Nice T-R-Y

Metabolic engineering of *Escherichia coli* for improved bio-based ethyl acetate production

Anna C. Bohnenkamp

Propositions

1. *Escherichia coli* outcompetes yeasts as bulk ethyl acetate production host. (this thesis)
2. Downstream processing is ignored in cell factory design. (this thesis)
3. Metabolic engineering cannot beat evolution.
4. Some equipment demands more attention than a pet at home.
5. Doing research is like fighting a hydra; one solved question results in at least two new ones.
6. Manners are lost in the digital age.
7. Providing an overflow of information is worse than providing too little information.

Propositions belonging to the thesis, entitled

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Nice T-R-Y

Metabolic engineering of *Escherichia coli* for improved bio-based ethyl acetate production

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Thesis

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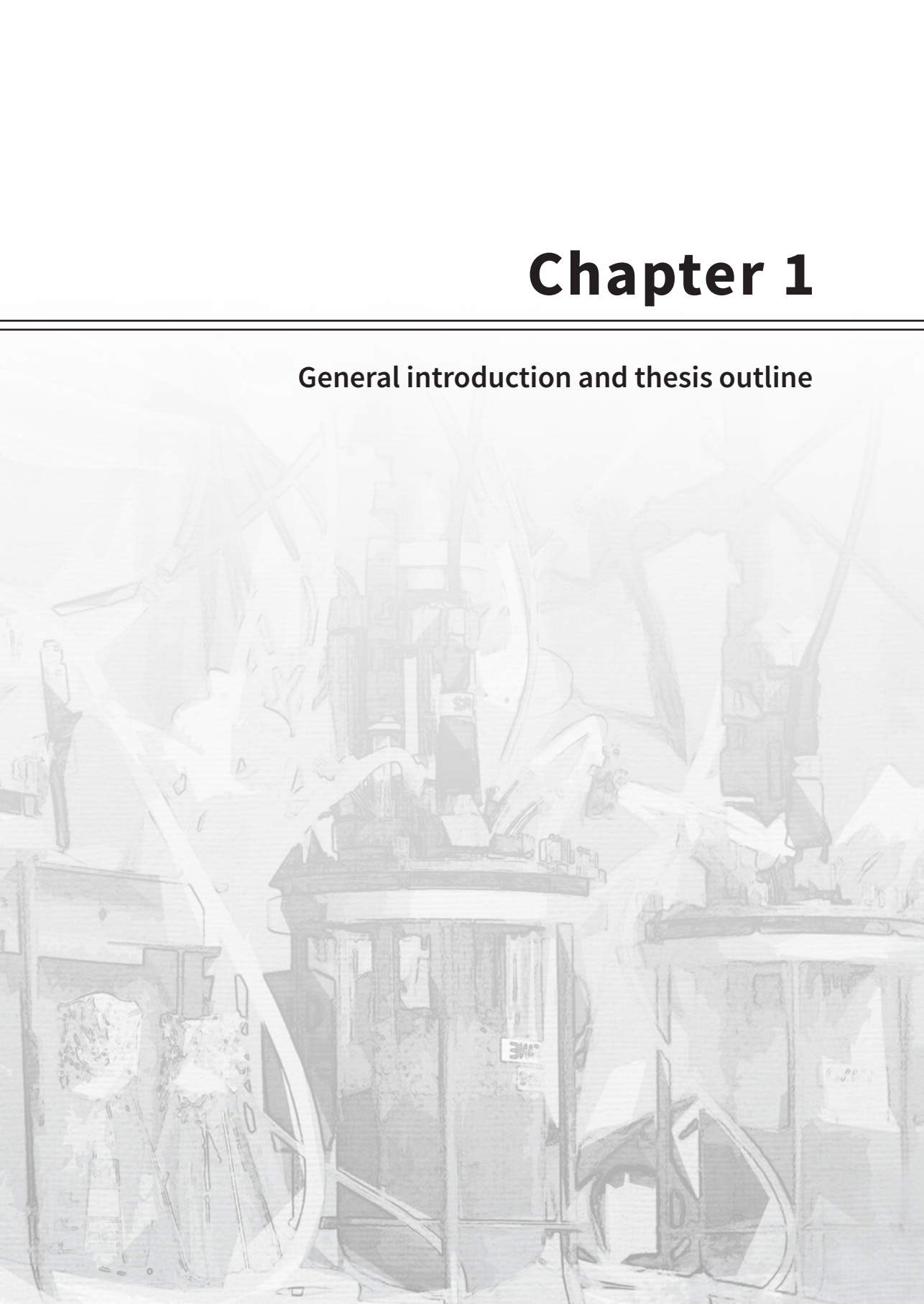
Chapter 1

General introduction and thesis outline



Biobased production of bulk chemicals

The use of renewable resources and sustainable manufacturing of products gained importance over the years. Concepts like circular economy, biobased economy and biorefinery have been suggested as the answer to diminishing fossil resources, increasing greenhouse gas emissions and climate change by relying on renewable resources, maximizing yields and minimizing waste streams (Ubando, Felix and Chen, 2020). Especially in the bulk chemical and transport sector depleting fossil resources and concerns about CO_2 emissions impact production costs and favor the introduction of bio-based alternatives (Van Dien, 2013).

While the concept of biological conversion of biomass has been exploited already more than hundred years ago with the fermentative production of acetone, butanol and ethanol it was limited by the cells natural metabolism and product range (Lopes, 2015). Consequently, only in few cases titers and productivities reached high enough levels for economic feasibility, so that optimization is inevitable in the development of competitive bio-based processes.

Microbial cell factories

Microbial cell factories opt at the controlled production of a desired product at high titers, rates and product yields. At first, organisms were selected on their ability to utilize a specific substrate, or to produce a desired compound such as *Zymomonas mobilis* for ethanol production, *Corynebacterium glutamicum* for amino acid production or *Thermoanaerobacterium* for its ability to degrade lignocellulosic feedstocks (Gronenberg, Marcheschi and Liao, 2013). Improvements based on phenotypic selection achieved by random mutagenesis were soon replaced by targeted genetic modifications (Lee, Mattanovich and Villaverde, 2012). The technical advances of past decades led to fast and inexpensive sequencing, targeted and efficient genome editing and rise of omics techniques and simulations and boosted cell factory development (Gustavsson and Lee, 2016). Especially *Escherichia coli* and *Saccharomyces cerevisiae* were exploited as cell factories due to their annotated genomes, well established genetic engineering tools and available metabolic models. Inactivation of competing pathways and overexpression of desired genes soon became standard procedures (Liao *et al.*, 2016). However, the regulatory mechanisms and metabolic interconnections are still not well understood so that the development of cell factories for novel products typically requires several iterative optimization and revision cycles, introduced as the “Design, Build, Test, Learn” concept (Nielsen and Keasling, 2016). Based on the above mentioned developments, both, the targeted products as well as production hosts increased in diversity and complexity (Lee *et al.*, 2011; Erickson, Nelson and Winters, 2012; Lopes, 2015). Still, the transfer from lab scale to commercialized process holds several pitfalls and failed upscaling tests can have dramatic effects on financial planning (Van Dien, 2013; Crater and Lievense, 2018).

Metabolic engineers have to carefully consider pros and cons of the anticipated cell factory, not only with respect to genetic accessibility or substrate range, but also process related factors like product tolerance or robustness towards harsh production environments (Gong, Nielsen and Zhou, 2017; Calero and Nikel, 2019). All considerations have to benefit the overall process feasibility and its impact on costs, as those are key for successful implementation of bulk chemicals or biofuels with overall low product value.

Key bottlenecks in biobased processes

As a rule of thumb, the downstream processing (DSP) strategy can take up to 70 % of the overall costs of the process. Therefore, the production process needs to be designed as efficient as possible, with high turnover of substrate into product. Generally, parameters such as titers, rates and yields (TRY) are considered key for developing successful bio-based processes, and thus are always subject to optimization.

Titers obtained by biological conversion often depend on the production host. Higher titers are beneficial for the DSP, and should ideally exceed 50 g/L (Van Dien, 2013). Especially for industrially relevant compounds, such as alcohols, organic acids and esters, product toxicity is a critical factor limiting achievable titers and reactor performance (Ingram, Vreeland and Eaton, 1980; Heipieper *et al.*, 1994; Straathof, 2003; Pereira, Verheijen and Straathof, 2016). With increasing understanding of metabolic networks and regulations, several mechanisms and proteins are identified as engineering targets for improved strain tolerance, no longer restricting the use of naturally tolerant strains such as *Clostridia* or *Pseudomonas putida* (Nicolaou, Gaida and Papoutsakis, 2010). Alternatively, *in situ* product removal offered an attractive work around and reduced the need to reach high titers while providing additional control and tunability on product concentration in the broth. Cell immobilization, gas stripping, two-phase systems and membrane extractions as reviewed for butanol production, can ease toxicity effects and have led to increased productivities (Ezeji *et al.*, 2005; Ezeji, Qureshi and Blaschek, 2007).

Keeping productivities or production rates high throughout the duration of the bioconversion is the next crucial parameter and should ideally exceed 2 g/L/h (Van Dien, 2013). Production rates determine the fermentation time and volume of the potential process. Higher rates can either lead to more product in less time or to smaller reactor volumes to achieve similar results. Rates may improve when all fluxes are redirected to the product of interest but often regulatory control restricts the full potential of the involved natural pathways (Nielsen and Keasling, 2016). Expression of entire pathways composed of one or a series of heterologous genes, or the use of heterologous promoters can be used to overrule natural regulation (Martin *et al.*, 2003; Yin *et al.*, 2007; Nielsen and Keasling, 2016; Wang *et al.*, 2019). The oxygen mass transfer rate is one of the most important parameter, setting the limits for large scale productions (Garcia-Ochoa and

Gomez, 2009). Therefore, productivities in aerobic systems are often significantly lower compared to anaerobic processes, which should be favored for low-value and high quantity products such as bulk chemicals (Weusthuis *et al.*, 2011).

Moreover, during anaerobic fermentation higher product yields can be obtained, as carbons can no longer be fully oxidized in the tricarboxylic acid (TCA) cycle and become lost as CO_2. Moreover, under anaerobic conditions less ATP is generated. Lower energy availability leads to less biomass, benefiting both product formation and product yield (J. Wang *et al.*, 2016). The product yield, or in other words the conversion efficiency from substrate into product, gives indications on substrate costs adding to the overall process economics. The maximum theoretical yield (Y^E) is calculated purely based on the degree of reduction of substrate and product, thus doesn't change. It assumes that all energy can be transferred to the product (Dugar and Stephanopoulos, 2011). The maximum pathway yield (Y^P) on the other hand, is determined by the metabolic pathway when considering all fluxes going to product formation. Consequently, the pathway yield changes among organisms using different pathways and can vary within an organism depending on the modifications that have been applied. Cell factories should be optimized to an extent in which the Y^P corresponds to at least 80 % of the Y^E (Van Dien, 2013).

It becomes apparent that the three key parameters TRY are interconnected and a choice favoring one parameter might have negative impacts for the other. It is advisable to not only reflect on the designed process but to also consider future scenarios and their impact on process related parameters, like feedstock availability, potential market share and abundancy of the product and eventual by-products (Noorman and Heijnen, 2017).

We want to use the example of esters and more specific that of ethyl acetate to explore the need and possible approaches towards bio-based solutions.

Ester production

Ester and specifically carboxylate esters, are a diverse group of compounds with a vast range of applications. In addition to flavor compounds in the food and beverage industry, they also find use in industrial applications such as lubricants, additives, or solvents (Durrans and Davies, 1971; Berger, 2009). Their basic composition is based on alcohol and carboxylic acid compounds with varying chain lengths and branches. Current ester production is predominantly based on petrochemical production processes. Conversion is performed via Fischer Speier Esterification, dictated by chemical equilibrium and enhanced by acid catalysts (Fischer and Speier, 1895; Jyoti *et al.*, 2018). Additionally, distillation or adsorption steps under high temperatures and high pressures, make the overall production an energy demanding process. The current process highlights some of the typical drawbacks of petrochemical production processes in the chemical industry.

The transition towards more sustainable production processes has been initialized and several bio-based esters are already commercially available (Table 1). However, in most cases bio-based production relies on the supply of bio-based alcohols and acid substrates, followed by the tradition chemical conversion into esters. While renewable resources are used, the downsides of chemical conversions remain unchanged. Another factor limiting this approach, is the bio-based production of the respective substrates. While for ethyl lactate, both pre-cursors ethanol and lactate have been successfully transferred to bio-based processes, for other alcohols and acids, production may be limited by high toxicity, consequently leading to unfeasible processes. Several studies have focused on assessing and understanding carboxylic acid toxicity (Royce *et al.*, 2013). Both, toxicity and low solubility especially for longer chains limit the achievable titers, thus threatening the economic feasibility of a biobased process due to high DSP costs.

TABLE 1: Examples of commercially available bio-based esters and their main conversion strategy.

Compound	Company	Strategy		Reference
Ethyl lactate	Vertec BioSolvents	Bio-based lactic acid and ethanol	Esterification reaction in patented membrane reactor	(Datta and Tsai, 1995; Vertec BioSolvents, 2019)
Ethyl, propyl and butyl lactate	Corbion / Purac Inc	Bio-based lactic acid	Esterification	(Datta and Henry, 2006; Corbion, 2019)
Ethyl acetate	Prairie Catalytic / Viridis	Bio-ethanol	Reactive distillation	(Strobel, 2021)
Ethyl acetate	Godavari Biorefineries Ltd.	Bio-ethanol	Esterification	(Godavari, 2021)

Industrial relevance of ethyl acetate

This thesis focuses on the development of a bioprocess for ethyl acetate, a short chain acetate ester. It is traditionally produced using ethanol and acetate as substrates. Ethyl acetate finds use in applications like paints, adhesives and is considered an environmentally benign solvent. The global market demand for ethyl acetate was 4.75 million tons and is expected to further increase due to rising market demands in the paints and coatings industry (Market Research Future, 2021).

Natural ester and bulk ethyl acetate production

Instead of focusing on providing bio-based substrates for chemical conversion of esters, one-pot conversions should be desired. This conversion is already taking place in several wine and beer fermentations where yeast ferment sugars not only to ethanol but also produce a wide range of esters that ultimately determine the taste and flavor profile of

the fermented beverage. However, ester concentrations usually settle in the range of parts per million (ppm) (Saerens *et al.*, 2010). Within a specific range the flavor profile is considered positive, while higher concentrations result in unpleasant characteristics (Liu, Holland and Crow, 2004).

With respect to ethyl acetate some natural bulk producers have been identified. Yeasts such as *Wickerhamomyces anomalus*, *Kluyveromyces marxianus* or *Kluyveromyces lactis* naturally produce high concentrations of ethyl acetate under specific conditions (Fredlund *et al.*, 2006; Löser *et al.*, 2011; Löser, Urit, Keil, *et al.*, 2015). Those strains have been investigated and optimized excessively in order to enhance bulk ethyl acetate production (Löser *et al.*, 2013). Most efforts however, focused on optimizing cultivation conditions as the main enzyme responsible for bulk ethyl acetate production remained elusive until recently.

Somewhat analogous to chemical production methods, enzymatic conversions catalyze the condensation of an alcohol and an acyl-group into the respective ester under physiological conditions. The enzymes responsible for this conversion belong to the group of alcohol acyltransferases (AAT). Several AATs are known with different substrate ranges and specificities, thus producing a range of esters resulting in the final ester profile. Atf1 and Atf2 are the most prominent yeast AATs and have been used for heterologous ester production (Verstrepen *et al.*, 2003; Horton and Bennett, 2006). Also Eht1 and Eeb1 exhibit AAT activity, while containing a α/β-hydrolase fold, that has been associated with hydrolysis reactions (Saerens *et al.*, 2006; Knight, Bull and Curnow, 2014). Regarding bulk ethyl acetate producers only about 50 % of the total ethyl acetate production could be assigned to these known AATs indicating that the group was incomplete.

In 2017, ethanol acetyltransferase 1 (Eat1) was identified as the missing member of the AAT family (Kruis *et al.*, 2017). In bulk ethyl acetate producers Eat1 indeed accounted for almost 50 % of the produced ethyl acetate, while in other yeasts like *S. cerevisiae* the capacity of Eat1 was lower. Characterization of Eat1 confirmed that also this AAT contained the active site consisting of Ser-Asp-His, typical for α/β - hydrolases. In fact, all AATs containing this fold, exhibit also esterase or thioesterase side activities. Esterase activity means the hydrolysis or breaking down of an ester into alcohols and acids, whereas thioesterase activity corresponds to the hydrolysis of an acyl-CoA into acid and free CoA. Fortunately, further testing of Eat1 showed that both side-activities are inhibited by ethanol, so that AAT activity can be secured (Kruis *et al.*, 2017).

With the discovery of Eat1 the list of ester-producing enzymes was expanded and especially the missing link for bulk ethyl acetate production was found. This opened the door for more directed engineering strategies for the bio-based production of esters and especially ethyl acetate.

Bioprocess design for ethyl acetate production

A first outline of the potential process and its requirements can be drawn by determining the Y^E and deriving the reaction equation. Based on the degree of reduction of glucose (24) and ethyl acetate (20), the Y^E based on glucose as carbon source yields 1.2 $mol_{ethyl\ acetate}/mol_{glucose}$ as maximum theoretical yield. A corresponding reaction equation indicates that this yield can be obtained without the addition of any external electron acceptors, theoretically enabling anaerobic production (Equation 1):

$$5\ C_6H_{12}O_6(aq) = 6\ C_4H_8O_2(aq) + 6\ CO_2(g) + 6\ H_2O(l) \qquad \text{(Eq. 1)}$$

Also calculation of the Gibbs free energy of the reaction ($\Delta G_r'^0 = -207.3 \pm 5.9$ kJ/$mol_{ethyl\ acetate}$) results in a negative value indicating thermodynamic feasibility of the reaction. A closer look to the chemical properties of ethyl acetate reveal that the compound is readily soluble in water, has a boiling point comparable to that of ethanol combined with a high vapor pressure (National Center for Biotechnology Information, 2021). Like most compounds that are synthesized by microorganisms, naturally only low titers are reached and after a certain critical concentration the product negatively impacts the cell with respect to growth and viability. There is increasing interest in estimating the critical concentration for industrially relevant compounds but only a few are well documented. For ethyl acetate critical concentrations range around 10 g/L for *E. coli* (Wilbanks and Trinh, 2017) and 17 g/L for natural producers such as *K. marxianus* (Joseph Tabachnick and Joslyn, 1953; Urit, Manthey, *et al.*, 2013).

Consequently, the desired titer threshold cannot be reached without severely impacting the fitness of the production host and an *in situ* product removal strategy needs to be developed. Based on its volatile character ethyl acetate can be removed from the fermentation broth via gas stripping (Urit *et al.*, 2011). Moreover, by changing stripping rates, stirring or temperatures, the stripping behavior can be fine-tuned and ethyl acetate concentrations in the liquid phase can be tightly controlled.

Anaerobic ethyl acetate production

Based on the reaction equation, we concluded that no oxygen is needed for the production of ethyl acetate. However, yeasts are not able to naturally produce ethyl acetate under complete anaerobic conditions. The production of ethyl acetate generates excess NADH and regeneration of NAD^+ needs to proceed via an external electron acceptor such as oxygen. Therefore, production improved in oxygen limited conditions, as they promote ethanol production but relieve the NAD^+ imbalance with oxygen. In addition, ethyl acetate production by bulk producers is strictly related to fermentation conditions such as oxygen or iron limitation and leads to complex process setups (Löser *et al.*, 2012). Other microorganisms use different pathways that enable redox balanced ethyl acetate

production under anaerobic conditions. Consequently, higher product yields could be obtained without much engineering effort.

To explore *E. coli* as ethyl acetate production platform different strategies are followed up on. The general ability to functionally express Eat1 and to produce ethyl acetate has been shown before (Kruis *et al.*, 2017). Overexpression of Eat1 and the supplementation of 5.86 g/L ethanol in addition to glucose as main carbon source led to the formation of 4.87 g/L ethyl acetate under aerobic conditions (Figure 1a). However, external supplementation of ethanol is not a feasible strategy as it significantly increases the costs for substrates. Therefore, an anaerobic production process is desired and further explored in this thesis. *E. coli* is able to generate ethyl acetate in a redox balanced manner once heterologous AAT is expressed, with a resulting pathway yield of 1 mol/mol (Figure 1b). The high flux through pyruvate formate lyase (Pfl), the key enzyme converting pyruvate into acetyl-CoA with the subsequent release of formate, results in the accumulation of high formate levels. Besides the potentially toxic effect of high formate levels, electrons are also lost with this by-product. To be economically viable, this issue should be tackled by valorizing formate. Co-production of hydrogen, an attractive biofuel could be an elegant solution (Figure 1c). Another approach to conserve these electrons is to metabolically engineer *E. coli* so that the theoretical maximum yield for ethyl acetate is achieved, consequently reducing by-product formation to a minimum (Figure 1d).

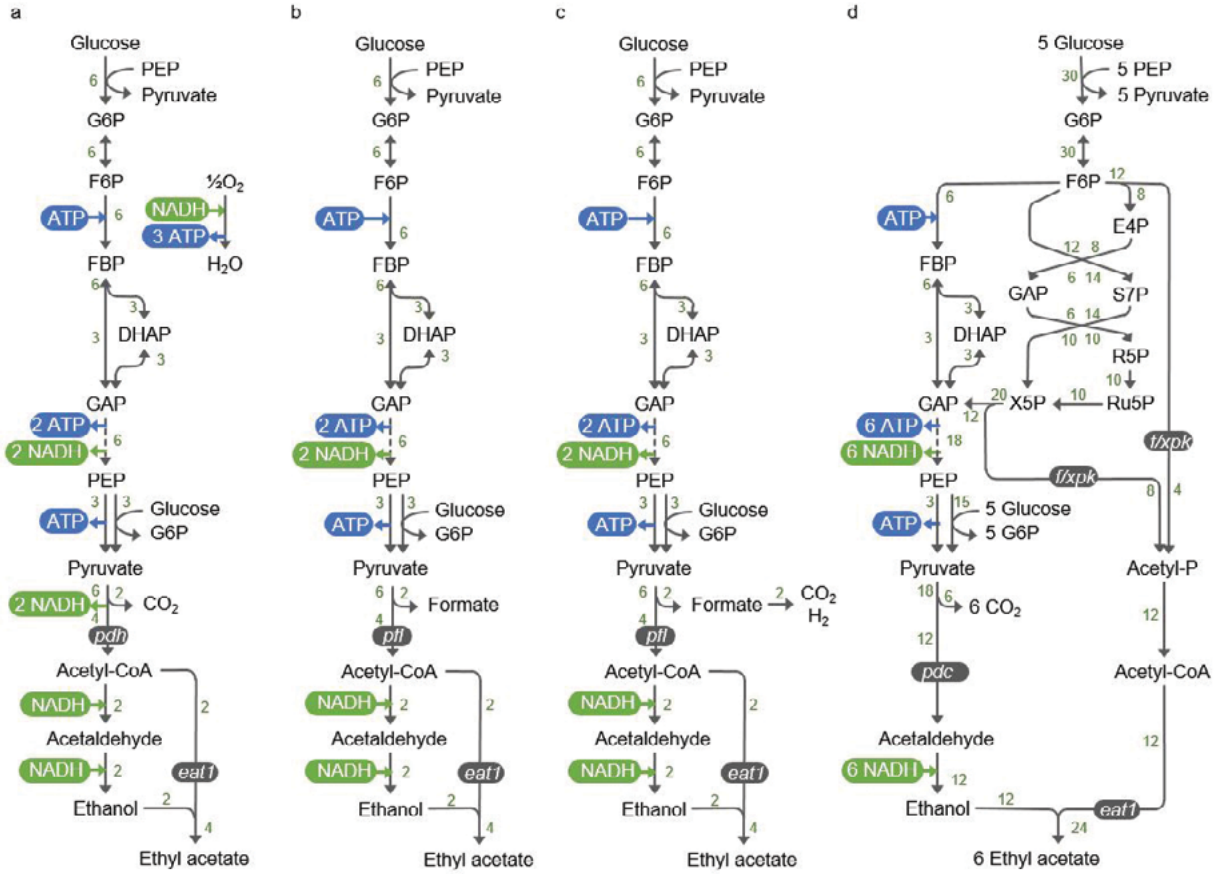


FIGURE 1: Schematic overview of different ethyl acetate production scenarios considering the central metabolism of *E. coli* and heterologous expression of Eat1. (a) Aerobic production and cofactor regeneration via the electron transport chain. (b) Anaerobic production relying on native enzymes of the fermentative pathway resulting in formate secretion. (c) Anaerobic production and electron recovery by co-producing green H_2 from formate via native formate hydrogen lyase. (d) Anaerobic production enabling the theoretical maximum yield by insertion of heterologous pyruvate decarboxylase and phosphoketolase for electron and carbon conservation.

Aim and outline of the thesis

The goal of this project was to design a microbial ethyl acetate production process and to improve the key parameters titers, rates and yields by applying different strategies.

We have introduced esters, and ethyl acetate in particular, as industrially relevant compounds that are in need of sustainable alternatives. In the case of ethyl acetate the factors titers, rates, yields are further sketched out and a proposition towards an *Escherichia coli* based production platform has been made that also suggests a suitable downstream processing strategy.

In **Chapter 2** we reviewed and updated the known ester producing enzymes in yeasts. We presented the different ester-producing mechanisms, with emphasis on alcohol acyltransferases (AATs). In addition, we summarized strain and metabolic engineering strategies towards ester producing microorganisms and highlighted in how far ester production could also benefit the production of other chemicals like diols, alcohols or carboxylic acids.

The enzyme Eat1 is responsible for more than 50 % of natural ethyl acetate production in yeast and is located in the yeast mitochondria. In **Chapter 3** we demonstrate that anaerobic ethyl acetate production is feasible by expressing *eat1* in *Escherichia coli*. Due to the lack of mitochondrial processing machinery in procaryotes, manual trimming of the pre-sequence was performed leading to insights into its effect on protein stability and heterologous expression.

In **Chapter 4** we evaluated the performance of two Eat1 homologs and the cytosolic AAT Atf1 and optimized their *in vivo* expression under anaerobic conditions. We hypothesized that generated ethyl acetate titers were limited by the buffering capacities of the medium. Using pH-controlled 1.5-L bioreactors we determined the impact of pH-control on ethyl acetate production and validated the suitability of gas stripping as *in situ* product removal strategy.

Chapter 5 focused on improving productivities of anaerobic ethyl acetate fermentation. To prevent inhibition by formate accumulation we exploited the ability of *Escherichia coli* to convert formate into hydrogen and carbon dioxide. Moreover, bio-based hydrogen can be an interesting co-product as it is considered a promising biofuel. Consequently, we evaluated the potential of co-producing hydrogen and ethyl acetate with respect to yields and productivities.

In order to achieve maximum theoretical yields, all carbons from the substrate need to be directed to product formation as much as possible. Aerobically the introduction of a heterologous phosphoketolase has benefitted the yield of several acetyl-CoA derived products. In **Chapter 6** we explored the option to use phosphoketolase for carbon

conservation under anaerobic conditions and aimed at producing ethyl acetate from two industrially relevant substrates near the theoretical maximum yield. To meet redox requirements we also employed heterologous pyruvate decarboxylase and evaluated the impact of all modifications on the central metabolism of *Escherichia coli.*

Chapter 7 will cover the general discussion on the results of this thesis. Moreover, the next steps necessary to reach the goal scenario will be outlined. Future challenges for bio-based ethyl acetate and ester production will be addressed. We will highlight the importance of integrated process design and the early integration of a downstream processing strategy and will re-evaluate Eat1 as key enzyme for bulk ethyl acetate production. Finally, findings and guidelines from this study will be transferred to a broader context.

Chapter 2

Microbial production of short and medium chain esters: Enzymes, pathways, and applications

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Abstract

Sustainable production of bulk chemicals is one of the major challenges in the chemical industry, particularly due to their low market prices. This includes short and medium chain esters, which are used in a wide range of applications, for example fragrance compounds, solvents, lubricants or biofuels. However, these esters are produced mainly through unsustainable, energy intensive processes. Microbial conversion of biomass-derived sugars into esters may provide a sustainable alternative. This review provides a broad overview of natural ester production by microorganisms. The underlying ester-forming enzymatic mechanisms are discussed and compared, with particular focus on alcohol acyltransferases (AATs). This large and versatile group of enzymes condense an alcohol and an acyl-CoA to form esters. Natural production of esters typically cannot compete with existing petrochemical processes. Much effort has therefore been invested in improving *in vivo* ester production through metabolic engineering. Identification of suitable AATs and efficient alcohol and acyl-CoA supply are critical to the success of such strategies and are reviewed in detail. The review also focusses on the physical properties of short and medium chain esters, which may simplify downstream processing, while limiting the effects of product toxicity. Furthermore, the esters could serve as intermediates for the synthesis of other compounds, such as alcohols, acids or diols. Finally, the perspectives and major challenges of microorganism-derived ester synthesis are presented.

Keywords: ester, metabolic engineering, alcohol acyltransferase, alcohol, carboxylic acid, bulk chemical, microbial synthesis.

Introduction

Carboxylate esters are versatile compounds that find various applications in the food and chemical industry. They are naturally produced by yeasts such as *Saccharomyces cerevisiae* and define the taste and odor of fermented beverages (Saerens *et al.*, 2010). As natural food additives they are used to enhance the flavor and odor profile of various food products (Berger, 2009). Ethyl acetate, isoamyl acetate or propyl acetate are common fragrance and aroma compounds, that also find application as bulk chemicals (Figure 1) (Rodriguez, Tashiro and Atsumi, 2014; Carroll, Desai and Atsumi, 2016). Various esters are used as industrial solvents due to their biodegradability and low toxicity, or as plasticizers and polymer additives (Durrans and Davies, 1971; Białecka-Florjańczyk and Florjańczyk, 2007; Löser, Urit and Bley, 2014). Further, they find applications as lubricants, coatings and are explored for their potential as drop-in fuels or biodiesels (Figure 1) (Kalscheuer, Stölting and Steinbüchel, 2006; Lange *et al.*, 2010; Chuck and Donnelly, 2014).

Traditional ester production processes make use of the Fischer-Speier esterification (Fischer and Speier, 1895). Alcohols and carboxylic acids, produced from fossil resources, are condensed in the presence of an acid catalyst at elevated temperatures (Jyoti *et al.*, 2018). Water is released in the process, which leads to the formation of the desired ester. The process however, is dictated by an equilibrium that reduces the reaction rate with time and prevents a complete conversion of all acid and alcohol substrates. In addition, water has been found to inhibit the catalytic activity of the acid catalyst (Liu, Lotero and Goodwin, 2006). To allow the reaction to proceed, the water is continuously removed from the system in energy intense distillation or adsorption steps. While transesterification of vegetable oils is evaluated for the production of biodiesel, this process has only limited applicability (Vyas, Verma and Subrahmanyam, 2010; Saba *et al.*, 2016). For other esters esterification of biobased alcohols and acids might be considered, but as equilibrium reactions remain part of the process other solutions should be evaluated.

Microbial conversion systems will be key in developing efficient ester-producing bioprocesses and have already received much attention for sustainable bulk chemical synthesis (Werpy and Petersen, 2004; van Haveren, Scott and Sanders, 2008; Vennestrøm *et al.*, 2011; Donate, 2014). Their vast array of enzymes is able to perform hundreds of chemical conversions at ambient conditions. To catalyze multiple metabolic pathways simultaneously, enzymes have evolved high specificity for their substrates, and the formation of by-products in industrial microbial bioprocesses is thereby limited (Quax, 2014).

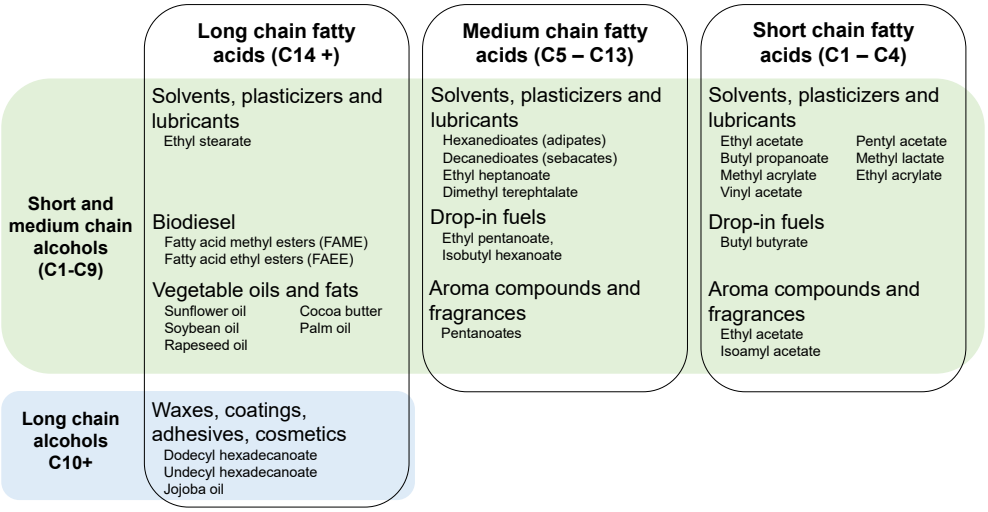

FIGURE 1: Applications of various esters as bulk chemicals or high value products. Due to their versatility, esters may be found in various groups of applications and are not limited to those mentioned here.

Bulk chemicals, such as esters used as solvents, polymers, drop-in fuels or lubricants, have low market prices and low profit margins compared to biopharmaceutical products or industrial enzymes (CIB, 2015; Marella *et al.*, 2018). To become economically competitive with their petrochemical counterparts, the biobased conversion processes must achieve high product titers, yields and volumetric productivities, and should be followed by efficient downstream processes. This is a major challenge in current biotechnology that has prevented many products from moving to large scale production (Van Dien, 2013)

Due to their broad application range, especially short chain esters such as acetate esters, lactate esters, butyrate esters or acrylate esters would benefit from sustainable production processes. Companies such as Corbion or Vertec BioSolvents are among the first to launch green lactate ester products for coatings, paints and cleaning (Corbion, 2019; Vertec BioSolvents, 2019). Many commercially interesting esters are important building blocks for various polymer structures, such as terephthalates. However, many are currently produced by chemical conversion processes and to date, no biobased approaches have been described. Such esters are therefore not further considered in this review (Banella *et al.*, 2016; Lee *et al.*, 2019).

This review article focuses on how and why microorganisms synthesize esters, particularly those that can be applied as commodity chemicals. We provide a detailed overview of the enzymatic reactions that produce esters in microorganisms and how these enzymes have been applied to improve ester formation. An overview of the metabolic engineering strategies aimed at increasing ester production is provided and notable examples are highlighted. We also consider the physical and chemical properties of esters and how

they might benefit the production of other valuable bulk chemicals, such as alcohols, carboxylic acids, and α,ω-diols. Lastly, the major challenges that lie ahead of biobased ester production are summarized.

Microbial ester production

Natural ester production by microorganisms, such as yeasts and lactic acid bacteria is well established and has historically been applied in food production. Volatile esters are among the most important aroma compounds in fermented foods, such as beer, wine and dairy products. In low concentrations, esters impart a sweet, fruity aroma, but are also considered as off-flavors when present in high amounts (Liu, Holland and Crow, 2004). Ethyl acetate is the most abundant volatile ester in food. Its concentration ranges from ~50 to 100 mg/L in dairy products and from ~8 to 64 mg/L in beer and wine (Liu, Holland and Crow, 2004; Saerens *et al.*, 2010). Other volatile esters, such as isoamyl acetate, phenylethyl acetate, ethyl hexanoate and many others do not exceed concentrations of 1 mg/L. These concentrations lie around or just above the human detection threshold (Saerens *et al.*, 2010; Dzialo *et al.*, 2017) and therefore greatly affect the aroma of food products. The amounts of esters naturally produced by microorganisms are typically low, although some exceptions exist, such as bulk ethyl acetate-producing yeast or wax-ester accumulation by *Euglena gracilis.* This microalga can accumulate wax esters to as much as 65 % of the cell dry weight under anaerobic conditions (Tucci *et al.*, 2010).

The ability of certain yeasts to produce high amounts of ethyl acetate was observed more than 120 years ago (Beijerinck, 1892). Yeasts such as *Kluyveromyces marxianus, Wickerhamomyces anomalus* and *Cyberlindnera jadinii* are able to synthesize ethyl acetate from sugars or ethanol (Tabachnick and Joslyn, 1953a; Tabachnick and Joslyn, 1953b; Armstrong, Martin and Yamazaki, 1984; Löser, Urit and Bley, 2014; Meersman *et al.*, 2016; van Rijswijck *et al.*, 2017). Growth under iron-limited conditions is the main trigger for bulk ethyl acetate production in yeasts (Armstrong and Yamazaki, 1984; Urit *et al.*, 2012; Kruis, *et al.*, 2018). In some yeasts, ethyl acetate formation has also been induced by oxygen limitation (Fredlund *et al.*, 2006; Kruis *et al.*, 2017). Ethyl acetate production in *K. marxianus* has been investigated in most detail. Several strains have been identified that form ethyl acetate from whey sugars (Kallel-Mhiri, Engasser and Miclo, 1993; Löser *et al.*, 2011), glucose (Willetts, 1989), and cassava bagasse supplemented with glucose (Medeiros *et al.*, 2001). *K. marxianus* is able to catabolize lactose and can utilize whey, a side stream of the cheese industry, to produce ethyl acetate. Moreover, this yeast is able to grow at elevated temperatures, which facilitates ethyl acetate removal from the fermentation broth. These traits make *K. marxianus* an attractive cell factory for the production of biobased ethyl acetate (Fonseca *et al.*, 2008; Löser *et al.*, 2011; Urit, Manthey, *et al.*, 2013; Löser, Urit and Bley, 2014). In one study, lactose was converted to ethyl acetate by *K. marxianus* at 42 °C.

The yield reached 56.2 % of the pathway maximum, which is the highest reported yield for a natural ethyl acetate-producer reported to date (Urit *et al.*, 2013).

