the precursors for the ‘activated’ form of fatty acid, the acyl-CoA chain. The reaction does not only form the entrance to the fatty acid synthase pathway, but is therefore also considered to function as a ‘gate keeper’ of the flux into this pathway.

$$\text{acetyl-CoA} + \text{ATP} + HCO_3^- \rightarrow \text{malonyl-CoA} + \text{ADP} + P_i + 2\,H^+ \qquad \text{(reaction 3.10)}$$

The reaction, in which malonyl-CoA is produced at the expense of ATP and carbon dioxide, occurs in two different compartments. The mitochondrial enzyme is encoded by *HFA1* whereas the cytosolic enzyme is encoded by *ACC1*. Acc1 is a multifunctional enzyme whose activity is controlled by biotin availability as it has been reported that reduced levels of this vitamin affect the synthesis of fatty acids [215]. The specific molecular composition of acyl-CoA and its downstream products directly regulate genes of the fatty acid synthesis pathway via feedback inhibition and by positive (Ino2p and Ino4p) and negative (Opi1p) regulatory factors [205, 216-218].

It is thought that Snf1-dependent phosphorylation attenuates the activity of acetyl-CoA carboxylase and maintains the appropriate acyl-CoA chain length distribution and the ratio of C16 versus C18 acyl-CoAs [219, 220]. In *S. cerevisiae*, Snf1 is involved in regulation of cellular stress response, pseudohyphal growth, aging, ion homeostasis and in regulation of specific pathways like gluconeogenesis, glyoxylate cycle, oxidation of fatty acids and therefore it was not possible to eliminate this regulatory factor [70]. However, to prevent post-translational regulation of Acc1 by phosphorylation in *S. cerevisiae*, amino acid 1157, verified as target by phosphoproteome analysis [221], and amino acid 659 (part of a potential phosphorylation recognition motif (Hyd-X-Arg-XX-Ser-XXX-Hyd) for Snf1) [222] both serines have been replaced by amino acid alanine [81, 216, 223]. Despite the fact that a higher content of free fatty acids (and related molecules) was yielded by the latter replacement [81] it was never proven that the acc1^{S1157A,S659A} was more active that its acc1 or acc1^{S1157A} counterpart.

3.5.2. Fatty acid synthase

The formation of acyl-CoA is catalyzed by fatty acid synthase, encoded by *FAS1* and *FAS2* both active in multiple compartments, including the cytosol. While the main function of mitochondrial fatty acid formation is the provision of lipoic acid co-factor for the pyruvate dehydrogenase complex [224], the majority of fatty acids are provided in the cytosole where they have a large (energy) storage and therefore they are more relevant for engineering applications [225]. Fas1 and Fas2 are multi-subunit proteins with all required enzyme activities for building long chain fatty acids from acetyl-CoA and malonyl-CoA [226, 227]. As shown in figure 3.7, acyl-CoA formation is established as followed: Acetyl-CoA is loaded to the acyl-carrier protein (ACP) by acyltransferase (AT), followed by repetitive elongation (of on average seven rounds) of the acyl-CoA chain by two carbon molecules a time, until a maximum of 16 carbons is reached [228]. The elongation cycle starts with loading a molecule of malonyl-CoA and sequential catalytic steps of β-ketoacyl-ACP synthesis, β-ketoacyl-ACP reduction, β-ketoacyl-ACP dehydration and enoyl-ACP reduction. Two molecules of NADPH are converted to $NADP^+ + 2\ H^+$ during the reducing steps [229]. During the final step, acyl-ACP and malonyl-CoA are transformed by malonyl transacylase (MPT) to form acyl-CoA and the activated malonyl-ACP, which is necessary for initiating the next acyl-CoA synthesis. More extensive reviews of the process of fatty acid synthesis and industrially relevant fatty acid derived products are reviewed elsewhere [214, 230].

The Fas1 and Fas2 enzyme system is tightly regulated by several cellular layers [205, 231, 232]. Due to the fact that the Fas1 and Fas2 are large enzymes with multiple subunits, communication between the different catalytic centers is required to enable the movement of the growing acyl-CoA chain. Both, *FAS1* and *FAS2* are constitutively activated by general transcription factors Rap1, Abf1 and Reb1 and similar to *ACC1*, both enzymes are activated by Ino2p and Ino4p [233-235]. The two enzymes also regulate each other as Fas1 controls *FAS2* mRNA levels [236]. Several engineering efforts have been performed to deregulate the

FAS1 control and to increase acyl-CoA derived products. A recent attempt, in which transcriptional factor *RPD3*, a negative regulator of phospholipid metabolism, was deleted, enabled an improved production of fatty alcohols 1.6- to 2.7-fold [237].

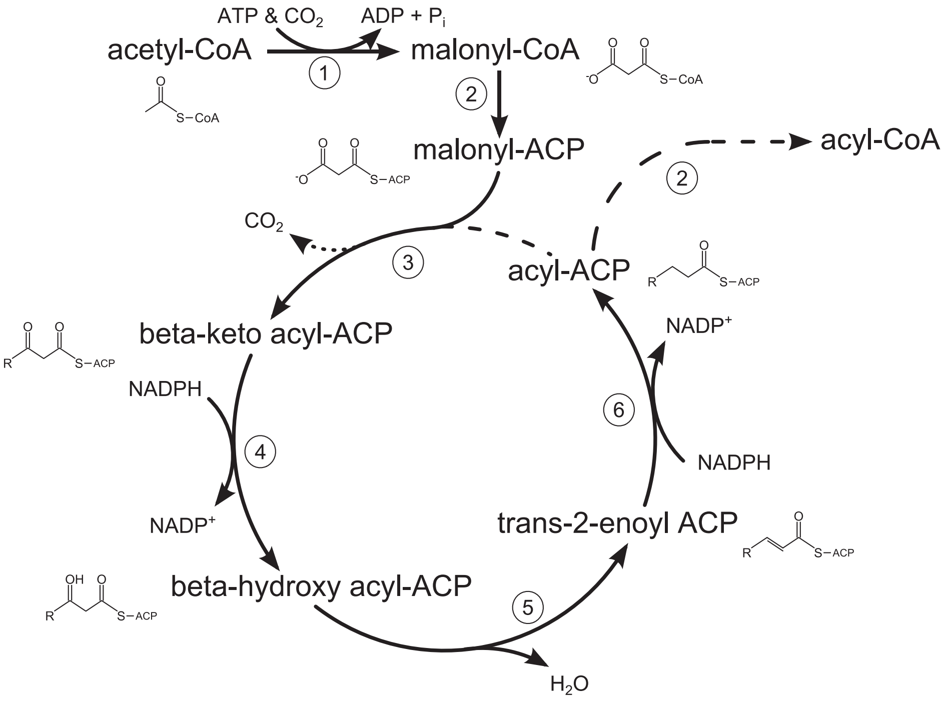

Figure 3.7: Overview of fatty acid biosynthase in *S. cerevisiae* of fatty acid biosynthase in *S. cerevisiae*: 1) acetyl-CoA carboxylase, 2) malonyl/palmitoyl transferase, 3) ketoacyl reductase, 4) dehydratase, 5) enoyl reductase and 6) keto acyl synthase.

Once the acyl-CoA chain is formed, it is bound by acyl-CoA binding protein (Acb1) which plays an important role for facilitating intracellular transport of acyl-CoA esters to the membrane of the endoplasmic reticulum and lipid bodies for phospholipid and TAG biosynthesis [238]. Additionally, it was reported that Acb1 had an attenuating effect on the negative feedback mechanism of acyl-CoAs on fatty acid synthase, acetyl-CoA carboxylase and long chain acyl-CoA synthetase [239-241]. *ACB1* has therefore been targeted for two opponent metabolic engineering strategies. Both the overexpression of the *ACB1* gene [84] to improve the attenuation of the negative feedback loop as well as its deletion to prevent transport of acyl-CoA towards unwanted cellular locations [82] resulted in a positive effect on FAEE production. It remains unclear which engineering strategy concerning *ACB1* resulted in the best outcome for FAEE production.

However, naturally several different pathways use acyl-CoA as a substrate for the formation of triacylglycerides (TAGs; storage lipids), sterol esters, phospholipids (membrane structure and function), sphingolipids or free fatty acids. Additionally, acyl-CoA can be degraded to acetyl-CoA by the β-oxidation pathway. It becomes clear that acyl-CoA competition plays a major role while implementing a new acyl-CoA consuming pathway, for industrial products like FAEEs, alkanes/alkenes, fatty alcohols and others.

Therefore the question rose whether or not pathways competing with wax ester conversion could be disrupted. Natural acyl-CoA derived products with cellular functionality for trafficking and for membranes could not be circumvented; however the acyl-CoA competing pathways leading towards triacylglycerols (TAGs), steryl esters (both serving as storage lipids) and the β-oxidation pathway, could be disrupted to prevent energy loss for the production of FAEEs. Therefore, these pathways have previously been inactivated by disruption of *DGA1*, *LRO1* (both encoding diacylglycerol acyltransferases), *ARE1*, *ARE2* (both encoding sterol acyltransferases) and *POX1* (encoding acyl-CoA oxidase) [32, 79]. The knock-out strain was used to express a wax ester synthase from *M. hydrocarbonoclasticus* (*ws2*) [80] and resulted in a yeast strain with the inability to synthesize storage lipids and an FAEE production of 17.2 mg/l [79].

Table 3.3: List of strains used in metabolic engineering strategy 3.