Because of the structural and chemical diversity of esters, no singular physiological role can be defined for ester synthesis and some are even still debated. Ethyl acetate is a major fermentation product of certain yeast species and contributes to balancing their central carbon metabolism under sub-optimal growth conditions (Fredlund, Blank, *et al.*, 2004; Löser, Urit and Bley, 2014; Kruis *et al.*, 2018). High concentrations of ethyl acetate also repress growth of competitive organisms (Fredlund *et al.*, 2002; Fredlund, Druvefors, *et al.*, 2004). Volatile esters serve as metabolic intermediates during growth on alkanes or cyclic alcohols in some bacterial species (Iwaki *et al.*, 2002; Kotani *et al.*, 2007). Some esters, such as isoamyl acetate, may help yeast to disperse in the environment by attracting insects (Christiaens *et al.*, 2014). Some specialized esters can even act as bacterial virulence factors (Onwueme *et al.*, 2005). Wax esters are produced as intracellular storage compounds in *Acinetobacter baylyi* and *Marinobacter hydrocarbonoclasticus* (Ishige *et al.*, 2003; Wältermann and Steinbüchel, 2005), or as an anaerobic fermentation product in *Euglena gracilis* (Müller *et al.*, 2012).

Ester-forming reactions

Four main enzymatic ways of ester biosynthesis have been described, using i) esterases (Figure 2a), ii) hemiacetal dehydrogenation (HADH, Figure 2b), iii) Baeyer-Villiger monooxygenases (BVMOs, Figure 1c) and iv) alcohol acyltransferases (AATs, Figure 2d). The reactions catalyzed by AATs and esterases are redox neutral, whereas, BVMOs and HADH require respectively NAD(P)H or NAD(P) (Figure 2).

Other ester-forming enzyme classes exist, but have not been applied extensively for ester synthesis. These include S-adenosyl methionine (SAM) dependent O-methyltransferases and polyketide synthase associated proteins. SAM dependent O-methyltransferases transfer the methyl group from SAM to free fatty acids to form FAME (Sherkhanov *et al.*, 2016). Polyketide associated proteins are better known for their role in the synthesis of secondary metabolites. One such protein, PapA5 is able to transfer alcohols to growing polyketide chains, forming complex esters with interesting biological activities in the process (Menendez-Bravo *et al.*, 2014, 2017).

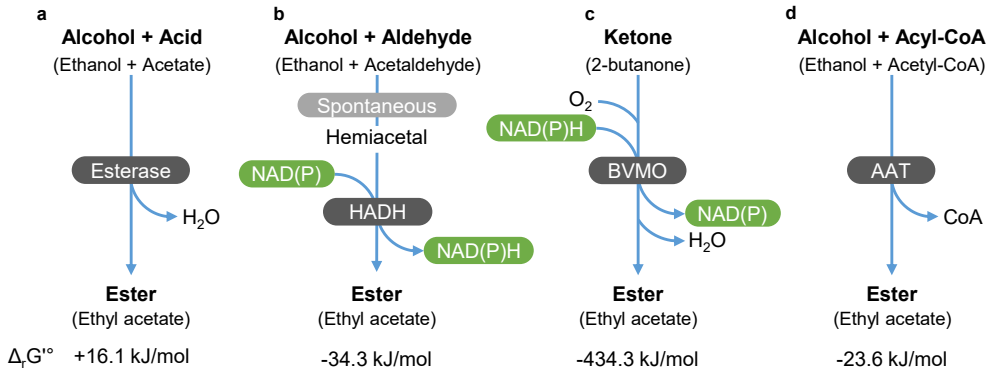

FIGURE 2: Enzymatic reactions that result in ester production. The ΔrG'° of the reactions were estimated using Equilibrator (Flamholz *et al.*, 2014) under standard conditions and pH 7. Abbreviations: AAT – alcohol acyltransferase, HADH – hemiacetal dehydrogenation, BVMO – Baeyer-Villiger monooxygenase

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Esterases

Esterases, including lipases, are ubiquitous enzymes that have been identified in all domains of life. In aqueous environments, they catalyze the hydrolysis of ester bonds, resulting in the formation of an alcohol and a carboxylic acid. Water activity, pH and substrate concentrations play a critical role in esterase reactions and special consideration should be given to the thermodynamics of the reaction. Under aqueous conditions, the reverse esterase reaction has a positive $\Delta_rG'°$, making the reaction thermodynamically unfavorable (Figure 2a). Hence, industrial production of esters *via* esterases is typically performed in non-aqueous systems using organic solvents or high substrate concentrations (Khan and Rathod, 2015; Bornscheuer, 2018). Nonetheless, several studies have reported *in vivo* reverse esterase activity as the cause of ester formation by microorganisms (Kashima *et al.*, 2000; Rojas *et al.*, 2002; Abeijón Mukdsi *et al.*, 2009; Costello *et al.*, 2013; Abeijón Mukdsi *et al.*, 2018; Hong *et al.*, 2018). In *Acetobacter pasteurianus*, disruption of the esterase-encoding gene *est1* eliminated ethyl acetate and isoamyl acetate production (Kashima *et al.*, 2000). The Est1 enzyme was characterized and showed significant reverse esterase activity *in vitro* (Kashima *et al.*, 1998).

Hemiacetal dehydrogenation

Hemiacetals are formed *in vivo* by spontaneous reaction of an aldehyde with an alcohol. The subsequent NAD(P)-dependent hemiacetal dehydrogenation results in ester formation (Figure 2b). The enzymes that catalyze the reaction are in some cases referred to as hemiacetal dehydrogenases (Kusano *et al.*, 1998, 1999). In a strict sense, this type of enzyme does not exist, since the HADH reaction is a side activity of certain alcohol dehydrogenases. It was proposed that the side activity is due to the resemblance of hemiacetals to secondary alcohols (Park, Shaffer and Bennett, 2009). Hemiacetal

dehydrogenation was first observed in methylotrophic yeast (Sakai *et al.*, 1995). When the yeasts were grown on methanol or ethanol as carbon source, high concentrations of formaldehyde and acetaldehyde accumulated, respectively, which are toxic for most organisms (Kunjapur and Prather, 2015). Hemiacetal dehydrogenation may act as mechanism to detoxify aldehydes by converting them to esters *via* hemiacetals (Yurimoto, Kato and Sakai, 2005). This activity has been observed for methyl formate synthesis in *Pichia methanolica*, *Candida boidinii* and *S. cerevisiae* (Murdanoto *et al.*, 1997; Kusano *et al.*, 1998; Yurimoto *et al.*, 2004). Hemiacetal dehydrogenation may also contribute to ethyl acetate formation in *Neurospora crassa, S. cerevisiae, Cyberlindnera jadinii,* and *K. marxianus*, but this has not been confirmed *in vivo* (Kusano *et al.*, 1998, 1999; Park, San and Bennett, 2007; Löbs *et al.*, 2017).

Baeyer-Villiger monooxygenases

BVMOs are flavin-containing enzymes that require NAD(P)H as cofactor. They catalyze the insertion of oxygen between a C-C bond in aldehydes and ketones (Figure 2c). BVMOs are characterized by a FXGXXXHXXXW(P/D) sequence motif and have been found in all domains of life (Fraaije *et al.*, 2002; Mascotti, Lapadula and Juri Ayub, 2015). In nature, BVMOs participate in the synthesis of secondary metabolites (Wen *et al.*, 2005; Frank *et al.*, 2007) or enable utilization of unconventional carbon sources, such as alkanes, ketones or cyclic alcohols (Britton and Markovetz, 1977; van der Werf and Boot, 2000; Iwaki *et al.*, 2002). They catalyze the conversion of ketones to esters, which are further hydrolyzed to readily metabolizable alcohols and acids. Such pathways enable *Gordonia sp.* or *Pseudomonas veronii* to grow on propane or methyl ketones, respectively (Kotani *et al.*, 2007; Onaca *et al.*, 2007; Völker *et al.*, 2008). The structures, functions and applications of BVMOs have been reviewed extensively elsewhere (Fraaije *et al.*, 2002; Kamerbeek *et al.*, 2003; de Gonzalo, Mihovilovic and Fraaije, 2010; Torres Pazmiño, Dudek and Fraaije, 2010; Leisch, Morley and Lau, 2011).

Alcohol acyltransferases

AATs are a large and diverse group of enzymes. They are the main ester-producing enzymes in plants, yeast, filamentous fungi and some bacteria (Nagasawa *et al.*, 1998; Aharoni *et al.*, 2000; Shalit *et al.*, 2001; Beekwilder *et al.*, 2004; Holland *et al.*, 2005; Saerens *et al.*, 2006; Stribny, Querol and Pérez-Torrado, 2016; van Mastrigt *et al.*, 2018). AATs form esters by transferring the acyl moiety from an acyl-CoA molecule to an alcohol (Figure 2d). They vary significantly in their specificities for their alcohol and acyl-CoA substrates (Table 1). As a result, a plethora of esters are produced in nature, ranging from short chain esters such as ethyl acetate, to long chain wax esters (Shi *et al.*, 2012; Stribny, Querol and Pérez-Torrado, 2016; Kruis *et al.*, 2017).

The most-studied microbial ester-producing AATs are derived from yeasts (Table 1). One of the few other well studied microbial AATs is the TAG and wax ester-producing AtfA (sometimes referred to as wax synthase – WS) from *Acinetobacter baylyi*. (Stöveken *et al.*, 2005; Röttig and Steinbüchel, 2013). Five yeast AATs have been described thus far, mostly in *S. cerevisiae*. The first yeast AATs with a determined protein sequence are the *S. cerevisiae* Atf1 and its paralog Atf2 (Fujii *et al.*, 1994; Nagasawa *et al.*, 1998). Atf1 in particular is responsible for 50 % of acetate ester production in *S. cerevisiae* (Verstrepen *et al.*, 2003), which was associated with attracting insects as a means of dispersal in the environment (Christiaens *et al.*, 2014). For *S. cerevisiae* Atf2, a role in sterol metabolism has also been proposed (Tiwari *et al.*, 2007). The second paralog-pair of *S. cerevisiae* AATs are Eht1 and Eeb1 (Saerens *et al.*, 2006). They produce MCFA ethyl esters as a way to detoxify MCFAs that accumulate during fatty acid synthesis (Tehlivets, Scheuringer and Kohlwein, 2007).

The most recently identified yeast AATs compose the Eat1 family. Homologs of this enzyme are responsible for 80 % of ethyl acetate synthesis in *K. lactis* and are present in all other known bulk ethyl acetate producing yeasts. A homolog of Eat1 also contributed to 50 % of ethyl acetate synthesis in *S. cerevisiae* (Kruis *et al.*, 2017). The role of Eat1 in ethyl acetate synthesis by *S. cerevisiae* was confirmed when the enzyme was identified through polygenic trait analysis (Holt *et al.*, 2018). The *S. cerevisiae* Imo32 is distantly related to Eat1 and also contributed to ethyl acetate production when overexpressed in *S. cerevisiae* lacking *atf1* (Holt *et al.*, 2018). Eat1 mainly contributes to the high amounts of ethyl acetate produced by yeasts such as *K. marxianus* and *W. anomalus* (Löser, Urit and Bley, 2014; Kruis *et al.*, 2017). Bulk ethyl acetate production was suggested to be associated with suboptimal growth conditions under which excess acetyl-CoA accumulates in yeast mitochondria (Thomas and Dawson, 1978; Löser, Urit, Keil, *et al.*, 2015). Ethyl acetate synthesis by Eat1 may relieve this accumulation by converting acetyl-CoA to ethyl acetate. The localization of Eat1 in yeast mitochondria supports this hypothesis (Huh *et al.*, 2003; Kruis *et al.*, *2018*; Löbs *et al.*, 2018). The cellular location of the remaining AATs has not been clearly linked to their proposed biological function. The *S. cerevisiae* Atf1 and Atf2 localize to the endoplasmic reticulum (ER), but are also associated to lipid droplets in the cytosol (Huh *et al.*, 2003; Verstrepen *et al.*, 2004; Lin and Wheeldon, 2014). Curiously, Atf1 and Atf2 homologs from *W. anomalus* and *K. lactis* did not localize to lipid droplets when expressed in *S. cerevisiae* (Lin and Wheeldon, 2014). Eht1 was traced to the ER, the outer mitochondrial membrane, and lipid bodies of *S. cerevisiae* (Huh *et al.*, 2003; Zahedi *et al.*, 2006; Zhu, Schwartz and Wheeldon, 2019), while its paralog is located in the mitochondria (Zhu, Schwartz and Wheeldon, 2019) (Table 1).

TABLE 1: Characteristics of the most studied and applied microbial AATs.

	AtfA (WS)	**Atf1**	**Atf2**
Source, *species examples*	Bacteria, *A. baylyi*	Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*
Typical ester product	Wax esters and TAG	Acetate esters of various alcohols	Acetate esters of various alcohols
Proposed biological functions	Synthesis of storage compounds (Rottig and Steinbuchel, 2013)	Attracting insects for dispersion (Christiaens *et al.*, 2014)	Sterol metabolism (Tiwari *et al.*, 2007)
Cellular location	Lipophilic inclusion in the bacterial cytosol (Stöveken *et al.*, 2005)	ER, Cytosolic lipid droplets (Lin and Wheeldon, 2014; Verstrepen *et al.*, 2004)	ER, Cytosolic lipid droplets (Lin and Wheeldon, 2014)
[1]Protein family	WES acyl-transferase-like domain (PF03007)	AATase domain (PF07247)	AATase domain (PF07247)
Catalytic or conserved regions	HXXXXD, DFGWG (Rottig and Steinbuchel, 2013)	HXXXXD (Nancolas *et al.*, 2017)	HXXXXD (Nancolas *et al.*, 2017)
Side activities	Unknown	Thioesterase (*in vitro*) (Nancolas *et al.*, 2017)	Unknown
[2]Alcohol specificity	Broad (C4-C20) unbranched primary alcohols (*in vitro*) (Stöveken *et al.*, 2005)	Broad towards primary alcohols (*in vitro*) (Nancolas *et al.*, 2017)	Primary alcohols (*in vitro*) (Stribny *et al.*, 2016) Sterols (*in vivo*) (Tiwari *et al.*, 2007)
[2]Acyl-CoA specificity	Preferred long acyl-CoAs (C14-C18) (*in vitro*), lower activity towards shorter and longer acyl-CoAs (Stöveken *et al.*, 2005)	Acetyl-CoA only (*in vitro*) (Nancolas *et al.*, 2017)	Acetyl-CoA (*in vivo*) (Tiwari *et al.*, 2007; Verstrepen *et al.*, 2003)

Eht1	Eeb1	Eat1
Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*	Yeast, *W. anomalus* *K. marxianus* *S. cerevisiae*
MCFA ethyl esters	MCFA ethyl esters	Ethyl acetate, other acetate esters
MCFA detoxification (Saerens *et al.*, 2006)	MCFA detoxification (Saerens *et al.*, 2006)	Relieving acetyl-CoA accumulation (Kruis *et al.*, 2018; Löser *et al.*, 2014)
ER, Mitochondrial outer membrane, lipid droplets (Huh *et al.*, 2003; Zahedi *et al.*, 2006; Zhu *et al.*, 2019)	Mitochondria (Zhu *et al.*, 2019)	Mitochondria (Huh *et al.*, 2003; Kruis *et al.*, 2018; Löbs *et al.*, 2018)
α/β-hydrolase family 4	α/β-hydrolase family 4	α/β-hydrolase family 6
Ser-Asp-His triad (Saerens *et al.*, 2006)	Ser-Asp-His triad (Saerens *et al.*, 2006)	Ser-Asp-His triad (Kruis *et al.*, 2017)
Esterase, thioesterase (*in vitro*) (Knight *et al.*, 2014; Saerens *et al.*, 2006)	Esterase (*in vitro*) (Saerens *et al.*, 2006)	Esterase (*in vitro*, *in vivo*) Thioesterase (*in vitro*) (Kruis *et al.*, 2017)
Ethanol, Phenylethyl alcohol (*in vivo*) (Knight *et al.*, 2014; Saerens *et al.*, 2006; Wang *et al.*, 2017)	Ethanol, (*in vivo*) (Saerens *et al.*, 2006)	Ethanol (*in vitro*) Primary alcohols (*in vivo*) (Kruis *et al.*, 2018)
MCFA-CoA (C4-C8) (*in vitro*) (Saerens *et al.*, 2006) MCFA-CoA (C6-C12) (Lin *et al.*, 2016) Caffeoyl-CoA (*in vivo*) (Wang *et al.*, 2017)	MCFA-CoA (C6-C10) (*in vitro*) (Lin *et al.*, 2016; Saerens *et al.*, 2006)	Acetyl-CoA (*in vitro*) (Kruis *et al.*, 2017) Propionyl-CoA (*in vivo*) (Kruis *et al.*, 2018)

Catalytic features of microbial AATs

The mechanisms that catalyze the AAT reaction are not entirely understood. There are no crystal structures of AATs available yet, although several computational models have been used to study their catalytic mechanisms (Morales-Quintana *et al.*, 2011; Galaz *et al.*, 2013; Navarro-Retamal *et al.*, 2016). Microbial AATs belong to two structurally unrelated protein families (Table 1). Atf1, Atf2 and AtfA share some characteristics of the BAHD O-acyltransferases, which are primarily found in plants and bacteria. The abbreviation BAHD is derived from the names of the first four members of this enzyme superfamily that were originally characterized in plants (D'Auria, 2006). Its members share the conserved HXXXD and DFGWG motifs (D'Auria, 2006; Galaz *et al.*, 2013). The HXXXD motif participates

in transferring the acyl group from the acyl-CoA substrate to the alcohol without forming a covalent acyl-protein intermediate (Figure 3ab) (Röttig and Steinbüchel, 2013; Navarro-Retamal *et al.*, 2016). Atf1 and Atf2 lack the DFGWG motif (Nancolas *et al.*, 2017), which stabilizes the solvent channel of the enzyme and is not directly involved in the catalytic mechanism (Morales-Quintana *et al.*, 2013; Morales-Quintana, Moya-León and Herrera, 2015). It should be noted that Atf1 and Atf2 are not closely related to AtfA (Röttig and Steinbüchel, 2013) and they belong to different AAT subfamilies (Table 1).

The second group of AATs consists of the paralogs Eht1 and Eeb1, and the recently discovered Eat1 AAT family. They are defined by an α/β-hydrolase fold and a Ser-Asp-His catalytic triad (Figure 3cde) (Saerens *et al.*, 2006; Knight, Bull and Curnow, 2014; Kruis *et al.*, 2017). This fold is typical for a large group of hydrolytic enzymes that includes proteases, esterases, lipases and peroxidases.

Eht1 and Eeb1 are only distantly related to Eat1 (Kruis *et al.*, 2017). The hypothetical catalytic mechanism of the α/β-hydrolase fold-containing AATs likely resembles the mechanism observed in other esterase-like acyltransferases. Unlike the catalytic mechanism of BAHD-like acyltransferases, a covalent protein-acyl intermediate is formed and transferred to an alcohol (Figure 3d). The preference for the acyltransferase reaction of AATs over hydrolysis is mediated by the specific environment within the three-dimensional protein structure (Rauwerdink and Kazlauskas, 2015). They can either exclude water from the active site, favor binding of alcohols over water, or decrease the reactivity of water compared to alcohols (Lejon *et al.*, 2008; Jiang *et al.*, 2011; Rauwerdink and Kazlauskas, 2015). It is tempting to speculate which mechanism promotes the AAT activity, but such information on the catalytic mechanism can only be obtained by studying the crystal structure of the enzymes and their acyl-protein intermediate (Mirza *et al.*, 2005).

Despite their structural differences, AATs show remarkably similar catalytic (mis)behavior. Thioesterase and/or esterase activities have been observed in Eht1, Eeb1 and Eat1 *in vitro* (Table 1). The hydrolytic activity of these AATs is likely related to their α/β-hydrolase fold, and has been described in unrelated acyltransferases containing the same fold as well (Rauwerdink and Kazlauskas, 2015). Thioesterase activity has also been demonstrated in the *S. cerevisiae* Atf1 (Nancolas *et al.*, 2017), even though Atf1 does not resemble α/β-hydrolases. It was able to act as a thioesterase on longer acyl-CoA substrates, but it could only utilize acetyl-CoA to produce esters in the AAT reaction. While hydrolytic side activities seem to be a characteristic of AATs in general, the interplay of AAT, thioesterase and esterase activities is poorly understood. In Eat1, both thioesterase and esterase activity are repressed by the presence of ethanol *in vitro* (Kruis *et al.*, 2017). The alcohol seems to be the preferred substrate that is able to displace water from the active site of the enzyme, but it is not clear how this occurs. Factors that control the reaction type in other AATs, such

as Eht1, Eeb1 and Atf1 have not been determined yet. The catalytic mechanism of AATs should be investigated further to prevent unwanted product or substrate degradation.

Substrate specificities and kinetic parameters of microbial AATs

In vitro data on substrate specificities is lacking for some AATs. For Eat1, only the specificity towards acetyl-CoA and ethanol has been determined *in vitro* (Kruis *et al.*, 2017). There are also no studies reporting *in vitro* alcohol specificities of the *S. cerevisiae* Eht1 and Eeb1. *In vivo* studies where AAT genes were expressed or deleted may offer some indications on their substrate specificities. However, factors like substrate availability or cellular localization of the AAT may mask the true substrate specificity of the enzyme, which can only be determined *in vitro*. Two types of enzymatic assays have been developed to measure AAT activity. The first couples the release of CoA (Figure 2d) to NADH formation or a colored reaction (Knight, Bull and Curnow, 2014; Lin, Zhu and Wheeldon, 2016; Kruis *et al.*, 2017). As AATs can often display thioesterase activity, care should be taken in interpreting the outcome of such coupled assays. It has been observed that the AAT and the thioesterase reaction can have different substrate specificities. For example, Atf1 could only accept acetyl-CoA as an AAT *in vitro*, but could hydrolyze longer acyl-CoA substrates as a thioesterase (Nancolas *et al.*, 2017). The second type of *in vitro* AAT assay measures the ester product directly (*e.g.* by gas chromatography). Such assays may provide a more reliable alternative to measuring CoA release (Stöveken *et al.*, 2005; Löbs *et al.*, 2016; Kruis *et al.*, 2017; Nancolas *et al.*, 2017). Both types of assays have been used to determine some enzyme kinetics of AATs.

AATs seem to be relatively unspecific towards the alcohol substrate (Table 1). For instance, the *S. cerevisiae* Atf1 and Atf2 have a broad specificity towards primary alcohols *in vitro* (Stribny, Querol and Pérez-Torrado, 2016; Nancolas *et al.*, 2017). This was reflected *in vivo* when the genes were overexpressed in *E. coli* and *S. cerevisiae,* leading to a broad increase in acetate ester production (Verstrepen *et al.*, 2003; Rodriguez, Tashiro and Atsumi, 2014). The overexpression of several *eat1* genes from different yeasts in *S. cerevisiae* also resulted in increased levels of various acetate esters (Kruis *et al.*, 2018). AtfA showed a particularly broad substrate specificity towards alcohols and accepted anything from short alcohols to long wax alcohols (Stöveken *et al.*, 2005). Less information is available for the alcohol specificities of the *S. cerevisiae* Eht1 and Eeb1. They are able to synthesize ethyl esters *in vivo* (Saerens *et al.*, 2006; Knight, Bull and Curnow, 2014; van Nuland *et al.*, 2017). Recently, Eeb1 was shown to utilize phenylethyl alcohol *in vivo*, indicating some degree of promiscuity of this enzyme (Wang *et al.*, 2017). Promiscuity towards the alcohol substrate was also observed in plant AATs (Beekwilder *et al.*, 2004; Lin, Zhu and Wheeldon, 2016). Even the antibiotic resistance marker chloramphenicol acetyltransferase (Cat),

which normally acetylates the antibiotic chloramphenicol, was able to form low amounts of aliphatic esters in *E. coli* (Rodriguez, Tashiro and Atsumi, 2014).

The specificity of AATs towards their acyl-CoA substrate is higher compared to alcohols in some cases. *S. cerevisiae* Atf1 exclusively utilized acetyl-CoA in *in vitro* AAT assays (Nancolas *et al.*, 2017). Other AATs, such as the *A. baylyi* AtfA are more flexible and can accept multiple long acyl-CoAs. However, the number of acyl-CoAs accepted by AtfA is lower compared to the variety of accepted alcohols (Table 1) (Stöveken *et al.*, 2005). The *S. cerevisiae* Eht1 and Eeb1 accept a broader range of MCFA-CoA substrates *in vitro* and *in vivo* (Table 1). It should be noted that the preferred acyl-CoA substrates of *S. cerevisiae* Eht1 and Eeb1 differ slightly among studies. For example, the *S. cerevisiae* Eht1 was initially named Ethanol hexanoyl-transferase because it showed the highest activity against hexanoyl-CoA (Saerens *et al.*, 2006), but appeared to be more active towards octanoyl-CoA in other studies (Knight, Bull and Curnow, 2014; Lin, Zhu and Wheeldon, 2016). Nevertheless, all studies agree that the *S. cerevisiae* Eht1 and Eeb1 have activity against a range of MCFA-CoA substrates. In one study, the *S. cerevisiae* Eht1 was even able to utilize caffeoyl-CoA, which contains an aromatic ring in its structure (Wang *et al.*, 2017).

Various enzymatic properties of AATs, such as K_M, or the K_{cat}/K_M ratio have been determined (Table 2). However, there is still a significant lack of detail in this area. For some AATs, such as Eeb1, no kinetic parameters have been determined to date. For other AATs, the knowledge is still incomplete. Based on information available for Atf1 and Eat1, it seems that these yeast AATs have lower affinity towards alcohols, compared to acetyl-CoA. In case of Atf1, the catalytic efficiency (K_{cat}/K_M) for isoamyl alcohol is also significantly lower relative to acetyl-CoA (Table 2). Whether such observations can be made in other families of AATs is still unknown. It is also unclear what the relevance of such low affinities is regarding *in vivo* ester synthesis.

Metabolic engineering for *de novo* ester synthesis

Competing with the petrochemical industry is challenging due to the low market prices of commodity chemicals. Bioconversion of substrates into products must therefore be as efficient as possible (Van Dien, 2013). The amounts of esters naturally produced by microorganisms are generally too low to support cost-competitive biobased processes and considerable metabolic engineering efforts have been invested to enhance this production. The crucial factors in any metabolic engineering strategy are the selection of a suitable catalyst and a sufficient supply of metabolic precursors. Most studies on ester production with microorganisms use sugars, particularly glucose as substrate and are the focus of this review.

TABLE 2: Enzyme kinetics of the most studies and applied microbial AATs.

AAT	Substrate	K_M(mM)	Kcat (s^{-1})	Kcat/K_M (s^{-1} M^{-1})	Co-substrate	Measurement method	Reference
AtfA	Palmitoyl-CoA	0.029	n.a.	n.a.	Hexadecanol	Detection of ester product	(Stöveken *et al.*, 2005)
Atf1	Isoamyl alcohol	32	n.a.	n.a.	Acetyl-CoA	Detection of ester product	(Stribny, Querol and Pérez-Torrado, 2016)
	Isoamyl alcohol	26	2.9	113	Acetyl-CoA	Coupled to CoA release	(Tai, Xiong and Zhang, 2015)
	Acetyl-CoA	0.061	0.4	6656	Ethanol	Coupled to CoA release	(Nancolas *et al.*, 2017)
Atf2	Isoamyl alcohol	22	1.6	74	Acetyl-CoA	Coupled to CoA release	(Tai, Xiong and Zhang, 2015)
Eht1	Octanoyl-CoA	0.002	0.28	150000	Ethanol	Detection of ester product	(Knight, Bull and Curnow, 2014)
Eat1	Ethanol	3.1	n.a.	n.a.	Acetyl-CoA	Detection of ester product	(Kruis *et al.*, 2017)
	Acetyl-CoA	2.4	n.a.	n.a.	Acetyl-CoA	Detection of ester product	(Kruis *et al.*, 2017)

Selection of the catalyst

Four main enzymatic reactions are available for engineering ester production *in vivo* (Figure 2). Esterases and lipases have been applied extensively for ester synthesis *via* transesterification or reverse esterase activity in nearly non-aqueous environments (Hari Krishna and Karanth, 2002; Levisson, van der Oost and Kengen, 2009; Stergiou *et al.*, 2013; Kumar *et al.*, 2016). However, the reverse esterase reaction is thermodynamically unfavorable in aqueous conditions under which microbial fermentations occur. Metabolic engineering of ester production using HADH has not been reported yet. To produce esters *via* this route, accumulation of aldehydes would be necessary. This may be challenging due to their toxicity (Kunjapur and Prather, 2015). Furthermore, hemiacetal formation is spontaneous and requires an acid catalyst, which is not present under physiological pH-neutral conditions.

Biotransformation of cyclic ketones into lactones (cyclic esters) by BVMOs have been studied extensively (Leisch, Morley and Lau, 2011). Several BVMOs exist that are also active towards linear ketones, but they are rarely applied *in vivo* (Rehdorf *et al.*, 2009; Ceccoli *et al.*, 2017; Pereira, van der Wielen and Straathof, 2018). Such *in vitro* processes rely on external supply of expensive cofactors, such as NAD(P)H. Direct synthesis of esters from cheaper substrates like sugars may therefore be more economical. BVMO-catalyzed ester production also depends on the supply of ketones, which are not common microbial metabolites.

AATs have dominated the field of metabolic engineering of *in vivo* ester production. This reaction is thermodynamically favorable and does not require the input of reducing equivalents (Figure 2d). AATs convert an alcohol and an acyl-CoA to an ester, releasing free CoA in the process. Both the alcohol and acyl-CoA are readily produced in biological systems and can serve as efficient precursors for ester synthesis. We here outline the general metabolic engineering strategies that have been applied to increase alcohol, acyl-CoA and ester synthesis in microorganisms *via* AATs, and highlight several notable achievements (Table 3).

The building blocks of ester synthesis

The AAT reaction determines the efficiency of the final catalytic step in ester formation. However, the reaction also depends on the supply of the alcohol and acyl-CoA substrates. In several studies precursors for ester synthesis have been added to the cultivation medium, such as acids or alcohols (Horton and Bennett, 2006; Singh, Bhadani and Singh, 2007; Rodriguez, Tashiro and Atsumi, 2014; Kruis *et al.*, 2017; Lin *et al.*, 2018). These studies have been useful for studying ester formation capacities of e.g. engineered strains. However, *de novo* ester synthesis from renewable substrates is often preferred. Ideally, carbon sources which do not compete with food and feed would be used for ester synthesis, for example

lignocellulosic biomass. Layton and Trinh have also proposed anaerobic waste digestions as platform for ester synthesis. The final products of such processes are a mix of MFCA. They, or their intermediates can be used to produce a mix of esters (Layton and Trinh, 2016a, 2016b). However, the majority of lab-scale studies on ester production have used crystalline glucose as carbon source, which will be the focus of this review (Figure 4).

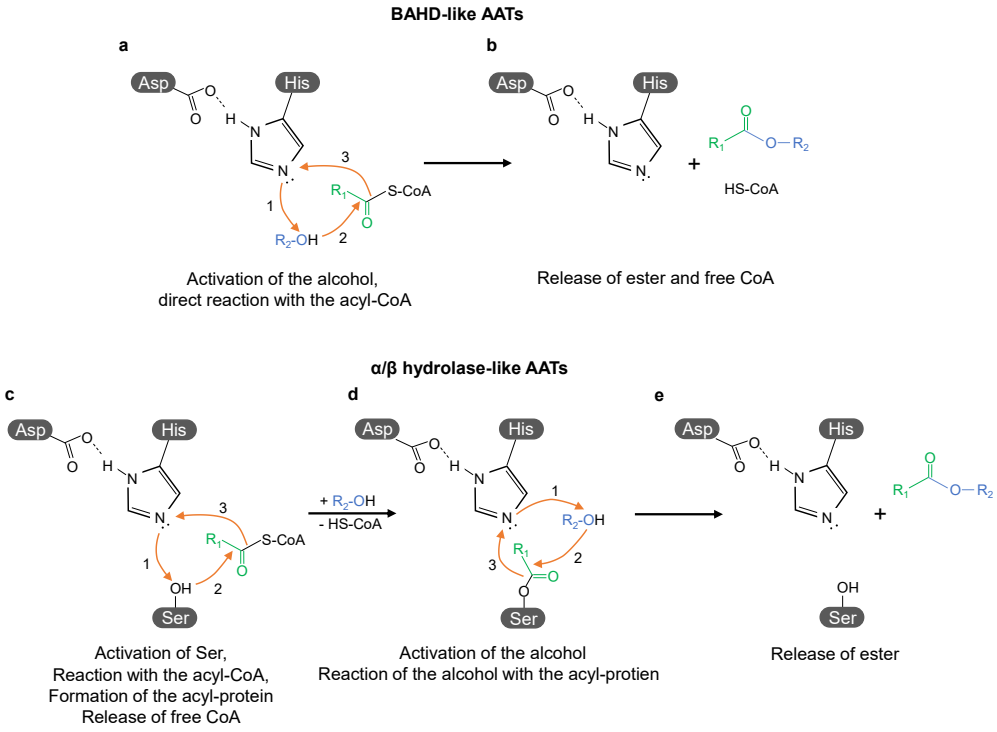

FIGURE 3: Hypothetical catalytic mechanisms of BAHD-like and α/β-hydrolase-like AATs. (a, b) – BAHD-like AATs form the ester by activating the alcohol, which reacts directly with the acyl-CoA. No covalent bonds with the enzyme are formed. (c, d, e) – AATs containing the α/β-hydrolase fold most likely perform the reaction via a covalent acyl-protein intermediate. The transition states in both hypothetical reaction mechanisms are not depicted to improve the figure clarity. Orange arrows indicate the sequence of the reaction transfer of electrons in the reactions. Numbers indicate the sequence of the reactions.

TABLE 3: Overview of metabolic engineering approaches for increasing microbial ester production.

Target ester	Organism	Goal/Strategy	AAT catalyst	Acyl-CoA supply
2-methyl-1-butyl acetate, 2-phenylethyl acetate	*E. coli*	Acetate ester prod.	Sce Atf1	Native Acetyl-CoA
Isobutyl acetate	*E. coli*	Isobutyl ac. prod.	Sce Atf1	Native Acetyl-CoA
Isobutyl acetate	*E. coli*	Isobutyl ac. prod.	Sce Atf1	Native Acetyl-CoA
Isoamyl acetate	*E. coli*	Isoamyl ac. prod.	Sce Atf1	Native Acetyl-CoA
Isoamyl acetate	*E. coli*	Increased CoA and acetyl-CoA supply	Sce Atf2	Increased CoA production (1), Combined with Pdh overexpression (2)
Ethyl acetate	*S. cerevisiae*	Pathway colocalization	Sce Atf1	Ald-Acs targeted to Atf1 (lipid droplet)
Butyl butyrate	*Clostridium acetobutylicum*	Butyl butyrate production	*Mallus sp.* (apple) AAT	Native butyryl-CoA
Isobutyl Isobutyrate	*E. coli*	Branched esters production	Sce Eht1 Cat	Isobutyryl-CoA synthesis via heterologous BC-2-KDH
MCFA ethyl ester	*S. cerevisiae*	Implement RBOX	Native AATs	Introduced RBOX
Butyrate ester platform	*E. coli*	Synthesis of various butyrate esters	Fan AAT (SAAT)	Butyryl-CoA module (*Clostridium* pathway)
Ethyl and isobutyl ester platforms	*E. coli*	Conversion of waste carboxylates to esters	Fan AAT (SAAT) or Fve AAT (VAAT)	Activation of carboxylates (e.g. acetate, propionate, pentanoate, hexanoate) to acyl-CoAs
Lactate esters platform	*E.coli*	Formation of lactate esters, focus on ethyl- and isobutyl lactate	Fve AAT (VAAT)	Activation of lactate to lactyl-CoA via Pct
1,6-diacetoxy hexane	*E. coli*	Diterminal acetylation of α,ω-alcohols	Sce Atf1	Native acetyl-CoA
Diethyl adipate	*E. coli*	Diterminal ethylation of α,ω-fatty acids	Sce Eeb1	Diterminal activation of adipic activation via acyl-CoA ligase
FASBE	*S. cerevisiae*	Combination of FAS and 2-keto pathway	*Marinobacter sp.* WS	Native FAS synthase; Deletion of *rpd3* and *opi1* (repressors of fatty acyl-CoA synthesis)

Alcohol supply	Effect/Titre	Notes	References
Overexpression of Lla 2-KDC gene	App. 20 mg/L		(Rodriguez *et al.*, 2014)
Overexpression of 2-keto pathway genes for isobutanol	17 g/L	80% theor. max. yield, Hexadecane layer as organic extraction phase	(Rodriguez *et al.*, 2014)
Overexpression of 2-keto pathway genes for isobutanol	36 g/L	42 % theor. max. yield	(Tai *et al.*, 2015)
Overexpression of 2-keto pathway genes for isoamyl alcohol	386 mg/L	11% theor. max.	(Tai *et al.*, 2015)
Isoamyl alcohol supplementation	2.3-fold increase (1) 6.2-fold increase (2)	Suppl. of pantothenic acid needed (CoA precursor)	(Vadali *et al.*, 2004)
Native ethanol	2-fold increase		(Lin *et al.*, 2017)
Native 1-butanol	45 mg/L	Butyl acetate and ethyl butyrate also detected	(Noh *et al.*, 2018)
Conversion of isobutyryl-CoA to isobutanol by native Adh	27 mg/L		(Rodriguez *et al.*, 2014)
Native ethanol	Increased MCFA ethyl esters	Quantity not reported	(Lian and Zhao, 2015)
Ethanol, isopropanol, isobutanol, butanol modules (2-keto pathway)	Production of ethyl-, isopropyl-isobutyl, and butyl butyrate esters	First engineered de *novo* synthesis of esters from glucose under anaerobic conditions; Decane as extraction phase	(Layton and Trinh, 2014)
Ethanol and isobutanol modules (2-keto pathway)	Production of a library of expected ethyl or isobutyl esters	Esters such as pentyl pentanoate were also produced	(Layton and Trinh, 2016)
Ethanol or isobutanol modules	App. 10 mg/L ethyl lactate and isobutyl lactate	First *de novo* synthesis of lactate esters from glucose	(Lee and Trinh, 2018)
Diterminal oxidation of hexane to hexanediol	4.3 g/L	Resting cells, supplementation of hexane, BEHP as organic extraction phase	(van Nuland *et al.*, 2017a)
Ethanol supplementation	20 mg/L		(van Nuland *et al.*, 2017b)
Overexpression of 2-keto pathway to isobutanol, isoamyl alcohol, amyl alcohol	230 mg/L FASBE		(Teo *et al.*, 2015)

Target ester	Organism	Goal/Strategy	AAT catalyst	Acyl-CoA supply
FASBE	*E. coli*	Combination of FAS and 2-keto pathway	Aba WS	Prevention of β-oxidation
FAEE	*E. coli*	Increased FAS activity	Aba AtfA	FAS – overexpressed thioesterase and MCAT (initial step in FAS)
FAEE	*S. cerevisiae*	Additional acetyl-CoA and NADPH	Mhy WS2	FAS Xpk+Pta bypass
FAEE	*Y. lipolytica*	Change cellular location of AAT	Per-AbaAtfA ER-AbaAtfA	Native acyl-CoA metab. in compartments
WE	*A baylyi*	Novel FAR	Native WS	Native fatty acyl-CoA
WE	*A baylyi*	Product diversification	Native WS	Native fatty acyl-CoA

Routine disruptions of common by-product pathways are not listed and can be found in the original references. Abbreviations: Aba – *Acinetobacter baylyi*, Adh – alcohol dehydrogenase, Acs – acetyl-CoA synthetase, Ald – aldehyde dehydrogenase, BC-2-KDH – branched chain 2-ketoacid dehydrogenase, BEHP – bis(2-ethylhexyl) phthalate, Cat – chloramphenicol acetyltransferase, ER – Endoplasmic reticulum, , Fan – *Fragarria x ananassa,* FAS – fatty acid synthase, FAR – fatty acid reductase, FASBE – fatty acid short- and branched-chain esters, Fve – *Fragarria vesca*, Lla – *Lactococcus lactis*, MCFA – medium chain fatty acid, Mhy – *Marinobacter hydrocarbonoclasticus*, Per – peroxisome, Pct – propionyl-CoA transferase, Pdh – pyruvate dehydrogenase, Pdc – pyruvate dehydrogenase, Pflu – *Pseudomonas fluorescens,* Pta – phosphotransacetylase, RBOX – reverse **β**-oxidation, Sce – *S. cerevisiae*, WS – wax synthase, WE – wax esters, Wan – *W. anomalus*, Xpk – phospoketolase

Alcohol supply	Effect/Titre	Notes	References
Overexpression of 2-keto pathway to isobutanol, isoamyl alcohol, amyl alcohol	1000 mg/L FASBE		(Guo *et al.*, 2014)
Introduced Pdc-Adh	674 mg/L FAEE (C12-C18)	9.4% of th. max yield	(Steen *et al.*, 2010)
Native ethanol	4.6 mg/g CDW	1.6-fold increase	(de Jong *et al.*, 2014)
Native ethanol	111 mg/L 137 mg/L	15-fold increase 19-fold increase	(Xu *et al.*, 2016)
Overexpression of novel FAR	450 mg/L	Highest WE titre to date on glucose only	(Lehtinen *et al.*, 2018)
Changed by Pflu LuxCDE	Shift from C18 to C16 WE		(Santala *et al.*, 2014)

Basic metabolites of ester synthesis

Metabolites, such as phosphoenolpyruvate (PEP), pyruvate and acetyl-CoA lie at the core of acyl-CoA, alcohol, and consequently ester synthesis. These carbon compounds are formed during the oxidation of glucose in the glycolysis. During this conversion, reducing equivalents (NADH, NADPH or Fd_{red}) and metabolic energy (ATP) are released (Figure 4a). In the presence of oxygen, ATP is also produced by oxidative phosphorylation. The carbon compounds, reducing equivalents, and ATP are subsequently used to produce acyl-CoAs (Figure 4b) and alcohols (Figure 4c). Finally, the ester is formed by an AAT (Figure 4d).