Strain	Genotype or relevant characteristics[†]	Engineered pathways*	Source
CEN.PK 113-11C	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1*	-	P. Kötter
BdJ10	CEN.PK113-11C *ws2*	WS	This study
BdJ11	CEN.PK113-11C *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P}	WS + EDP	This study
BdJ12	CEN.PK113-11C *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P} $ACC1^{S659A,S1157A}$ *ACB1*↑	WS + EDP + FASP	This study
JV04	*MAT*a *MAL2-8*^{c} *SUC2 ura3-52 his3-Δ1 are1Δ dga1Δ are2Δ lro1Δ pox1Δ*	-	This study
BdJ13	JV04 *ws2*	WS	This study
BdJ14	JV04 *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P}	WS + EDP	This study
BdJ15	JV04 *ws2 ADH2*↑ *ALD6*↑ acs_{se}^{L641P} $ACC1^{S659A,S1157A}$ *ACB1*↑	WS + EDP + FASP	This study

[†] native overexpressed genes are market with ↑, *Engineered pathways are defined as followed: WS: wax ester synthesis, EDP: ethanol degradation pathway, FASP: fatty acid synthesis pathway.

3.5.3. Experimental outcome

Here (**paper III**), a combination of multiple successful FAEE engineering strategies was investigated by stable (over)expression in *S. cerevisiae*. Therefore, the endogenous alcohol dehydrogenase (encoded by *ADH2*), acetaldehyde dehydrogenase (encoded by *ALD6*) and a heterologous acetyl-CoA synthetase variant from *S. enterica* insensitive to regulation by acetylation (encoded by acs_{SE}^{L641P}) were over-expressed for the improvement of the supply of the precursor acetyl-CoA and cofactor NADPH [164]. This was combined with overexpression of $ACC1^{S1157A,S659A}$ and *ACB1* to increase the acyl-CoA pool [81], which was converted into FAEEs by wax ester synthase Ws2 [80]. These stable chromosomal integrations were introduced in wild type *S. cerevisiae* as well as in a mutant strain lacking acyl-CoA competitive but non-vital pathways (*are1Δ dga1Δ are2Δ lro1Δ pox1Δ*) [79]. The constructed strains are described in table 3.3 and figure 3.8 gives an overview of the modified genes in the metabolic pathway.

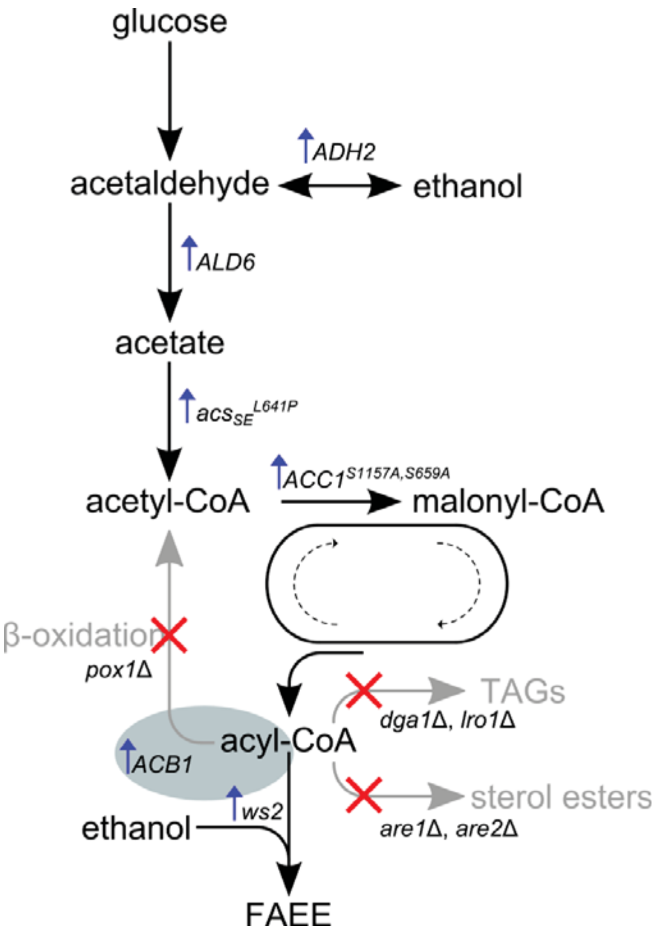
glucose
ADH2
acetaldehyde
ethanol
ALD6
acetate
acs_{SE}^{L641P}
$ACC1^{S1157A,S659A}$
acetyl-CoA
malonyl-CoA
β-oxidation
pox1Δ
TAGs
dga1Δ, lro1Δ
acyl-CoA
ACB1
ws2
sterol esters
are1Δ, are2Δ
ethanol
FAEE

Figure 3.8: Overview of metabolic engineering approaches of FAEE in *S. cerevisiae*. Blue arrow indicates overexpressing of the gene. This scheme is simplified and does not show co-factors or compartmentalization.

The expression of chromosomal integrated *ws2* in a CEN.PK113-11C background strain resulted in a production of 261 ± 57 µg total FAEEs gCDW^{-1}. Additional expression of *ADH2*, *ALD6* and acs_{SE}^{L641P} resulted in an FAEE production of 463 ± 77 µg total FAEEs gCDW^{-1} and further chromosomal integration of $ACC1^{S1157A,S659A}$ and *ACB1* resulted in a strain producing 570 ± 130 µg total FAEEs gCDW^{-1}. Compared to the expression of *ws2* alone, the latter two strains improved the yield 1.8 fold and 2.2 fold (see figure 3.9 A). The additional expression of $ACC1^{S1157A,S659A}$ and *ACB1* improved the FAEE yield 1.2 fold.

Chromosomal overexpression of *ws2* inside a background strain with disrupted conversion of free fatty acids towards sterol esters, triacylglycerides and acetyl-CoA via the β-oxidation pathway resulted in 506 ± 58 µg total FAEEs gCDW^{-1}. In the same strain, additional expression of genes *ADH2*, *ALD6*, acs_{SE}^{L641P}, yielded 797 ± 69 µg total FAEEs gCDW^{-1}, which was a 1.6 fold improvement. The strain in which the strategies of expressing *ws2, ADH2*, *ALD6*, acs_{SE}^{L641P}, $ACC1^{S1157A,S659A}$, *ACB1* and eliminating the fatty acid competing pathways were combined, resulted in a yield of 1072 ± 160 µg total FAEEs gCDW^{-1} which was the highest FAEE yield detected here (figure 3.9 B). This most extensively engineered strain resulted in a 4.1 fold improvement of the FAEEs yield compared with the reference strain, in which *S. cerevisiae* only carried the *ws2* gene.

Overall, overexpression of genes in combination with the disruption of the acyl-CoA competing pathways resulted in an 1.85 ± 0.11 fold improvement in FAEEs production compared with the respective counterpart strains based on CEN.PK113-11C (figure 3.9 A and 3.9 B). However, the mutant strains without these acyl-CoA competing pathways resulted in a lower final biomass concentration after substrate depletion (data not shown). Therefore, while comparing the titers of the different strains, the first strains showed titers of, respectively, 1.3 ± 0.3, 2.3 ± 0.4 and 3.5 ± 0.8 mg l^{-1} (figure 3.9 C) versus FAEEs titers of the latter strains of respectively 2.0 ± 0.2, 2.2 ± 0.2 and 4.4 ± 0.7 mg l^{-1} (figure 3.9 D).

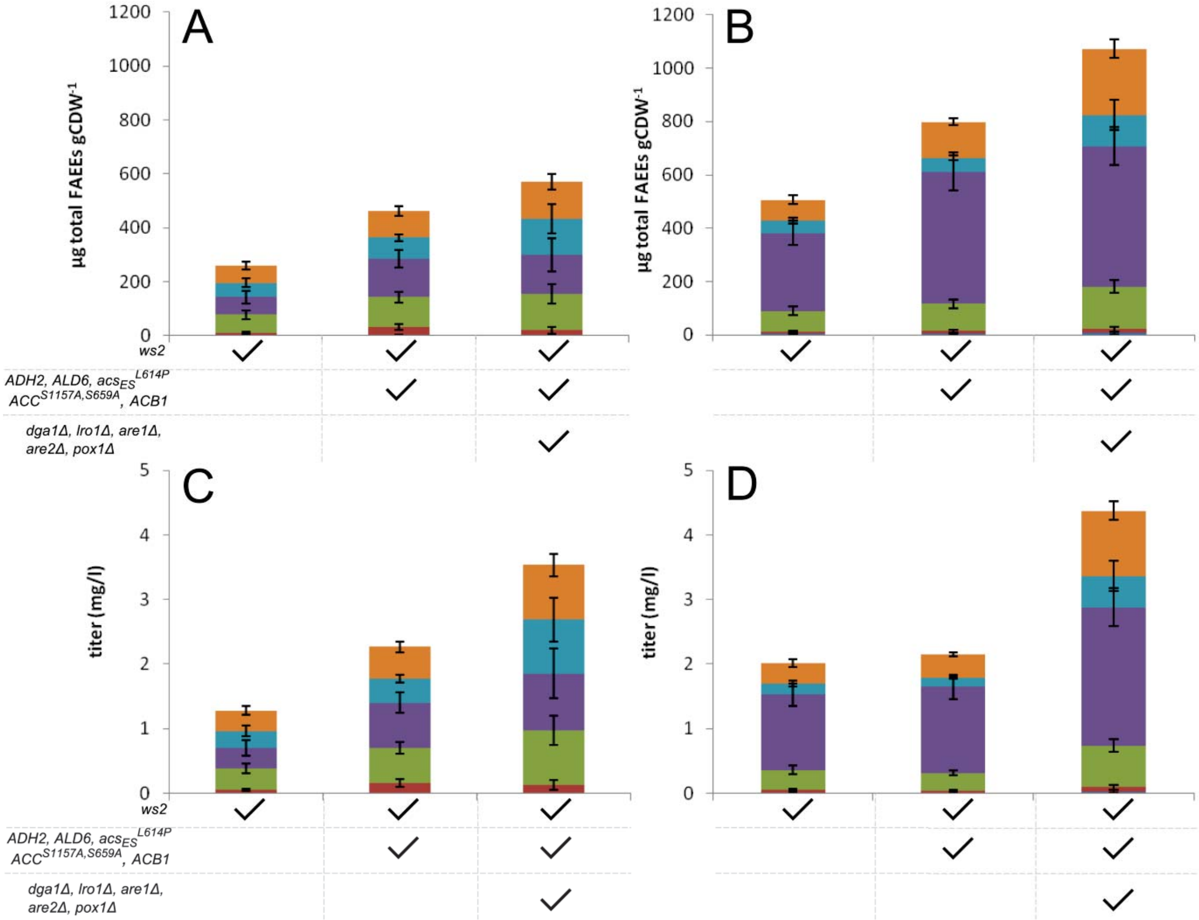

FIGURE 3.9: FAEE yields of engineered strains based on background CEN.PK.113-11C (A) and background strain JV04 with disrupted conversion of acyl-CoAs towards sterol esters, triacylglycerides and acetyl-CoA via the β-oxidation pathway (B) as well as FAEE titers (g/l) of FAEEs producing strains based on background CEN.PK113.11C (C) and JV04 (D). The distribution of acyl moieties in the different FAEEs pools is indicated by the color code. Results represent the average ± standard deviation of duplicate performed shake flask experiments with biological triplicates [151].

3.5.3.1. Chain length distribution

All constructed strains contain a wax ester synthase, which catalyzes the transesterification reaction that converts ethanol and acyl-CoA into FAEEs. In *S. cerevisiae*, the predominant fatty acids are sixteen and eighteen carbon atoms long and exist in their saturated form as well as in their mono-unsaturated form [216]. The most obvious change in FAEEs chain length distribution occurred for unsaturated 16:1 FAEEs after elimination of the fatty acid competing pathways (figure 3.9). Before the deletion the share of unsaturated 16:1 FAEE was 27 ± 3.5 %

whereas after the deletion 56 ± 6.5 % was unsaturated 16:1 FAEE. This increase is almost solely responsible for the improved production of total FAEEs. On the other hand, the fraction of saturated 16:0 FAEE and saturated 18:0 FAEE in the same strains were reduced from, respectively, 24 ± 1 % to 14 ± 3 % and from 20 ± 1% to 8 ± 2 %. No significant difference for the 18:1 unsaturated FAEE content was detected. Also the implementation of genes *ADH2*, *ALD6* and acs_{SE}^{L641P} or $ACC1^{S1157A,S659A}$ and *ACB1* did not show any significant difference for the distribution of the carbon-chain length (see figure 3.9).

3.6. Overview of the metabolic engineering strategies

During this chapter, three different metabolic engineering strategies to convert *S. cerevisiae* from ethanol producer into a FAEE producing cell factory were discussed. First, two different strategies, the ethanol degradation pathway and the heterologous phosphoketolase pathway were tested in *S. cerevisiae*.

The overexpression of genes *ADH2*, *ALD6* and acs_{SE}^{L641P} in combination with *ws2* from *M. hydrocarbonoclasticus* showed a 3-fold improvement compared to sole expression of the wax ester (p-value: 0.03). This lower fold improvement (compared to the application of the strategy for the production of other compounds) might be caused due to regulation of downstream pathways leading to the formation of FAEEs.

Also the expression of *ack* from *A. nidulans* or *pta* from *B. subtillis* combined with expression of *xpkA* from *A. nidulans* and *ws2* from *M. hydrocarbonoclasticus* represents a successful strategy for increasing FAEEs production by *S. cerevisiae*. The maximal improvement was achieved by the combined expression of *xpkA*, *pta* and *ws2* which led to a FAEE yield of 105 ± 30 µg $gCDW^{-1}$ which was 5.7 fold higher than the strain with solely *ws2* expression (p-value: 0.03).

Further the results from a combination of several engineering strategies, which were stably integrated into the *S. cerevisiae* chromosome was discussed here. This most extensively engineered strain, with overexpression of genes *ADH2*, *ALD6* and acs_{SE}^{L641P} or $ACC1^{S1157A,S659A}$, *ACB1* and *ws2* and simultaneous deletion of acyl-CoA competitive but non-vital pathways (*are1Δ dga1Δ are2Δ lro1Δ pox1Δ*) resulted in stable expression and a 4.1 fold improvement of the FAEEs yield compared with the reference strain, in which *S. cerevisiae* only carried the *ws2* gene. This strain represents a very good starting point for further engineering, e.g. evaluation of the effect of increased expression of the wax ester synthase, which is likely to be limiting production by the engineered strains.

4. Phenotypic characterization

The design of a new cell factory, irrelevant whether or not constructed by rational modifications or by evolutional adaptations, will result in a new phenotype of the cell factory. It is the task of the researcher to understand the new cellular behavior, to compare it with the expectations and to connect the outcomes of different analyses and to draw conclusions. In that sense, fundamental knowledge of cellular behavior is a requirement for applied engineering strategies. This chapter will discuss several different analytical methods used for gaining insight into the (global) behavior of microbial cell factories.

4.1. Quantification of batch cultivations

The aim that is mostly pursued for microbial cell factories is to reach high product formation, within a short amount of time and inside a small reaction volume. These parameters are of crucial importance for industrial process economics and therefore the main engineering targets of cell factories. In order to access the performance of a cell factory it is important to quantitatively access its titer, rate and yield, and the simplest way to obtain this quantitative information for cell factories is the use of a so called 'black-box' model. In this case, a black-box is a system in which it is precisely defined what goes into the microbial cell factory and accurately measured what is excreted from the system, while the microbial cells are only considered as self replicating catalysts. Mass balances are therefore indispensable tools offering valuable information on reaction rates such as biomass formation, substrate uptake or product formation rates, but also for example, enable the determination of unknown metabolic products. The same way, not only carbon balances, but also redox or electron balances can be formed.

A typical batch growth of *S. cerevisiae*, consists of a glucose consumption phase, a diauxic shift in which metabolism switches from glucose to ethanol consumption, followed by an ethanol consumption phase. The growth on each of the substrates is characterized by a lag, acceleration, exponential, deceleration and stationary phase (see experimental data figure 4.3). An important parameter is the speed of growth of the microbe. With sufficient nutrients, cellular growth is exponential. The specific growth rate is described by μ (h^{-1}) and in a closed batch system it equals

$$\mu = \frac{1}{c_x}\frac{dc_x}{dt} \qquad \text{(equation 4.1)}$$

with c_x being the biomass concentration. And the doubling time t_d (h), which also resembles the generation time $\boldsymbol{\tau}$ can directly be derived from integration of reaction 4.1

$$t_d = \frac{\ln 2}{\mu} = \frac{0.693}{\mu} \qquad \text{(equation 4.2)}$$

The specific growth rate of an organism is dependent on the nutrient availability, the assumption that one substrate is limiting, as formulated by Monod (1949) results in:

$$\mu = \mu_{\max} \frac{c_s}{c_s + K_s} \qquad \text{(equation 4.3)}$$

Here, c_s is the substrate concentration of the limiting substrate in the environment (g L^{-1}), μ_{max} the maximum specific growth rate (h^{-1}) and K_s is the limiting substrate concentration at which the specific growth rate is half its maximum value. Notably, the K_s value in the Monod model does not exactly represent the saturation constant for substrate uptake, but only an overall saturation constant for the whole growth process. However, K_s values mostly do not differ significantly from K_m values of the enzymes involved in substrate uptake, because substrate uptake is often closely connected to the control of substrate metabolism. During the exponential growth phase of a batch cultivation it can be assumed that $K_s << c_s$, and therefore, during exponential growth the following equation is valid

$$\mu = \mu_{max} \qquad \text{(equation 4.4)}$$

To obtain the best possible experimental outcome, the μ_{max} can be calculated by linear regression and least square fitting of $\ln(c_x)$ over time during the exponential growth phase.

Mass balances for substrate (s) and product (p) can be set the same way as described for biomass (x):

$$-q_s = \frac{1}{c_x} \frac{dc_s}{dt} \qquad \text{(equation 4.5)}$$

$$q_p = \frac{1}{c_x} \frac{dc_p}{dt} \qquad \text{(equation 4.6)}$$

in which c_s and c_p are the concentrations of substrate and product, respectively.

Next to the microbial growth rate, one of the most important parameters is the amount of product produced per amount of substrate, the yield: Y_{sp}. Yield can be assigned to each compound in the process and is performed depending on the piece of information necessary for process improvement (for example Y_{xp} in kg/kg but mostly mole/mole or Cmol/Cmol). The theoretical yield of a process, compared to the current process yield, demonstrates the available capacity for improvement of the process. For a specific time in a process the yield of, in this case substrate on product, can be described as

$$Y_{sp} = \frac{r_p}{-r_s} = \frac{q_p}{-q_s} \qquad \text{(equation 4.7)}$$

In the examples above, the rates specific to biomass are indicated by q (Cmol p (Cmol s)$^{-1}$ h^{-1}). However, as described in equation 4.7, rates of conversion can also be indicated per volume: r (kg m^{-3} h^{-1} or Cmol m^{-3} h^{-1}) which describes the rate of utilization of the reactor.