Several variants of glycolysis exist (Figure 4a). The main differences between them are the number and types of reducing equivalents produced, and their ATP yield (Stincone *et al.*, 2015; Chen *et al.*, 2016). The Embden-Meyerhof-Parnas (EMP) pathway is the conventional glycolytic pathway, which produces NADH and yields 2 ATP per glucose (Figure 4a). Parallel glycolytic routes include the pentose phosphate pathway (PPP) and the Entner-Doudoroff (ED) pathway, which produce both NADPH as well as NADH (Figure 4a) (Rutkis *et al.*, 2013; Sudarsan *et al.*, 2014). In the PPP pathway carbon is lost in the form of CO_2. The final product of these glycolytic pathways is pyruvate, which can be used for e.g. alcohol synthesis (Figure 4c), or converted further to acetyl-CoA (Figure 4a).

Acetyl-CoA can be used directly to produce a variety of acetate esters, or it can be condensed into longer acyl-CoA moieties (Figure 4b) (Park, Shaffer and Bennett, 2009; Barney, 2014). The oxidative decarboxylation of pyruvate to acetyl-CoA has been engineered and reviewed extensively (Krivoruchko *et al.*, 2015; van Rossum, Kozak, Pronk, *et al.*, 2016). Pyruvate formate lyase (Pfl), pyruvate dehydrogenase (Pdh) and

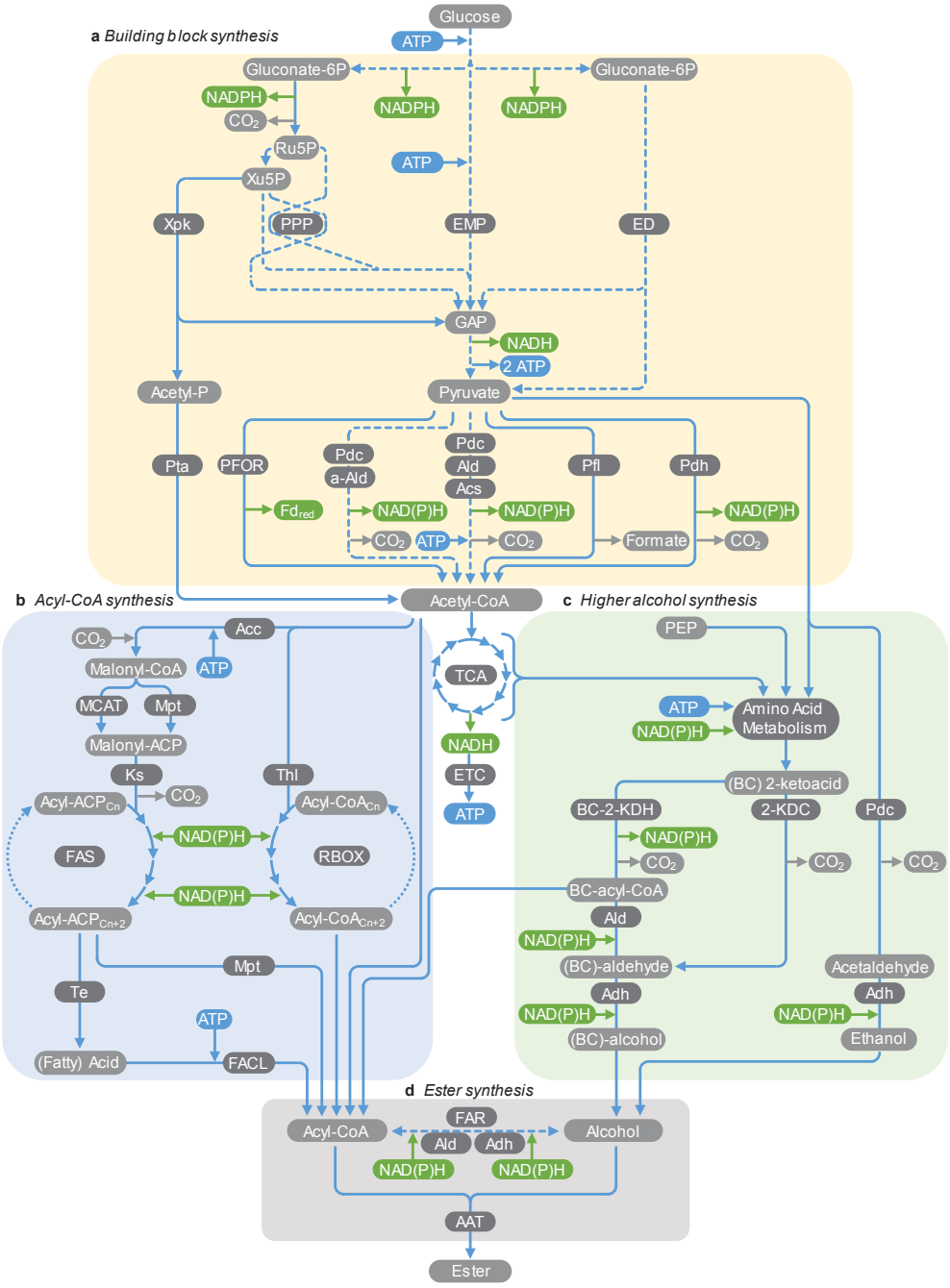

FIGURE 4: Overview of the general metabolic pathways converting glucose to esters. (a) – Formation of basic carbon building blocks, redox equivalents and ATP from glucose. (b) – Generalized formation of longer chain acyl-CoAs from acetyl-CoA. (c) – Generalized formation of branched alcohols from 2-ketoacids. (d) The final ester formation reaction catalyzed by an AAT. Also shown is the interconversion of acyl-CoAs and alcohols. Dashes

indicate multi-enzyme conversion pathways. Abbreviations: a-Ald – acetylating acetaldehyde dehydrogenase, AAT – alcohol acyl transferase, Acc – acetyl-CoA carboxylase, ACP – Acyl carrier protein, Acs – acetyl-CoA synthetase, Adh – alcohol dehydrogenase, Ald – acetaldehyde dehydrogenase, BC – branched chain, BC-2-KDH – branched-chain 2-ketoacid dehydrogenase, ED-Entner-Doudoroff pathway, EMP – Embden-Meyerhof-Parnas pathway, ETC – electron transport chain, FACL – fatty acid CoA-ligase, FAR – Fatty acid reductase, FAS – fatty acid synthase, Fd – Ferredoxin, GAP – Glycerol 3-phosphate, 2-KDC – 2-ketoacid decarboxylase, Ks – ketoacyl synthase, MCAT – Malonyl-CoA acyl carrier protein transacylase, Mpt – malonyl/palmitoyl transferase, Pdc – Pyruvate decarboxylase, Pdh – pyruvate dehydrogenase, PEP - Phosphoenolpyruvate, Pfl – pyruvate formate lyase, PFOR - pyruvate-ferredoxin oxidoreductase, PPP – Pentose phosphate pathway, Pta – phoshotransacetylase, RBOX – reverse β-oxidation, R5P – Ribulose 5-phosphate, TCA – tricarboxylic acid cycle, Te - thioesterase, Thl – thiolase, Xpk – phosphoketolase, Xu5P – Xylulose 5-phosphate.

pyruvate-ferredoxin oxidoreductase (PFOR) catalyze the conversion in a single enzymatic step. Other pathways convert pyruvate to acetyl-CoA in a series of reactions (Figure 4a). These include the coupling of pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase (Pdc-Ald-Acs, respectively). This pathway is sometimes referred to as the Pdh bypass. An alternative pathway couples Pdc and the acetylating acetaldehyde dehydrogenase (Pdc - a-Ald) to produce acetyl-CoA directly (Figure 4). In most cases, CO_2 and a reduced cofactor are produced. The exception is Pfl, which functions in bacteria under anaerobic conditions and releases formate instead (Knappe and Sawers, 1990) (Figure 4a). Most naturally occurring acetyl-CoA-forming pathways release NADH as the cofactor, although variants of NADP-dependent Pdh and Ald have been described and engineered (Figure 4a) (Inui *et al.*, 1989; Bocanegra, Scrutton and Perham, 1993; Miyagi, Kawai and Murata, 2009).

Most acetyl-CoA forming reactions do not consume ATP, with the exception of acetyl-CoA formation *via* Pdc-Ald-Acs (Figure 4a). This pathway is the main acetyl-CoA forming pathway in the *S. cerevisiae* cytosol (Zaldivar, Nielsen and Olsson, 2001). Many industrially interesting compounds, including esters rely on the supply of cytosolic acetyl-CoA in this yeast. Engineering of energy-efficient bypass reactions has therefore been the focus of several studies. These bypass mechanisms include cytosolic expression of the acetylating acetaldehyde dehydrogenase (a-Ald) or Pdh (Figure 4a) (Kozak *et al.*, 2014; van Rossum, Kozak, Niemeijer, *et al.*, 2016). The availability of cytosolic acetyl-CoA in yeast has also been increased by introducing the ATP-citrate lyase, which converts mitochondrial citrate to acetyl-CoA and oxaloacetate at the cost of ATP. Expression of a phosphoketolase (Xpk) also resulted in an increased flux towards acetyl-CoA (de Jong *et al.*, 2014). This enzyme cleaves acetyl-P units from xylulose-5P and fructose-6P (Bergman *et al.*, 2016). The acetyl-P can then be converted to acetyl-CoA *via* phosphotransacetylase (Pta) (Figure 4a). The availability of acetyl-CoA in *E. coli* could also be manipulated by upregulating the production of the cofactor CoA. Overexpression of pantothenate kinase, which limits CoA synthesis in *E. coli*, resulted in improved production of CoA as well as acetyl-CoA (San *et al.*, 2002).

The amounts of acetyl-CoA, reducing equivalents, and ATP that are required for ester biosynthesis are determined by the subsequent biosynthetic pathways for the AAT substrates acyl-CoA and alcohol (Figure 4cb). These compounds are biotechnologically relevant in their own right for a number of applications, such as production of sustainable chemical building blocks, fragrances or biodiesels. The metabolic engineering approaches that were used for engineering acyl-CoA and alcohol supply for these products can also be applied to ester synthesis.

Synthesis of long and branched acyl-CoA substrates

The microbial metabolism is able to produce a variety of structurally diverse acyl-CoAs, which can serve as a substrate for AATs. Linear acyl-CoAs are produced from acetyl-CoA and are based on fatty acid metabolism (Figure 4b). Two main pathways have been used to produce linear (fatty) acyl-CoAs *de novo*; fatty acid synthesis (FAS) and the reversed β-oxidation (RBOX) (Figure 4b). The FAS pathway is native to most organisms and relies on Acyl-ACP carriers (Acyl-Activated Carrier Protein) to produce fatty acids. The cycle is initiated by the carboxylation of acetyl-CoA to malonyl-CoA at the cost of ATP, and converting malonyl-CoA to malonyl-ACP (Figure 4b). Iterative cycles of acetyl-CoA carboxylation to malonyl-CoA/ACP, the condensation of malonyl-ACP with an existing Acyl-ACP, and subsequent reduction of the intermediate elongate the Acyl-ACP moiety by two carbon atoms per cycle (Figure 4b). The termination reaction determines the final product of the FAS. If malonyl/palmitoyl transferase (Mpt) terminates the reaction, a fatty acyl-CoA is released. If the reaction is terminated by a thioesterase (Te), a free fatty acid is released (Schweizer and Hofmann, 2004; Johansson *et al.*, 2009). The free fatty acid can be activated to an acyl-CoA by a fatty acid CoA-ligase (FACL) at the cost of ATP (Figure 4b) (White *et al.*, 2005). The net cost of the FAS cycle per acetyl-CoA incorporated is thus 2 NADPH and 1 ATP (release *via* Mpt), or 2 NADPH and 2 ATP (release *via* Te).

Due to the high energetic demand of fatty acid elongation, the activity of the FAS complex is tightly regulated. Increasing the activity of this complex has been the target of many studies aimed at increasing *de novo* fatty acid and fatty acyl-CoA production. Strategies include increasing the activity of rate limiting steps, such as acetyl-CoA carboxylase (Acc) (Figure 4b), or removing the regulatory mechanisms that repress the FAS cycle (Duan *et al.*, 2011; Runguphan and Keasling, 2014; Valle-Rodríguez *et al.*, 2014; Beld, Lee and Burkart, 2015; Pan *et al.*, 2017).The efficiency of FAS can also be limited by the supply of acetyl-CoA. Implementation of alternative acetyl-CoA generating routes, such as Pfl or Xpk-Pta (Figure 4a) in the cytosol of *Yarrowia lipolytica* increased the lipid titer 1.5 and 1.6-fold, respectively (Xu *et al.*, 2016). The operation of the FAS cycle also requires high supply of NADPH. In yeast such as *Y. lipolytica*, NADPH is derived from the PPP pathway, which limits the maximum lipid yield due to the decarboxylation step in the pathway (Figure 4a). To circumvent carbon loss, the metabolism of the yeast was rewired for more efficient NADPH supply, resulting in the highest fatty acid titer to date of 99 g/L (Qiao *et al.*, 2017).

The reversed β-oxidation (RBOX) provides an energetically efficient alternative pathway for acyl-CoA synthesis (Dellomonaco *et al.*, 2011). As the name suggests, the RBOX is the reversal of the β-oxidation pathway that normally oxidizes fatty acyl-CoAs (Houten and Wanders, 2010). Elements of the RBOX pathway are naturally present in *E. gracilis* and are the main source of acyl-CoA moieties used for the synthesis of wax esters under anaerobic conditions (Müller *et al.*, 2012). The key difference between the FAS and RBOX is the reaction used for the entry of acetyl-CoA into the cycle (Figure 4b) (Kallscheuer *et al.*, 2017). The RBOX uses the thiolase (Thl)-catalyzed entry of acetyl-CoA into the cycle which avoids the ATP-consuming malonyl-CoA formation (Figure 4b). In most engineered RBOX systems NADH instead of NADPH is used to reduce the acyl-CoA intermediate. However, by combining the Thl reaction with the FAS II system in *E. coli*, an NADPH-dependent RBOX metabolism was engineered (Clomburg *et al.*, 2018). The RBOX operates *via* CoA, and not ACP intermediates (Figure 4b). An acyl-CoA is thus the direct final product of the RBOX pathway, which can be used for further synthesis. The net cost of an RBOX cycle per acetyl-CoA incorporated is 2 NAD(P)H while the reaction does not consume ATP. As a result, glucose can be converted to acyl-CoAs at the theoretical maximum yield while still generating ATP (Dellomonaco *et al.*, 2011). Implementation of the RBOX in *E. coli* or *S. cerevisiae* required extensive modifications of the regulatory mechanisms that control the native β-oxidation (Dellomonaco *et al.*, 2011; Lian and Zhao, 2015). The pathway has been used to produce a variety of acyl-CoAs from butyryl-CoA (C4) to decanoyl-CoA (C10), which are precursors for a variety of MCFA, dicarboxylic acids, and other biotechnologically relevant compounds, including esters (Dellomonaco *et al.*, 2011; Clomburg *et al.*, 2015; Kim, Cheong and Gonzalez, 2016; Kallscheuer *et al.*, 2017).

Another class of acyl-CoAs are the branched chain (BC) acyl-CoAs. The formation of these precursors is tightly linked to the generation of higher alcohols (Figure 4c). They are derived from BC-2-ketoacids, which are in turn derived from the amino acid metabolism (Hazelwood *et al.*, 2008). The reaction is catalyzed by the BC-2-ketoacid dehydrogenase (BC-2-KDH) and is analogous to acetyl-CoA formation by Pdh (Figure 4ac) (Mooney, Miernyk and Randall, 2002). Introduction of this pathway in *E. coli* enabled the production of isovaleryl-CoA, 3-methylvaleryl-CoA and isobutyryl-CoA from leucine, isoleucine and valine, respectively (Rodriguez, Tashiro and Atsumi, 2014). BC-acyl-CoAs can also be generated by converting BC-alcohols *via* an appropriate aldehyde dehydrogenase (Ald) and alcohol dehydrogenase (Adh) (Rodriguez, Tashiro and Atsumi, 2014). Alcohols and acyl-CoA can generally be interconverted (Figure 4d), although the reaction from acyl-CoA to alcohol is thermodynamically more feasible (Flamholz *et al.*, 2014).

Synthesis of higher and fatty alcohols

The biosynthesis of alcohols is linked to the production of pyruvate, acyl-CoAs and the amino acid metabolism (Figure 4abc). Ethanol is a simple alcohol and a common microbial fermentative product. In yeast and *Zymomonas mobilis*, ethanol is produced *via* the

non-oxidative decarboxylation of pyruvate to acetaldehyde, which is then reduced with 1 NAD(P)H to ethanol. Pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) catalyze the reactions, respectively (Figure 4ac). In *E. coli*, ethanol is produced *via* oxidative decarboxylation of pyruvate to acetyl-CoA by pyruvate dehydrogenase (Pdh) (Figure 4a). In the next step, acetyl-CoA is reduced with 2 NAD(P)H, first to acetaldehyde, and then to ethanol (Figure 4d). The reactions are catalyzed by an aldehyde dehydrogenase (Ald) and an alcohol dehydrogenase (Adh), respectively. In *E. coli*, the bifunctional alcohol/acetaldehyde dehydrogenase (AdhE) performs both reactions in one step.

The catalytic steps that form higher and fatty alcohols are analogous to the conversion of pyruvate to ethanol *via* acetyl-CoA. For example, the 1-butanol produced during the Clostridial ABE fermentation is formed from butyryl-CoA *via* an Ald and Adh at the cost of 2 NADH (Figure 4cd) (Becerra, Cerdán and González-Siso, 2015). Fatty acyl-CoAs that are produced in the FAS and RBOX cycles (Figure 4b) can be converted to fatty alcohols *via* the same reactions. However, this conversion is usually performed by a single enzyme, the fatty acid reductase (FAR) (Akhtar, Turner and Jones, 2013) (Figure 4d). By introducing FAR genes into *S. cerevisiae*, fatty alcohols, such as 1-hexadecanol could be produced from fatty acyl-CoAs (G. Wang *et al.*, 2016).

Some alcohols, such as 1-propanol, isobutanol, isoamyl alcohol, 1-butanol and 2-phenylethanol can be produced from 2-ketoacids that are derived from the amino acid metabolism (also referred to as the 2-keto pathway) (Peralta-Yahya *et al.*, 2012). This pathway is the source of higher alcohol synthesis in yeast such as *S. cerevisiae*. The specific 2-ketoacids are first decarboxylated to an aldehyde by a 2-ketoacid decarboxylase (2-KDC) and then reduced to an alcohol by Adh. The reactions are analogous to the conversion of pyruvate to ethanol *via* Pdc and Adh (Figure 4c) (Hazelwood *et al.*, 2008). Typical approaches for increasing amino acid-derived alcohol production are the disruption of by-product formation, overexpression of 2-ketoacid biosynthetic genes, and the introduction of appropriate 2-KDC and Adh enzymes (Rodriguez, Tashiro and Atsumi, 2014; Tai, Xiong and Zhang, 2015; Yuan, Mishra and Ching, 2016). This approach enabled the production of 22 g/L isobutanol from glucose at 86 % of the maximum yield (Atsumi, Hanai and Liao, 2008). The synthesis of amino acid-derived alcohols relies on the supply of NADPH (Figure 4c). Engineering additional supply of this cofactor in *S. cerevisiae* improved the production of isobutanol and isoamyl alcohol (Matsuda *et al.*, 2013).

Engineering microbial ester production

Ester production requires the supply of alcohols, acyl-CoAs and the selection of a suitable AAT. Most metabolic engineering efforts aimed at improving ester production have focused on improving the supply of alcohols and acyl-CoAs. A common initial strategy is increasing the availability of basic cellular building blocks, such as acetyl-CoA. This is typically achieved

by channeling the carbon flux away from competing pathways e.g. by disrupting lactate and acetate production in *E. coli* (Shen *et al.*, 2011; Wu *et al.*, 2017). The carbon flux has also been channeled towards the relevant metabolic precursors by introducing alternative or more efficient metabolic pathways. For example, FAEE synthesis in *S. cerevisiae* is limited by the availability of cytosolic acetyl-CoA, as well as NADPH, which fuel the FAS cycle. To overcome the limitation, the Xpk-Pta pathway (Figure 4a) was expressed in the *S. cerevisiae* cytosol, improving FAEE production by 1.6-fold (Table 3) (de Jong *et al.*, 2015). In *E. coli*, acetyl-CoA supply was improved by increasing the availability of the cofactor CoA. This approach increased isoamyl acetate production in *E. coli* by 6.3-fold when combined with overexpression of the Pdh complex (Vadali, Bennett and San, 2004) (Table 3).

The synthesis of more complex esters, such as isoamyl- or isobutyl-acetate relies on the activity of pathways that produce the appropriate alcohols. The 2-keto pathway (Figure 4c) is commonly used to produce such higher alcohols and is naturally present in organisms such as yeast and some lactic acid bacteria. The key enzyme of the pathway is 2-KDC (Figure 4c) that converts 2-ketoacids to aldehydes. When 2-KDC from *Lactococcus lactis* was introduced in *E. coli* along with the *S. cerevisiae* Atf1, up to 20 mg/L acetate esters were produced (Rodriguez, Tashiro and Atsumi, 2014). Efficient ester synthesis only commenced after the entire 2-keto pathway towards isobutanol production was introduced (Atsumi, Hanai and Liao, 2008; Rodriguez, Tashiro and Atsumi, 2014). By using this pathway, isobutyl acetate was produced from glucose at 80 % of the maximum theoretical yield. Other authors have reported similar improvements for the production of isobutyl and isoamyl acetate *via* the 2-keto pathway, using the *S. cerevisiae* Atf1 as the catalyst (Tai, Xiong and Zhang, 2015). Some *Clostridia* utilize an alternative pathway *via* reactions that resemble the RBOX to generate higher alcohols, particularly 1-butanol (Figure 4c). By introducing an AAT from apple (*Malus sp.*), the natural 1-butanol production was used to produce butyl butyrate from glucose (Table 3) (Noh *et al.*, 2018). Introducing novel pathways for the synthesis of uncommon acyl-CoA, such as lactyl-CoA has enabled the production of lactate esters (Lee and Trinh, 2018). This pathway relies on the activation of lactate to lactyl-CoA *via* propionate-CoA transferase (Pct), in a reaction that consumes acetyl-CoA. By coupling the synthesis of lactyl-CoA with ethanol and isobutanol production modules, ethyl lactate and isobutyl lactate were produced *de novo* from glucose (Table 3).

The production of FAEE in *E. coli* and yeast can be limited by the supply of fatty acids. The activity of FAS is often the limiting factor, as this complex is tightly regulated. Its activity in *E. coli* was improved by overexpressing one of the first steps of the pathway, malonyl CoA-acyl carrier protein transacylase (MCAT) as well as the thioesterase (Te) that terminated the FAS cycle (Figure 4b). This allowed FAEE production at almost 10 % of the theoretical maximum on glucose (Table 3) (Steen *et al.*, 2010). The production of FAEE could also be improved by disrupting the transcriptional repressors that downregulate the synthesis of acyl-CoAs, or by preventing their degradation in the β-oxidation pathway (Guo *et al.*,

2014; Teo *et al.*, 2015). Alternatively, the RBOX pathway was used to supply acyl-CoAs instead, resulting in MCFA ethyl ester production in *S. cerevisiae* (Lian and Zhao, 2015). Increased fatty acid supply could also be achieved by translocating the *A. baylyi* AtfA to the endoplasmic reticulum or peroxisome of *Y. lipolytica*. The fatty acid precursors are more abundant in this organelle, which resulted in a 15- to 19-fold improved FAEE synthesis in this yeast (Table 3).

Beyond the supply of metabolic precursors, the selection of a suitable AAT determines the final ester product. Microbial AATs (Table 1) have been used most extensively for improving ester production in microbial hosts. Plant AAT genes have also been used to evoke ester production. These enzymes belong to the BAHD family of AATs and share structural similarities to AtfA, Atf1 and Atf2 (Table 1). Two members, SAAT and VAAT, have often been used for engineering ester production in microbial hosts (Layton and Trinh, 2016a, 2016b; Noh *et al.*, 2018). SAAT originates from the garden strawberry (*Fragaria x ananassa*) and VAAT was derived from the wild strawberry (*Fragaria vesca*). SAAT and VAAT can accept a variety of alcohols ranging from linear alcohols, such as ethanol, butanol, and isoamyl alcohol to aromatic alcohols like geraniol (Beekwilder *et al.*, 2004). They are also flexible in the acyl-CoA moiety and have been used to synthesize a variety of acetate, propanoate, butanoate, pentanoate, hexanoate, as well as lactate esters (Layton and Trinh, 2016a; Lee and Trinh, 2018; Noh *et al.*, 2018). Often, homologs of the same enzyme originating from different organisms exhibit altered kinetic parameters or substrate specificities (Röttig, Zurek and Steinbüchel, 2015; Stribny, Querol and Pérez-Torrado, 2016). The first step in many studies has therefore been bioprospecting of several AAT candidates before selecting the optimal one (Rodriguez, Tashiro and Atsumi, 2014; Röttig, Zurek and Steinbüchel, 2015; Tai, Xiong and Zhang, 2015). Other engineering approaches targeting the AAT enzyme or reaction are not commonplace, but they can be effective. For example, co-localizing the *S. cerevisiae* Atf1 with the acetyl-CoA generating Ald-Acs pathway (Figure 4a) led to a metabolic channeling effect and resulted in a 2-fold increase in ethyl acetate formation (Table 3) (Lin, Zhu and Wheeldon, 2017).

The flexibility of the AAT reaction hypothetically allows for any alcohol to react with any acyl-CoA. This has enabled the development of modular *E. coli* cell factories, where each metabolic pathway towards the synthesis of an alcohol or acyl-CoA constitutes a submodule (Layton and Trinh, 2014). By combining a metabolic module for butyryl-CoA synthesis with various alcohol modules, the synthesis of ethyl butyrate, propyl butyrate, isobutyl butyrate, and butyl butyrate from glucose was observed (Table 3). A similar principle was applied to convert various carboxylates (waste products of anaerobic digestion of lignocellulose) to ethanol or isobutanol esters (Layton and Trinh, 2016a). The acyl-CoA module was based on activating the carboxylates *via* FACL (Figure 4b) while ethanol and isobutanol were produced through their respective pathway submodules (Table 3).

In *S. cerevisiae* and *E. coli*, the 2-keto production pathway towards isobutanol or isoamyl alcohol was combined with the synthesis of fatty acid acyl-CoA to give rise to a number of fatty acid short and branched-chain esters (FASBE) (Guo *et al.*, 2014; Teo *et al.*, 2015; Wierzbicki *et al.*, 2016), which can be applied as biodiesels. In *A. baylyi*, the spectrum of wax esters could be adapted by expressing alternative FAR enzymes. These enzymes converted the fatty acyl-CoAs produced by the organism to fatty alcohols. By using an alternative FAR system from *Pseudomonas fluorescens*, the product spectrum shifted from C18 wax esters to C16 wax esters (Table 3) (Santala *et al.*, 2014). The versatility and broad substrate specificities of some AATs also allow for the design of novel esters that may not exist in nature (Table 3). For example, the *S. cerevisiae* Atf1 is able to acetylate both termini of various α,ω-diols, such as hexanediol and pentanediol (van Nuland *et al.*, 2017). Similarly, Eht1 could react ethanol with activated α,ω-fatty acids, resulting in the production of di-ethyl esters (van Nuland, Eggink and Weusthuis, 2017).

Recent understanding and engineering of (heterologous) ester production has enabled the development of *in silico* models, such as MODCELL and ModCell2 (Trinh, Liu and Conner, 2015; Garcia and Trinh, 2019). These tools are able to predict and design modular cells and metabolic pathways, that can couple the production of several ester platforms, based on their common metabolic reactions. By this approach, genetic engineering for the design of novel ester producing strains can be combined and optimized, reducing the overall workload.

Beyond esters as final products

The various metabolic engineering strategies and increased understanding of microbial ester synthesis should boost developments towards production of biobased esters as high value compounds and bulk chemicals. As esters can be easily hydrolyzed to organic acids and alcohols, which are valuable bulk chemicals in their own right (Chen and Nielsen, 2016), the biobased production of these compounds may profit from enhanced ester production as well, e.g. by decreasing product toxicity or facilitating product removal. In addition, esterification of intermediates improves microbial production of α,ω-diols as they act as a protective group during conversions.

Esters for the production of alcohols and carboxylic acids

By adding a hydrolysis step, the organic acid and alcohol portion of the produced esters can be readily recovered in a potential bioprocess. The following section highlights a few cases in which alcohol or acid production *via* an ester intermediate might be beneficial based on their physical properties.

A minimum product titer of 50 g/L is considered acceptable when implementing a biobased process or the downstream processing (DSP) steps would become too cost intense (Van Dien, 2013). Product toxicity often prevents reaching sufficiently high titers during microbial production of chemicals, especially regarding alcohols and acids. The presence of 4 g/L (50 mM) 2-butanol already negatively affected the growth rate of *S. cerevisiae, E. coli* and *B. subtilis*, while concentrations of 16 g/L butanol even inhibit growth and continuation of the fermentation in solventogenic *Clostridia* (Ezeji *et al.*, 2005; Pereira, Verheijen and Straathof, 2016). A similar effect is observed for organic acids such as acetic acid, propionic acid, or butyric acid where growth is completely inhibited at concentrations exceeding 5 g/L, 11 g/L or 6 g/L, respectively (Vázquez *et al.*, 2011). Only for ethanol, fairly tolerant hosts were found, like *S. cerevisiae*, where ethanol tolerances may exceed 100 g/L ethanol (Casey and Ingledew, 1986; Ghareib, Youssef and Khalil, 1988).

Based on their physical characteristics, a relation has been found between compound hydrophobicity, expressed in the $\log P_{o/w}$ value, and microbial toxicity. The $\log P_{o/w}$ value describes how well a compound distributes over an octanol phase in comparison to an aqueous phase (Harnisch, Möckel and Schulze, 1983) but can also be linked to the molecular toxicity of the compound for microorganisms during fermentation (Laane *et al.*, 1987; Inoue and Horikoshi, 1991; Heipieper *et al.*, 1994; Straathof, 2003). Straathof and colleagues correlated both the $\log P_{o/w}$ and the aqueous solubility of a compound to the experimentally determined critical concentration C^{aq}_{crit} retrieved from various literature, at which cell growth is no longer possible. While this value might slightly vary with the microorganism of choice, the general correlation between toxicity and polarity stays valid (Straathof, 2003). The critical concentrations derived from the $\log P_{o/w}$ correlation of some industrially important esters, acids and alcohols highlight the above-mentioned bottleneck of reaching insufficient titers and dealing with rather severe toxic effects of the products (Table 4). As a rule of thumb, compounds with a $\log P_{o/w}$ between 0.7 and 4 are considered toxic to an organism. At higher $\log P_{o/w}$ values the molecular toxicity effect is avoided as the compound is so apolar that it forms a second phase, thus, is no longer interfering with the aqueous system of the microbe. Then, however, one has to account for the toxicity that the second phase imposes on the microorganism, the phase toxicity (Bar and Gainer, 1987). At lower $\log P_{o/w}$ values the toxic effects are circumvented as the compound is so hydrophilic that it is no longer interfering with the cell membrane.

Most of the industrially relevant alcohols, acids, as well as esters (Figure 1) are in the toxic range based on their corresponding $\log P_{o/w}$ values (Table 4). Nevertheless, microbial production of these esters may be more promising than microbial production of their alcohol or acid precursors. In line with the $\log P_{o/w}$ values, C2 to C8 esters were generally less toxic to *E. coli* than alcohol or acid equivalents (Wilbanks and Trinh, 2017). Moreover, the C-mol-based Heat of Vaporization at standard conditions ($H_{vap}°$), the energy needed to evaporate a compound of interest, shows that short and medium chain lengths esters

consistently require less energy input than their acid or alcohol counterpart (Table 4). *In situ* product removal (ISPR) by, for instance gas stripping or phase extraction has proven an efficient way to increase final yields and titers and is better applicable to esters than to alcohols and acids. Introducing a biphasic system using hexadecane, enabled a yield of 80 %, reaching a final titer of 17 g/L isobutyl acetate (Rodriguez, Tashiro and Atsumi, 2014). With gas stripping, isobutyl acetate yields could be increased from 28.8 % without stripping to 42 % of the theoretical maximum, and reached 50 % of the theoretical maximum yield in an ethyl acetate stripping experiment (Urit *et al.*, 2011; Tai, Xiong and Zhang, 2015). For ethyl acetate this would correspond to liquid titers twice as high as the predicted critical concentration, for isobutyl acetate the increase is even more than 10-fold (Table 4). Therefore, this approach offers a way to keep up high productivities by avoiding accumulation of inhibitory product concentrations.

Acetic acid, ethanol and ethyl acetate are abundant industrial compounds, with moderate toxicity ($logP_{o/w}$ <0.7). While a lot of research has been performed on improving ethanol production itself, the latest advances focus on ISPR *via* gas stripping (Taylor *et al.*, 1998; Qureshi *et al.*, 2012). A similar approach has been pursued for ethyl acetate production (Urit *et al.*, 2011). In direct comparison to ethanol, higher volatilities for ethyl acetate relate to a more favorable Henry coefficient. As a consequence, the liquid/gas distribution of ethyl acetate, even at 25 °C is approximately 25-fold more beneficial compared to ethanol (Sander, 2015). For acetic acid in turn, gas stripping cannot be recommended as low volatility paired with a high boiling point and good solubility push the equilibrium to the liquid phase. Considering applications of acetic acid as a bulk chemical however, only few studies obtained sufficiently high titers to encourage further research on a biobased process (Huang and Yang, 1998). Latest technologies and challenges for the bulk production of acetic acid have been summarized recently and predict yields, DSP efficiency and costs as major challenges (Vidra and Németh, 2017). Therefore, the production of acetic acid *via* hydrolysis of the ester intermediate could be an attractive alternative, as it leads to a second valuable by-product, ethanol.