4.2. Fermentation mode

A microbial cell factory can be characterized well in fully controlled fermentors, but such a process is time consuming and expensive. Therefore, microorganisms are mostly cultured in different ways. A fast, uncontrolled and imprecise method of growing and screening cellular behavior is performed with, for example, shake flasks in which only constant mixing and temperature of the culture is ensured. In industry, large numbers of modified strains have to be tested and to speed up the process of strain screening, the volume (and consequently the costs) is scaled down to less than a milliliter with systems like for example the commercial BioLector® (m2p-labs, Baesweiler, Germany). This and similar systems ensure a larger throughput of samples and enable the monitoring of (few) parameters, but are not able to react to changes in the fermentation broth (like pH). Neither is it possible to control culture modes differently than batch-cultures or to take (larger) samples [242]. Additionally, some of the measured parameters provide crucial information but lack precision and accuracy demanded for drawing calculated conclusions. Therefore, it is challenging to create high-through-put methods of cell culturing which trustfully transfer knowledge of the microbial physiology without missing out data.

However, the physiological data and the ability to regulate the parameters in the reactor are important reasons for investing time and money into controlled cultivation. Mainly three different cultivation modes (many more exist), the batch, fed-batch and chemostat reactors are used (see figure 4.1). During batch, all nutrients are available from the beginning of the cultivation and the process will continue until the substrate is depleted or growth is inhibited by external factors. A fed-batch is started like a batch reactor but will increase the fermentation volume with controlled inflow of substrates so that the maximum product formation will be yielded when the capacity of the reactor is reached. The rate of nutrient addition can depend on different parameters of regulation. A chemostat culture or continued culture, has a constant inflow rate of nutrients which equals the outflow rate of fermentation broth, while the reactor volume is constant. The rate and composition of the inflow can be regulated and therefore parameters like the specific growth rate or the limitation of nutrients can be influenced.

In large scale industrial set-ups most processes are run in a batch or fed-batch mode despite the advantages of continuous cultures. In industrial practice, the latter system is tackled down by the fact that a continuous system is sensitive to contamination by other, faster growing microbes. However, on a lab scale, a chemostat provides a valuable basis for investigating reactor and microbial cultivation properties because of the fact that the process remains in a pseudo steady state. For this reason it is the ideal state to measure the difference between different culture conditions. More information can be found the text book of Bioreaction Engineering Principles [44].

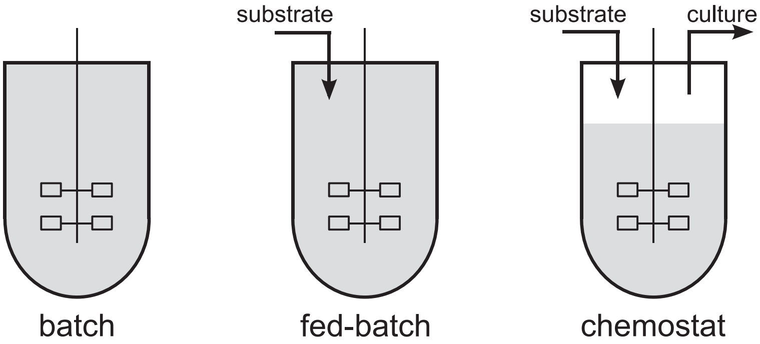

Figure 4.1: Graphical illustration of the three mostly used fermentation systems: batch, fed-batch and chemostat.

4.3. Experimental data

During this work, several strains with plasmid-based modifications of the PHK-pathway and the EDP-pathway were grown in shake flasks in which, general physiological traits were monitored (**paper II**). In **paper IV**, different FAEE producing strains of *S. cerevisiae* were cultivated in controlled batch fermentation to characterize the physiological behavior and to sample for global transcriptional analysis.

In **paper II**, the native pathway in *S. cerevisiae* towards cytosolic acetyl-CoA synthesis was metabolically engineered to increase the flux towards acetyl-CoA required for the formation of FAEEs. Ethanol, the second metabolite for synthesis of FAEEs, forms the major byproduct during FAEE production in *S. cerevisiae*. To re-channel the carbon flux towards the synthesis of acetyl-CoA, Adh2 and Ald6, which respectively catalyze the conversion of ethanol to acetaldehyde and acetaldehyde to acetate, were overexpressed together with the heterologous acs_{SE}^{L641P}, which encodes a de-regulated acetyl-CoA synthetase that catalyzes the conversion of acetate to acetyl-CoA. Also the introduction of the PHK pathway was thought to potentially increase the yield of FAEEs in *S. cerevisiae* due to its ability to generate two moles of NADPH per mole of glucose, which would provide additional NADPH for acyl-CoA synthesis. Therefore, two genes, *xpkA* and *ack*, both descending from *A. nidulans*, were expressed to catalyze, respectively, the conversion of xylulose-5-phosphate to acetyl phosphate and glyceraldehyde-3-phosphate and acetyl phosphate to acetate with the gain of one ATP. A second strain was constructed, in which the gene *pta* from *B. subtilis* replaced *ack*. Pta converts acetyl phosphate directly to acetyl-CoA. Details about the metabolic pathways and the strain construction were shown in chapter 2 and chapter 3.

Wild type *S. cerevisiae* is a fast growing and robust microorganism. However, the genetic modifications leading to improved FAEE production could have an impact on its physiology. Cell growth, glucose consumption as well as ethanol formation and consumption were therefore monitored during shake flask cultivations (Table 4.1 and Figure 4.2). The final biomass of the engineered strains was reduced compared to the reference strain (BdJref) and also the maximum specific growth rate (μ_{max}) for both PHK pathway expressing strains and ethanol degradation pathway engineered strains showed a reduction of up to 40% (BdJ08). The main difference between the reference strain and engineered strains for increased ethanol degradation was a slower maximal consumption rate of ethanol (q_{eth}^{max}) for the modified strains. Especially the strains overexpressing genes *ADH2*, *ALD6*, *acs*$_{SE}^{L641P}$ and *ws2* showed a reduced ethanol consumption rate of, respectively, 45% and 70% compared to the reference strain. It seemed unlikely that FAEEs are toxic to *S. cerevisiae* at the concentrations produced here. There was also no negative effect on cell growth when 1 g/l of myristic acid ethyl esters were added to the medium (data not shown). Therefore, the reduced ethanol consumption rates observed during the ethanol degradation pathway might most likely be explained by the rapid conversion of the ethanol to cytosolic acetyl-CoA which hence reduces the amount of ethanol that can be oxidized in the mitochondria. It is a presumable assumption that the metabolic changes were caused by the stress caused by overexpression of several genes.

Table 4.1 Physiological characteristics of modified strains

Strain	**(Over)expressed genes (HIS3 plasmid) & (URA3 plasmid)**	**Final FAEE yield (µg gCDW^{-1})**	**Maximal specific growth rate (μ_{max}) (h^{-1})**	**Final biomass (OD 600)**	**Glucose rate (q_{smax}) (h^{-1})**	**Ethanol rate (q_{ethmax}) (h^{-1})**
BdJref	--- & ---	16.3 ± 3.5	0.190 ± 0.010	5.84 ± 0.14	-1.534 ± 0.054	-0.204 ± 0.015
Phosphoketolase pathway						
BdJ01	ws2 & ---	18 ± 4.8	0.159 ± 0.006	5.51 ± 0.16	-1.448 ± 0.057	-0.200 ± 0.021
BdJ02	ws2 & xpkA, ack	28.0 ± 3.5	0.168 ± 0.009	5.27 ± 0.13	-1.357 ± 0.084	-0.208 ± 0.016
BdJ03	ws2 & xpkA, pta	105 ± 30	0.136 ± 0.025	4.55 ± 0.11	-1.357 ± 0.078	-0.197 ± 0.014
Ethanol degradation pathway						
BdJ07	--- & ws2	133 ± 113	0.192 ± 0.003	5.14 ± 0.22	-1.403 ± 0.035	-0.174 ± 0.003
BdJ08	ws2, ALD6, ADH2, acsSEL641P & ---	408 ± 270	0.115 ± 0.026	3.97 ± 0.42	-1.265 ± 0.365	-0.113 ± 0.002
BdJ09	ALD6, ADH2, acsSEL641P & ws2	359 ± 128	0.136 ± 0.035	3.67 ± 1.82	-1.208 ± 0.495	-0.061 ± 0.023

For **paper IV**, three different mutant strains of *S. cerevisiae*, which were engineered to produce FAEEs, were compared with each other and with a wild type yeast strain, auxotrophic CEN.PK113-7D. For characterization of the strains, biological triplicates were grown in controlled batch fermentation. As described in earlier chapters, strain BdJ12 carried overexpressed endogenous as well as heterologous genes *ws2*, *ADH2*, *ALD6* and *acs*$_{SE}^{L641P}$, *ACC1*^{S1157A,S659A} and *ACB1* integrated into its chromosome [151]. The modifications were implemented to enable FAEE production and to ensure an efficient precursor supply. Strain BdJ15 additionally carried five different gene deletions to prevent the conversion of free fatty acids to sterol esters, triacylglycerides and their degradation to acetyl-CoA via the β-oxidation pathway (*dga1Δ lro1Δ are1Δ are2Δ pox1Δ*) [151]. The last FAEE producing strain, CB2I20, carried about six copies of the gene *ws2*, which were integrated into the chromosome [84]. See figure 3.8, 4.5 and table 2.1. The growth of all strains was followed until the stationary phase and the concentrations of glucose, ethanol, glycerol and acetate were measured during the same time period (Figure 4.3). The biological triplicates of each strain showed similar characteristics with small standard deviations for all values, the carbon balances are shown in table 4.2.