When comparing other compounds of the alcohol, acid or ester family, most of them have a poor volatility paired with a boiling point well above the one of water. This makes product removal *via* gas stripping less feasible. Nevertheless, when comparing butanol and butyric acid with esters such as butyl butyrate or ethyl butyrate, biobased production *via* an ester intermediate might again be an interesting solution. Looking at the respective extractabilities of these compounds by an organic phase such as decane, the outcome favors the ester derivative once again, not in the least due to the absence of functional polar groups (Table 4). Despite the fact that all compounds are fairly toxic to any production host ($logP_{o/w}$ below 4), ISPR by applying an organic phase might be applied to keep the dissolved concentration low. The potential for liquid/liquid extraction of butanol *via* an organic phase was already mentioned before when different ISPR

strategies were compared (Groot, van der Lans and Luyben, 1992; Ezeji *et al.*, 2005; Xue *et al.*, 2014). Moreover, Oudshoorn *et al.* (2009) concluded, the energy requirements for steam stripping and distillation were approximately 66% of the combustion energy of recovered 1-butanol while extraction and adsorption showed the lowest energy costs with still significant losses of 25 and 22%, respectively (Oudshoorn, Van Der Wielen and Straathof, 2009). For butyl butyrate the extractability by decane is more than 1000-fold better than for butanol, indicating even more potential for energy savings when taking a route *via* the ester intermediate.

TABLE 4: Characteristic parameters of several esters and their alcohol and acid derivatives. $logP_{o/w}$ values and the heat of vaporization at standard conditions ($H_{vap}°$) were retrieved from PubChem and Chemeo database, the critical concentration (C_{rit}) was calculated based on the Straathof correlation obtained from experimental data (Straathof, 2003). The partition coefficient on decane/water was estimated using the LSER approach (Vitha and Carr, 2006). Solute parameters were calculated by ACDLabs (ACD-Labs, 2018) while solvent parameters were exported from (Stovall, Acree and Abraham, 2005).

Compound	#C	$logP_{o/w}$	C_{crit} (M)	$H_{vap}°$ (kJ C-mol^{-1})	Partition coefficient (decane/water)
Ethanol	2	-0.31	2.0700	21.20	9.60E-03
Acetic Acid	2	-0.17	1.6787	25.80	1.10E-03
Ethyl acetate	4	0.73	0.1686	8.83	1.94E+00
Butyric acid	4	0.79	0.1343	14.50	n.a.
Butanol	4	0.88	0.1604	13.10	1.69E-01
Butyl acetate	6	1.78	0.0228	7.17	3.63E+01
Ethyl butyrate	6	1.85	0.0149	7.00	3.91E+01
Hexanoic acid	6	1.92	0.0861	11.88	1.02E+00
Hexanol	6	2.03	0.0577	10.17	2.53E+01
Ethyl hexanoate	8	2.40	0.0044	6.08	1.45E+03
Butyl butyrate	8	2.83	0.0021	5.32	7.43E+02
Octanol	8	3.00	0.0024	8.66	n.a.
Dodecanoic acid	12	4.20	0.0001	5.48	n.a.
Hexyl Hexanoate	12	4.40	<0.0001	4.29	4.06E+05
Dodecanol	12	5.10	<0.0001	7.50	n.a.
Oleic acid	18	6.50	<0.0001	4.39	5.27E+06
Ethyl oleate	20	8.00	<0.0001	3.46	7.44E+09

n.a. – not available

Due to the low solubility or good volatility, esters can be extracted from the system with less effort. If these benefits in ester production and extraction are sufficient to compensate for the energy spend on subsequent hydrolysis and recovery of acid and alcohol derivatives, remains to be answered. Especially for the above-mentioned examples, all classes (esters,

acids and alcohols) are settled in the most toxic range according to their $logP_{o/w}$ values. Microbial production will therefore always be limited by strain robustness and efficiency of ISPR. For these reasons, it might be worthwhile to focus on esters outside the critical $logP_{o/w}$ range. These include, but are not limited to hexyl hexanoate ($logP_{o/w}$ = 4.4), ethyl decanoate ($logP_{o/w}$ = 4.6) or butyl decanoate ($logP_{o/w}$ = 5.4). Extraction of these compounds in microbial production systems might be straightforward and energy efficient, as an organic phase is formed spontaneously. The effect of phase toxicity of the different compounds needs to be evaluated.

One benefit of the proposed approaches however, is undebatable in all cases: it enables tunable processes with esters as fermentation output or the co-production of carboxylic acids and alcohols upon hydrolysis. This provides great flexibility on industrial scale and enables fast responses to changing market demands.

Esters for the production of α,ω-bifunctional monomers

Esterification can also be exploited for the production of α,ω-bifunctional monomers (BMs). These compounds contain a functional group (e.g., hydroxy, carboxy, amine) on both terminal carbon atoms, which allow them to be used as building blocks for a vast array of polymers. BMs are industrially interesting due to their high market demands which are higher compared to molecules with a single functionality. Examples of BMs are α,ω-diols and α,ω-dicarboxylic acids, which are currently produced by multistep, energy-intensive processes. Hence, much research has been devoted to the development of more environmentally friendly production routes. For medium-chain molecules, the major challenge is the introduction of a second functional group (e.g., hydroxy, carboxy) on the ω-position. Alkane monooxygenases can ω-oxidize primary alcohols and fatty acids, but efficiencies are low with medium-chain molecules (Fujii *et al.*, 2006; Scheps *et al.*, 2011; Gudiminchi *et al.*, 2012; Honda Malca *et al.*, 2012; Clomburg *et al.*, 2015; van Nuland, Eggink and Weusthuis, 2017). This is likely caused by the presence of the polar group that hampers entry into the hydrophobic binding pockets of the monooxygenase. Esterification of fatty acids hide the polar group and allows alkane monooxygenase AlkB to efficiently ω-oxidize esterified fatty acids (Schrewe *et al.*, 2011, 2014; Julsing *et al.*, 2012; van Nuland, Eggink and Weusthuis, 2017). Instead of adding esterified fatty acids directly, the fatty acids can also be esterified *in vivo* and then be combined with a monooxygenase to achieve ω-oxidation. To achieve this, *E. coli* was equipped with acyl-CoA ligase AlkK and either *A. baylyi* AtfA or *S. cerevisiae* Eeb1 to produce esters from medium-chain fatty acids. The resulting esters were efficiently converted to mono-ethyl dicarboxylic acids. Also, di-ethyl esters accumulated (van Nuland, Eggink and Weusthuis, 2017). A challenge for such process is the inherent toxicity of medium-chain fatty acids to microorganisms. This could be circumvented by fed-batch addition of the fatty acid, or to produce them *de novo* with

subsequent esterification. The same concept can be applied to produce the mono-ethyl dicarboxylic acids from n-alkanes (van Nuland, Eggink and Weusthuis, 2017). Ideally, this approach should be coupled or combined to microbial alkane production from glucose, which is reviewed elsewhere (Jiménez-Díaz *et al.*, 2017).

For the production of α,ω-diols another challenge has to be tackled. Alkane monooxygenases tend to overoxidize primary alcohols to aldehydes and carboxylic acids. Furthermore, under aerobic conditions, alcohol and aldehyde dehydrogenases that are present in the production host can do the same. Esterification of the alcohols is a promising strategy to prevent overoxidation. This protective group chemistry is common in organic chemistry, and has been applied to protect overoxidation of inactivated sp^3 C-H bonds (Desai, Hull and Sanford, 2004; Hashiguchi *et al.*, 2014; Konnick *et al.*, 2014).

Challenges and perspectives

This review provides an overview of microbial ester production on a fundamental level, with special attention towards the potential applications in the bulk chemical arena. There are some major challenges ahead before biobased ester production can move to the industrial level. The key ester producing enzyme AAT is not understood well, and studies that focus on fundamental aspects of its function are scarce. For example, it is still not clear why many AATs display thioesterase and esterase activities, and how to control these activities. In case of Eat1, ethanol is the determining factor that shifts the enzyme from a hydrolase to an AAT (Kruis *et al.*, 2017). The controlling factors in other AATs have not been established yet. It is furthermore unclear how efficiently AATs are expressed and translated in heterologous hosts, which may hinder the development of efficient bioprocesses.

Another major challenge is how to fully employ the vast array of AATs that are available in nature. Metabolic engineering studies have hitherto utilized a relatively limited subset of AATs. However, it has been shown that even homologs of the same AAT can show remarkable differences in their substrate specificities. Mining the unknown AATs may provide us with enzymes that are able to perform conversions that are currently either not possible or inefficient. AATs with narrower substrate specificities than the ones employed currently should also be identified. Catalytic promiscuity can be useful as the same enzyme can form different products. This can simplify the development of ester producing-strains, particularly in laboratory research where many metabolic engineering strategies are typically tested at the same time. However, promiscuity can also be a disadvantage, especially on large scale, where a single fermentation product is desired. Special consideration should also be given to discovering catalytically efficient AATs. In many studies aimed at ester production, the titers achieved were relatively low. The

reasons for this are not well understood, but it is likely that some AATs may be catalytically inefficient to allow metabolic flux towards ester synthesis. To enable development of ester-producing processes, efficient AATs should be discovered, or alternatively evolved from known AATs.

The advances in metabolic engineering of ester production are closely linked to the developments in engineering towards production of acyl-CoA (acids) and alcohols. The metabolic diversity of the alcohol and acyl-CoA pathways indicate that a practically limitless number of esters can be designed, assuming an AAT with the appropriate specificity is available. The real challenge of engineering ester production will be to balance the supply of the alcohol and acyl-CoA substrates. Ideally, both substrates will be produced in a 1:1 ratio to ensure efficient conversion of sugars into esters. This is challenging as both substrates are produced in complex and intertwined metabolic pathways.

The commercialization of ester production will require efficient DSP development. Based on the physical properties of esters, they can be removed by gas stripping, extracted into an organic phase, or may even form their own phase. In comparison to their alcohol or acid precursor ISPR is benefitting from the absence of polar groups, making esters generally easier to remove from aqueous systems. Small, volatile esters can freely diffuse from the cell. However, this is not the case for large, insoluble esters, such as wax esters of FAEE. These esters mostly accumulate in the cell and need to be extracted, adding costs to the DSP.

Finally, the true potential of esters as a platform chemical for alcohol and carboxylic acids should be investigated. From a bioprocess engineering perspective, ester formation could be favorable to alcohol and acid formation due to lower toxicity and easier extractability. Esterification could also facilitate the production of high value compounds such as α,ω-diols and α,ω-dicarboxylic acids and unleash the full potential of biobased ester production.

Chapter 3

From Eat1 to trEat1: Engineering the mitochondrial Eat1 enzyme for enhanced ethyl acetate production in *Escherichia coli*

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Abstract

Genetic engineering of microorganisms has become a common practice to establish microbial cell factories for a wide range of compounds. Ethyl acetate is an industrial solvent that is used in several applications, mainly as a biodegradable organic solvent with low toxicity. While ethyl acetate is produced by several natural yeast species, the main mechanism of production has remained elusive until the discovery of Eat1 in *Wickerhamomyces anomalus*. Unlike other yeast alcohol acetyl transferases (AATs), Eat1 is located in the yeast mitochondria, suggesting that the coding sequence contains a mitochondrial pre-sequence. For expression in prokaryotic hosts such as *E. coli*, expression of heterologous proteins with eukaryotic signal sequences may not be optimal.

Unprocessed and synthetically truncated eat1 variants of *Kluyveromyces marxianus* and *Wickerhamomyces anomalus* have been compared *in vitro* regarding enzyme activity and stability. While the specific activity remained unaffected, half-life improved for several truncated variants. The same variants showed better performance regarding ethyl acetate production when expressed in *E. coli.*

By analyzing and predicting the N-terminal pre-sequences of different Eat1 proteins and systematically trimming them, the stability of the enzymes *in-vitro* could be improved, leading to an overall improvement of *in-vivo* ethyl acetate production in *E. coli*. Truncated variants of *eat1* could therefore benefit future engineering approaches towards efficient ethyl acetate production.

Keywords: Eat1, Alcohol acetyl transferase (AAT), mitochondria, *Escherichia coli*, ethyl acetate.

Background

Ethyl acetate production in yeast is catalyzed by alcohol acetyltransferases (AATs), which synthesize ethyl acetate from acetyl-CoA and ethanol, releasing free CoA (Kruis et al., 2019). The first described ethyl acetate-producing AAT was the *Saccharomyces cerevisiae* Alcohol acetyltransferase 1 (Atf1) (Minetoki *et al.*, 1993). However, its homologs in *Wickerhamomyces anomalus* and *Kluyveromyces marxianus* appeared to have only a minor role in bulk ethyl acetate production (Kruis *et al.*, 2017; Löbs *et al.*, 2017). Instead, these microorganisms use the recently identified ethanol acetyltransferase (Eat1) to produce ethyl acetate. All ethyl acetate-producing yeasts were shown to possess at least one functional Eat1 homolog (Kruis *et al.*, 2017). Besides AAT activity, Eat1 enzymes exhibit esterase and thioesterase activities as well, hydrolyzing ethyl acetate and acetyl-CoA, respectively. The hydrolyzing activities of *W. anomalus* Eat1 could be suppressed *in vitro* by sufficiently high levels of ethanol (Kruis *et al.*, 2017; Nancolas *et al.*, 2017).

A key difference between Atf1 and Eat1 is their cellular location in yeast. Atf1 localizes to lipid particles in the cytosol (Verstrepen *et al.*, 2004; Lin and Wheeldon, 2014), while Eat1 homologs are located in yeast mitochondria (Kruis, Mars, *et al.*, 2018; Löbs *et al.*, 2018). Most mitochondrial enzymes like Eat1 are encoded on the nuclear genome and synthesized in the cytoplasm. They are transported into the mitochondria via the translocase of the outer mitochondrial membrane (TOM) complex, based on the presence of mitochondrial targeting signals. These targeting sequences contain amphipathic helices, which partially destabilize the nascent proteins and facilitate the cross-membrane transport into the mitochondria (Wiedemann and Pfanner, 2017). Most mitochondrial proteins contain N-terminal pre-sequences that are cleaved by mitochondrial proteases, with mitochondrial processing peptidase (MPP) being the most prominent. In some cases, other peptidases like Oct1 or Icp55 initiate additional cleavage events of the pre-protein (Mossmann, Meisinger and Vögtle, 2012). Icp55 cleaves one additional amino acid (AA) from MPP-generated N-termini, while Oct1 removes another 8 AA after cleavage by MPP or Icp55 (Vögtle *et al.*, 2009, 2011).

Mitochondrial cleavage events of Eat1 in native yeasts have not been studied in much detail. In the *K. marxianus* Eat1, removal of the initial 19 AA prevented localization to the native yeast mitochondria. It is unclear whether this is the final, mature form of Eat1, or if additional cleavage events occur after MPP cleavage (Löbs *et al.*, 2018). Imo32, a *S. cerevisiae* homolog of Eat1 (Kruis *et al.*, 2017), is processed by both MPP and Oct1 (Vögtle *et al.*, 2011). It is possible that multiple processing events occur in other Eat homologs as well. However, the precise final form of Eat proteins can only be determined through experiments in the native hosts, such as isolation of mature Eat1 from yeast mitochondria. These procedures include isolation of intact yeast mitochondria through differential centrifugation, which is laborious. Furthermore, the purification of Eat1 would likely

require an *in vitro* assay. To this point, the AAT activity of Eat1 in cell free extracts has not been reported, which makes this approach difficult.

Ethyl acetate production by native yeasts on an industrial scale is limited by the yeast metabolism due to the specific environmental conditions that are required for ester synthesis. These include iron or oxygen limitation, which are difficult to control on an industrial scale (Urit, Li, *et al.*, 2013; Kruis *et al.*, 2017; Kruis, Mars, *et al.*, 2018). Alternative hosts, especially bacteria and archaea could be used instead. However, prokaryotic hosts are unable to cleave mitochondrial pre-sequences, which may lead to hampered heterologous expression of *eat1* and impaired ethyl acetate production. Introduction of the mature, final forms of the mitochondrial Eat1 in prokaryotes would likely improve *in vivo* ethyl acetate production. In this study we improved ethyl acetate production in *Escherichia coli* by truncating the N-terminus of Eat1 enzymes from *W. anomalus* and *K. marxianus*. To determine the optimal truncation position, 16 Eat1 variants were produced in *E. coli*, and tested *in vivo* and *in vitro* for their effect on ethyl acetate production.

Results

In silico analysis of Eat1 N-terminal sequences

Optimal function of Eat1 in *E. coli* likely depends on introducing the mature, truncated form of the enzyme. This form is not known for any of the Eat1 homologs that are able to produce ethyl acetate and can only be determined in the native yeast hosts through laborious experiments. Instead, we searched *in silico* for predicted cleavage sites within the N-termini of 15 Eat homologs from various yeast species using MitoFates (Fukasawa *et al.*, 2015). All but the *S. cerevisiae* Eat1 N-termini contained an amphipathic region that is typically observed in N-terminal sequences of mitochondrial proteins (Figure 1a). Several sequences also had predicted MPP/Icp55 cleavage sites. Curiously, the predicted MPP/Icp55 cleavage sites would not fully remove the destabilizing amphipathic region of their respective Eat1 N-termini. Since the amphipathic regions destabilize protein folding (Wiedemann and Pfanner, 2017), they are presumably removed during enzyme processing in the mitochondria. This may indicate that additional cleavage events, such as Oct1 cleavage in Cja Eat1 (Figure 1a) occur in Eat1.

We focused on N-termini of the *Wickerhamomyces anomalus* (Wan) Eat1 and *Kluyveromyces marxianus* (Kma) Eat1. Both enzymes are derived from yeasts that are able to produce high amounts of ethyl acetate. Efficient ethyl acetate synthesis by unmodified (but codon-harmonized) Wan Eat1 has already been demonstrated in *E. coli* (Kruis *et al.*, 2017). However, the composition of the Wan and Kma Eat1 N-termini is remarkably different. The longer Kma Eat1 contained a clear pre-sequence and recognition sites for

two mitochondrial peptidases, MPP and Icp55 at amino acid (AA) positions 19 and 20, respectively (Figure 1a). The shorter N-terminus of Wan still showed the characteristic amphipathic region, but no clear mitochondrial peptidase motifs were detected (Figure 1a). We therefore initially focused on optimizing the N-terminus of the Kma Eat1. We designed 14 truncated versions of Kma Eat1 (trEat1) based on predicted cleavage sites, as well as arbitrary positions within the N-terminus. The truncated variants are denoted by the first AA appearing after the cleavage position (Figure 1b), although in reality, all proteins begin with M.

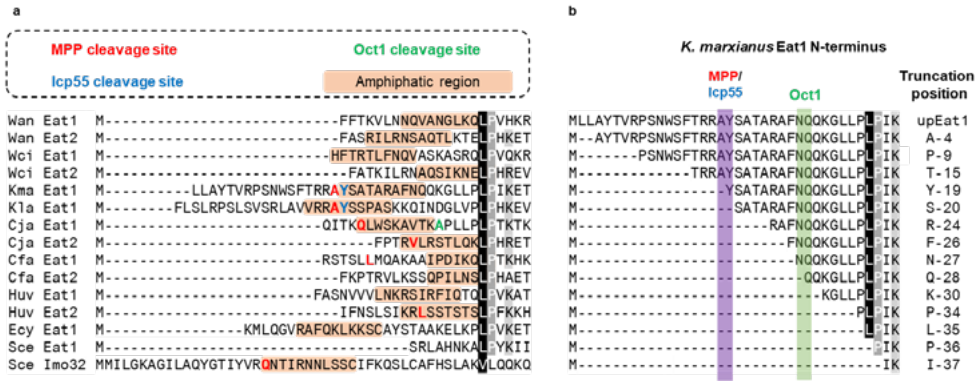


FIGURE 1: *In silico* analysis of 15 Eat homologs from various yeasts using MitoFates (Fukasawa *et al.*, 2015). (a) Prediction of amphipathic regions and putative cleavage sites in Eat N-termini. (b) Design of 15 Kma trEat1 variants. Abbreviations: Wan - *Wickerhamomyces anomalus*, Wci - *Wickerhamomyces ciferrii*, Kma - *Kluyveromyces marxianus*, Kla - *Kluyveromyces lactis*, Cja - *Cyberlindnera jadinii*, Cfa - *Cyberlindnera fabianii*, Huv - *Hanseniaspora uvarum*, Ecy - *Eremothecium cymbalariae,* Sce - *Saccharomyces cerevisiae.*

Expression of truncated Kma Eat1 variants in *E. coli*

Ethyl acetate production from glucose by the truncated Kma Eat1 (Kma trEat1) variants was assessed in *E. coli.* The cells were grown under anaerobic conditions to stimulate production of ethanol, which is required by Eat1 to produce ethyl acetate. The carbon flux was channeled towards ethyl acetate production by disrupting the lactate dehydrogenase (*ldhA*) and acetate kinase (*ackA*) genes. This eliminated lactate production and lowered acetate formation, respectively (results not shown). The resulting *E. coli* BW25113 *ΔldhAΔackA* (DE3) strain was used to express the *eat1* gene variants under the control of the LacI/*T7* promoter.

We induced gene expression with 0.01 mM IPTG and 0.1 mM IPTG (Figure 2). At the lowest concentration, a profoundly positive effect on the final ethyl acetate titer was observed with several truncated variants compared to the untruncated (up) Eat1 (Figure 2ab). At 0.1 mM IPTG, the differences in ethyl acetate titers produced by Kma upEat1 and the

Kma trEat1 variants were less apparent (Figure 2cd). Since 0.1 mM IPTG is a high inducer concentration, it is likely that ethyl acetate production was not limited by the AAT activity of Eat1, but instead by other metabolic bottlenecks. However, the low ethyl acetate production at 0.01 mM IPTG suggests that ethyl acetate production was limited by the activity of Kma Eat1. Any changes in the ethyl acetate production can therefore be linked directly to the *in vivo* activity of the enzymes.

The BW25113 Δ*ldhA*Δ*ackA* (DE3) strains producing Kma trEat1 F-26, N-27, Q-28 and K-30 all formed ethyl acetate within 24h of cultivation, whereas no ethyl acetate was detected in the strains producing the unprocessed Kma Eat1 and most other Kma trEat1 variants (Figure 2a). During the second time point (144 hours) all Eat1 variants produced detectable amounts of ethyl acetate, except Kma trEat1 T-15, P-36 and I-37. Nevertheless, Kma trEat1 F-26, N-27, Q-28 and K-30 produced substantially more ethyl acetate compared to the unprocessed control and other Eat1 variants. The best performer was *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing Kma trEat1 K-30, which formed 11.8-fold more ethyl acetate than the unprocessed variant corresponding to 2.07 mM or 182.4 mg/L (Figure 2b). *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing Kma trEat1 P-9, Y-19, S-20, P-34 and P-35 formed approximately the same amounts of ethyl acetate as the unprocessed Kma1 which produced around 0.18 mM (15.5 mg/L) ethyl acetate (Figure 2).

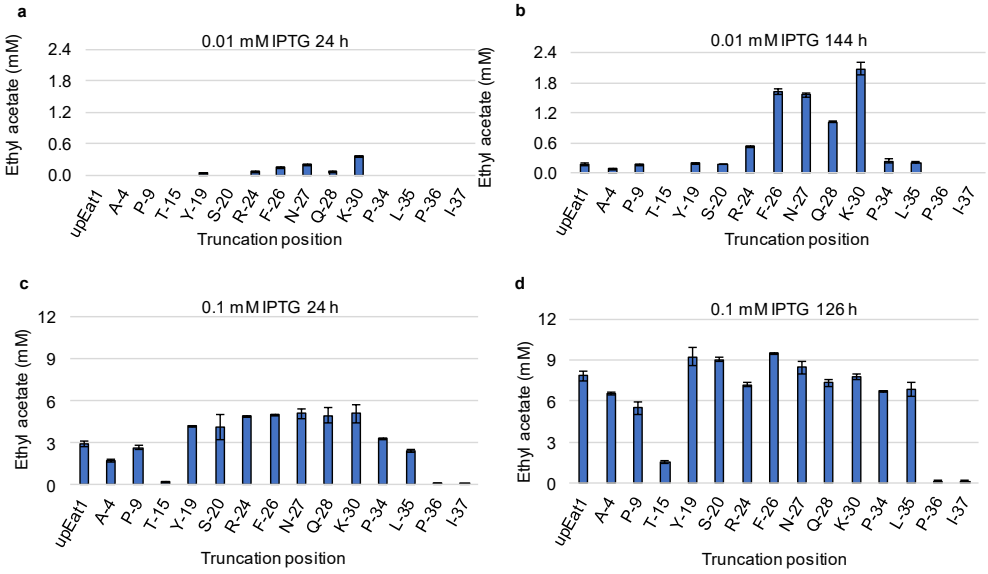


FIGURE 2: Improved ethyl acetate production by N-terminal truncated versions of Kma Eat1. (a,b) – Ethyl acetate titers reached by cultures producing Kma trEat1 variants at 0.01 mM IPTG after (a) 24 h and (b) 144 h. (c,d) - Ethyl acetate titers reached by cultures producing Kma trEat1 variants at 0.1 mM IPTG after (c) 24 h and (d) 126 h. Strains were grown under anaerobic conditions in modified M9 medium. Genes were expressed from a series of pET26b plasmids. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: Kma – *K. marxianus*

Most trEat1 variants either led to increased ethyl acetate production or did not affect it significantly (Figure 2ab). The exceptions were the strains producing Kma trEat1 T-15, P-36 and I-37, which formed only traces of ethyl acetate. The Kma trEat1 P-36, and I-37 removed the first conserved region that is present in all Eat1 homologs from various yeasts (Kruis *et al.*, 2017), which indicates that this conserved region is critical for ethyl acetate formation by Eat1. It is unclear why ethyl acetate formation was severely reduced in the strain producing Kma trEat1 T-15 (Figure 2).

Improved *in vivo* performance of Kma trEat1 F-26 and K-30 may be linked to improved protein solubility. To test this, soluble and insoluble fractions of cell free extracts (CFE) were prepared during ethyl acetate formation by unprocessed Kma Eat1 and trEat1 K-30 (Supplementary Figure 1a). Gene expression was induced with 0.01 mM IPTG. Most of the Kma Eat1 was in the insoluble fraction. Truncating the protein did not affect this, indicating that improved ethyl acetate production by Kma trEat1 K-30 was not caused by improved protein solubility.

An alternative explanation may be that truncating the 5' coding sequence of Kma *eat1* affected the translation initiation rates of the ribosome binding sites (RBS) used for protein translation. To exclude this possibility, we calculated the translation initiation rates for each Kma *trEat1* gene using the RBS Calculator (Salis, Mirsky and Voigt, 2010). We compared the translation initiation rates with the ethyl acetate titers achieved by *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing the Kma trEat1 variants with 0.01 mM IPTG (Figure 2a) and found little correlation between them (r = -0.14, Supplementary Figure 2). This supports the hypothesis that truncating the N-terminus of Kma Eat1 affected its function on the protein level.

Improved *in vitro* stability of Kma trEat1 variants

The improved ethyl acetate production was presumably caused by changes to Eat1 on the protein level, either by a higher specific activity or by an enhanced stability. To test this, we purified the unprocessed Kma Eat1, Kma trEat1 F-26, and Kma trEat1 K-30, and measured their initial 1-naphthyl acetate (1-NA) hydrolysis rates at 30 °C, 35 °C and 40 °C based on the esterase activity of Eat1 (Figure 3a). Hydrolysis of 1-NA releases free 1-naphthol, which can be measured spectrophotometrically. The 1-NA assay was used in place of direct measurement of ethyl acetate synthesis (AAT activity) since the method is considerably less laborious and more sensitive. The specific esterase activities of the three proteins moderately increased with temperature, with a 10 °K increase leading to a 3-fold higher specific activity (Figure 3a). The truncated variants of Kma Eat1, however, did not exhibit a higher specific activity compared to unprocessed Eat1. We then tested whether truncating Kma Eat1 affected the stability of the proteins by determining their half-lives at 45 °C, 50 °C and 55 °C. For both Kma trEat1 F-26 and K-30, the half-lives were significantly higher at all

tested temperatures compared to the unprocessed Kma Eat1 (Figure 3b, Supplementary Figure 3). Kma trEat1 F-26 and K-30 were thus more thermostable. Apparently, the N-terminal region has a weakening effect on the thermostability of Eat1.

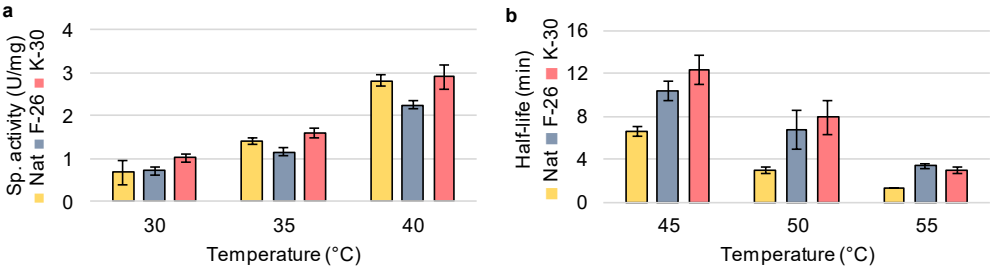


FIGURE 3: Improved stability of N-terminal truncated versions of Kma Eat1. (a) – *In vitro* specific activity and (b) half-life, of purified unprocessed Kma Eat1, Kma trEat1 F-26 and K-30 at various temperatures. Esterase activity was measured by following the release of 1-naphthol at 320 nm from 1-naphthyl acetate. Measurements were performed as technical triplicates; error bars represent the standard deviation.

3

Expression of truncated Wan Eat1 variants in *E. coli*

We examined whether the function of Wan Eat1 could also be improved by truncating its N-terminus. Predicting the structure of the N-terminal localization sequence of Wan Eat1 using MitoFates did not result in clearly defined protease cleavage positions. Therefore, we used the conserved region at AA positions 36 and 37 within the Kma N-terminus as a guide to create two Wan trEat1 variants. Kma trEat1 Q-28 and K-30 were used to generate their Wan trEat1-V11 and N-13 counterparts, respectively (Figure 4a). The variants were produced in *E. coli* BW25113 *ΔldhAΔackA* (DE3) under the control of the LacI/*T7* promoter. Interestingly, ethyl acetate titers exceeded measured values of Kma Eat1 already after 24 h when induced with 0.01 mM IPTG. All three strains producing the Wan Eat1 variants formed approximately 4 mM ethyl acetate (352 mg/L), and no significant difference could be observed between unprocessed and truncated Eat1s (Figure 4b). After 120 h of fermentation, ethyl acetate concentrations varied between 9 and 11 mM or 792 and 968 mg/L, which was higher than all values obtained with the Kma Eat1 variants at 0.1 mM IPTG (Figure 2cd, 3b). This suggests that 0.01 mM IPTG was sufficient to fully induce Wan *eat1* expression to a point where the activity of Eat1 did not limit ethyl acetate synthesis.

To more accurately study the effect of the truncations, we lowered the IPTG concentration to 0.001 mM IPTG (Figure 4c). The ethyl acetate formation trends in *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing Wan trEat1 N-13 at 0.001 mM IPTG were similar to those observed in strains producing the Kma K-30 at 0.01 mM IPTG (Figures 2ab and 4c). At both sampling points, the strain producing Wan trEat1 N-13 reached a 2-fold higher ethyl

acetate concentration than the unprocessed Wan Eat1 (Figure 4b). No difference was found between the Wan trEat1 V-11 and the unprocessed Wan Eat1 after 24 h, while over a longer time period the truncated variant even produced less ethyl acetate than the other two tested strains (Figure 4b, c). The CFE extracts of cultures producing unprocessed Wan Eat1 and Wan trEat1 N-13 were also analyzed by SDS-PAGE during ethyl acetate formation (Supplementary Figure 1b). Similar to Kma Eat1. The overall solubility of Wan Eat1 did not improve after the protein was truncated.

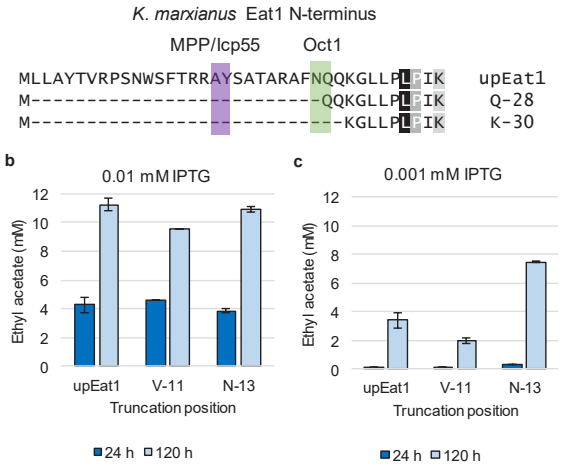


FIGURE 4: Improved ethyl acetate production by N-terminal truncated versions of Wan Eat1 (a) - truncated variants of the Wan Eat1 N-terminus based on the Kma trEat1 Q-28 and K-30. The highlighted LP residues indicate the first region that is conserved in all known Eat1 proteins. (b) – Ethyl acetate titers reached by cultures producing Wan trEat1 variants at 0.01 mM IPTG induction levels after 24 h (dark blue) and 120 h (light blue). (c) – Ethyl acetate titers reached by cultures producing Wan trEat1 variants at 0.001 mM IPTG induction levels after 24 h (dark blue) and 120 h (light blue) Strains were grown under anaerobic conditions in modified M9 medium. Genes were expressed from a series of pET26b plasmids. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: Wan – *W. anomalus*, upEat1 – unprocessed Eat1.

Discussion

We described the optimization of functional expression of the mitochondrial Eat1 enzyme from yeasts in a prokaryotic host. The *in vivo* function of the mitochondrial Eat1 proteins in *E. coli* could be improved by removing the destabilizing N-termini of the proteins. The Kma trEat1 F-26, N-27, Q-28 and K-30 contained potential cleavage sites that are located 7-11 AA residues after the predicted MPP cleavage site of Y-19 and S-20, indicating that one or more of them may be the mature form of Eat1. By removing the N-terminus, *in vivo* ethyl acetate production was improved as much as 11.8-fold in Kma trEat1 K-30 and 2-fold higher in Wan trEat1 N-13. Because the specific activity of the truncated versions was not significantly different from the non-truncated control, as shown for Kma variants,

improved ethyl acetate production must have been caused by improved stability of the enzyme. This higher stability most likely leads to a higher number of active Eat1 proteins *in vivo*, causing the higher ethyl acetate titers. The prediction of the RBS strength of each individual truncated Kma trEat1 gene showed no correlation to the ethyl acetate titer, supporting this explanation.

While the cultures producing unprocessed Eat1 variants were analyzed by SDS-PAGE, it became apparent that most of the enzyme was located in the insoluble fraction of the CFE. Truncation of Eat1 did not affect this distribution to a measurable extent. A similar trend was observed when *S. cerevisiae* Atf1 was expressed in *E. coli* and largely formed insoluble aggregates with reduced specific activity (Zhu *et al.*, 2015). This suggests that parts of the Eat1 as well as the trEat1 proteins remain unfunctional, which gives room for further improvement of ethyl acetate production by Eat1.

In yeast, the N-termini are removed by mitochondrial peptidases during protein translocation from the cytosol to the mitochondria, releasing the mature and stable protein (Dolezal *et al.*, 2006; Vögtle *et al.*, 2009). *E. coli* and other prokaryotic hosts cannot perform these processing events. Cleavage sites were only predicted within the N-terminus of Kma Eat1. The strains producing Kma trEat1 variants that were truncated at the predicted positions (Kma trEat1 Y-19 and S-20) did not show substantially improved ethyl acetate production relative to the unprocessed Kma Eat1. However, removing 19 AA from Kma Eat1 (Kma trEat1 S-20) was indeed sufficient to fully prevent Eat1 from being targeted to the mitochondria of *K. marxianus* (Löbs *et al.*, 2018). The ethyl acetate production only improved when an additional 7-11 AA residues were removed from the N-terminus of Kma Eat1. These variants were the Kma trEat1 F-26, N-27, Q-28, and K-30. They were chosen based on the processing events that occur in the *S. cerevisiae* Imo32, which may be a distant homolog of the Kma Eat1 (Vögtle *et al.*, 2011; Kruis *et al.*, 2017). This may suggest that similar events occur during the processing of Eat1 in *K. marxianus*. The fact that Kma trEat1 S-20 is unable to migrate to the mitochondria in *K. marxianus* (Löbs *et al.*, 2018), but did not show improved performance in *E. coli* supports this hypothesis. It is likely that Eat1 homologs from other yeasts undergo different processing events as well. For example, the N-terminus of Wan Eat1 had no predicted cleavage sites and is roughly half the length of its *K. marxianus* counterpart, while truncation improved ethyl acetate production in Wat Eat1 N-13. Confirming the true final forms of Eat1 proteins is only possible by analyzing the proteins in the native yeasts and may help to identify the most functional trEat1 variants.

The strains producing Wan Eat1 variants consistently formed 10-15 % more ethyl acetate *in vivo* compared to strains producing Kma Eat1. They also required about 2-fold lower induction levels to achieve this. It has been shown that inducer concentrations affect growth and impose an additional metabolic burden to the cell, next to plasmid maintenance (Diaz Ricci and Hernández, 2000; Malakar and Venkatesh, 2012). Lower

induction levels are therefore more desirable and screening for optimum levels is strongly recommended.

In this study, up to 4 mM or 352 mg/L ethyl acetate was produced within 24 h of anaerobic cultivation. While cultivation conditions, cell densities and working volumes affect the final outcome, reported titers for ethyl acetate production in *E. coli* settled in the 20 mg/L range (Rodriguez, Tashiro and Atsumi, 2014; Layton and Trinh, 2016a). In contrast, isobutyl acetate production by *E. coli* using the *S. cerevisiae* Atf1 reached 17-36 g/L (Rodriguez, Tashiro and Atsumi, 2014; Tai, Xiong and Zhang, 2015). This may be related to a lower affinity of the AATs used in those studies with respect to ethanol or acetyl-CoA and supports choice of Eat1 for efficient ethyl acetate production in *E. coli.* Unprocessed Wan Eat1 was used in *E. coli* to produce 5 g/L ethyl acetate under aerobic condition and ethanol supplementation (Kruis *et al.*, 2017). The truncated Eat1 variants have the potential to increase this further. Whether Eat1 can also be used to produce other esters in *E. coli* has not been confirmed. Given that Eat1 contributes to acetate ester production in *S. cerevisiae* in general, this seems plausible (Kruis, Gallone, *et al.*, 2018).

The better performance of Wan Eat1 compared to Kma Eat1 may also originate from its temperature optimum. The yeast *W. anomalus* is routinely cultivated at temperatures between 25-30 °C (Joseph Tabachnick and Joslyn, 1953; Fredlund, Blank, *et al.*, 2004; Kurita, 2007; Sabel *et al.*, 2014). In contrast, the yeast *K. marxianus* produced ethyl acetate more efficiently at 42 °C compared to lower temperatures (Urit, Li, *et al.*, 2013). As we cultivated the *E. coli* BW25113 Δ*ldhA*Δ*ackA* (DE3) strains at 30 °C, it is possible that Kma Eat1 was less efficient in *E. coli* BW25113 *ΔldhAΔackA* (DE3) due to suboptimal cultivation temperatures for the enzyme. Our enzyme assays showed that the specific activity of Kma Eat1 was much higher at 40 °C than at 30 °C, supporting this hypothesis. While at low induction levels truncated variants performed consistently better, the benefits were less prone in anaerobic fermentations at higher inducer concentrations. Further analysis of the Kma trEat1 F-26 and K-30 *in vitro* revealed that the specific activity of the enzyme was unaffected by the truncations. Instead, their stability was improved as shown by the increased half-life at all tested temperatures compared to the unprocessed variant. *In* vivo, this improved stability of the trEat1 variants is reflected by the earlier appearance of ethyl acetate synthesis during the fermentation, and the higher difference in ethyl acetate production at the lowest IPTG concentrations. The limited stability of the unprocessed Eat1 enzymes can probably be compensated by higher IPTG concentrations, as *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing the unprocessed Eat1 or the truncated trEat1 variants reached comparable ethyl acetate titers when induced with higher IPTG concentrations.

Conclusion

Expression of heterologous genes generally requires further optimization. By acknowledging the mitochondrial origin of Eat1 we improved stability and *in vivo* performance of the enzyme in prokaryotic cells.

Systematic trimming of the N-terminus was performed on *eat1* originating from *K. marxianus* and *W. anomalus* leading to truncated variants with enhanced performance. Cleavage events exceeding the pre-sequence on the other hand, led to the loss of activity, highlighting the importance of the conserved region for functionality of the proteins.

Ethyl acetate production by *E.* coli BW25113 *ΔldhAΔackA* (DE3) was improved by 10-15 % for variants, that were trimmed 7-11 AA residues next to the predicted MPP cleavage site. While *in vitro* enzyme activities remained unaffected, half-life of the truncated enzymes increased, indicating higher stability. Expression of variants Kma trEat1 F-26 and K-30, as well as the corresponding Wan trEat1 V-11 and N-13, resulted in highest ethyl acetate titers *in vivo*. Additionally, the level of induction could be reduced, compared to the unprocessed variants.

Removal of the mitochondrial pre-sequence therefore contributed to better *in vivo* performance of the Eat1 enzyme in *E. coli.* The results can benefit further engineering approaches using *E. coli* as expression system for the efficient production of ethyl acetate.

Materials and methods

Strain and plasmid construction

The strains and plasmids used in this study are shown in Tables 1 and 2, respectively. Gene disruptions were performed with CRISPR-Cas9 (Jiang *et al.*, 2015) using 50 bp homologous regions immediately upstream and downstream of the ATG and stop codon, respectively. The pTarget and pET26b plasmids were assembled using the 2X HiFi assembly master mix (NEB) according to supplier instructions. All *K. marxianus* and *W. anomalus eat1* genes were cloned with a Strep-tag or 6-His-tag, respectively, to facilitate protein purification. The pTarget sequences containing homologous regions and the gRNA module were ordered synthetically as gBlocks (IDT). PCR amplifications were performed with Q5 polymerase (NEB) according to supplier instructions. Plasmids carrying truncated versions of *eat1* genes were constructed by PCR-amplifying either pET26b-hKmaEat1 or pET26b-hWanEat1 with primers that excluded the appropriate part of the 5' sequence of the *eat1* gene. The reverse primer included the ATG codon and was phosphorylated at the 5' end. The linear PCR product was circularized using T4 ligase (Thermo Scientific) according to manufacturer instructions.

TABLE 1: Strains used in this study.

Strain	Characteristics	Source
Escherichia coli BW25113 (DE3)	Wild type with integrated DE3 lysogen	(Vuoristo *et al.*, 2015)
Escherichia coli BW25113 *ΔldhAΔackA*	Disruption of lactate and acetate production (via *ackA*)	This study
Escherichia coli T7 Express	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS *λ DE3* = *λ* sBamHIo ΔEcoRI-B int::(LacI::PlacUV5::T7 gene1) i21 Δnin5	NEB
Escherichia coli NEB® 5-alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB

TABLE 2: Plasmids used in this study.

Plasmid	Promoter	Gene/Protein	Source
pET26b	LacI/*T7*	/	This study
pET26b:hWanEat1	LacI/*T7*	Codon harmonized *eat1* from *Wickerhamomyces anomalus* DSM 6766	(Kruis *et al.*, 2017)
pET26b:hKmaEat1	LacI/*T7*	Codon harmonized *eat1* from *Kluyveromyces marxianus* DSM 5422	This study
pET26b:hKma trEat1A-4	LacI/*T7*	Kma Eat1 truncated at A-4	This study
pET26b:hKma trEat1 P-9	LacI/*T7*	Kma Eat1 truncated at P-9	This study
pET26b:hKma trEat1 T-15	LacI/*T7*	Kma Eat1 truncated at T-15	This study
pET26b:hKma trEat1 Y-19	LacI/*T7*	Kma Eat1 truncated at Y-19	This study
pET26b:hKma trEat1 S-20	LacI/*T7*	Kma Eat1 truncated at S-20	This study
pET26b:hKma trEat1 R-24	LacI/*T7*	Kma Eat1 truncated at R-24	This study
pET26b:hKma trEat1 F-26	LacI/*T7*	Kma Eat1 truncated at F-26	This study
pET26b:hKma trEat1 N-27	LacI/*T7*	Kma Eat1 truncated at N-27	This study
pET26b:hKma trEat1 Q-28	LacI/*T7*	Kma Eat1 truncated at Q-28	This study
pET26b:hKma-trEat1-K30	LacI/*T7*	Kma Eat1 truncated at K-30	This study
pET26b:hKma-trEat1-P34	LacI/*T7*	Kma Eat1 truncated at P-34	This study
pET26b:hKma-trEat1-L35	LacI/*T7*	Kma Eat1 truncated at L-35	This study
pET26b:hKma-trEat1-P36	LacI/*T7*	Kma Eat1 truncated at P-36	This study
pET26b:hKma-trEat1-I37	LacI/*T7*	Kma Eat1 truncated at I-37	This study
pET26b:hWan-trEat1-V11	LacI/*T7*	Wan Eat1 truncated at V-11	This study
pET26b:hWan-trEat1-N13	LacI/*T7*	Wan Eat1 truncated at N-13	This study
pCas9	/		(Jiang *et al.*, 2015)
pTarget	/		(Jiang *et al.*, 2015)
pTarget-*ackA*	/		This study
pTarget-*ldhA*	/		This study

Cultivation

E. coli strains were routinely grown on LB medium supplemented with kanamycin (50 µg/mL) or spectinomycin (50 µg/mL). Anaerobic experiments were performed in 250-mL serum bottles containing 50 mL modified M9 medium, which contained M9 salts (Difco, 1X), glucose (55 mM), $MgSO_4$ (2 mM), $CaCl_2$ * 2 H_2O (0.1 mM), MOPS (100 mM) and 1 mL 1000X trace elements and vitamins each according to Verduyn et al, 1992. The serum bottles were made anaerobic by flushing with nitrogen gas. Precultures were made by transferring single colonies to 10 mL LB medium in a 50-mL tube and grown overnight at 30 °C and 250 rpm. The next day, 1-2 mL of the LB culture was transferred to 50 mL modified M9 medium in a 250-mL Erlenmeyer flask. The culture was grown overnight aerobically at 30 °C and 250 rpm. The anaerobic serum bottles were inoculated to an initial OD of 0.2 and incubated at 30 °C and 150 rpm. When appropriate, isopropyl β-D-1-thiogalactopyranoside - IPTG (0.01-0.5 mM) was added to induce gene expression. Experiments were performed as biological duplicates. Ethyl acetate production in serum bottles was measured only in the liquid phase.

Visualization of cell-free extracts by SDS-PAGE analysis

Cell lysates were prepared from frozen culture samples using B-PER (Thermo Scientific) in combination with DNase I and Lysozyme according to the manufacturer's protocol. The soluble and insoluble fractions were separated, and the latter was washed once with 1 mL of a 10X diluted B-PER – 20 mM TrisHCl (pH 7.5) solution, and one time with 20 mM Tris-HCl (pH 7.5). The pellet was then resuspended in 20 mM TrisHCl (pH 7.5) in a similar volume as the soluble fraction. Total protein concentrations were estimated using the DC Protein Assay (BIO RAD) according to the supplier's manual. The soluble and insoluble fraction were diluted to equal concentrations using 20 mM TrisHCl buffer (pH 7.5) and treated with 1X Laemmli buffer (1 % SDS, 10 % glycerol, 0.01 % bromophenol blue, 250 mM Tris-HCl, pH 6.8, 1 % beta-mercaptoethanol) for 5 min at 95 °C. A total amount of 20 µg protein sample was loaded on a BIO-RAD Criterion TGX Stain-Free Gel (4-15 %), run at 200 V and stained with Bio-Safe Coomassie G-250 Stain (BIO RAD) according to manufacturer instructions.

Protein purification

The *K. marxianus* Eat1 and its variants were purified by Strep-tag purification. *E. coli* cultures were grown aerobically in 250-mL Erlenmeyer flasks containing 100 mL modified M9 minimal medium supplemented with 100 µg/mL kanamycin. The flasks were inoculated to a starting OD_{600} of 0.05 from an overnight LB pre-culture and incubated at 25 °C and 250 rpm. After 3 h of growth, IPTG was added to a final concentration of 0.05 mM. Cultures were harvested by centrifugation at 4500 x *g* and 4 °C. The pellets were resuspended in

1 mL BufferW (Strep-Tactin® XT Spin Column Kit, IBA Life Sciences). The cell suspension was transferred to a tube containing Lysing matrix E (MP Biomedicals) and lysed by beat-beating for 30 s at 6500 rpm using a FastPrep®-24 apparatus (MP Biomedicals). The lysed cells were centrifuged for 10 min at 20000 *x g.* The supernatant was transferred to an Eppendorf tube and re-centrifuged for 10 min at 20000 *x g*. The resulting supernatant was used for protein purification. All further purification steps were performed in accordance with the Strep-Tactin® XT Spin Column Purification Kit high protein yield protocol (IBA Life Sciences). The eluent containing purified protein was transferred to a Vivaspin® 500 10,000 MWCO PES column (Sartorius) and concentrated by centrifugation at 15000 x *g* for 10-15 min. Protein concentration was determined with the Micro-Lowry (Onishi & Barr modification) total protein kit (Sigma-Aldrich) according to supplier instructions. A calibration curve using bovine serum albumin (BSA) was used to determine protein concentration. The *W. anomalus* Eat1 protein was purified by 6X His-tag purification as described previously (Kruis *et al.*, 2017).

Enzyme assays

The hydrolysis of 1-naphthyl acetate (1-NA) was measured spectrophotometrically by monitoring the release of 1-naphthol at 320 nm (He, 2003) in a Synergy MX temperature regulated plate reader (BioTek) at the desired temperature. Assays were performed in 96-well flat-bottom plates (Greiner) in a final volume of 100 µL. The well contained sodium phosphate buffer (50 mM, pH 7.5), NaCl (100 mM) and 1-naphthyl acetate (0.5 mM). The reaction was initiated by adding purified protein to a final concentration of 10 µg/mL. Residual esterase activity assays were performed by incubating the enzymes in sodium phosphate buffer (50 mM, pH 7.5) containing NaCl (100 mM) in a PCR thermocycler at the desired temperature. Aliquots were taken at various incubation times (0-90 min) and assayed for 1-naphthyl acetate hydrolysis at 40 °C. A calibration curve was used to calculate the concentration of 1-naphthol released in the reaction. Specific activity was defined as the amount of protein (mg) required to form 1 µmol of 1-naphthol, per min. Measurements were performed as technical triplicates.

Bioinformatics

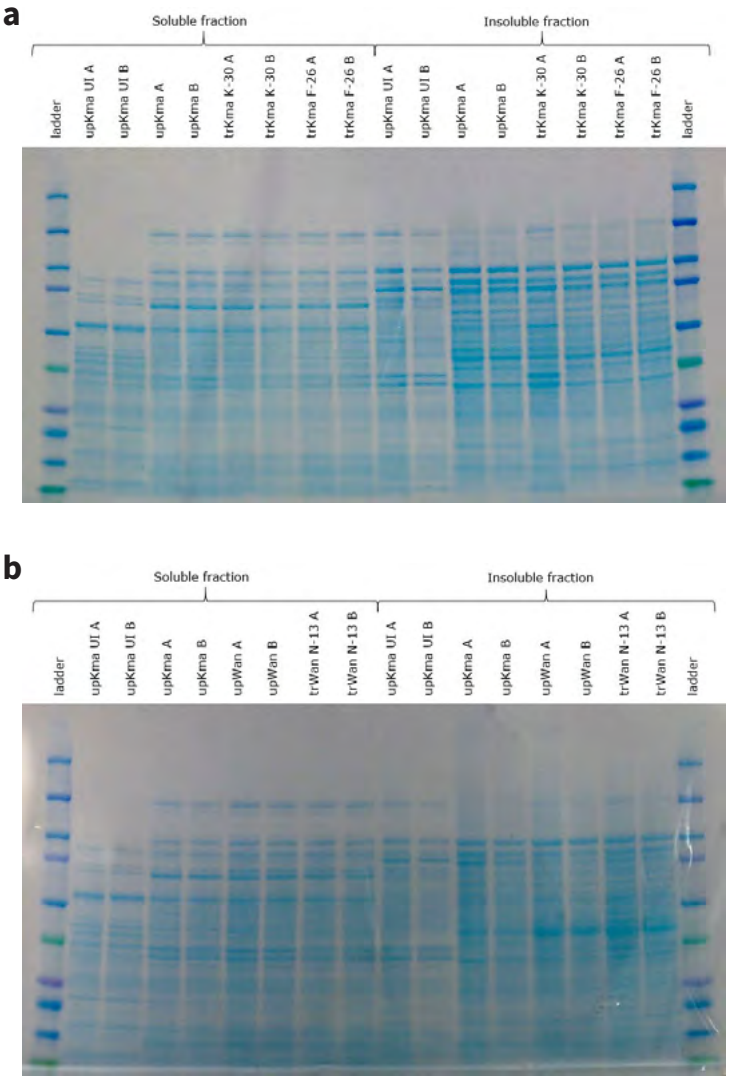
Mitochondrial pre-sequences and amino acid (AA) positions of typical cleavage sites were identified using the prediction tool MitoFates (Fukasawa *et al.*, 2015). The translation initiation rates of ribosome binding sites were predicted with the RBS Calculator v2.1. The predictions were performed for *E. coli* MG1655 (ACCTCCTTA).

Analytical

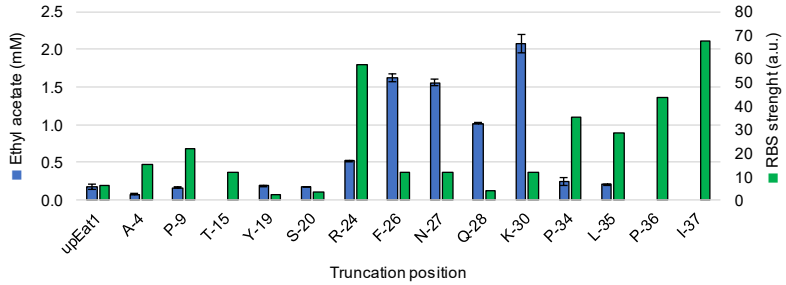
Glucose and organic acids were analyzed by high performance liquid chromatography (HPLC) on an Agilent 1290 LC II system, equipped with an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity Autosampler, Agilent 1290 Infinity diode array detector operated at 210 nm, and an Agilent 1260 Infinity RI detector operated at 45 °C. Either an Aminex HPX-87H (Bio-Rad) or an Rezex ROA-Organic Acid H+ (Phenomenex) column were used with a mobile phase of 0.008 mM H_2SO_4. The HPLC was operated at 0.8 mL/min and 60 °C. Propionic acid (50 mM) was used as internal standard.

Ethyl acetate and ethanol in liquid samples were measured by an Agilent 7890B gas chromatograph equipped with a flame ionization detector (GC-FID) and an Agilent 7693 autosampler. Samples were analyzed by injecting 0.5 µL of liquid sample onto a Nukol™ column (30 m x 0.53 mm, 1.0 µm coating, Supelco). The column temperature was maintained at 50 °C for 2 min and increased to 200 °C at a rate of 50 °C/min. The split ratio was 10. 1-Butanol (2 mM) was used as internal standard.

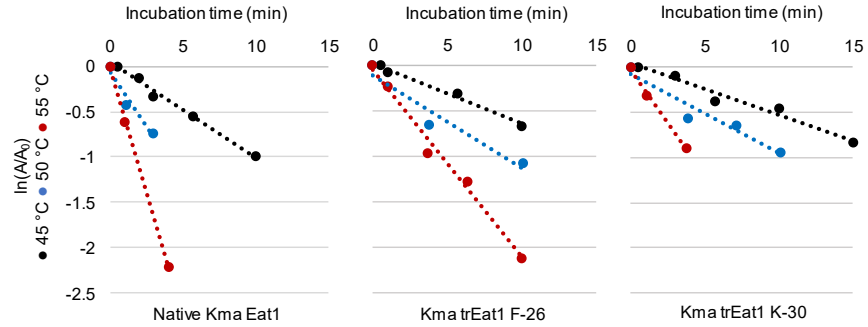
Supplementary Material



SUPPLEMENTARY FIGURE 1: SDS Page analysis of CFE of *E. coli* cultures producing various Eat1 variants. Soluble and insoluble fractions of CFE were prepared from cultures induced with 0.01 mM IPTG after 70 h of anaerobic cultivation. Two biological replicates (A and B) of each culture were analyzed. Uninduced cultures were used as a control. The Precision Plus Protein Kaleidoscope Standard (BIO RAD) was added in lanes 1 and 18. Kma Eat1 variants are shown in (a) and Wan Eat1 variants are shown in (b). The Eat1 proteins are expected at a band size of approximately 42 kDa. Truncation of Eat1 had only a minor effect on the size of the protein, that was not detectable by SDS-PAGE.



SUPPLEMENTARY FIGURE 2: Lack of correlation between ethyl acetate formation and predicted strength of the RBS controlling the production of *K. marxianus* trEat1. Ethyl acetate titers were obtained from *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing *K. marxianus* trEat1 variants at 0.01 mM IPTG concentration (Figure 2b). The translation initiation rates of the RBS were predicted with the RBS calculator (Salis, Mirsky and Voigt, 2010).



SUPPLEMENTARY FIGURE 3: Thermal inactivation measurements used to determine the inactivation constants (k_i) of three *K. marxianus* Eat1 variants at 45 °C, 50 °C and 55 °C.

Chapter 4

Multilevel optimization of anaerobic ethyl acetate production in engineered *Escherichia coli*

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Abstract

Ethyl acetate is a widely used industrial solvent that is currently produced by chemical conversions from fossil resources. Several yeast species are able to convert sugars to ethyl acetate under aerobic conditions. However, performing ethyl acetate synthesis anaerobically may result in enhanced production efficiency, making the process economically more viable.

We engineered an *E. coli* strain that is able to convert glucose to ethyl acetate as the main fermentation product under anaerobic conditions. The key enzyme of the pathway is an alcohol acetyltransferase (AAT) that catalyzes the formation of ethyl acetate from acetyl-CoA and ethanol. To select a suitable AAT, the ethyl acetate-forming capacities of Atf1 from *Saccharomyces cerevisiae,* Eat1 from *Kluyveromyces marxianus* and Eat1 from *Wickerhamomyces anomalus* were compared. Heterologous expression of the AAT-encoding genes under control of the inducible LacI/*T7* and XylS/*Pm* promoters allowed optimization of their expression levels.

Engineering efforts on protein and fermentation level resulted in an *E. coli* strain that anaerobically produced 42.8 mM (3.8 g/L) ethyl acetate from glucose with an unprecedented efficiency, i.e. 0.48 C-mol/C-mol or 72 % of the maximum pathway yield.

Keywords: Eat1, anaerobic, alcohol acetyl transferase (AAT), *Escherichia coli*, ethyl acetate, bioreactor, fermentation.

Background

Ethyl acetate is used on a large scale as an industrial solvent for the production of paints, coatings and resins (Löser, Urit and Bley, 2014), as well as in the flavors and fragrances industry (Dzialo et al., 2017; Kruis et al., 2019). The global production of the ester was estimated at 3.5 million tons in 2015 (The Market Publishers, 2014). Currently, ethyl acetate is produced from petrochemicals in energy intensive and unsustainable processes. Traditional Fischer Speier esterification makes use of equilibrium reactions and energy used for elevated temperatures and continuous water removal are adding to the costs (Fischer and Speier, 1895; Liu, Lotero and Goodwin, 2006; Jyoti *et al.*, 2018). A sustainable alternative is the use of biobased processes in which yeasts produce ethyl acetate from sugars or ethanol at high yields (Armstrong, Martin and Yamazaki, 1984; Fredlund, Blank, *et al.*, 2004; Löser *et al.*, 2012). The most prominent and well-studied yeast is *Kluyveromyces marxianus,* which produces ethyl acetate from whey sugars at more than 50 % of the maximum pathway yield of 1 $mol_{ethyl\ acetate}/mol_{glucose}$ (Löser *et al.*, 2013; Urit, Li, *et al.*, 2013). Other examples include *Wickerhamomyces anomalus* and *Kluyveromyces lactis* (Kruis *et al.*, 2017).

Ethyl acetate production in yeast is catalyzed by alcohol acetyltransferases (AATs), which synthesize ethyl acetate from acetyl-CoA and ethanol, releasing free CoA in the reaction (Kruis et al., 2019). The first described ethyl acetate-producing AAT was the *Saccharomyces cerevisiae* Alcohol acetyltransferase 1 (Atf1) (Minetoki *et al.*, 1993). However, its homologs in *W. anomalus* and *K. marxianus* appeared to have only a minor role in bulk ethyl acetate production (Kruis *et al.*, 2017; Löbs *et al.*, 2017). Instead, they use the recently identified Ethanol acetyltransferase 1 (Eat1) to produce ethyl acetate (Kruis *et al.*, 2017). Eat1 is targeted to the mitochondria of yeasts due to the presence of an N-terminal mitochondrial pre-sequence (Kruis, Mars, *et al.*, 2018). In yeasts these pre-sequences are cleaved upon arrival in the mitochondrion by cleavage proteins such as mitochondrial processing peptidase, Oct1 or Icp55 peptidases (Vögtle *et al.*, 2011; Mossmann, Meisinger and Vögtle, 2012). Only after all processing events have occurred the proteins fold into their mature and fully functional form. It has recently been shown that the stability of Eat1, when expressed in *E. coli,* can be improved by using N-terminally truncated versions without significantly affecting the specific activity *in vitro* (Kruis *et al.*, 2020). An undesirable characteristic of yeast AATs is that they also exhibit thioesterase or esterase side activities, which implies that it is able to hydrolyze acetyl-CoA – the substrate for ethyl acetate production – but also ethyl acetate itself, respectively (Figure 1). In Eat1, however, these side activities could be subdued by sufficiently high levels of ethanol (Kruis *et al.*, 2017; Nancolas *et al.*, 2017).

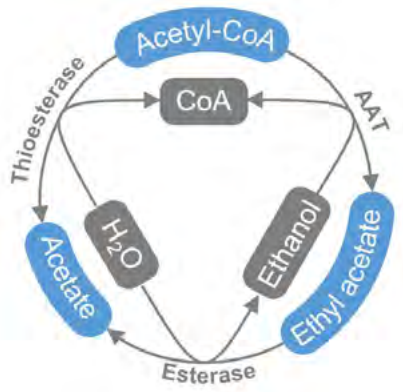

FIGURE 1: Schematic representation of the three catalytic activities of the Eat1 enzyme for the case ethyl acetate. The AAT activity of Eat1 catalyzes the condensation of acetyl CoA and ethanol into ethyl acetate, ethyl acetate is degraded into ethanol and water as consequence of esterase activity, and acetyl CoA is converted into acetate, CoA and water exhibiting thioesterase activity.

The production of ethyl acetate from glucose results in an NADH surplus (Davies *et al.*, 1951; Peel, 1951; Löser, Urit and Bley, 2014; Löser, Urit, Keil, *et al.*, 2015). Yeasts are only able to dispose of this surplus by respiration, rendering ethyl acetate production an aerobic process (Figure 2a). Under these conditions, a significant part of glucose is oxidized in the TCA cycle, leading to lower product yields (Weusthuis *et al.*, 2011). Moreover, large scale aerobic cultivations are often rate limited by the oxygen transfer rate, due to the low solubility of oxygen (Garcia-Ochoa and Gomez, 2009). Another commonly observed problem in yeast cultivation, particularly in *S. cerevisiae,* is the Crabtree effect, the undesired production of ethanol, consequently lowering the yield on the desired product (van Dijken, Weusthuis and Pronk, 1993; Löser *et al.*, 2013). *E. coli* and other bacteria can avoid this redox imbalance anaerobically by using pyruvate formate lyase (Pfl). Instead of forming NADH, the excess redox equivalents are secreted as formate. In the overall pathway, 1 mol glucose is converted via the EMP pathway to 2 mol pyruvate and 2 mol NADH. Pyruvate is then converted to 2 mol acetyl-CoA and 2 mol formate by Pfl. To maintain the cellular redox balance, the 2 mol NADH produced in glycolysis are regenerated by converting 1 mol acetyl-CoA to ethanol via the bifunctional alcohol/aldehyde dehydrogenase (Adh). A heterologous AAT then condenses the remaining acetyl-CoA with ethanol to form ethyl acetate. This allows redox-neutral production of 0.67 $C\text{-}mol_{ethyl\ acetate}/C\text{-}mol_{glucose}$ under anaerobic conditions (Figure 2b). The only by-product of the pathway is formate which can be further converted to CO_2 and hydrogen by formate-hydrogen lyase (Fhl). The latter can also be considered a valuable product that has potential as a biofuel (Stephen *et al.*, 2017). The resulting anaerobic process requires less energy for cooling and lower stirring rates due to the absence of transfer limitations, and can be upscaled to larger reactor volumes (Blanch, 2012).

A critical step in enabling heterologous ethyl acetate production in *E. coli* is the selection of an efficient AAT catalyst. Both Atf1 and Eat1 have been used to increase ethyl acetate production (Rodriguez, Tashiro and Atsumi, 2014; Kruis *et al.*, 2017). AATs from fruit have

also been used to evoke ethyl acetate synthesis in *E. coli* under anaerobic conditions and show various affinities for a range of esters (Layton and Trinh, 2016a; Lee and Trinh, 2018, 2019). However, these enzymes have not yet been compared in the same metabolic background.

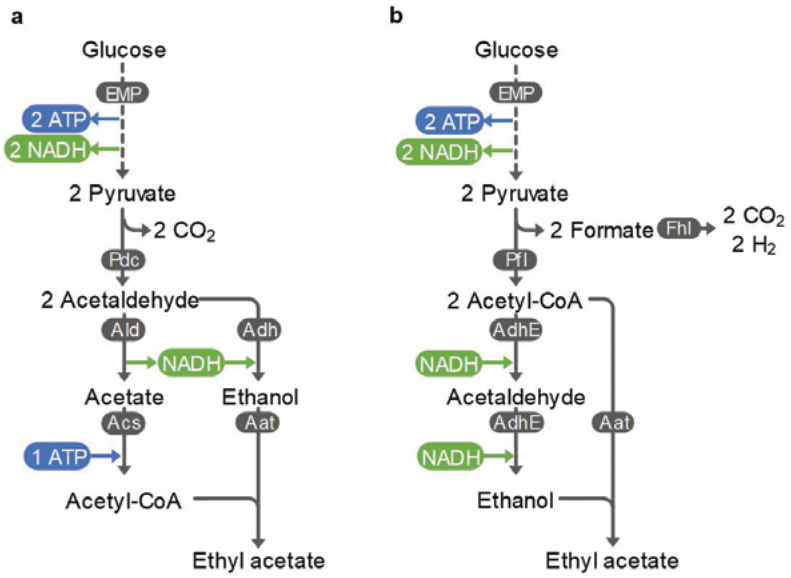

FIGURE 2: Two anaerobic variants of ethyl acetate production from glucose via the Embden-Meyerhof-Parnas (EMP) pathway. An alcohol acetyltransferase (AAT) catalyzes the production of ethyl acetate from acetyl-CoA and ethanol. a – Ethyl acetate production in yeast. Acetyl-CoA is formed in a series of three reactions: pyruvate decarboxylase (Pdc), acetaldehyde dehydrogenase (Ald) and acetyl-CoA synthetase (Acs). Ethanol is formed from acetaldehyde by an alcohol dehydrogenase (Adh). CO_2 is produced as a by-product. b – Ethyl acetate production in bacteria *(e.g. E. coli*). Acetyl-CoA is formed directly from pyruvate via pyruvate formate lyase (Pfl). Ethanol is formed from acetyl-CoA via the bifunctional alcohol/aldehyde dehydrogenase (AdhE). Formate is produced as a by-product that can be converted to CO_2 and H_2 via formate-hydrogen lyase (Fhl).

In this study we optimized anaerobic production of ethyl acetate in *Escherichia coli.* We compared and evaluated ethyl acetate production by three AATs from different yeasts after reducing the formation of by-products by creating knock-out strains. We optimized gene expression levels by using two inducible promotors in combination with several inducer concentrations, and also used N-terminally truncated variants. Final experiments in 1.5-L pH-controlled bioreactors with continuous gas stripping resulted in ethyl acetate production at high yield.

Results

Anaerobic ethyl acetate production in *E. coli* and reduction of by-product formation

To enable ethyl acetate production in *E. coli* BW25113 (DE3), we introduced the *K. marxianus eat1* (Kma *eat1*) under the control of the IPTG-inducible LacI/*T7* promoter. Under anaerobic conditions the strain produced 2.7 ± 0.1 mM ethyl acetate, representing a yield of 0.03 ± 0.00 C-mol$_{ethyl\ acetate}$/C-mol$_{glucose}$ (Figure 3ac). Due to the formation of by-products, particularly lactate and acetate, the ethyl acetate titer was low. To maximize the metabolic flux towards ethyl acetate, we disrupted the acetate kinase (*ackA*) and lactate dehydrogenase (*ldhA*) genes to reduce acetate and lactate formation, respectively. This increased the ethyl acetate titer to 9.1 ± 0.3 mM (Figure 3b). The final ethyl acetate yield increased to 0.13 ± 0.00 C-mol$_{ethyl\ acetate}$/C-mol$_{glucose}$, or 21.4 % of the maximum pathway yield (Figure 3c). Lactate production was almost completely abolished. Acetate yields did not decrease significantly despite the *ackA* disruption (Figure 3c). A possible explanation is that acetate is produced via the hydrolysis of ethyl acetate or acetyl-CoA by the esterase and thioesterase side activity of eat1, respectively.

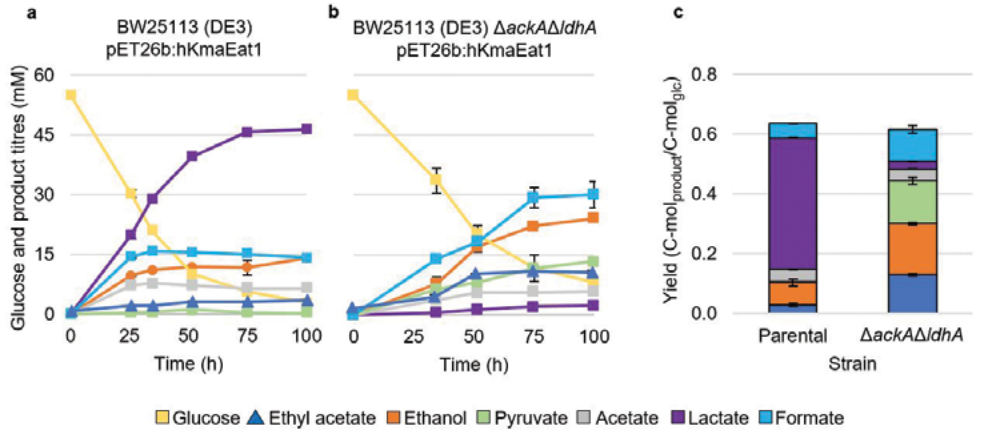

FIGURE 3: Anaerobic ethyl acetate production in *E. coli* BW25113 (DE3) and *E. coli* BW25113 *ΔldhAΔackA* expressing the Kma *eat1* from pET26b:hKmaEat1. a – Fermentation profile of *E. coli* BW25113 (DE3) (pET26b:hKmaEat1). b – Fermentation profile of *E. coli* BW25113 *ΔldhAΔackA* (DE3) (pET26b:hKmaEat1). c – Product yields during anaerobic ethyl acetate production. Experiments were performed as biological duplicates. Strains were grown in sealed and N_2 flushed serum bottles under anaerobic conditions in modified M9 medium at 30 °C and 150 rpm. Gene expression was induced with 0.05 mM IPTG. Error bars represent the standard deviation. Ethyl acetate concentration in the headspace, CO_2 and H_2 were not measured.

Since lactate production can no longer act as sink of NADH, ethanol synthesis should fulfil this role. The conversion of ethanol together with acetyl-CoA to ethyl acetate would basically consume all available NADH and make the entire process redox neutral. However,

the accumulation of ethanol and also pyruvate suggests that synthesis of ethyl acetate is limited and that Eat1 activity is the bottleneck of the process (Figure 3bc). We therefore focused on optimizing the activity of the AAT step.

Selection of ethyl acetate-producing AAT and gene expression optimization

We compared the ethyl acetate-production capacity of *S. cerevisiae atf1* (Sce *atf1*), Kma *eat1* and *W. anomalus eat1* (Wan *eat1*) genes in *E. coli* BW25113 Δ*ldhA*Δ*ackA* (DE3) cultivated in anaerobic serum bottles. The genes were placed under the control of the inducible LacI/*T7* (Figure 4abc) or XylS/*Pm* promoter (Figure 4def) to allow modulation of their expression levels. To induce gene expression, IPTG or m-toluate were added at various concentrations.

Increased AAT activities will reduce the accumulation of pyruvate, increase the production of ethyl acetate when it is active as an AAT, and increase the production of acetate when it is active as either an esterase or thioesterase.

Strains expressing Wan *eat1* (Figure 4be) showed the highest ethyl acetate yields compared to the other AAT genes controlled by the same promoter. High yields of ethyl acetate were also reached by strains expressing Kma *eat1* under control of the lac-*T7* promoter (Figure 4a). Surprisingly, strains expressing Kma *eat1* under the XylS/*Pm* promoter produced only traces of ethyl acetate under all induction levels (Figure 4d). Sce *atf1* also evoked ethyl acetate production, but the yields were significantly lower compared to the two *eat1* genes (Figure 4cf). Pyruvate accumulation decreased significantly, indicating that Atf1 was active, but primarily as an esterase/thioesterase as the acetate yields increased. These results show that Eat1 homologs are better catalysts than Sce Atf1 for *in vivo* ethyl acetate production in *E. coli* BW25113 *ΔldhAΔackA* (DE3) under the tested conditions.

The expression of Wan and Kma *eat1* under the control of the LacI/*T7* promoter resulted in 0.2 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$ or higher. However, Wan *eat1* required 10-fold less IPTG to reach the same or higher ethyl acetate yields than Kma *eat1* (Figure 4ab). Moreover, the strains expressing Wan *eat1* under the control of the XylS/*Pm* promotor produced up to 0.16 ± 0.01 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$ (Figure 4e), while Kma *eat1* produced almost no ethyl acetate (Figure 4d). This difference may be explained by the fact that the XylS/*Pm* promoter is weaker compared to the LacI/*T7* promoter (Balzer *et al.*, 2013). The higher yield obtained with lower gene expression levels indicates that Wan Eat1 was more active than its *K. marxianus* homolog under these cultivation conditions.

Selecting the best AAT gene and optimizing its expression level diminished the metabolic bottleneck present in ethyl acetate production, but pyruvate still accumulated (Figure 4a-f). This indicated that the conversion efficiency of Eat1 was still insufficient to handle the EMP metabolic pathway flux.

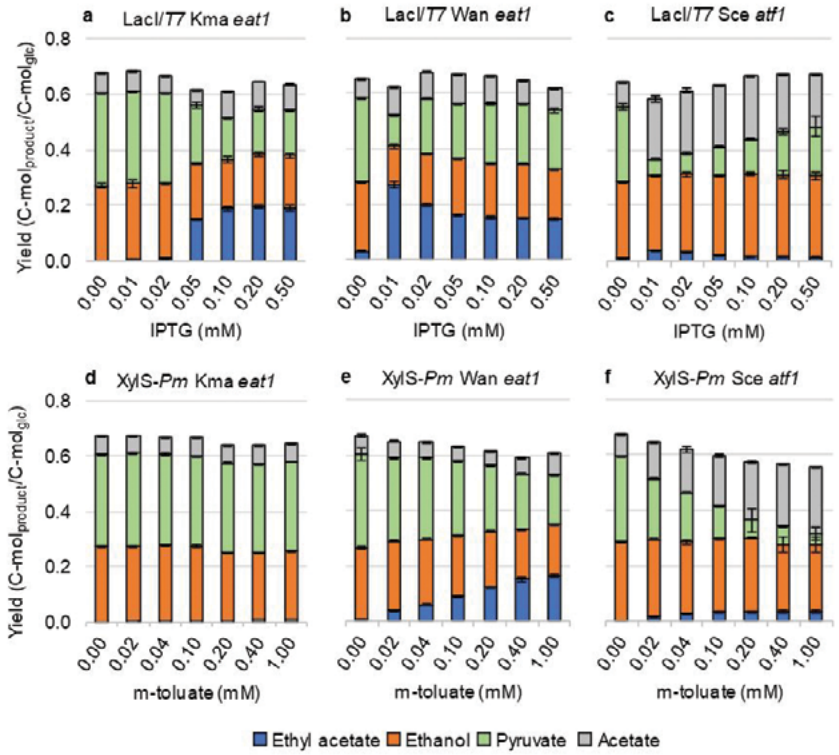

FIGURE 4: Comparison of three ethyl acetate producing AAT genes under various gene expression levels, induced by 0.01-0.5 mM IPTG or 0.02-1 mM m-toluate. (a, b, c) – Fermentation product yields of strains expressing the Kma *eat1,* the Wan *eat1* and the Sce *atf1*, respectively, under the control of the LacI/*T7* promoter after 120 h of cultivation. Gene expression was induced with 0.01-0.5 mM IPTG. (d, e, f) – Fermentation product yields of strains expressing the Kma *eat1,* the Wan *eat1* and the Sce *atf1*, respectively, under the control of the XylS/*Pm* promoter after 120 h of cultivation. Strains were grown in sealed and N_2 flushed serum bottles under anaerobic conditions in modified M9 medium at 30 °C and 150 rpm. Genes were expressed in *E. coli* BW25113 *ΔldhAΔackA* (DE3) from a series of pET26b plasmids. Succinate and formate were detected but are not shown. Experiments were performed as biological duplicates; error bars represent the standard deviation.