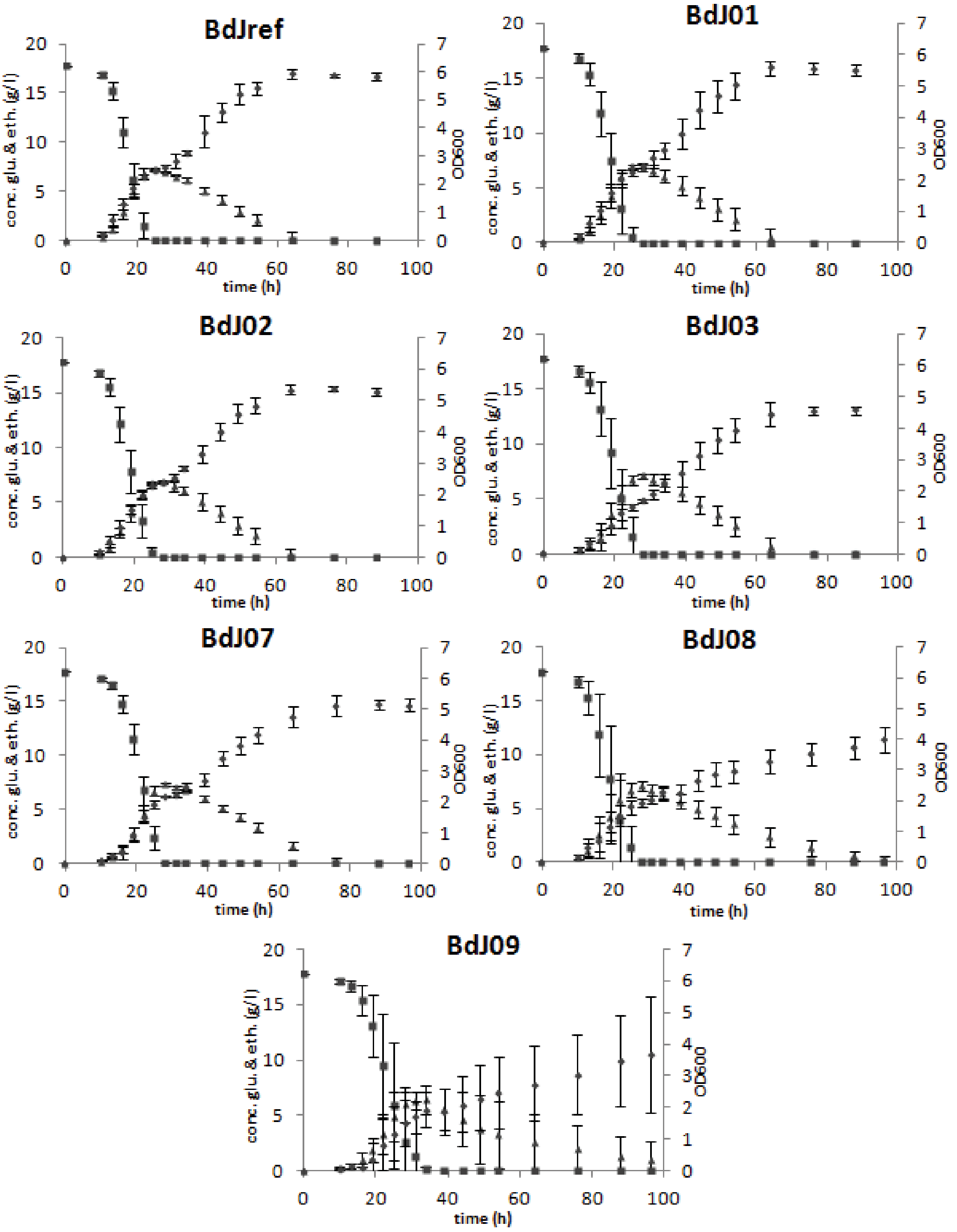

Figure 4.2: Cell growth and substrate consumption of recombinant strains during shake flask cultivation. The strains BdJref and BdJ01-BdJ06 were analyzed in biological triplicates. The glucose concentration (g l^{-1}) is indicated by squares, the ethanol concentration (g l^{-1}) is indicated by triangles and cell growth (OD 600) is shown by diamonds.

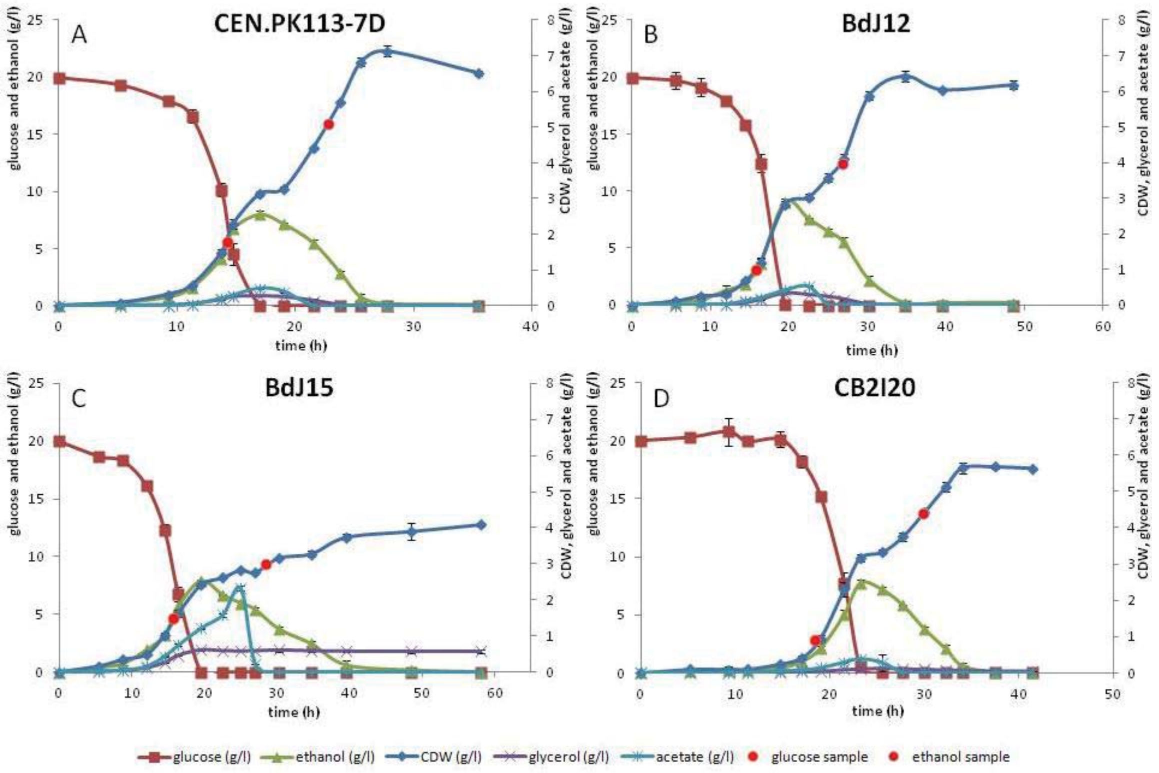

Figure 4.3: Physiological parameters from biological triplicates (standard deviations) of strain CEN.PK113-7D (A), BdJ12 (B), BdJ15 (C) and CB2I20 (D) grown in controlled batch reactors. The red dots show the time point in which samples were taken for transcriptional analysis.

It could be observed that glucose was completely consumed, resulting in maximal specific growth rates (μ_{max}) which were reduced for the modified strains (table 4.2). While glucose was consumed and biomass was formed, three different byproducts were detected, ethanol, glycerol and acetate. Once glucose was consumed the diauxic shift occurred from glucose to ethanol consumption. It was eye catching that the growth rate of strain BdJ15 was much lower than the growth of the other strains on ethanol, which could also be seen in the much longer duration of the ethanol phase of that strain. While the reference strain accumulated the largest total amount of biomass (7.1 g l^{-1}), it was closely followed by strains BdJ12 and CB2I20, whereas strain BdJ15 reached only about 4 g l^{-1} of final biomass (see figure 4.3).

Table 4.2: Physiological parameters from biological triplicates of strain CEN.PK113-7D, BdJ12, BdJ15 and CB2I20 grown in controlled batch reactors.

strain	**max. growth rate on glucose μ (h^{-1})**	**max. growth rate on ethanol μ_{eth} (h^{-1})**	**glucose uptake rate q_g (h^{-1})**	**ethanol uptake rate q_{eth} (h^{-1})**	**carbon balance**
CEN.PK113-7D	0.45 ± 0.01	0.39 ± 0.01	-1.77 ± 0.16	-0.89 ± 0.01	1.05 ± 0.00
BdJ12	0.34 ± 0.01	0.24 ± 0.01	-0.56 ± 0.10	-0.46 ± 0.10	0.96 ± 0.05
BdJ15	0.26 ± 0.00	0.06 ± 0.00	-1.03 ± 0.06	-0.36 ± 0.02	0.92 ± 0.02
CB2I20	0.42 ± 0.04	0.27 ± 0.01	-1.17 ± 0.17	-0.78 ± 0.04	0.89 ± 0.01

Another clear difference between the four strains was the large accumulation of acetate during the glucose consumption phase of strain BdJ15. 2.5 g l^{-1} of acetate was accumulated during the BdJ15 batch culture, which was five times higher than the accumulation of acetate during cultivation of the other strains. Together with ethanol, acetate was co-consumed in all yeast strains (figure 4.4).