The ethyl acetate yields increased with rising inducer concentrations (Figure 4ef), reached a plateau (Figure 4a) and even began to decline at higher inducer concentration (Figure 4bc). Determining the optimal inducer concentrations thus resulted in significantly improved ethyl acetate yields. For example, optimized IPTG concentrations used for gene induction in *E. coli* BW25113 Δ*ldhA*Δ*ackA* (DE3) (pET26b:hKma Eat1) led to an increase of the ethyl acetate yield from 0.13 ± 0.00 (Figure 3c) to 0.19 ± 0.00 C-mol$_{ethyl\ acetate}$/C-mol$_{glucose}$ (Figure 4a). The highest ethyl acetate yield was achieved in *E. coli* BW25113 Δ*ldhA*Δ*ackA* (DE3) (pET26b:hWan Eat1) that was induced with 0.01 mM IPTG. It produced 0.27 ± 0.01 C-mol$_{ethyl\ acetate}$/C-mol$_{glucose}$ or 40.7 % of the theoretical pathway maximum (Figure 4b).

Using truncated Eat1 variants

Removal of the mitochondrial pre-sequences of *K. marxianus* and *W. anomalus* Eat1 resulted in a higher stability of the enzyme when expressed in *E. coli* (Kruis *et al.*, 2020). We tested if this elevated stability also led to more ethyl acetate production. Optimization of gene expression levels for Kma trEat1 F-26 and K-30 resulted in a lower accumulation of pyruvate compared to the unprocessed version (Figure 5abc), suggesting a higher efficiency of the truncated Eat1 variants. However, this did not result in a higher ethyl acetate yield; only the acetate yield increased.

Nevertheless, the optimum inducer concentration shifted to 0.05 mM IPTG, which was 50 % lower compared to the native Eat1. Ethyl acetate production was also higher at 0.01 and 0.02 mM IPTG, indicating that the *in vivo* production capacity of ethyl acetate improved. At the same time, the acetate yields increased for induction levels above 0.05 mM IPTG, while the pyruvate yields decreased (Figure 5bc).

As discussed above, a limiting Eat1 efficiency resulted in the accumulation of ethanol. Pyruvate was produced by *E. coli* BW25113 *ΔldhAΔackA* (DE3) to counter the redox imbalance caused by ethanol accumulation. The higher stability of the truncated Eat1 versions did indeed result in a decreased pyruvate yield (Figure 5bcef) but the ethyl acetate yield did not increase accordingly. Instead, the acetate yield increased, likely due to the esterase and thioesterase side activities of Eat1.

At the lowest IPTG concentration, the strains producing the truncated Wan Eat1 (Wan trEat1 N-13) reached a 3.5-fold higher ethyl acetate yield on glucose than the unprocessed Wan Eat1 (Wan Eat1) (Figure 5ef). However, at higher IPTG concentrations these differences were absent. The acetate yield in the strain producing Wan trEat1 N-13 also increased relative to the strain producing Wan Eat1. (Figure 5df). The increase in acetate production was not as pronounced as with the Kma trEat1 F-26 and K-30 (Figure 5abc). No difference was found between the Wan trEat1 V-11 and the unprocessed Wan Eat1 (Figure 5de).

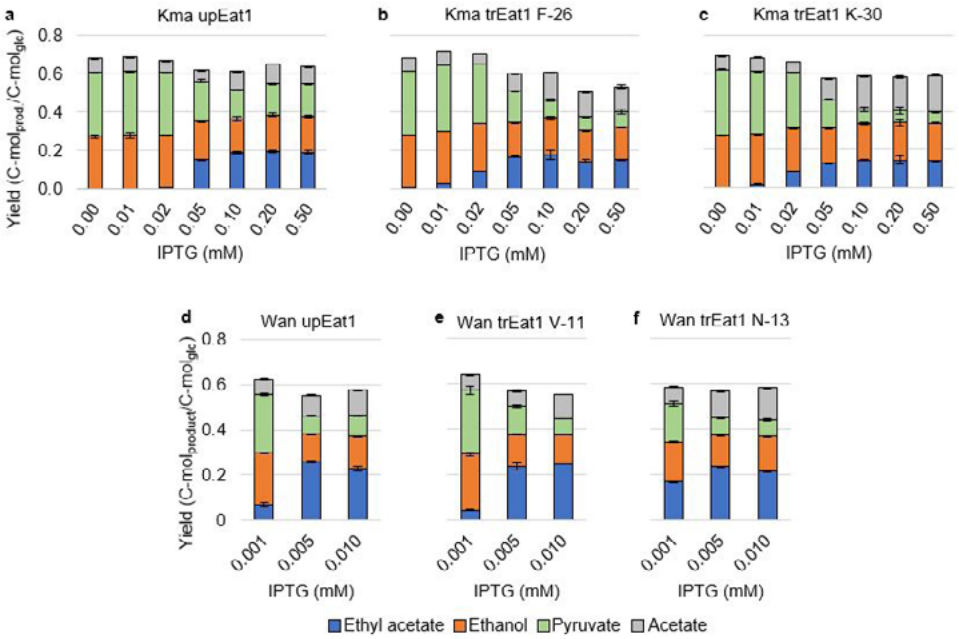

FIGURE 5: Comparison of truncated eat1 genes under various gene expression levels Induced by 0.01-0.5 mM IPTG or 0.001-0.01 mM IPTG. (a, b, c) – Fermentation product yields of strains expressing the Kma *Eat1,* the Kma trEat1 F-26 and the Kma trEat1 K-30, respectively, under the control of the LacI/*T7* promoter after 120 h of cultivation. Gene expression was induced with 0.01-0.5 mM IPTG. (d, e, f) – Fermentation product yields of strains expressing the Wan Eat1*,* the Wan *trEat1 V-11* and the Wan trEat1 N-13, respectively, under the control of the LacI/*T7* promoter after 120 h of cultivation. Gene expression was induced with 0.001-0.01 mM IPTG. Strains were grown in sealed and N_2 flushed serum bottles under anaerobic conditions in modified M9 medium at 30 °C and 150 rpm. Genes were expressed in *E. coli* BW25113 *ΔldhAΔackA* (DE3) from a series of pET26b plasmids. Succinate and formate were detected but concentrations are not shown. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: Kma – *K. marxianus*, Wan – *W. anomalus,* Eat1 – unprocessed Eat1, trEat1 – truncated Eat1

Improving ethyl acetate production with H_2 co-production in controlled bioreactors

In all serum bottle experiments described above, glucose consumption was incomplete, most likely caused by the accumulation of organic acids, especially formate, and the associated pH decrease due to a limited buffering capacity of the medium. To avoid limitations caused by medium acidification, additional cultivations were performed in pH-controlled reactors under anaerobic conditions. To limit the accumulation of formate even further, Na_2SeO_3 was added to stimulate the conversion of formate into H_2 and CO_2 by Fhl. A constant flow of nitrogen gas was applied to keep the culture conditions anoxic. This resulted in stripping of ethyl acetate, H_2 and CO_2 from the broth and the concentrations of these compounds in the exhaust gas were therefore analyzed.

We cultivated *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing several Eat1 variants. Gene expression was induced with the optimal IPTG concentration of each strain based on the findings of previous experiments (Figures 4, 5). In contrast to the shake flask experiments, glucose was fully consumed at the end of the batch fermentations and ethyl acetate production proceeded until glucose was depleted (Figure 6ab). Formate was converted into CO_2 and H_2 by *E. coli* BW25113 *ΔldhAΔackA* (DE3), but conversion percentages were inconsistent and the conversion was incomplete (Figure 6cd). Strains converted between 6 % and 27 % of the formate into CO_2 and H_2 while in most fermentations the conversion averaged around 10 %. There was no correlation between the conversion efficiency, the strain, or reactor vessel. Between 93.0 and 103.7 % of the carbon was recovered in all runs performed when biomass formation and the main fermentation products ethyl acetate, ethanol, acetate, pyruvate, formate, succinate and CO_2 were included (Figure 7ab).

Consistently, all strains cultivated in pH-controlled bioreactors showed improved performance compared to the serum bottle cultivations. Once the unprocessed Kma Eat1 was induced with an optimal 0.1 mM IPTG, a beneficial effect on ethyl acetate yield was apparent. A yield of 0.35± 0.01 C-mol$_{ethyl\ acetate}$/C-mol$_{glucose}$ corresponded to a 1.8-fold increase when compared to a serum bottle yield of 0.19 ± 0.01 C-mol$_{ethyl\ acetate}$/C-mol$_{glucose}$, reaching about 50 % of the maximum pathway yield. A similar yield was obtained in strains producing Kma trEat1 K-30 in the presence of 0.05 mM IPTG (Figure 7a).

The best producers tested in pH-controlled bioreactors were *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing Kma trEat1 K-30 and Wan trEat N-13. They formed 27.6 ± 3.7 mM (2.4 ± 0.3 g/L) and 42.8 ± 3.3 mM (3.8 ± 0.3 g/L) ethyl acetate from 55.6 ± 2.5 mM (10.0 ± 0.5 g/L) glucose, respectively (Figure 6, Supplementary Table). Generally, ethyl acetate yields were between 1.6- to 2.8-fold higher in bioreactors compared to serum bottles. The highest yield was obtained by the strain producing Wan trEat1 N-13, reaching 0.48 ± 0.03 C-mol$_{ethyl\ acetate}$/C-mol$_{glucose}$, or 72.3 % of the maximum pathway yield.

The yields of ethanol and pyruvate decreased with increasing ethyl acetate yields (Figure 7ab). Strains producing the unprocessed Kma Eat1 and Kma trEat1 K-30 in the presence of optimal IPTG concentrations accumulated 66 % less pyruvate (Figure 7a) compared to cultivations in serum bottles (Figure 5 ac). For the strains producing unprocessed Wan Eat1 and Wan trEat1 N-13 pyruvate accumulation was almost entirely abolished (Figures 6b, 7b). The ethyl acetate yield for Wan Eat1 was consequently higher compared to the strains producing the Kma Eat1 variants. It should be noted that a statistically significant difference in ethyl acetate yields (p-value = 0.03) was only found for the strain producing Wan trEat1 N-13 (Figure 7ab). This strain converted approximately 72 % of glucose to ethyl acetate based on the maximum pathway yield.

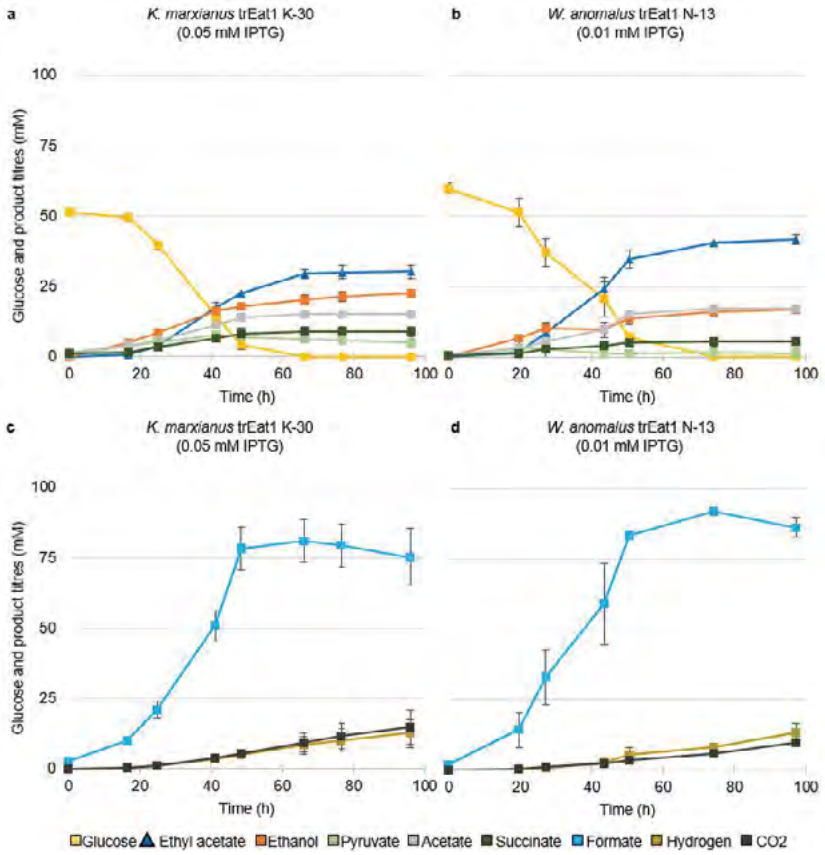

FIGURE 6: Ethyl acetate production in pH-controlled bioreactors with continuous gas stripping. Two examples of controlled batch fermentations are shown. (a,c) – Fermentation profile of the strain producing Kma trEat1 K-30 in the presence of 0.05 mM IPTG. (b,d) Fermentation profile of the strain producing Wan trEat1 N-13 in the presence of 0.01 mM IPTG. Strains were grown under anaerobic conditions in minimal medium containing 55 mM glucose. Genes were expressed in *E. coli* BW25113 *ΔldhAΔackA* (DE3) from a series of pET26b plasmids. The cumulative mass of ethyl acetate, CO_2 and H_2 removed by gas stripping was divided by the culture volume of the reactor and in case of ethyl acetate added to concentrations measured in the liquid. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: trEat1 – truncated Eat1.

Not only did the trEat1 variants require lower induction levels and accumulated less by-products, glucose was also depleted faster. As a result, the volumetric productivity of ethyl acetate (Q_{EA}) was higher. The Q_{EA} of the strain producing Kma trEat K-30 (0.05 mM IPTG) was 35 % higher (p-value = 0.013) compared to the strain producing unprocessed Kma Eat1 (0.1 mM IPTG) (Figure 7c). A similar trend was present in *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing unprocessed Wan Eat1 and trEat1 N-13 in the presence of 0.01 mM IPTG. The Q_{EA} of the latter strain was 26 % higher (p-value = 0.042) compared to the strain producing the unprocessed Wan Eat1 (Figure 7d).

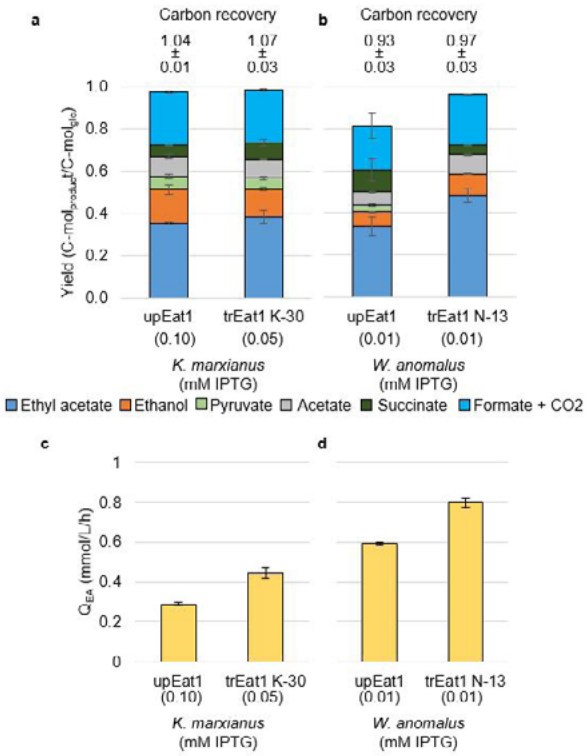

FIGURE 7: Effect of pH-control and continuous ethyl acetate stripping on product yield and volumetric productivity. (a) – Final product yields achieved by cultures producing unprocessed Kma Eat1 and trEat1 K-30 in the presence of 0.05 or 0.1 mM IPTG. The numbers above the bars represent the carbon recovery of the fermentations. (b) – Final product yields achieved by cultures producing unprocessed Wan Eat1 and trEat1 N-13 in the presence of 0.01 mM IPTG. The numbers above the bars represent the carbon recovery of the fermentations. (c, d) – The volumetric productivity of ethyl acetate (Q_{EA}) of the fermentation shown in (a) and (b), respectively. Strains were grown under anaerobic conditions in minimal medium containing 55 mM glucose. Genes were expressed in *E. coli* BW25113 *ΔldhAΔackA* (DE3) from a series of pET26b plasmids. The cumulative mass of ethyl acetate, CO_2 and H_2 removed by gas stripping was divided by the culture volume of the reactor and in case of ethyl acetate added to concentrations measured in the liquid. Formate and CO_2 yields were lumped together to compensate for the variation in H_2 formation. Experiments were performed as biological duplicates or triplicates; error bars represent the standard deviation. Abbreviations: Eat1 – unprocessed Eat1, trEat1 – truncated Eat1.

The hydrolysis of ethyl acetate by the side activity of Eat1 might be restricted by efficiently removing all ethyl acetate by gas stripping. But due to low gas flow rates ethyl acetate still accumulated in the liquid during the fermentation. At times of maximum productivities, liquid ethyl acetate concentrations ranged from 2.87 ± 0.1 mM for Kma Eat1 with 0.05 mM IPTG induction to up to 14.7 ± 0.4 mM for Wan trEat1 N-13 with 0.01 mM IPTG induction (data not shown).

Discussion

We describe the engineering of *E. coli* for the anaerobic production of ethyl acetate and the different optimization efforts to further improve the product yield. In all cultivations of the metabolically streamlined *E. coli* BW25113 ΔldhAΔackA (DE3) (pET26b:hKmaEat1) substantial amounts of ethanol and pyruvate were formed, in addition to ethyl acetate. This redox-neutral accumulation of pyruvate and ethanol indicated that the *in vivo* activity of Eat1 was insufficient to cope with the supply of acetyl-CoA and ethanol.

Screening of *E. coli* strains expressing three different AATs revealed that their capacity to produce ethyl acetate under anaerobic conditions varied significantly. The expression of Sce *atf1* evoked acetate production, which may be related to its thioesterase activity (Nancolas *et al.*, 2017). Alternatively, Atf1 may act as an esterase, but this has not been determined. It is unknown whether ethanol inhibits the hydrolytic activity of Sce Atf1 in the same way as was demonstrated for Eat1 (Kruis *et al.*, 2017). It was observed before that Atf1 exhibits low affinity for the catalysis of ethyl acetate despite external ethanol addition (Horton and Bennett, 2006). On the other hand, Sce Atf1 enabled isobutyl acetate production at 80 % of the pathway maximum (Rodriguez, Tashiro and Atsumi, 2014), which indicates that it can be an effective AAT in *E. coli*. Thus, the inefficient ethyl acetate production by Sce Atf1 may have been caused by differences in substrate specificity or fermentation conditions. However, Atf1 in *S. cerevisiae* is most active under anaerobic conditions due to higher gene expression (Fujiwara *et al.*, 1999), suggesting that anoxic conditions should not be a bottleneck in Atf1 activity. Nevertheless, the results of this study, disqualified it as catalyst for effective ethyl acetate production under the tested conditions.

Next to plasmid maintenance also inducer compounds are commonly imposing an additional burden to the cells (Diaz Ricci and Hernández, 2000; Malakar and Venkatesh, 2012). While the *LacI/T7* promoter system is widely applied in molecular engineering studies, it is known to have a strong expression as well as exhibiting some leaky behavior under non-induced conditions (Tabor and Richardson, 1985). Moreover, inclusion bodies may form if translation rates are too high and can been a bottleneck in the heterologous expression of AATs (Yin *et al.*, 2007; Zhu *et al.*, 2015). It is possible that lower IPTG concentrations increased the amount of correctly folded protein and led to higher ethyl acetate production. In contrast, the XylS/*Pm* promoter system is weaker, but remarkably tight and titratable (Balzer *et al.*, 2013). In the present case however, the strong LacI/*T7* promoter system more efficiently triggered *eat1* activity and ethyl acetate production. Only for Wan Eat1 ethyl acetate formation was observed with the XylS/Pm system. This may result from an overall higher efficiency of the Wan Eat1 variant, compared to Kma Eat1.

Consistently, Wan Eat1 and its truncated variant were most efficient. Strains producing Wan Eat1 variants formed up to 15 % more ethyl acetate *in vivo* compared to strains producing

Kma Eat1. Optimizing the Eat1 efficiency by selecting a better Eat1 variant and improving the expression indeed led to a significant decrease in pyruvate accumulation. Performing similar optimizations on truncated variants, had similar effects, but interestingly also led to lower induction levels for similar or better results. Manual cleavage of the N-termini affected the enzymes cellular localization in yeasts and diminished or enhanced catalytic performance in *E. coli*, emphasizing the importance of those pre-sequences (Löbs *et al.*, 2017; Kruis *et al.*, 2020). Proper cleavage likely improved protein stability, which was reflected by the lower required inducer concentration.

While especially the transfer to pH-controlled reactor systems boosted general performance of the presented ethyl acetate production process, production of other dissimilatory products, like succinate, ethanol and acetate needs to be further minimized. As fermentations were performed under anaerobic conditions, ethanol and acetate could not be assimilated for additional ethyl acetate formation but remained as by-products of the fermentation. The disruption of *ackA* did not block acetate synthesis completely. The predominant acetate-forming route under anaerobic conditions is the conversion of acetyl-CoA to acetyl-P and further to acetate (Wolfe, 2005). Two genes are involved in this pathway, phosphotransacetylase (*pta*) and acetate kinase (*ackA)*, while other enzymes with similar catalytic activities, such as propionate kinase are able to perform the same reaction (Heßlinger, Fairhurst and Sawers, 1998). Disrupting *pta* and additional acid kinases might block acetate production completely.

Acetate accumulation by the *ackA* knockout strain, and to some extent ethanol accumulation, may also result from the hydrolytic side-activities of Eat1. It has been shown that this esterase and thioesterase activity is prevented above a critical ethanol concentration (Kruis et al., 2017). Below this critical concentration, there was no net ethyl acetate synthesis and ethanol and acetate were produced instead. Under the tested conditions, Atf1 exhibited more esterase and thioesterase activities, barely producing ethyl acetate. The tested eat1 variants of *W. anomalus* and *K. marxianus* also showed increased acetate levels at higher induction levels. Better understanding of the protein structure and catalytic mechanisms is needed to streamline the desired catalytic activities even further.

A build-up of high formate levels could be detrimental to cell growth and function and might have inhibited the serum bottle fermentations (Warnecke and Gill, 2005). In batch bioreactors, this problem might be tackled by applying pH control. Moreover, converting formate to CO_2 and H_2 via the Fhl complex would allow for co-production of ethyl acetate and H_2, the latter also being a valuable biofuel (Brentner, Peccia and Zimmerman, 2010). In our experiments, however, formate was only partially converted (between 6 % and 27 %), which was also experienced in other studies (Penfold, Forster and Macaskie, 2003; Yoshida *et al.*, 2005). The reason for the high variability is not clear, but it may be due to the complex transcriptional regulation of the 15 genes that are required to form an

active Fhl complex (Zinoni *et al.*, 1984; Birkmann *et al.*, 1987; Rossmann, Sawers and Böck, 1991). The issue might be prevented in the future by constitutively overexpressing *fhlA*, the transcriptional activator of the Fhl system to improve H_2 production (Rossmann, Sawers and Böck, 1991; Yukawa *et al.*, 2007). Addition of nickel may also be explored as this compound is required in the functional Fhl system (McDowall *et al.*, 2014).

In situ product removal via gas stripping has already been applied in some yeast production systems (Urit *et al.*, 2011; Löser, Urit, Gruner, *et al.*, 2015). Primarily, it improves downstream processing or can be used to prevent product inhibition during fermentations (Urit, Manthey, *et al.*, 2013; Kruis *et al.*, 2019). While no critical concentrations of ethyl acetate were reached in the performed fermentations, gas stripping could benefit the fermentations by limiting ethyl acetate hydrolysis. However, temporary accumulation and hydrolysis of ethyl acetate in the medium could not be avoided by the applied stripping rates, particularly for efficient ethyl acetate producers such as Kma trEat1 K-30 or Wan trEat1 N-13. Therefore, performances of the respective strains may still improve when higher stripping rates are applied.

While the reduction of degradation of the product by gas stripping improves the performance in the current research, it primarily aims at preventing product toxicity (Kruis 2019). Currently the reached titers of ethyl acetate are well below toxic levels for *E. coli* (Wilbanks and Trinh, 2017) but higher inoculation densities and the switch to fed-batch systems should benefit the final product titers. If the volumetric productivities can also compete with those reached by aerobic systems, is another factor that needs to be evaluated in the future.

The yield of 72 % of the maximum pathway yield did already exceed the best ethyl acetate yield reported for *K. marxianus* converting whey sugars (predominantly lactose) to ethyl acetate under aerobic conditions, reaching 56 % of the theoretical maximum pathway yield (Urit, Li, *et al.*, 2013).

Conclusion

We demonstrated that *E. coli* can be engineered to efficiently convert glucose to ethyl acetate as the primary fermentation product, which may serve as a point of reference for future development of biobased ethyl acetate-production processes in which Eat1 serves as the AAT catalyst. The combined effects of several rounds of metabolic, protein and process engineering resulted in an up to 14.3-fold increase in ethyl acetate yield. The highest ethyl acetate yield was achieved with *E. coli* BW25113 Δ*ldhA*Δ*ackA* (DE3) producing Wan trEat1 N-13 in the presence of 0.01 mM IPTG. This strain formed 0.49 ± 0.03 $C\text{-}mol_{ethyl\ acetate}/C\text{-}mol_{glucose}$, which corresponds to ~ 72 % of the theoretical pathway maximum.

Materials and methods

Strain and plasmid construction

The strains and plasmids used in this study are listed in Tables 1 and 2, respectively. The pET26b-XylS/*Pm* plasmids were obtained by replacing the LacI/*T7* promoter of pET26b with the XylS/*Pm* promoter (Balzer *et al.*, 2013) using 2X HiFi assembly master mix (NEB) according to the supplier protocol. All *K. marxianus* and *W. anomalus eat1* genes were cloned with a Strep-tag or 6-His-tag, respectively, to facilitate protein purification. PCR amplifications were performed with Q5 polymerase (NEB) according to supplier instructions.

TABLE 3: Strains used in this study.

Strain	Characteristics	Source
Escherichia coli BW25113 (DE3)	Wild type with integrated DE3 lysogen	(Vuoristo *et al.*, 2015)
Escherichia coli BW25113 *ΔldhAΔackA*	Disruption of lactate and acetate production (via *ackA*)	(Kruis *et al.*, 2020)
Escherichia coli T7 Express	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS *λ DE3* = λ sBamHIo ΔEcoRI-B int::(LacI::PlacUV5::T7 gene1) i21 Δnin5	NEB
Escherichia coli NEB® 5-alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB

TABLE 4: Plasmids used in this study.

Plasmid	Promoter	Gene/Protein	Source
pET26b	LacI/*T7*	/	This study
pET26b:hWanEat1	LacI/*T7*	Codon harmonized *eat1* from *Wickerhamomyces anomalus* DSM 6766	(Kruis *et al.*, 2017)
pET26b:hKmaEat1	LacI/*T7*	Codon harmonized *eat1* from *Kluyveromyces marxianus* DSM 5422	(Kruis *et al.*, 2020)
pET26b:opSceAtf1	LacI/*T7*	Codon optimized atf1 from *Saccharomyces cerevisiae* (Rodriguez, Tashiro and Atsumi, 2014)	This study
pET26b: XylS/*Pm* -hWanEat1	XylS/*Pm*	Codon harmonized *eat1* from *Wickerhamomyces anomalus* DSM 6766	This study
pET26b: XylS/*Pm* hKmaEat1	XylS/*Pm*	Codon harmonized *eat1* from *Kluyveromyces marxianus* DSM 5422	This study
pET26b: XylS/*Pm* opSceAtf1	XylS/*Pm*	Codon optimized atf1 from *Saccharomyces cerevisiae*	This study
pET26b:hKma trEat1 F-26	LacI/*T7*	Kma Eat1 truncated at F-26	(Kruis *et al.*, 2020)
pET26b:hKma-trEat1-K30	LacI/*T7*	Kma Eat1 truncated at K-30	(Kruis *et al.*, 2020)
pET26b:hWan-trEat1-V11	LacI/*T7*	Wan Eat1 truncated at V-11	(Kruis *et al.*, 2020)
pET26b:hWan-trEat1-N13	LacI/*T7*	Wan Eat1 truncated at N-13	(Kruis *et al.*, 2020)

Cultivation

Routinely, *E. coli* strains were grown on LB medium supplemented with kanamycin (50 µg/mL) or spectinomycin (50 µg/mL). Sterile 250-mL serum bottles were filled with 50 mL modified M9 medium, consisting of M9 salts (Difco, 1X), glucose (55 mM), $MgSO_4$ (2 mM), $CaCl_2$ * 2 H_2O (0.1 mM), MOPS (100 mM) and 1 mL 1000X trace elements and vitamins each according to Verduyn, 1992, and used for anaerobic cultivation experiments. The serum bottles were made anaerobic by flushing with nitrogen. For precultures single colonies were transferred to 10 mL LB medium in a 50-mL tube and grown overnight at 30 °C and 250 rpm. A second overnight cultivation under same conditions was performed after 1-2 mL of the LB culture was transferred to 50 mL modified M9 medium in a 250-mL Erlenmeyer flask. The anaerobic serum bottles were inoculated to an initial OD of 0.2 and incubated at 30 °C and 150 rpm. The inducing reagents isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.01-0.5 mM) or m-toluate (0.02-1 mM) were added to induce gene expression when appropriate. Experiments were performed as biological duplicates. Ethyl acetate production in serum bottles was measured only in the liquid phase.

Batch fermentations

Anaerobic fermentations were performed in 1.5 L bioreactors (Applikon) in 0.5 L defined medium. The fermentation medium contained glucose (55 mM), $(NH4)_2SO_4$ (37.8 mM), KH_2PO_4 (22 mM), NaCl (171 mM), kanamycin (100 µg/mL) and Na_2SeO_3 (0.3 mg/L) to promote hydrogen formation, unless mentioned otherwise. The medium was supplemented with vitamins and trace elements (Verduyn *et al.*, 1992). The fermentation broth was stirred at 400 rpm with a Rushton turbine controlled by an ADI 1012 Motor Controller (Applikon). pH was kept constant at 7.0 by automatic addition of 3 M KOH or 0.5 M H_2SO_4. The temperature was controlled at 30 ˚C by a Thermo Circulator ADI 1018 (Applikon). Anaerobic conditions were maintained by using oxygen-impermeable Marprene tubing (Watson-Marlow) and constant sparging with 3 L/h N_2. Inocula were prepared by transferring 0.5 mL fresh overnight LB pre-culture to 50 mL modified M9 medium in a 250-mL Erlenmeyer flask. The culture was grown overnight aerobically at 30 °C and 250 rpm. The reactors were inoculated to an initial OD_{600} of 0.4. Metabolites in the liquid phase were measured by high performance liquid chromatography (HPLC) and gas chromatography coupled to a flame ionization detector (GC-FID). Online measurements of volatile compounds and gases removed from the vessel by gas stripping were performed with an δB Process Mass Spectrometer (MS, Thermo Scientific™).

Calculations

The gaseous concentration of ethyl acetate, CO_2 and H_2 ($C_{C,gas}$, mol/L) in the outflow were calculated based on the ideal gas law according to Eq.1 follows:

$$C_{C,gas} = \frac{\frac{X_{C,gas}}{P_{atm}}}{R*T} \quad \text{(Eq. 1)}$$

With C representing the corresponding compound (ethyl acetate, CO_2 and H_2) $X_{C,gas}$ the volumetric fraction of compound C in the gas (-), P_{atm} the atmospheric pressure (Pa), R the ideal gas constant (m^3 Pa/mol/K) and T the temperature (K). The cumulative mass of compound C ($m_{C,gas}$, mol) stripped up to each time point (t_n, h) was calculated using Eq. 2

$$m_{C,gas}(t_n) = \frac{C_{C,gas}(t_{n-1}) + C_{C,gas}(t_n)}{2} * \frac{F_{gas,out}(t_{n-1}) + F_{gas,out}(t_n)}{2} * \Delta t + m_{C,gas}(t_{n-1}) \quad \text{(Eq. 2)}$$

Where the average gaseous concentration of the product between time points t_{n-1} and t_n is calculated from Eq. 1 (mol/L), $F_{gas,out}$ is the total volumetric gas flow rate leaving the reactor (L/h), Δt the time between two time points (h) and $m_{C,gas}(t_{n-1})$ the amount of compound C stripped up to the previous time point (mol). $F_{gas,out}$ was calculated assuming N_2 as inert gas and knowing the total volumetric gas flow into the reactor ($F_{gas,in}$) and the volumetric fractions of N_2 in the corresponding in- and outflows ($X_{N2,in}$, $X_{N2,out}$) at time point t using Eq.3.

$$F_{gas,out} = \frac{X_{N2,in}}{X_{N2,out}} * F_{gas,in} \quad \text{(Eq. 3)}$$

The cumulative mass of stripped ethyl acetate after Eq. 3 was divided by the culture volume in the bioreactor and added to the current ethyl acetate concentration in the liquid. The resulting value is an apparent ester concentration at time t_n which would be found in the culture medium if no stripping was applied .

Carbon balances were calculated according to Eq. 4.

$$C_{balance} = \frac{\text{C-mol products formed}}{\text{C-mol glucose consumed}} \quad \text{(Eq. 4)}$$

The compounds included in the calculation were glucose, ethyl acetate, ethanol, acetate, succinate, pyruvate, formate and CO_2. Biomass formation was included in the calculation assuming a biomass composition of $CH_2O_{0.5}N_{0.2}$ and an experimentally determined conversion factor of 0.3232 from OD_{600} to g/L dry weight (data not shown).

Analytical

Glucose and organic acids were analyzed by HPLC on an Agilent 1290 LC II system, equipped with an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity Autosampler, Agilent 1290 Infinity diode array detector operated at 210 nm, and an Agilent 1260 Infinity RI detector operated at 45 °C. Either an Aminex HPX-87H (Bio-Rad) or an Rezex ROA-Organic Acid H+ (Phenomenex) column were used with a mobile phase of 0.008 mM H_2SO_4. The HPLC was operated at 0.8 mL/min and 60 °C. Propionic acid (50 mM) was used as internal standard.

Ethyl acetate and ethanol in liquid samples were measured by an Agilent 7890B gas chromatograph equipped with a flame ionization detector (GC-FID) and an Agilent 7693 autosampler. Samples were analyzed by injecting 0.5 µL of liquid sample onto a Nukol™ column (30 m x 0.53 mm, 1.0 µm coating, Supelco). The column temperature was maintained at 50 °C for 2 min and increased to 200 °C at a rate of 50 °C/min. The split ratio was 10. 1-Butanol (2 mM) was used as internal standard.

Supplementary Material

SUPPLEMENTARY TABLE 1: Overview of pH-controlled batch fermentations in 1.5-L Applikon bioreactors with continuous gas stripping. Measured and calculated concentrations of main fermentation products, carbon balance and C-mol yields at end of fermentations are represented as average (AV) with standard deviations (SD) for each duplicate. *E. coli* BW25113 *ΔldhAΔackA* (DE3) producing Eat1 variants from pET26b plasmids were grown under anoxic conditions in minimal medium containing 55 mM glucose. Expression of Eat1 was induced by IPTG.

Strain		Kma upEat1		Kma trEat1 K30		Wan upEat1		Wan trEat1 N13	
	Unit	**AV**	**SD**	**AV**	**SD**	**AV**	**SD**	**AV**	**SD**
Ethyl acetate	mM	27.8	0.6	29.5	1.7	27.6	3.7	42.8	3.3
Ethanol	mM	25.3	2.6	20.3	1.7	11.7	0.0	17.7	0.4
Pyruvate	mM	6.6	0.7	5.5	0.9	3.2	0.4	0.0	NA
Acetate	mM	14.8	1.1	13.5	0.9	10.4	0.7	16.1	0.3
Lactate	mM	1.0	0.1	1.0	0.1	0.6	0.1	1.4	0.0
Succinate	mM	5.7	1.2	8.0	1.2	11.5	6.0	5.6	0.1
Formate+CO_2	mM	79.5	1.2	77.4	3.1	68.4	19.7	84.0	0.2
Carbon balance	-	1.04	0.05	1.07	0.03	0.93	0.03	0.97	0.03
Yield EA/Glc	Cmol/Cmol	0.35	0.01	0.38	0.03	0.34	0.05	0.48	0.03
Yield EtOH/Glc	Cmol/Cmol	0.16	0.02	0.13	0.01	0.07	0.00	0.10	0.00
Yield Pyr/Glc	Cmol/Cmol	0.06	0.01	0.05	0.01	0.03	0.00	0.00	NA
Yield Ac/Glc	Cmol/Cmol	0.09	0.01	0.09	0.00	0.06	0.00	0.50	0.00
Yield Suc/Glc	Cmol/Cmol	0.05	0.00	0.08	0.01	0.11	0.05	0.05	0.00
Yield For+CO_2/Glc	Cmol/Cmol	0.25	0.01	0.25	0.00	0.21	0.06	0.24	0.00

Chapter 5

Co-production of hydrogen and ethyl acetate in *Escherichia coli*

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Abstract

Ethyl acetate ($C_4H_8O_2$) and hydrogen (H_2) are industrially relevant compounds that preferably are produced via sustainable, non-petrochemical production processes. Both compounds are volatile and can be produced by *Escherichia coli* before. However, relatively low yields for hydrogen are obtained and a mix of by-products render the sole production of hydrogen by micro-organisms unfeasible. High yields for ethyl acetate have been achieved but accumulation of formate remained an undesired but inevitable obstacle. Coupling ethyl acetate production to the conversion of formate into H_2 may offer an interesting solution to both drawbacks. Ethyl acetate production requires equimolar amounts of ethanol and acetyl-CoA, which enables a redox neutral fermentation, without the need for production of by-products, other than hydrogen and CO_2.

We engineered *Escherichia coli*, towards improved conversion of formate into H_2 and CO_2 by inactivating the formate hydrogen lyase repressor (*hycA*), both uptake hydrogenases (*hyaAB*, *hybBC*) and/or overexpressing the hydrogen formate lyase activator (*fhlA*) in an acetate kinase (*ackA*) and lactate dehydrogenase (*ldhA*) deficient background strain. Initially 10 strains, with increasing number of modifications were evaluated in anaerobic serum bottles with respect to growth Four reference strains *ΔldhAΔackA*, *ΔldhAΔackA p3-fhlA, ΔldhAΔackAΔhycAΔhyaABΔhybBC and ΔldhAΔackAΔhycAΔhyaABΔhybBC p3-fhlA* were further equipped with a plasmid carrying the heterologous ethanol acyltransferase (Eat1) from *Wickerhamomyces anomalus* and analyzed with respect to their ethyl acetate and hydrogen co-production capacity. Anaerobic co-production of hydrogen and ethyl acetate via heterologous Eat1 was achieved in 1.5-L pH controlled bioreactors. The cultivation was performed at 30°C in modified M9 medium with glucose as sole carbon source. Anaerobic conditions and gas stripping were established by supplying N_2 gas.

We showed that the engineered strains co-produced ethyl acetate and hydrogen to yields exceeding 70 % of the pathway maximum for ethyl acetate and hydrogen, and propose *in situ* product removal via gas stripping as efficient technique to isolate the products of interest.

Keywords: ethyl acetate, hydrogen, co-production, fermentation, *Escherichia coli*, Eat1, formate hydrogen lyase.

Background

Esters are a diverse group of compounds important not only for the food industry but also for various industrial purposes (Kruis *et al.*, 2019). Ethyl acetate is among the most relevant esters with respect to industrial use. It is considered relatively environmentally friendly and thus a popular solvent used in paints and adhesives, and other applications.

Yeasts are natural producers of a variety of esters, including ethyl acetate. Efforts have been made to understand and direct ester production and composition, focusing on bulk producers of ethyl acetate, including *Kluyveromyces marxianus* and *Wickerhamomyces anomalus* (Sabel *et al.*, 2014; Löser, Urit, Keil, *et al.*, 2015; Kruis *et al.*, 2017). Especially *K. marxianus* has been exploited and optimized with respect to efficient ethyl acetate production. In fermentations on whey based medium a yield of 0.265 $g_{ethyl\ acetate}/g_{sugar}$, corresponding to 50 % of the maximum yield, was reached in a 70-L reactor, demonstrating the scalability of the system (Löser *et al.*, 2013). Recently, we have shown that a heterologous expression system in *Escherichia coli* can compete with natural producers in terms of ethyl acetate yields (Bohnenkamp *et al.*, 2020). A streamlined *E. coli* strain harboring a truncated ethanol acetyltransferase (*eat1*) gene from *W. anomalus* reached 72 % of the maximum pathway yield on glucose under anoxic conditions. This is the highest reported yield to date.

In contrast to yeasts that use pyruvate decarboxylase to convert pyruvate to acetaldehyde, *E. coli* uses pyruvate formate lyase to produce acetyl-CoA during anaerobic conditions (Bohnenkamp *et al.*, 2020). This ultimately results in a redox and carbon balanced pathway under anoxic conditions, contributing to the overall efficiency of the process as less carbon is lost to biomass or respiration (Weusthuis 2011). However, as *E. coli* uses pyruvate formate lyase, one mole of formate is coproduced with every conversion of pyruvate into acetyl-CoA, coproducing two moles of formate per generated mole of ethyl acetate (Figure 1).

Formate is accumulating during the fermentation process, acidifying the medium and causing inhibiting effects on the *E. coli* cells. While the acidification of the medium can be prevented by pH-control of the reactor, buildup of formate to inhibiting concentrations may nevertheless negatively affect performance of the system. Formate concentrations below 100 mM already severely hamper *E. coli* growth, and concentrations of 50 mM have been reported to cause growth inhibition of 50 % (Zaldivar, Martinez and Ingram, 1999). One way *E. coli* counteracts these negative side-effects of formate, is by converting it to CO_2 and H_2 by a membrane bound formate hydrogen lyase (Fhl) after formate concentrations exceed a certain threshold (McDowall *et al.*, 2014).

Hydrogen is considered an attractive, environmentally friendly energy carrier, but 95 % of the current production is still derived from non-renewable resources (Balat and Kirtay,

2010; Nikolaidis and Poullikkas, 2017). In order to benefit from hydrogen as future fuel also its production needs to rely on sustainable methods paving the path for green or bio-hydrogen (Levin and Chahine, 2010; Dincer and Acar, 2014; Stephen *et al.*, 2017). Regarding microbial hydrogen production attention has been paid to increasing yields and productivity by means of genetic engineering, with a strong focus on *E. coli*. While *E. coli* primarily secretes formate and naturally is a poor hydrogen producer, the complexity and transcriptional regulation of the Fhl complex with the involvement of around 15 genes is well understood (Zinoni *et al.*, 1984; Birkmann *et al.*, 1987; Rossmann, Sawers and Böck, 1991). Due to its annotated genome and well established genetic engineering tools, several targets and strategies for improving hydrogen production have been identified (Maeda, Sanchez-Torres and Wood, 2012).

Several studies used formate as substrate for the production of bio-hydrogen from *E. coli* (Penfold, Forster and Macaskie, 2003; Yoshida *et al.*, 2005). Inactivating the Fhl repressor *hycA* was among the first modifications to promote Fhl activity, thus enhancing hydrogen production (Penfold, Forster and Macaskie, 2003). Combining *hycA* deactivation and overexpression of the formate hydrogen lyase transcriptional activator (FhlA) further improved strain performance (Yoshida *et al.*, 2005). In addition, Maeda and colleagues studied the effect of various modifications concerning hydrogen production and uptake, extensively (Maeda, Sanchez-Torres and Wood, 2007). They found that besides inactivating *hycA* and overexpressing *fhlA*, inactivation of hydrogen uptake by knocking out hydrogenase 1 (*hyaB*) and 2 (*hybC*) further benefitted hydrogen production. Moreover, inactivating *hycA hyaB hybC* together with inactivating the formate transporter *focA* did not impact growth of *E. coli* under aerobic conditions, while leading to an almost 5-fold increased hydrogen production capacity with respect to wild-type *E. coli* (Maeda, Sanchez-Torres and Wood, 2008).

However, to date microbial hydrogen production with sole focus on generation of bio-hydrogen is considered rather unfeasible mainly due to the low conversion efficiency and low maximum yields obtained (Nikolaidis and Poullikkas, 2017). Therefore coupling it to the production of another relevant product may improve the overall feasibility of such process as shown with the example of ethanol (Sundara Sekar *et al.*, 2016; Stephen *et al.*, 2017; Lopez-Hidalgo, Balderas and de Leon-Rodriguez, 2021). However, ethanol and hydrogen are competing for electrons and maximum yields for one product will automatically decrease the achievable yield for the other product.

This study investigates in how far redox-balanced co-production can benefit the bio-based generation of two industrially relevant compounds with respect to yields and rates. Here, we describe the efficient co-production of ethyl acetate and bio-hydrogen using an engineered *E. coli* strain, while restricting product accumulation by *in situ* product removal.

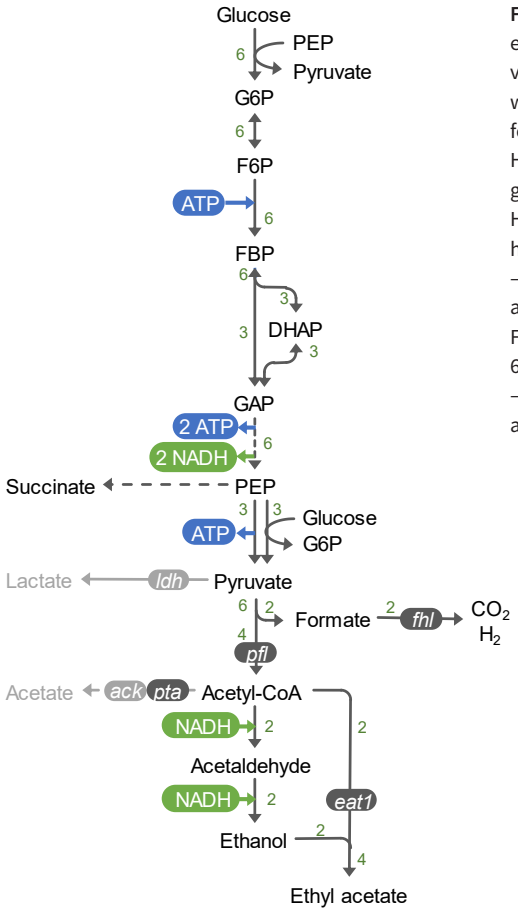


FIGURE 1: Schematic representation of anaerobic ethyl acetate production from glucose in *E. coli* via the Embden-Meyerhof-Parnas (EMP) pathway with hydrogen co-production. Lactate and acetate formation is limited by *ackA* and *ldhA* inactivation. Heterologous alcohol acetyltransferase Eat1 generates ethyl acetate from ethanol and acetyl-CoA. Hydrogen co-production is achieved via formate hydrogen lyase (Fhl). Ack – acetate kinase, DHAP – dihydroxyacetone phosphate, eat1 – ethanol acetyltransferase, FBR – fructose 1,6-bisphosphate, F6P – fructose 6-phosphate, G6P – glucose 6-phosphate, PEP – phosphoenolpyruvate, GAP – glyceraldehyde 3-phosphate, pta – phosphate acetyltransferase.

Results

Increasing hydrogen gas production

A series of modifications to a *BW25113 ΔldhAΔackA* (BW25113 *ΔΔ)* background strain were applied in order to improve the conversion of formate into hydrogen. Sequential inactivation of the Fhl repressor hycA, and the uptake hydrogenases *hyaAB* and *hybBC*, were combined with overexpression of the Fhl activator FhlA. A first evaluation of strains took place in anaerobic serum bottles with ethanol, pyruvate and formate as main fermentation outputs. Due to the *ackA* knockout in *BW25113 ΔΔ*, NADH requirements for ethanol formation cannot be balanced by co-production of acetate but are met by secretion of the intermediate metabolite pyruvate.

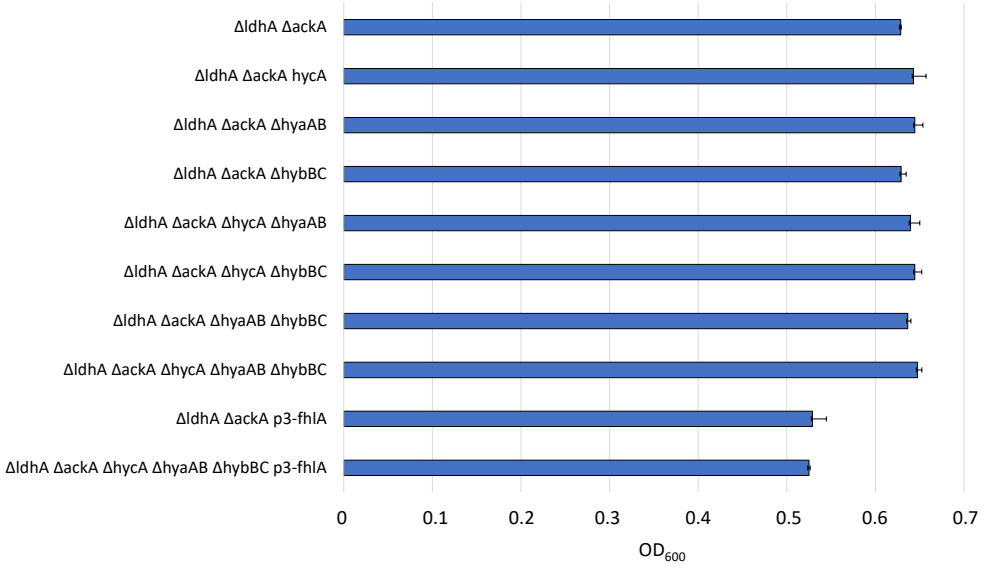


FIGURE 2: OD_{600} after 72 h of cultivation under anaerobic conditions with glucose as carbon source of a BW25113 *ΔldhAΔackA* background strain containing additional KOs and/or overexpressing FhlA for improved hydrogen production. Initial OD_{600} was 0.2. Data and error bars indicate averages and standard deviations among duplicates

5

Neither the three individual knock-out events, nor a combination thereof, did have any effect on growth rates of the resulting strains when compared to their parental strain BW25113 *ΔΔ* (Figure 2). After 72 h of cultivation all strains reached an OD_{600} of around 0.64. Overexpression of fhlA was achieved by introduction of the *p3* promoter in front of the start codon of the native *fhlA*. This modification slightly affected growth of the double knockout strain BW25113 *ΔldhAΔackA p3-fhlA (*BW25113 *ΔΔ p3-fhlA*) as well as in the quintuple knockout strain BW25113 *ΔldhAΔackAΔhycAΔhybBCΔhyaAB p3-fhlA* (BW25113 *ΔΔΔΔΔ p3-fhlA*) (Figure 2). Overexpression of fhlA led to a reduced OD_{600} after 72 h, 15 % lower compared to parental strains relying on native expression of fhlA.

At the same time strains overexpressing FhlA consumed about 30 % less glucose, resulting in less ethanol, pyruvate and formate production (Figure 3abcd). Despite knocking out *ackA* some acetate production could not be avoided and reached levels around 6 mM for all strains tested (Figure 3e). Succinate titers reached 3.96 ± 0.2 mM for the parental strain BW25113 *ΔΔ*, but were increased by 10 % to 50 % by strains with additional modifications towards hydrogen production, likely due to increased CO_2 availability (Figure 3f).

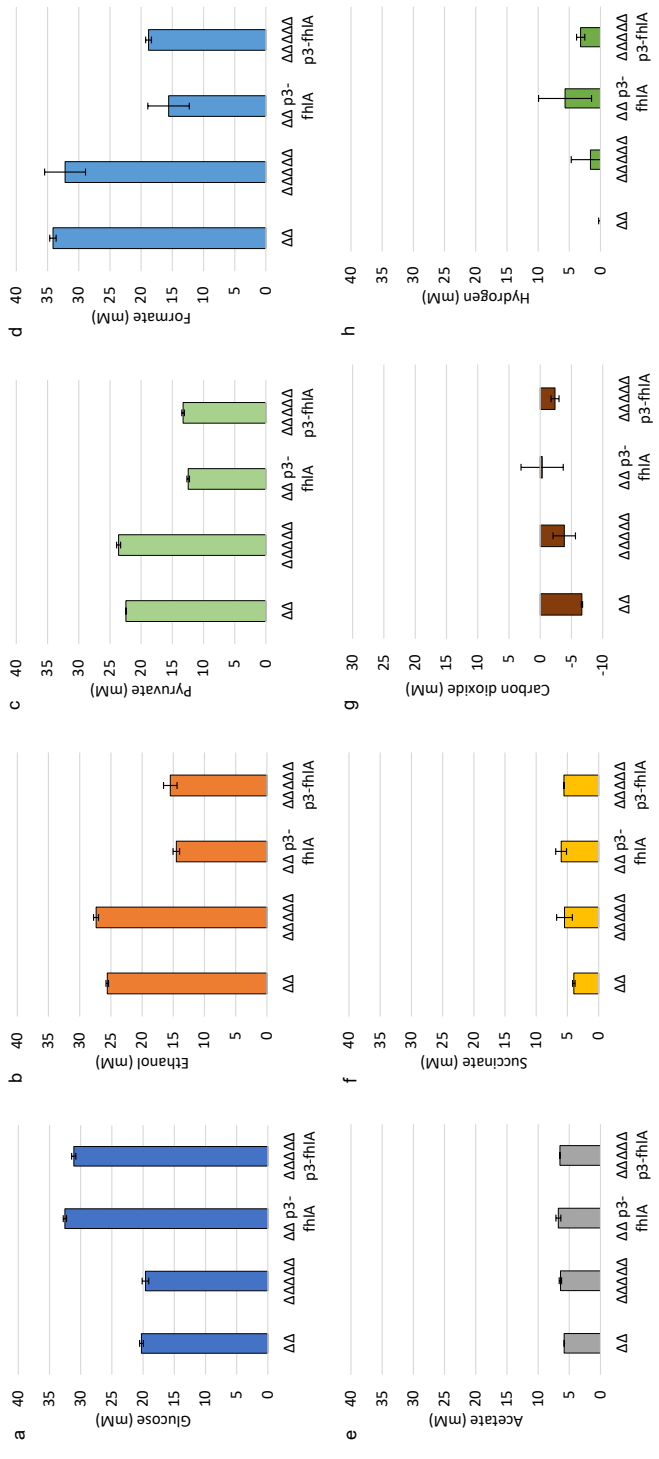


FIGURE 3: Concentrations of glucose and products after 72 h of anaerobic cultivation for strains with a *ΔldhAΔackA* (*ΔΔ*) background and further modifications for improved hydrogen production, from left to right: inactivation of *hycA*, *hyaAB* and *hybBC* (*ΔΔΔΔ*), overexpression of *fhlA* (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔ p3-fhlA*). For CO_2 and H_2, data represent calculated concentrations. Data shows average values and standard deviations from biological duplicates.

It is difficult to determine hydrogen and carbon dioxide gas production accurately in serum bottles. The effect of the genetic modifications on the production of both gasses was therefore estimated indirectly, by subtracting the amount of formate produced from the amount of ethanol plus acetate formed to obtain a calculated hydrogen concentration (mM). For estimating CO_2, fixation for succinate synthesis was included, but not CO_2 production associated with biosynthesis. This resulted in slightly negative calculated CO_2 concentrations (Figure 3g). While for the parental strain no H_2 could be calculated, the other strains generated between 2 and 8 mM (Figure 3h). However, variations in formate accumulation and conversion among duplicates led to large error bars in calculated concentrations.

Ethanol yields on glucose dropped by 12 % for strains overexpressing FhlA in respect to BW25113 *ΔΔ* and BW25113 *ΔΔΔΔΔ* for which yields of about 0.8 $mol_{ethanol}/mol_{glucose}$ were obtained (Figure 4). However, succinate yields significantly increased and doubled for BW25113 *ΔΔ p3-fhlA* and BW25113 *ΔΔΔΔΔ p3-fhlA* ($p<0.05$). For strain BW25113 *ΔΔΔΔΔ* the hydrogen yield on glucose was only 0.02 $mol_{hydrogen}/mol_{glucose}$. Both strains overexpressing fhlA reached a higher yield, around 0.1 and 0.25 $mol_{hydrogen}/mol_{glucose}$ respectively. However, due to variations in the replicas only BW25113 *ΔΔΔΔΔ* and BW25113 *ΔΔΔΔΔ p3-fhlA* showed significant increase in hydrogen yields ($p<0.05$).

Concluding, the effect of the subsequent inactivation steps in strain BW25113 *ΔΔΔΔΔ* remains elusive while overexpression of FhlA supports hydrogen production. On the other hand, overexpression causes a reduction in biomass formation and slower glucose consumption.

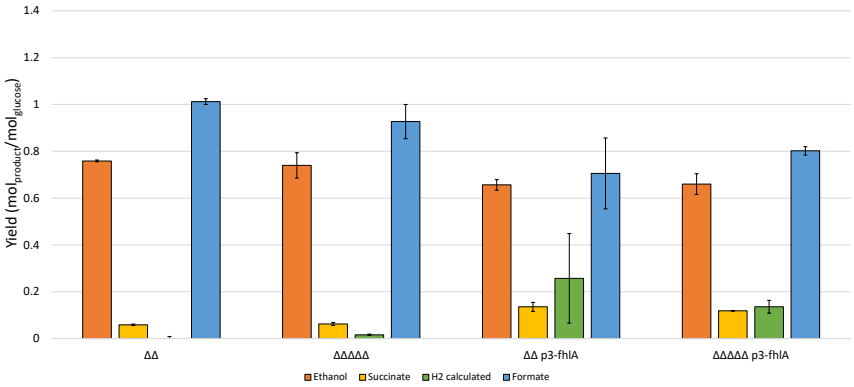


FIGURE 4: Product yield on glucose on selected products after 72h of anaerobic fermentation for strains based on *ΔldhAΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of *hycA, hyaAB* and *hybBC* (*ΔΔΔΔΔ*), overexpression of *fhlA* (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*). Values are averages of two biological replicates and error bars represent standard deviations.

Combining hydrogen gas and ethyl acetate production

After initial screening experiments and indirect performance assessments, three strains were generated with the purpose of co-producing hydrogen and ethyl acetate from glucose as carbon source. Strains BW25113 *ΔldhAΔackA* p3-fhlA *(*BW25113 *ΔΔ p3-fhlA)*, BW25113 *ΔldhAΔackAΔhycAΔhyaABΔhybBC (*BW25113 *ΔΔΔΔΔ)* and BW25113 *ΔldhAΔackAΔhycAΔhyaABΔhybBC p3-fhlA (*BW25113 *ΔΔΔΔΔ p3-fhl)* were equipped with the plasmid that encoded the ethanol acetyltransferase, pET26b:LacI/*T7-trEat1 Wan N13 (*trEat1*)* and gene expression was induced by 0.01 mM IPTG. Anaerobic ethyl acetate and hydrogen co-production were assessed in pH-controlled 1.5-L bioreactors with a continuous N_2 gas flow of 100 mL/min coupled to online MS measurements of the off gas. In this way stripped ethyl acetate, as well as produced CO_2 and H_2 could be measured and quantified.

Similar to observations during the serum bottle experiments, overexpression of FhlA led to a decrease in maximum OD_{600} and slower glucose conversion (Figure 5ab). In contrast, however, knocking out the formate hydrogen lyase repressor and both uptake hydrogenases improved overall fermentation performance of BW25113 *ΔΔΔΔΔ* trEat1 including a reduced total fermentation time by about 35 %. Expression of Eat1 and synthesis of ethyl acetate in a redox balanced way, apparently lifted the earlier observed NADH shortage and therefore prevented pyruvate excretion almost completely (Appendix). Gas stripping kept overall ethyl acetate levels in the fermentation broth well below 10 mmol and resulted in a cumulative amount of stripped ethyl acetate near to 20 mmol (Figure 5c). Formation of other by-products such as ethanol, acetate and succinate were mostly similar among all strains and did not exceed 10 mmol per compound (Figure 5def). However, BW25113 *ΔΔΔΔΔ* trEat1 did accumulate more than twice as much succinate as the remaining strains. Formate secretion was reduced for all engineered strains, while H_2 and CO_2 accumulated to 4-times higher levels than the control strain without modifications in Fhl regulation or hydrogenases (BW25113 *ΔΔ* trEat1) (Figure 5ghi).

With respect to product yields no significant differences in ethyl acetate yields on glucose could be found. With yields ranging from 0.63 ± 0.03 to 0.71 ± 0.04 $mol_{ethyl\ acetate}/mol_{glucose}$ about 70% of the pathway maximum was reached (Figure 6a). Knocking out *hycA, hyaAB, hybBC*, as well as overexpressing *fhlA* significantly improved hydrogen yields, reaching 50 % and more of the pathway maximum. For the strain overexpressing *fhlA* (*BW25113 ΔΔ p3-fhlA* trEat1) the highest hydrogen yield was obtained with 1.47 ± 0.11 $mol_{hydrogen}/mol_{glucose}$, corresponding to 73 % of the pathway maximum.

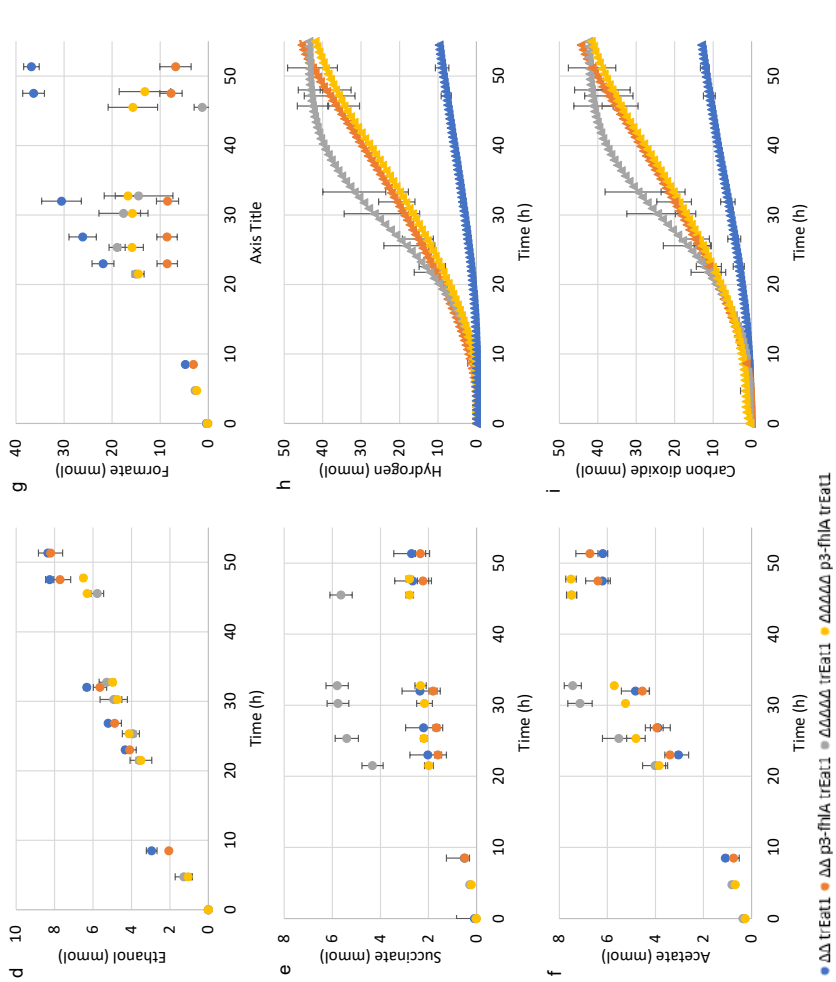


FIGURE 5: Fermentation profile of four strains engineered for ethyl acetate and hydrogen co-production in pH-controlled bioreactors with continuous gas stripping. Strains based on *Δ1dhAΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of *hycA, hyaAB* and *hybBC* (*ΔΔΔΔΔ*), overexpression of *fhlA* (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*) producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and cultivated under anaerobic conditions in minimal medium with 55 mM glucose as carbon source.. Experiments were performed as biological duplicates; error bars represent the standard deviation. Circles – compounds in liquid broth, triangle – compounds in off-gas.

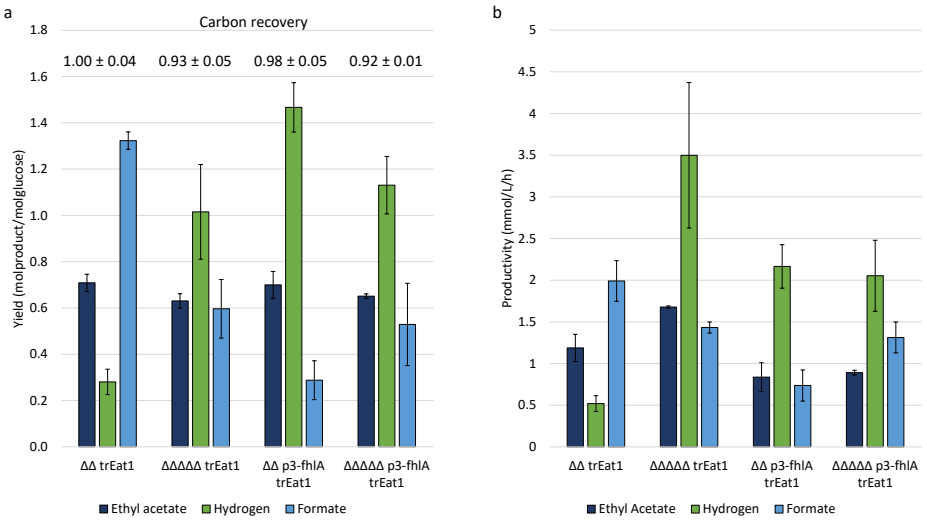


FIGURE 6: Effect of modifications towards improved hydrogen production on product yields and productivities for main fermentation products, with from left to right: inactivation of *hycA, hyaAB* and *hybBC*, overexpression of *fhlA* and a combination of knockouts and overexpression. Strains producing trEat1 *Wan* N-13 were induced by 0.01 mM IPTG and grown under anaerobic conditions in minimal medium containing 55 mM glucose using pH-controlled bioreactors with 0.5-L working volume. (a) Product yields for ethyl acetate, hydrogen and formate in $mol_{product}/mol_{glucose}$ after glucose depletion. The numbers above the bars represent the carbon recovery of the fermentations. (b) Volumetric productivities for ethyl acetate, hydrogen, and formate in mmol/L/h. Experiments were performed as biological duplicates or triplicates; error bars represent the standard deviation. Abbreviations: trEat1 – truncated Eat1 Wan N-13.

Despite that the product yield for ethyl acetate was rather similar, productivity of ethyl acetate did differ among the different strains. BW25113 *ΔΔΔΔΔ* trEat1 showed an improved ethyl acetate production by 41 % (p=0.052), while both FhlA overexpression strains showed a drop in productivity by 25-30 %, which was however, not statistically significant (Figure 6b). Regarding the co-production of hydrogen, all modifications led to a significant increase in conversion of formate into hydrogen and concomitantly CO_2 (p<0.05). The highest hydrogen productivity of 3.5 mmol/L/h was reached by BW25113 *ΔΔΔΔΔ* trEat1. Unexpectedly, overexpression of FhlA led to hydrogen production rates of only 2 mmol/L/h regardless whether only *fhlA* was overexpressed or additional knockouts were carried out.

Discussion

The current study demonstrates how anaerobic ethyl acetate production can be coupled to efficient hydrogen co-production thereby improving overall fermentation performance of the system. With an ethyl acetate yield on glucose close to 70 % of the pathway yield *E. coli* can compete with natural producers, like *K. marxianus* (Löser *et al.*, 2013) and performs close to earlier reported values using a truncated version of *W. anomalus* Eat1 (Bohnenkamp *et al.*, 2020).

Inactivation of the uptake hydrogenases (*hyaAB* and *hybBC*) and the Fhl repressor (*hycA*) led to 4-times higher hydrogen production rates relative to the control strain. While other studies found that those modifications did not negatively affect growth rates, here, the strain performance was even slightly improved during batch reactor fermentations (Maeda, Sanchez-Torres and Wood, 2008). This is likely a consequence of reduced formate concentrations, that may impose inhibitory effects to the cells (Zaldivar, Martinez and Ingram, 1999).

Hydrogen yields realized by modified strains ranged from 1 – 1.47 $mol_{hydrogen}/mol_{glucose}$, thus the improvements are comparable to earlier reported values around 1.15-1.8 $mol_{hydrogen}/mol_{glucose}$ (Yoshida *et al.*, 2006; Maeda, Sanchez-Torres and Wood, 2007; Kim *et al.*, 2009; Mathews, Li and Wang, 2010; Sundara Sekar *et al.*, 2016). Overexpression of the Fhl activator *fhlA* using the *p3* promoter, led to the highest hydrogen yields on glucose in BW25113 *ΔΔ p3-fhlA* trEat1, with a product yield of 1.47 $mol_{hydrogen}/mol_{glucose}$ respectively. However, for this strain also reduced biomass formation and reduced production rates of hydrogen and ethyl acetate were observed. In previous research, overexpression of fhl from a low copy number plasmid improved growth rates and hydrogen production from formate (Yoshida *et al.*, 2005). Also on glucose no impact of overexpression was noted using an IPTG-inducible expression system while the plasmid insertion itself did reduce the growth rate of the strain and also impacted growth rates during aerobic cultivation on formate (Maeda, Sanchez-Torres and Wood, 2007, 2008). Therefore, finetuning the overexpression with different promoters or inducible expression systems, combined with adaptation seems necessary to keep the hydrogen overexpression strains competitive. While the applied modifications reportedly improve hydrogen (co-) production, there are still options to inactivate formate exporters (focA) or other formate consuming enzymes including formate dehydrogenase-N (FdnG), dehydrogenase-O (FdoG), or nitrate reductase A (NarG) that positively impacted hydrogen production (Maeda, Sanchez-Torres and Wood, 2007).

In the mentioned studies, efficient hydrogen producing strains also carried an *frdAB* inactivation to eliminate succinate formation, which should be considered when optimizing further towards the maximum pathway yield of 2 $mol_{hydrogen}/mol_{glucose}$. Especially for strain BW25113 *ΔΔΔΔΔ* trEat1 the succinate yield was 2-times higher than the parental

strain and may have masked the positive effects of hydrogen production as carbons were deviated from the intended co-product ethyl acetate.

Complete suppression of acetate formation is challenging and inactivation of *ackA* or *pta* often leads to a reduction in acetate accumulation only (Vuoristo *et al.*, 2015; Li *et al.*, 2017). Inactivation of the full *ackA-pta* operon, could help to lower acetate accumulation to negligible amounts (Vadali *et al.*, 2004; Seol *et al.*, 2014). Additionally, acetate may originate from Eat1 thiolysis or esterase side-activities converting ethyl acetate or acetyl-CoA into acetate (Bohnenkamp *et al.*, 2020; Patinios *et al.*, 2020). Eliminating side-activities by protein engineering may be one way to overcome this drawback of Eat1. Here, we applied gas stripping to remove ethyl acetate more efficiently and reduce the residence time in the fermentation broth. Next to product degradation, product toxicity is another factor tackled with this strategy (Löser, Urit, Gruner, *et al.*, 2015; Kruis *et al.*, 2019). Like most products, ethyl acetate can accumulate to toxic concentrations, thereby imposing inhibitory effects to the cells. For *E. coli* the threshold is estimated for ethyl acetate titers above 110 mM (Wilbanks and Trinh, 2017). While this concentration was not and could not be reached under the tested conditions, gas stripping will become more important once the process is further upscaled. Moreover, the production of H_2 and CO_2 instead of formate, also benefits from gas stripping and enables continuous removal of both products of interest.

Low hydrogen yields during fermentation in expression hosts like *E. coli* combined with a mix of other fermentation products is a major drawback in microbial hydrogen production (Maeda, Sanchez-Torres and Wood, 2012; Stephen *et al.*, 2017). Besides efficient production of hydrogen, production of only one other main fermentation product remains challenging Especially high yield production of ethanol is often limited by NAD(P)H availability. Since NAD(P)H is only produced during the EMP pathway (GAP oxidation), ethanol formation can only amount to 1 $mol_{ethanol}/mol_{glucose}$, with the concomitant formation of 1 $mol_{acetate}/mol_{glucose}$. Higher ethanol yields requires additional NAD(P)H. Various engineering approaches have been used to generate extra NAD(P)H; Sundara Sekar et al. (2016) employed a partial pentose phosphate pathway, which resulted in co-production of ethanol and hydrogen, with limited by-products formation or loss of growth, reaching yields for ethanol and hydrogen on glucose of 1.4 $mol_{ethanol}/mol_{glucose}$ and 1.88 $mol_{hydrogen}/mol_{glucose}$ respectively. Others made use of a pyruvate dehydrogenase instead of the pyruvate formate lyase yielding more NAD(P)H and reaching ethanol yields of 1.8 $mol_{ethanol}/mol_{glucose}$ (Zhou, Iverson and Grayburn, 2008). The latter obviously occurs at the expense of formate or hydrogen. Thus, optimal co-production of hydrogen and one other product requires a redox balanced acetyl-CoA conversion. The production of ethyl acetate as demonstrated here enables such redox neutral acetyl-CoA conversion and simultaneously co-production of hydrogen at its theoretical maximum of 2 $mol_{hydrogen}/mol_{glucose}$. With the co-production of ethyl acetate and hydrogen from glucose of 0.71

$mol_{ethyl\ acetate}/mol_{glucose}$ and 1.47 $mol_{hydrogen}/mol_{glucose}$ for strain BW25113 *ΔldhAΔackA p3-fhlA* pET26b:Eat Wan N13 we successfully provide a first outlook on the applicability of this strategy towards another industrially relevant compound. Especially with respect to green hydrogen, co-production strategies offer an elegant way to improve the economic feasibility of a microbial production route and should be further pursued.

Conclusion

Modification of the Fhl regulation system is an effective way to improve hydrogen production in *E. coli*. Overexpression of the Fhl activator *fhlA*, but also the inactivation of the Fhl repressor *hycA* and hydrogenases 1 and 2 by knocking out *hyaAB* and *hybBC* improved hydrogen production 4-fold. During anaerobic fermentation of BW25113 *ΔldhAΔackA p3-fhlA* pET26b:LacI/*T7-trEat1 Wan* N-13 on glucose 70 % of the pathway yields for ethyl acetate and hydrogen, 0.695 $mol_{ethyl\ acetate}/mol_{glucose}$ and 1.44 $mol_{hydrogen}/mol_{glucose}$ respectively, were obtained. Cultivation of BW25113 *ΔldhAΔackAΔhycAΔhyaABΔhybBC* pET26b: LacI/*T7-trEat1 Wan* N-13 led to highest ethyl acetate and hydrogen production rates, being 1.41- and 4-fold higher than the parental strain that mainly accumulated formate. Coupled to *in situ* product removal by gas stripping both products can efficiently be produced and recovered, offering attractive downstream processing opportunities for co-production of bio-based ethyl acetate and green hydrogen by *E. coli*.