The co-production of glycerol during production of ethanol in yeast is a consequence of redox balancing of the yeast cell. Here it could be observed that the reference strain (CEN.PK113-7D) and strain BdJ12 accumulated about 0.3 g l^{-1} glycerol, which was completely consumed during growth on ethanol. These values differentiated from the glycerol accumulation in strain BdJ15, which was 2-fold higher (0.6 g l^{-1} and the glycerol accumulation in strain CB2I20, which was reduced (0.15 g l^{-1}) (see figure 4.4). Another observation was the lack of glycerol consumption of BdJ15 and the lack of complete glycerol consumption of CB2I20. The difference of glycerol metabolism might indicate cellular stress or difficulties of maintaining the cellular redox balance. However, the latter argument could be excluded, because of a degree of reduction (κ) balance calculation, which indicated no significant redox imbalance [44].

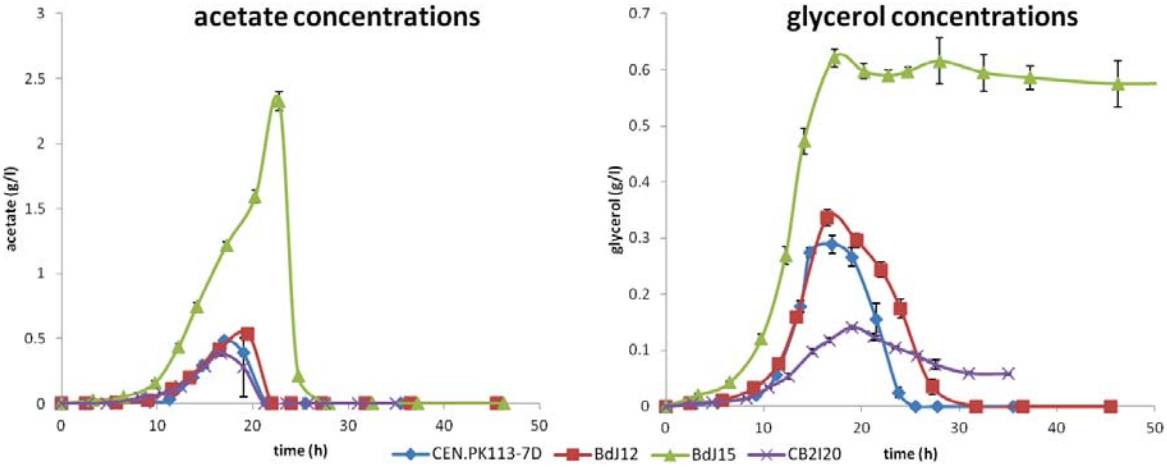

Figure 4.4: Acetate and glycerol concentration of reference and FAEEs producing mutant strains.

From the physiological data from **paper II** it became clear that the introduction of the modifications slowed down the maximal specific growth rates of the different strains. Additionally, some of the strains showed some reduced ethanol consumption rate, which was possibly caused by stress. In **paper IV** the main physiological observation was the reduced ethanol consumption rate of strain BdJ15, in combination with a large acetate and glycerol production, which are most probably caused by the disruption of the acyl-CoA competing pathways. Also the growth rate was reduced due to the implementation of the modifications. It became clear that the data from the uncontrolled shake flasks cultivation and the data from the controlled batch fermentation are not comparable, which was inter alia due to the use of different media.

4.4. Quantification of FAEEs

Accurate product detection and quantification is crucial for the evaluation of a cell factory. Additionally, the quantification needs to be performed with the same high-throughput manner as the other factors in the strain developing pipe-line. During this work, intracellular accumulation of FAEEs was measured with a gas chromatograph-mass spectrometer (GC-MS) which could precisely detect the quantity of different (carbon-) chain length of FAEEs. Here, 12:0, 14:0, 16:0, 16:1, 18:0 and 18:1 were detected. Quantification was done using the addition of a known amount of internal standard of heptadecanoic acid ethyl ester (17:0) to the sample. However, the quantification of FAEEs is time consuming and far from high-through-put practice. Sample preparation, in which it was challenging to exclude systematic errors, involved sample washing, freeze-drying, extracting of total lipids, separation of the lipids by thin liquid chromatography (TLC) and final collection of the FAEEs for the separation and quantification by GC-MS. A precise description was given in the material and methods section of **paper II** [164].

It is inevitable that the detection and evaluation of the product formation should be performed in a cleverer way. To keep the precision and accuracy of the product quantification, one might want to continue with the use of the GC-MS system for the detection of the FAEEs. However, the sampling to detection pipeline needs to be shortened. Solutions could be twofold: First, of all, the sample preparation pipeline could be shortened by the implementation of other techniques for the separation of FAEEs (as an example the use of head-space detection). A second option would be to establish extracellular detection of FAEEs, which would circumvent most of the time consuming extraction from the yeast cells during sample preparation. The use of a dodecane overlay during cultures might help the excretion of the product and in future larger amounts of product might stress the cell for excretion of the FAEEs. The detection and quantification of indirect indicators for FAEE, which are fast to analyze, would be another option. Examples could be a biosensor systems, which are often coupled to fast detection of fluorescence by flow cytometry [243]. However, the risk of indirect product detection is the loss of accuracy of the quantification.

4.5. System wide profiling of cell factories

System wide screening and therefore the use of -omics techniques became indispensable for the analytical evaluation of microbial cell factories. As described in chapter 1, different -omics techniques can cover all cellular layers of the central dogma. These methods are especially valuable for understanding complex cellular systems and new phenotypes created by metabolic engineering. During this work, **paper IV**, global transcriptional profiling of three different *S. cerevisiae* mutants (strains BdJ12, BdJ15 and CB2I20) was performed with the help of microarray technology to find out new metabolic engineering strategies for the improvement of FAEE producing cell factories. Detailed description of the genetic changes of the strains can be found in chapter 3 while the physiological behavior was described above in this chapter. Specifically the expected transcriptional changes (p-value 0.01), due to the applied genetic modifications of all three strains were compared to reference strain CEN.PK113-7D, which was not able to produce FAEEs. In addition, the influence of the gene deletions on the global transcriptional level was investigated by comparing differential expression of strains BdJ12 and BdJ15.

It could be observed that genes *ADH2* and *ALD6* in BdJ12 and BdJ15 showed increased expression compared to the reference strain during glucose consumption. But despite the introduction of the extra copy, it could also be observed that *ADH2* showed lower expression in BdJ15 than the reference strain during consumption of ethanol (figure 4.5). This finding was not expected although it is known that expression of the alcohol dehydrogenase is also strongly regulated by the carbon source on post-translational level [195], but possibly this resulted from a changed expression of the wild type gene copy in this strain. Acetyl-CoA synthase is catalyzing the conversion of acetate to acetyl-CoA, encoded by the combination of endogenous genes (*ACS1* and *ACS2*) and the heterologously expressed acs_{SE}^{L641P}. As expected, expression of the heterologous gene did not lead to major expression changes in the endogenous genes, which may be partly due to the known regulation of the protein by acetylation [174].

Cytosolic acyl-CoA formation is catalyzed by fatty acid synthase, encoded by genes *FAS1* and *FAS2*. The synthesis of these metabolites starts with loading acetyl-CoA to the acyl carrier protein (ACP) by ACP acyltransferase (AT) and is followed by repetitive elongation under consumption of malonyl-CoA [225]. Malonyl-CoA is formed from acetyl-CoA by acetyl-CoA carboxylase (reviewed by [214]). Here it was shown that both Endegenous acetyl-CoA carboxylase encoded by gene *ACC1*, the heterologous overexpressed variant $ACC1^{S1157A,S659A}$ and fatty acid synthase (*FAS1* and *FAS2*) were upregulated in all three engineered strains during glucose consumption. During the ethanol phase no significant change was detected despite the overexpression of the *ACC1* variant in strains BdJ12 and BdJ15 (figure 4.5).

In strain BdJ15, genes *DGA1*, *LRO1*, *ARE1*, *ARE2* and *POX1*, which are involved in acyl-CoA consuming pathways, were disrupted, which also became evident from the transcriptional analysis (figure 4.5). Genes *DGA1*, *ARE1* and *ARE2* were up-regulated during both glucose and ethanol phase, while gene *LRO1* showed decreased expression during all measurements in both strains BdJ12 and CB2I20. β-oxidation pathway genes *POX1*, *POT1* and *ECI1* in strains BdJ12 and CB2I20 were highly up-regulated during glucose phase while during ethanol phase, the β-oxidation pathway was down-regulated in strain CB2I20. An interesting observation was that genes *FOX1*, *POT1* and *ECI1* in strain BdJ15 were highly expressed, especially during ethanol phase, despite the *POX1* deletion.

ACB1 (coding for acyl-CoA binding protein) was up-regulated in all FAEE producing strains, but the fold change was higher in strains BdJ12 and BdJ15, in which the gene was actually overexpressed (figure 4.5). During growth on glucose the glycerol production pathway, encoded by four genes (*GDP1*, *GDP2*, *GPP1* and *GPP2*), was down-regulated in all three engineered strains, but upregulated during growth on ethanol in strain BdJ12 and BdJ15. PP pathway genes *GND2*, *SOL4* and *TKL2* (and in lower amounts *ZWF1*) were up-regulated in strain BdJ12 during both glucose and ethanol phase. The same genes were more mitigated during growth on ethanol in strain BdJ15 and during the glucose phase in strain CB2I20.

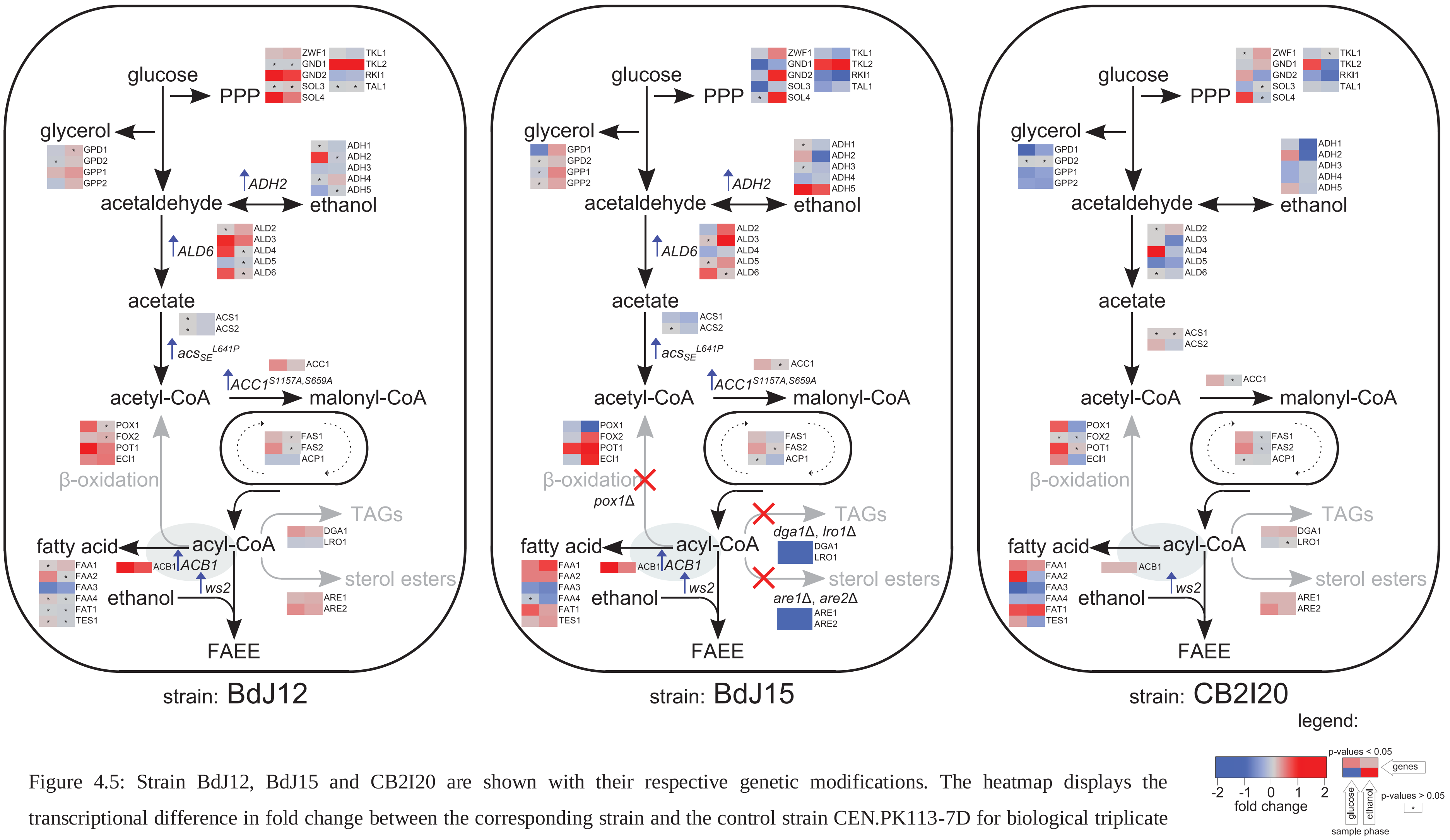
glucose
PPP
glycerol
acetaldehyde
ethanol
acetate
acetyl-CoA
malonyl-CoA
β-oxidation
fatty acid
acyl-CoA
TAGs
sterol esters
FAEE
ADH2
ALD6
acs$_{SE}^{L641P}$
ACC1^{S1157A,S659A}
ACB1
ws2
pox1Δ
dga1Δ, lro1Δ
are1Δ, are2Δ
ZWF1
GND1
GND2
SOL3
SOL4
TKL1
TKL2
RKI1
TAL1
GPD1
GPD2
GPP1
GPP2
ADH1
ADH2
ADH3
ADH4
ADH5
ALD2
ALD3
ALD4
ALD5
ALD6
ACS1
ACS2
ACC1
POX1
FOX2
POT1
ECI1
FAS1
FAS2
ACP1
DGA1
LRO1
ARE1
ARE2
FAA1
FAA2
FAA3
FAA4
FAT1
TES1
strain: BdJ12
strain: BdJ15
strain: CB2I20
legend:
-2 -1 0 1 2
fold change
p-values < 0.05
genes
p-values > 0.05
glucose
ethanol
sample phase

Figure 4.5: Strain BdJ12, BdJ15 and CB2I20 are shown with their respective genetic modifications. The heatmap displays the transcriptional difference in fold change between the corresponding strain and the control strain CEN.PK113-7D for biological triplicate samples taken during the glucose and ethanol consumption phase.

4.5.1. GO-term analysis, reporter metabolites and consensus scoring of gene sets

In strain BdJ15 additional carbon became available for the formation of FAEEs because of the disruption of metabolic pathways leading towards TAGs (*dga1Δ lro1Δ*), sterol esters (*are1Δ are2Δ*) and acetyl-CoA via the β-oxidation pathway (*pox1Δ*) [151]. Although the consequences of these modifications on the regulation of the global metabolism were unclear, with the observation of physiological changes of i.e. growth rate during ethanol consumption and acetate and glycerol accumulation, it became interesting to investigate the global transcriptional changes caused by the disruption of these genes. Therefore a 'GO-term', 'Reporter Metabolite' analysis and consensus scoring of different gene sets were performed of the differentially transcribed genes of strains BdJ12 and BdJ15.

Three major changes between strain BdJ12 and strain BdJ15 were observed during growth on glucose. First of all, transcriptional changes within major mitochondrial processes suggested oxidative stress and difficulties maintaining the demand of energy in the form of ATP via oxidative phosphorylation. Secondly, reporter metabolite analysis highlighted transcriptional changes around 6-phospho-D-gluconate (PPP, oxidative part, connected genes: *SOL3*, *SOL4, GND1* and *GND2*) and sedoheptulose-7-phosphate (PPP, non-oxidative part, connected genes: *TKL1*, *TKL2* and *TAL1*), which are possibly related to the provision of cofactor NADPH, required for reduction during fatty acid biosynthesis. Gene *GRE2*, which is involved in the oxidation of NADPH to NADP$^+$, in general induced by stress [244, 245], was transcriptional down-regulated which could indicated a lack of NADPH or an increased stress level. This transcriptional change caused by cellular stress seemed to be in consensus with the physiological results. It was interesting that the complete ergosterol biosynthesis was down-regulated in strain BdJ15 during glucose consumption. Important roles, i.a. a role in fluidity of lipid membranes and contribute to vital cellular processes like cellular sorting, the cytoskeleton organization or mating (reviewed by [246, 247]), but also play an important role in thermotolerance [248] are dedicated to the different sterols derived from the ergosterol pathway. It can be speculated that despite the down-regulation of the pathway, a sufficient supply of sterols for the vital cellular processes was provided. Earlier research also indicated that the ergosterol pathway was down-regulated under carbon limitation [249] and that the oxygen uptake rate was also related to the ergosterol pathway.

Other transcriptional changes caused by the gene disruptions highlighted an increased expression of elongase (*ELO1*), which catalyzes the extension of unsaturated acyl-CoAs (carbon length 12-16) towards very long chain fatty acids and transcriptional activity of the genes connected to the metabolite octadecanoyl-CoA, an intermediate in the beta-oxidation pathway in the peroxisome. Both expressional changes could have a relation to the final chain length and saturation of the FAEEs.

4.6. Conclusion

The above described characterized strains represent the largely modified *S. cerevisiae* cell factories designed for the production of FAEEs. From both, physiological and transcriptional results it became clear that the disruption of acyl-CoA competing pathways caused cellular stress, which influenced the cellular growth. Additionally, it could be seen that there was a demand for NADPH, required for reduction steps during fatty acid synthase and therefore alternative NADPH supplying strategies could be targeted. Future metabolic engineering strategies on genes which were known to be suppressed by cellular regulation might also want to address the regulatory mechanism of the genes. A sum of several engineering strategies will absolutely improve the productivity of FAEEs in future.

5. Conclusion and future perspectives

Currently there is a need for sustainable solutions to replace fossil fuels, like diesel. One alternative are FAMEs, however, for several reasons the production method is not sustainable. A more sustainable production method is based on microbial cell factories. The aim of this PhD-thesis was the investigation and improvement of a FAEE producing microbial cell factory based on a *S. cerevisiae* host.

5.1. Metabolic engineering strategies

During this research, three metabolic engineering strategies improved the production of FAEEs in the *S. cerevisiae* host. The first strategy included the improvement of the native cytosolic pyruvate dehydrogenase bypass including the regulation of the acetaldehyde branch point and expression of a wax ester synthase which resulted in a 2.7 fold yield of FAEEs (see chapter 3.3 and **paper II**). A second metabolic engineering strategy was performed to re-channel the carbon flux via the PP pathway and the heterologous PHK pathway towards acetate or acetyl-CoA and eventually to FAEEs. The expression of *xpkA*, *pta* and *ws2* debouched into a 5.7 fold improved of the FAEE yield (see chapter 3.4 and **paper II**). Many different metabolic engineering strategies have been employed individually to improve *S. cerevisiae* cell factories for fatty acid derived products. There against, in the third strategy, combined overexpression of *ws2*, *ADH2*, *ALD6*, acs_{SE}^{L641P}, $ACC1^{S1157A,S659A}$ and *ACB1*, which were stably integrated into the chromosome, was investigated together with the disruption of acyl-CoA competing pathways for the production of FAEEs (see chapter 3.5 and **paper III**). This resulted in a 4.1 fold improvement compared to sole *ws2* expression.

The above mentioned engineering strategies all demonstrated successful improvements in the yield of FAEEs in *S. cerevisiae*. However, despite this success, the final yields and titers of the engineered strains did not result in competitive values. The evaluation of the above strategies leads to a couple of essential future engineering targets to improve the production of FAEEs in *S. cerevisiae*.

The first bottleneck faces the key-enzyme of this cell factory, the wax ester synthase. During this work the gene *ws2* from *M. hydrocarbonoclasticus* was used because of its superior conversion of alcohols (including ethanol) and acyl-CoA into FAEEs, compared to other known enzymes. It was thought that the modest yield of FAEEs, despite the major engineering efforts (described above), was partly blamed by the conversion capacity of the wax ester synthase. Wax ester synthase engineering strategies could e.g. lead to improved substrate affinities. Strain BdJ15 represents a very good starting point for further engineering, e.g. evaluation of the effect of increased expression of the wax ester synthase.

Another important engineering target is the improvement of the acyl-CoA biosynthesis in yeast. While acetyl-CoA carboxylase, acyl-CoA binding protein and fatty acid synthase [82] have been engineering targets before, the improvements were only limited. The enzymes are tightly regulated by several cellular layers and therefore improvement of this pathway will go hand in hand with fundamental understanding of the regulatory mechanisms. For example, screening enzyme libraries from oleaginous organisms for homology might point out enzymes parts which are sensitive to regulation.

The largest byproduct of the FAEE cell factory in *S. cerevisiae* is the natural product ethanol. Despite the overexpression of *ADH2*, which encodes the only alcohol dehydrogenase that converts ethanol into acetaldehyde, no reduction of ethanol was observed. Complete deletion of the alcohol dehydrogenase complex is not a suitable metabolic engineering strategy, because of the fact that ethanol is one of the substrates for FAEE. Production of other fatty acid derived products, i.e. fatty alcohols, is additionally, dependent on alcohol dehydrogenase activity. Therefore, a solution to remove ethanol to an extend that it is only available for product formation could be by the partial deletion or down-regulation of alcohol dehydrogenases involved in the formation of ethanol. Another, rather complicated, option could be an extensive engineering strategy including several heterologous pathways. While the cytosolic conversion of pyruvate to acetaldehyde (catalyzed by the pdc enzyme family) would be disrupted, several heterologous pathways, like the pyruvate dehydrogenase complex (PDH) from *Enterococcus faecalis* [176], the PFL pathway [177] and the PHK pathway [164] could lead towards the formation of acetyl-CoA and further conversion to acyl-CoA. Additional introduction of the *A-ALD* [177], naturally expressed in several prokaryotes, which catalyze the reversible reaction converting acetyl-CoA into acetaldehyde could regain the formation of ethanol. Circumventing endogenous pathways by the introduction of heterologous pathways, which often come with an advantage of cofactor or energy yield, has still many pitfalls, as for example the functionality of the heterologous enzymes in *S. cerevisiae*.

Another metabolic engineering strategy for the improvement of FAEE production in *S. cerevisiae* might be the deletion of acetyl-CoA competing pathways, which has been used earlier by deletion of *CIT2* and *MLS1* [166]. The transcriptional analysis performed in **paper IV** suggested the additional demand of NADPH. As indicated earlier [198], the metabolic flux through the PHK pathway was only limited and therefore this pathway should become more attractive for metabolic flux.

5.2. Pathway assembly and construction of microbial cell factories

During this work, large effort was consumed by the construction of the metabolic pathway which had to be implemented into the *S. cerevisiae* host. In theory, all constructed strains could have been assembled within a many times shorter period. Unfortunately a large gap existed between the theoretical time of pathway assembly described by the individual methods (see chapter 2) and the practical attempts to assemble pathways by using different (cloning) techniques. It becomes clear that there is a high demand of high-throughput pathway assembly methods including criteria as efficiency, reliability, flexibility, time of assembly, fragment size, size of the total pathway, regulation and expression platform. As described before, a clear result from a comparative research [86] attributes the assembly advantage to ligase cycling reaction cloning and homologous recombination. In agreement with this study, homologous recombination was used for successful pathway assembly and integration during this work (**paper III**). The success of pathway expression and consequently cellular phenotyping is also leaning on the expression platform and the pathway regulation (mostly dependent on different promoter types) provided. Chromosomal expression should be an integrative part of pathway engineering instead of the plasmid based expression to circumvent instability problems occurring with the implementation of larger pathways. Nevertheless, hopefully will the ongoing development of pathway assembly result in methods to evaluate the microbial phenotype much sooner in future.

Considering the efforts described above, it was amusing but mostly interesting reading some very daring future perspectives on cell factory design of one of the professors acting in the forefront of the field of metabolic engineering [40]. Jay Keasling envisioned on a future with tailor made, synthetically designed chromosomes including biosynthetic pathways constructed from a parts registry containing all known enzymes to maximize yield and to minimize the time required to grow the organism, including a genetic control system controlling gene expression at the correct time and appropriate levels. Additionally, he would like to see the synthetic designed chromosomes implemented in customized cellular envelopes, designed to withstand industrial processes to produce the desired chemical compound from the desired starting material. The combination of skills from the fields of metabolic engineering and synthetic chemistry would enable these fully functional self-replicating cell factories to minimize the time required to grow and to maximize the product yield. While this idealistic view covers a more futuristic view, it can already been seen that first steps of a combined synthetic and biological pathway assembly methods are performed now-a-days in which part of the DNA sequence is chemically synthesized and others are ‘reused’ by cloning techniques. Here, I could imagine a slowly growing larger share for the synthetic and computational technologies and a continuing joined venture between the different expertises towards a similar view as described above. A recent work on synthesizing a minimized genome by the J. Craig Venter Institute (JCVI) might be another step towards above described future [250].

A final comment on the pathway assembly paragraph is that despite the perspective of future cell factory engineering, current methods, using well known microorganisms with tractable genetic systems are highly relevant, and the development of pathway assembly methods, tools and fundamental knowledge for existing hosts, like *S. cerevisiae*, will enable the competitiveness of many microbial cell factories.

5.3. Feasibility

Transportation fuels, like FAEEs, have by far the highest volume and lowest economical margin of all metabolically engineered products. The economical feasibility of production of FAEEs will be dependent on availability of low-cost starting materials such as starch, sucrose, or cellulosic biomass. Apart from a cheap feedstock, the commercial success of FAEE production in yeast cell factories is dependent on the fatty acid derived products platform. Because of the high developing costs, it is important to strategically position the bulk biodiesel product within a platform of higher value compounds like for example pharmaceuticals and flavors. The product portfolio of Amyris Inc is a good example for this. Another essential criterion for the production of transportation fuels is the product excretion from the microbes and the ease of recovery of the product. This is not only necessary to close the tight economical margin on bulk products, but also for fast and accurate quantification, needed for evaluation of newly designed cell factories. High through-put methods to analyze cell factory systems throughout the central dogma will therefore remain crucial to increase the speed and reduce the costs of the development of cell factories.

All together, as long as there will be a transportation platform based on diesel engines, I am convinced that FAEEs, sustainably produced in a *S. cerevisiae* cell factory will become an economically competitive production method. Together, technological development, improved fundamental knowledge and commercial interest will push FAEEs, as part of the fatty acid derived products platform, towards a world of mobilization.

6. Acknowledgement

Unpublished Unpublished research recently revealed that the acknowledgement was selected to be the most read chapter of a thesis and from that point of view and due to the omniscient audience, the most important part of this work. Personally I don't mind that much about unpublished research, but appreciate the freedom of writing, given to the author in this last chapter of the thesis and therefore I am writing this chapter with much joy. The short version of this chapter is that I appreciated the last four years a lot, but that it is now time to move on. Without the supervision, expertise, help, and laughter from the people around me I could not have completed this work and therefore I can only recommend you to continue reading.

At the moment of writing this acknowledgement, I can look back on four of the most bustling, atmospheric, knowledge enriching, mood changing, lively, 'lagom', multicultural and (learning to become) humble years, during which I was free falling into some PhD valleys and climbed on top of the figurative PhD peaks. I was also enriched by several families during this time. I remember the yeast genomics family (which finished the polish vodka storage), metabolic engineering society, and most of all the Nielsens' lab family, coming together for the reunion in 2013. Being a member of the lattest, does not only make me proud, but also enabled me to enter a world of science and engineering with many in-house discussions and many nice places around the world to visit for discussion about the great evolution of microbial cell factory research. A personal favorite was, for obvious reasons, the 'Cell Factories and Biosustainability' conference.

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Nice, very nice!

Anonymous optimist

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