5

Methods

Strain and plasmid construction

All strains and plasmids used can be found in Tables 1 and 2. Generation of genomic knockouts and insertion of *p3*-promoter (Mutalik *et al.*, 2013) was achieved by CRISPR-Cas9 (Jiang *et al.*, 2015). To generate the corresponding pTarget plasmid, a sequence containing gRNA module and the homologous sequences of 50 bp immediately upstream the start codon and downstream the stop codon were ordered as synthetic gBlocks (IDT) (Supplementary Table 3). For insertion of the p3-promoter sequence, the homologous sequences were located 35 bp upstream and beginning with the start codon for the downstream sequence. Using 2X HiFi assembly master mix (NEB) according to manufacturer's instructions plasmids were assembled and propagated in competent NEB® 5-alpha cells. The pET26b:LacI/*T7-trEat1 Wan N-13* plasmid was inserted by following instructions from the Mix&Go *E. coli* Transformation Kit (ZYMO Research). PCR amplification was performed using Q5 polymerase (NEB).

TABLE 5: Strains used in this study.

Strain	Characteristics	Source
Escherichia coli BW25113 (DE3)	Wild type with integrated DE3 lysogen	(Vuoristo *et al.*, 2015)
Escherichia coli BW25113 *ΔldhAΔackA*	Disruption of lactate and acetate production (via *ackA*)	(Kruis *et al.*, 2020)
Escherichia coli BW25113 *ΔackAΔldhA p3-fhlA*	Disruption of lactate and acetate production (via *ackA*) and overexpression of formate hydrogen lyase transcriptional activator (fhlA)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhycA*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (*hycA*)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhyaAB*	Disruption of lactate and acetate production (via *ackA*) and inactivation of uptake hydrogenase (hyaAB)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of uptake hydrogenase (hybBC)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhycAΔhyaAB*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenase (hyaAB)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhycAΔhybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenase (hybBC)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhyaABΔhybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of uptake hydrogenases (hyaAB, hybBC)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhycAΔhyaABΔhybBC*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenases (hyaAB and hybBC)	This study
Escherichia coli BW25113 *ΔldhAΔackAΔhycAΔhyaABΔhybBC p3-fhlA*	Disruption of lactate and acetate production (via *ackA*) and inactivation of Fhl repressor (hycA) and uptake hydrogenases (hyaAB and hybBC) with overexpression of Fhl activator (fhlA)	This study
Escherichia coli T7 Express	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS *λ DE3 = λ* sBamHIo ΔEcoRI-B int::(LacI::PlacUV5::T7 gene1) i21 Δnin5	NEB
Escherichia coli NEB® 5-alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB

TABLE 6: Plasmids used in this study.

Plasmid	Promoter	Gene/Protein	Source
pET26b	LacI/*T7*	/	This study
pET26b:hWan trEat1 N-13	LacI/*T7*	Codon harmonized *eat1* from *Wickerhamomyces anomalus* DSM 6766	(Kruis *et al.*, 2020)
pCas9	/		(Jiang *et al.*, 2015)
pTarget	/		(Jiang *et al.*, 2015)
pTarget-*hycA*	/		This study
pTarget-*hyaAB*	/		This study
pTarget-*hybBC*	/		This study
pTarget-p3	/		This study

Cultivation

Strains were routinely cultured on LB medium with supplementation of spectinomycin (50 µg/mL) and/or kanamycin (50 µg/mL) when appropriate. Preculturing of strains started by plating glycerol stocks stored at -80°C onto LB agar plates. From single colonies, overnight cultures for transformations or experiments were inoculated into 10 mL LB medium in a 50-mL tube and grown at 30°C and 250 rpm. For pre-cultures and anaerobic experiments, 250-mL Erlenmeyer flasks or serum bottles were filled with 50 mL modified M9 medium consisting of M9 salts (Difco, 1X), glucose (55 mM), $MgSO_4$ (2 mM), $CaCl_2$ * 2 H_2O (0.1 mM), MOPS (100 mM) and 1X trace elements and vitamin solutions based on Verduyn *et al.*, 1992. The serum bottles were capped and flushed with nitrogen gas for anoxic conditions. From overnight cultures 1-2 mL were transferred to 50 mL modified M9 medium in 250-mL Erlenmeyer flasks and grown at 30°C and 250 rpm. Strains for anaerobic experiments were inoculated as biological duplicates at an initial OD_{600} of 0.2 and incubated at 30°C and 150 rpm (Kruis *et al*., 2020).

Batch reactor fermentation

Batch fermentations were performed in 1.5-L bioreactors (Applikon, The Netherlands) with a working volume of 0.5 L as described before (Bohnenkamp *et al.*, 2020). Defined medium contained glucose (55 mM), $(NH_4)_2SO_4$ (37.8 mM), KH_2PO_4 (22 mM), NaCl (171 mM), kanamycin (100 µg/mL), Na_2SeO_3 (0.3 mg/L) and 1X trace elements and vitamin solutions (Verduyn *et al.*, 1992). Eat1 gene induction was achieved by addition of 0.01 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Stirring at 400 rpm with a Rushton turbine was controlled by a ADI 1012 Motor Controller (Applikon), the target pH of 7 was maintained by automated addition of 3 M KOH solution and a temperature of 30°C was achieved by a Thermo Circulator ADI 1018 (Applikon). Oxygen impermeable Marprene tubing (Watson-Marlow, UK) and a gas flow of 6 L/h N_2 set the framework for anaerobic

conditions. Pre-cultures were prepared as stated above and used to inoculate the reactors to a starting OD_{600} of 0.4. Liquid samples were taken regularly via a sampling port to assess optical density and composition of the fermentation broth. Metabolites were analyzed by high performance liquid chromatography (HPLC) and gas chromatography coupled to a flame ionization detector (GC). The off-gas composition was determined by online measurements of a δB Process Mass Spectrometer (MS, Thermo Scientific™, USA).

Calculations

During anaerobic serum bottle experiments, H_2 and CO_2 production was estimated indirectly. Calculated H_2 and CO_2 concentrations (C in mol/L) were derived by assuming that significant production of either compound is solely attributed to Fhl activity, thus following the stoichiometric relation as shown in Equation 1:

$$n\,CH_2O_2 \rightarrow n\,CO_2 + n\,H_2 \qquad \text{(Eq. 1)}$$

The deficit in formate measured and formate expected due to acetate and ethanol formation, combined with Equation 1 leads to Equation 2 with C_C (mol/L):

$$C_{H_2} = \left(C_{C_2H_5OH} + C_{CH_3COOH}\right) - C_{CH_2O_2} \qquad \text{(Eq. 2)}$$

For CO_2 calculations also the incorporation of CO_2 during the synthesis of succinate needs to be accounted for. Therefore Eq. 2 is expanded to Eq. 3 for calculated CO_2 concentrations (mol/L):

$$C_{CO_2} = \left(C_{C_2H_5OH} + C_{CH_3COOH}\right) - C_{CH_2O_2} - C_{C_4H_6O_4} \qquad \text{(Eq. 3)}$$

In batch reactor fermentations the off-gas composition was analyzed via online measurements via MS. Nitrogen, carbon dioxide, hydrogen, oxygen, ethanol and ethyl acetate fractions in the gas phase were considered and the cumulative amounts calculated as described in earlier research (Bohnenkamp *et al.*, 2020).

Carbon yields were estimated for all experiments according to Eq. 4 including glucose as substrate; ethyl acetate, ethanol, pyruvate, lactate, acetate, succinate, formate and CO_2 as products and biomass based on a conversion factor of 0.3232 from OD_{600} to g/L dry weight (Bohnenkamp *et al.*, 2020) and assuming a biomass composition of $CH_2O_{0.5}N_{0.2}$

$$Y_{Carbon} = \frac{\text{C} - \text{mol products formed}}{\text{C} - \text{mol glucose consumed}} \qquad \text{(Eq. 4)}$$

Volumetric productivities (Q_P) were calculated in mmol/L/h by taking the slope of a linear trendline including at least four data points. For ethyl acetate productivity only three data points could be included (Appendix).

Statistical significance was assessed by using a two-sided students t-test assuming equal variance and $p<0.05$.

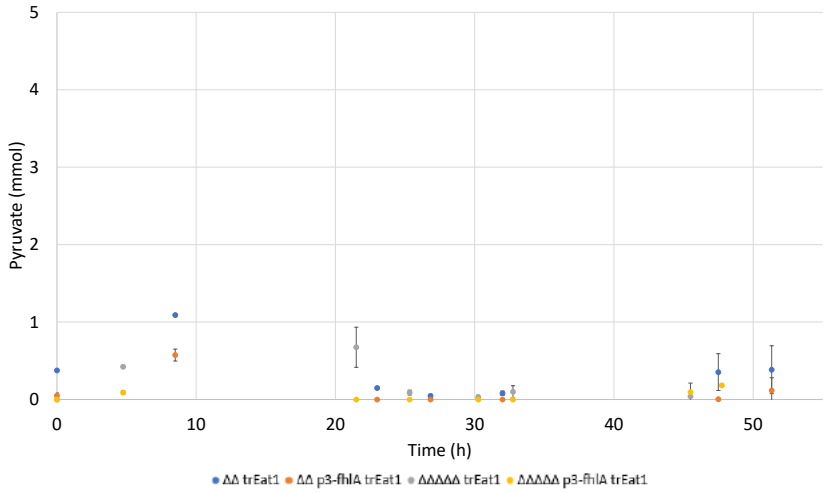
Analytics

Liquid samples, including 50 mM propionic acid as internal standard, were analyzed with respect to glucose and organic acids using an Agilent 1290 LC II system (Agilent, USA), with an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity Autosampler, Agilent 1290 Infinity diode array detector operated at 210 nm, and an Agilent 1260 Infinity RI detector operated at 45 °C (Bohnenkamp *et.al.*, 2020). The HPLC was operated with an Aminex HPX-97H (Bio-Rad, USA) column at 60°C and 0.008 mM H_2SO_4 as mobile phase at 0.8 mL/min as flow rate.

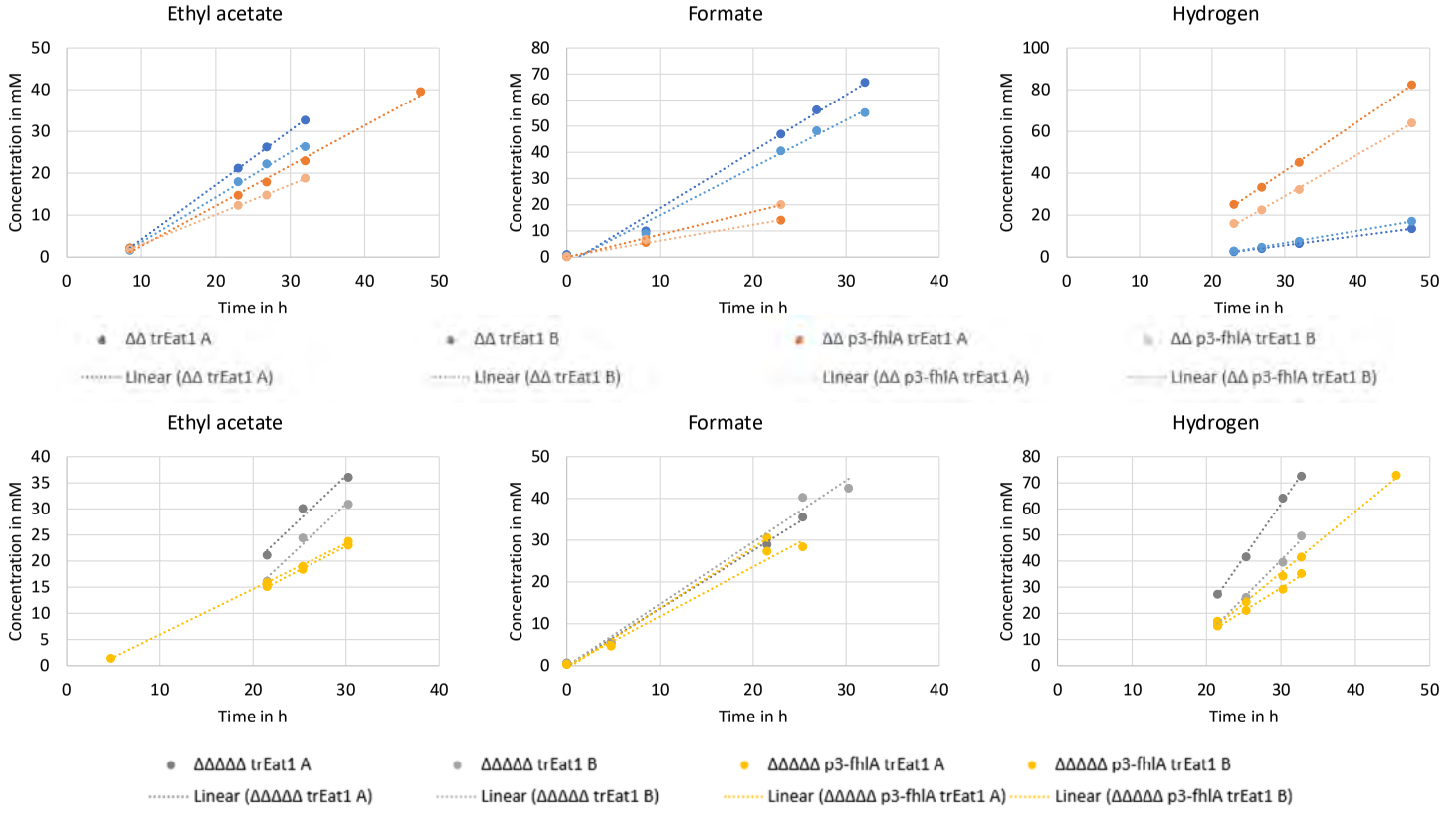
Analysis of ethanol and ethyl acetate in the liquid phase was carried out by an Agilent 7890B gas chromatograph (Agilent, USA) equipped with a flame ionization detector (GC-FID) and an Agilent 7693 autosampler. Samples were injected into a NukolTM column (30 m x 0.53 mm, 1.0 µm coating, Supelco, USA). Column temperature was maintained at 50 ˚C for 2 min, then increased to 200 ˚C at the rate of 50 ˚C/min, with a split ratio of 10. As internal standard 2 mM 1-butanol was added.

Online measurements of volatile compounds and gases removed from the reactor vessel by gas stripping were performed with an δB Process Mass Spectrometer (MS, Thermo Scientific™) (Bohnenkamp *et.al.*, 2020).

Supplementary Material



SUPPLEMENTARY FIGURE 1: Fermentation profile for pyruvate in pH-controlled bioreactors with continuous gas stripping. Strains based on *ΔldhAΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of *hycA, hyaAB* and *hybBC* (*ΔΔΔΔΔ*), overexpression of *fhlA* (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*) producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and cultivated under anaerobic conditions in minimal medium with 55 mM glucose as carbon source. Experiments were performed as biological duplicates; error bars represent the standard deviation. Circles – compounds in liquid broth.



SUPPLEMENTARY FIGURE 2: Product formation rates for strains co-producing ethyl acetate and hydrogen in pH-controlled reactors under anaerobic conditions. Rates are estimated by the slope of a linear trendline for cumulated product (mmol) per reactor volume (0.5 L) vs. time (h) to obtain rates in mmol/L/h. The rates and its corresponding R^2 value per replicate is listed by compound in Supplementary Table 2.

SUPPLEMENTARY TABLE 1: Product and carbon yield in C-mol$_{product}$/C-mol$_{glucose}$ for strains cultivated in pH-controlled bioreactors with constant gas stripping after glucose depletion. Strains based on *ΔldhAΔackA* (*ΔΔ*) with further modifications for improved hydrogen production, from left to right: inactivation of *hycA*, *hyaAB* and *hybBC* (*ΔΔΔΔΔ*), overexpression of *fhlA* (*ΔΔ p3-fhlA*) and a combination of knockouts and overexpression (*ΔΔΔΔΔ p3-fhlA*) producing trEat1 Wan N-13 were induced by 0.01 mM IPTG and cultivated under anaerobic conditions in minimal medium with 55 mM glucose as carbon source.

	ΔΔ trEat1	**ΔΔΔΔΔ trEat1**	**ΔΔ *p3-fhlA* trEat1**	**ΔΔΔΔΔ *p3-fhlA* trEat1**
Ethyl acetate	0.47 ± 0.02	0.42 ± 0.02	0.47 ± 0.04	0.43 ± 0.01
Ethanol	0.10 ± 0.01	0.06 ± 0.01	0.10 ± 0.00	0.07 ± 0.00
Pyruvate	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Acetate	0.08 ± 0.00	0.09 ± 0.00	0.08 ± 0.00	0.08 ± 0.00
Lactate	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Succinate	0.05 ± 0.01	0.10 ± 0.01	0.04 ± 0.00	0.05 ± 0.00
Formate	0.22 ± 0.01	0.10 ± 0.02	0.05 ± 0.01	0.09 ± 0.03
CO_2	0.07 ± 0.01	0.16 ± 0.04	0.24 ± 0.02	0.19 ± 0.02
Y_{Carbon}	1.00 ± 0.04	0.93 ± 0.05	0.98 ± 0.05	0.92 ± 0.01

SUPPLEMENTARY TABLE 2: Product formation rates and R^2 values of generated trendlines.

Compound		**Ethyl acetate**		**Formate**		**Hydrogen**	
Strain		**Rate (mmol/L/h)**	**R^2**	**Rate (mmol/L/h)**	**R^2**	**Rate (mmol/L/h)**	**R^2**
ΔΔ trEat1	A	1.3031	0.999	2.1632	0.9862	0.4527	0.9995
	B	1.0721	0.9961	1.8179	0.9876	0.5875	0.9980
ΔΔ *p3-fhlA* trEat1	A	0.9592	0.9955	0.6044	0.9994	2.3493	0.9996
	B	0.714	0.9996	0.8691	0.9983	1.9801	0.9988
ΔΔΔΔΔ trEat1	A	1.6875	0.9658	1.385	0.9971	4.1158	0.9975
	B	1.6687	0.9802	1.4792	0.9897	2.8801	0.9915
ΔΔΔΔΔ *p3-fhlA* trEat1	A	0.8703	0.9996	1.4444	0.9947	1.7516	0.9935
	B	0.9112	0.9993	1.1814	0.9890	2.3546	0.9964

SUPPLEMENTARY TABLE 3: Information on gRNA and homologous sequences used for creating the pTarget vectors for genomic knockouts and insertions as described in Materials and Methods. gRNA – guide RNA, USR – upstream homologous region, DSR – downstream homologous region

pTarget	Part	Sequence
hycA	gRNA	aagatggcgaagacaaacag
	USR	gcacaaaaaatgcttaaagctggcatctctgttaaacgggtaacctgaca
	DSR	gctgaggctttgcccgttttgcaggcgttacgcctgtttggggatgggcg
hyaAB	gRNA	tgaaattgtcaaaatccacg
	USR	cataagcgcccggtgtcctgccggtgtcgcaaggaggagagacgtgcgat
	DSR	cagcgaaggagaatcatcatgcaacagaaaagcgacaacgttgtcagcca
hybBC	gRNA	gccgcacattcagaacctgg
	USR	gctggttcgtcgcaacaccaaaaacgaccatcacgacggaggagacgatc
	DSR	atgcgtattttagtcttaggggtcggcaatattttgctgaccgatgaagc
P3-insertion	gRNA	tgacctttgcaccgctttg
	USR	gtatatgctaataaaattctaaatctcctatagttagtcaatgacctttt
	insertion	agtctcaagcaccgctttgcggtgctttccaaaaaatttatttgcttattaatcatccggctcgtataatgtgtggaattc gaaagataaggaggtagcaca
	DSR	atgtcatatacaccgatgagtgatctcggacaacaagggttgttcgacat

Chapter 6

Ethyl acetate production near the theoretical maximum yield: Solutions and challenges

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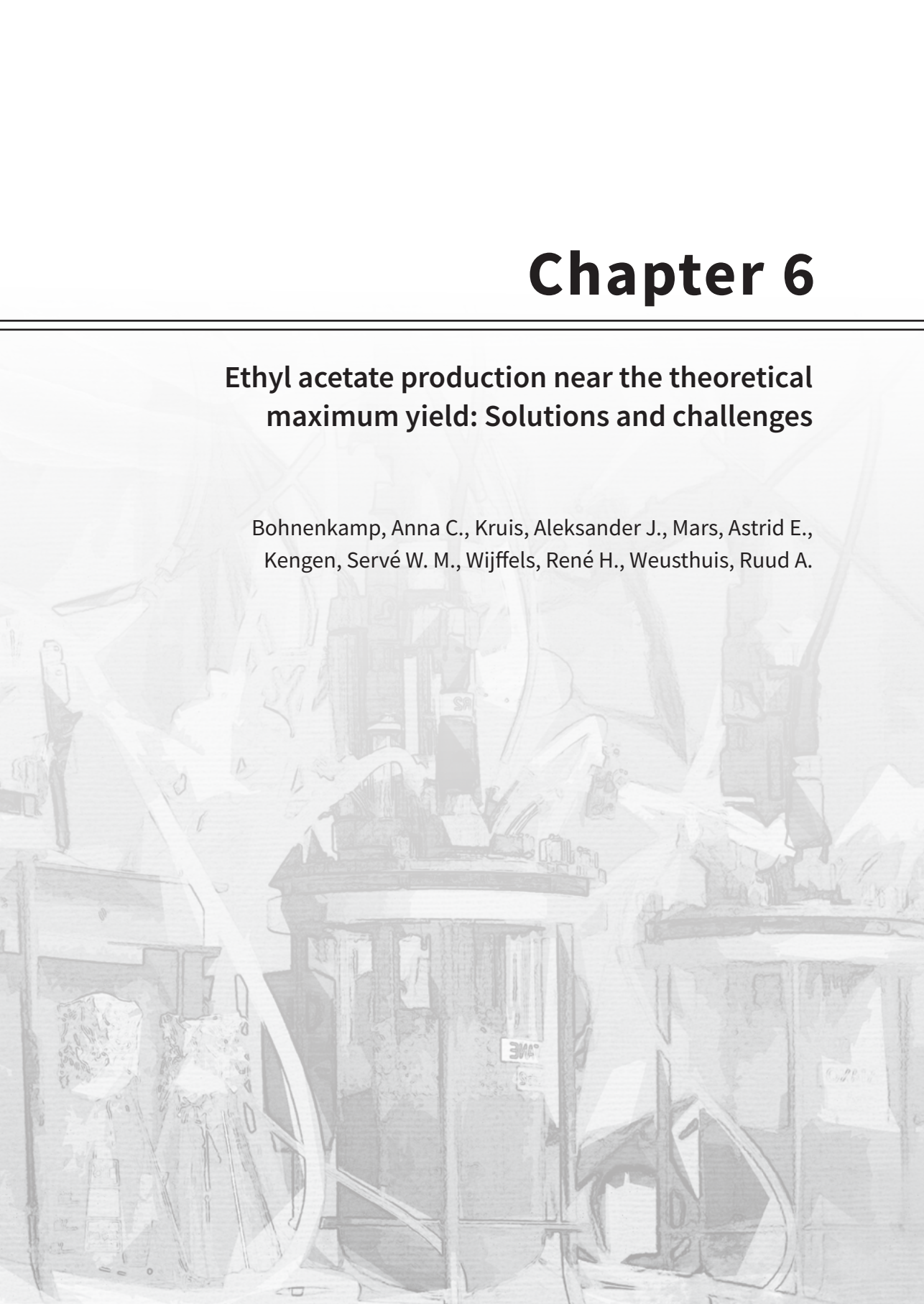


Abstract

The sustainable production of bulk chemicals and other industrially relevant compounds gained attention throughout the years. In order to compete with existing processes, bio-based production needs to proceed at high efficiency.

The product yield on substrate is one common indicator for efficiency and should be as high as possible. The maximum theoretical yield is dictated by the degree of reduction and is reached once all electrons are directed to product formation in the most effective way. However, in a physiological context the maximum theoretical yield is barely reached. In the present study, we engineered the central *E. coli* metabolism to enable ethyl acetate production at the maximum theoretical yield and discussed remaining challenges of the approach.

Using three metabolic modules, we conserved electrons and carbons and ensured a redox balanced pathway for anaerobic ethyl acetate production. Moreover, we explored the applicability of the approach using xylose and glucose as substrate. Phosphoketolase was employed to conserve electrons and carbons and carried a good flux during anaerobic growth on xylose. Pyruvate decarboxylase restricted the loss of electrons to formate and was crucial for redox balanced production of ethanol. Ethyl acetate production relied on functional heterologous expression of ethanol acetyltransferase 1, condensing ethanol and acetyl-CoA. Whereas the functionality of all separate modules was successfully demonstrated, their concerted action did not lead to the expected increase in product yield. Possible bottlenecks and remaining challenges were discussed, which may also be of value for improving the yield of other industrial compounds.

Keywords: ethyl acetate, theoretical maximum yield, bifido shunt, phosphoketolase, fermentation, *Escherichia coli.*

Introduction

Ethyl acetate is a short carboxylate ester and bulk chemical, used as industrial solvent in paints, adhesives, and various other applications. To date, production mainly relies on the petroleum-based Fischer Speier esterification, requiring high temperatures and pressures (Fischer and Speier, 1895). As the process is based on an equilibrium reaction, no complete conversion can be achieved and continuous water removal is required to prevent inhibition of the acid catalyst, adding another energy intensive step to the process (Liu, Lotero and Goodwin, 2006). In the past years progress towards bio-based ester and – in particular - ethyl acetate production, has been made (Mason and Dufour, 2000; Löser, Urit and Bley, 2014; Kruis *et al.*, 2019). In order to compete with conventional processes several factors in a biobased process have to be considered on an overall level, including cultivation conditions, feedstocks, complexity of downstream processing (Van Dien, 2013), but also on a cell level. Yields should be increased, a sufficient tolerance against the product should exist, and formation of by-products should be minimized (Ezeji, Qureshi and Blaschek, 2007; Weusthuis *et al.*, 2011).

In an earlier study we discovered a family of alcohol acetyltransferases (AAT) responsible for bulk ethyl acetate production in yeast (Kruis *et al.*, 2017). The mitochondrial enzyme ethanol acetyltransferase (Eat1) condenses ethanol and acetyl-CoA into ethyl acetate (Kruis, Mars, *et al.*, 2018). In addition to its AAT activity, Eat1 exhibits esterase and thioesterase side-activities, as well strong alcoholysis and thiolysis activities (Patinios *et al.*, 2020). By removing the signaling peptide responsible for targeting to the mitochondrion we improved the enzyme's stability and expression in *Escherichia coli* (Kruis *et al.*, 2020). Insertion of the truncated genes (*trEat1*) in *E. coli* and subsequent optimization of the expression enabled a 72 % maximum pathway yield of 1 mol/mol based on glucose as carbon source under anaerobic conditions. The maximum theoretical yield however, calculated based on the degree of reduction of 24 for glucose and 20 for ethyl acetate is 1.2 mol/mol. The associated product equations (Equations 1 and 2) result in Gibbs free energy of the reaction under standard conditions ($\Delta G_r'^0$) of -201.7 ± 7.8 kJ/$mol_{ethyl\ acetate}$ for a pathway yield of 1 mol/mol, and -207.3 3 ± 5.9 kJ/$mol_{ethyl\ acetate}$ for reaching the maximum theoretical yield, using glucose as carbon source, respectively. This indicates thermodynamic feasibility for both cases.

$$C_6H_{12}O_6(aq) = C_4H_8O_2(aq) + 2CH_2O_2(aq) \quad \text{(Eq. 1)}$$

$$5C_6H_{12}O_6(aq) = 6C_4H_8O_2(aq) + 6CO_2(g) + 6H_2O(l) \quad \text{(Eq. 2)}$$

Despite the fact that glucose plays a central role in early research, it is not the only suitable carbon source for production of bulk chemicals. Generally 2nd generation feedstocks, such as lignocellulosic or other waste streams are preferred (Tuck, 2012). C5 sugars such as

xylose and arabinose make up significant parts of lignocellulosic material and engineering efforts have been done which focused on the efficient utilization of xylose by yeasts and bacteria for chemicals production (Sonderegger, Schümperli and Sauer, 2004; Van Maris *et al.*, 2007; Zhou, Iverson and Grayburn, 2008). Utilizing the rearrangement reactions of the pentose phosphate pathway (PPP) *E. coli* generates five moles of glyceraldehyde-3-phosphate (GAP) from three moles of xylose, enabling the redox balanced production of two molecules of ethyl acetate, together with formate and lactate, leading to a pathway yield of 0.83 $mol_{ethyl\ acetate}/mol_{xylose}$ (Figure 1a). However, based on the degree of reduction a maximum theoretical yield of 1 $mol_{ethyl\ acetate}/mol_{xylose}$ can be achieved. Similarly to the glucose case, both reaction equations lead to a negative $\Delta G_r'^0$ values, -217.0 ± 8.7 kJ/ $mol_{ethyl\ acetate}$ and -220.0 ± 6.7 kJ/ $mol_{ethyl\ acetate}$ respectively, indicating thermodynamic feasibility (Equation 3 and 4).

$$6C_5H_{10}O_5(aq) = 5C_4H_8O_2(aq) + 10CH_2O_2(aq) \quad \text{(Eq. 3)}$$

$$C_5H_{10}O_5(aq) = C_4H_8O_2(aq) + CO_2(g) \quad \text{(Eq. 4)}$$

Valuable electrons and carbons are lost in the conversion of pyruvate into acetyl-CoA. Anaerobic conversion catalyzed by pyruvate formate lyase (Pfl) co-produces formate, but also the aerobic conversion via pyruvate dehydrogenase (Pdh) complex generates CO_2 and NADH. Such carbon and electron losses need to be lowered and an alternative pathway at least partially by-passing the native pyruvate to acetyl-CoA conversion is required to approach maximum yields. In recent years the use of phosphoketolases (Pkt) to increase product yields of acetyl-CoA derived products has received much attention. Phosphoketolases are known to either break down fructose-6-phosphate (F6P) into acetyl-P and erythrose-4-phosphate (E4P) or to exhibit additional activity for xylulose-5-phosphate (Xu5P) which is converted into acetyl-P and GAP (Meile *et al.*, 2001). They are the key enzymes in the heterofermentative phosphoketolase pathway and the bifido shunt used by several lactic acid bacteria, bifidobacteria and clostridia (Yin *et al.*, 2005; Liu *et al.*, 2012; Folch, Bisschops and Weusthuis, 2021). Generated acetyl-P can be transformed into acetyl-CoA via reverse activity of phosphate acetyltransferase (Pta) ($\Delta G_r'^0$ = -8.7 kJ/ mol) thereby offering an alternative to the conventional conversion of pyruvate into acetyl-CoA at the expense of one carbon atom (Figure 1a). Various Pkt's have been heterologously expressed in yeasts to enhance acetyl-CoA pools for production of PHB, fatty acid ethyl esters, propionic acid and other acetyl-CoA derived products (Sonderegger, Schümperli and Sauer, 2004; de Jong *et al.*, 2014; Bergman *et al.*, 2016; Qin *et al.*, 2020). The functional implementation of *Bifidobacterium adolescentis* phosphoketolase in *E. coli* has also been shown (Bogorad, Lin and Liao, 2013; Krüsemann *et al.*, 2018). Strains solely relying on Pkt activity for catabolism, can convert one glucose molecule into three acetyl-P molecules via the synthetic non-oxidative-glycolysis (NOG) pathway thereby enabling near to complete carbon conservation (Bogorad, Lin and Liao, 2013; Lin *et al.*, 2018).

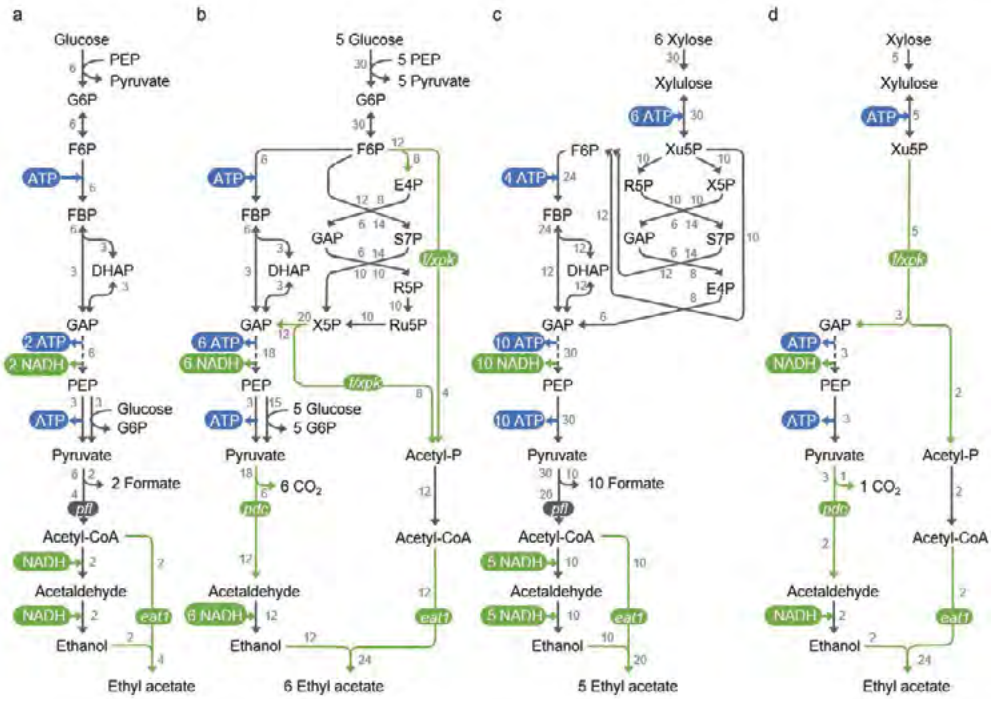


FIGURE 1: Schematic representations of anaerobic ethyl acetate production pathways based on AAT conversion of ethanol and acetyl-CoA into ethyl acetate. (A) Production on glucose with a maximum pathway yield of 1 mol/mol. (B) Production on glucose with a maximum pathway yield of 1.2 mol/mol. (C) Production on xylose with a maximum pathway yield of 0.83 mol/mol. (D) Production on xylose with a maximum pathway yield of 1mol/mol. Strategies B and D equal the theoretical maximum yield and rely on carbon conservation via Pkt and redox balancing via Pdc.

To establish ethyl acetate production at high yields in *E. coli* we chose a *Bifidobacterium breve* phosphoketolase (*f/xpk*) with dual specificity for Xu5P and F6P substrates for carbon conservation (Suzuki *et al.*, 2010; Bergman *et al.*, 2016). As the subsequent conversion of acetyl-P into acetyl-CoA by native Pta appeared to be a critical step (Sonderegger, Schümperli and Sauer, 2004; de Jong *et al.*, 2014; Bergman *et al.*, 2019), we co-expressed f/*xpk* always with *pta*.

For ethyl acetate synthesis, next to acetyl-CoA also an efficient ethanol supply needs to be established. *E. coli* uses pyruvate formate lyase (Pfl) to produce ethanol, coproducing one formate. However, formate coproduction has to be reduced as it makes it impossible to reach the maximum theoretical yield. (Figure 1 ab). One successful way of ethanol production without formate coproduction exploits *Zymomonas mobilis'* pyruvate decarboxylase (Pdc) (Bräu and Sahm, 1986; Ingram *et al.*, 1987; Ingram and Conway, 1988; Ohta *et al.*, 1991). By converting pyruvate into acetaldehyde and releasing CO_2, the overall need of NADH for ethanol production is reduced, thus ethanol production as sole

fermentation product from GAP is enabled. As indicated by the reaction equation above, some CO_2 loss cannot be prevented. To ensure that the proposed pathway is used, native Pfl will be inactivated.

Here, we describe the modular generation of an engineered *E. coli* strain theoretically able to produce ethyl acetate close to the maximum theoretical yield. The different hurdles that we encountered are presented and discussed.

Results

In order to achieve the desired strain able to produce ethyl acetate close to the theoretical maximum yield, modifications were gradually combined. By creating modules effects of Pkt, Pdc, Pdc combined with inactivation of Pfl and Eat1 were evaluated individually and in combination with others. Both D-xylose and glucose were used as carbon and energy source.

Using xylose as substrate

To test the functionality of heterologous *B. breve* phosphoketolase a strain with a D-xylose growth deficit was generated by knocking out ribulose-phosphate-3-epimerase (*rpe).* Disabling the interconversion of D-xylulose-5-phosphate (Xu5P) and D-ribulose-5-phosphate (Ru5P) prevents utilization of the non-oxidative branch of the pentose phosphate pathway, rendering growth on D-xylose impossible. By co-expressing the *f/xpk* and *pta* operon growth can be restored as D-xylulose-5-phosphate (Xu5P) can be converted into acetyl-CoA and GAP and subsequently converted into other metabolites (Lyngstadaas, Sprenger and Boye, 1998).

Introduction of the phosphoketolase module

An inducible *XylS/Pm* promoter system as well as constitutive expression via the *E. coli pgi* promoter were assessed on their ability to restore growth under anaerobic conditions. The WT control strain was readily able to utilize D-xylose as sole carbon source and significant increases in OD_{600} could be observed within 24 h under the tested conditions (Figure 2a). In accordance, no growth could be observed for the *rpe* deficient control strain *E. coli BW25113 Δrpe pCDF:EV* (Figure 2b).

The strain expressing the inducible system required a longer period to start growing, as no significant change in OD_{600} could be observed within the first 24 h (Figure 2c). Growth of *E. coli* BW25113 *Δrpe* pCDF:*XylS/Pm-f/xpk Bbr-pta Eco* was delayed and reached only one third of the WT OD_{600} increase after 73 hours of anaerobic cultivation, respectively. In contrast, strain *E. coli* BW25113 *Δrpe* pCDF:*pgi-f/xpk Bbr-pta Eco* could compete with the WT control, showing comparable values, if not exceeding the OD_{600} change of the wild type *E. coli* BW25113 carrying an empty vector control (Figure 2d).

After validation of the basic functionality of the heterologous phosphoketolase a closer look on its effect on anaerobic metabolism was taken. Due to the higher biomass production only the constitutive expression system pCDF:*pgi-f/xpk Bbr-pta Eco* (f/xpk-pta) was evaluated further.

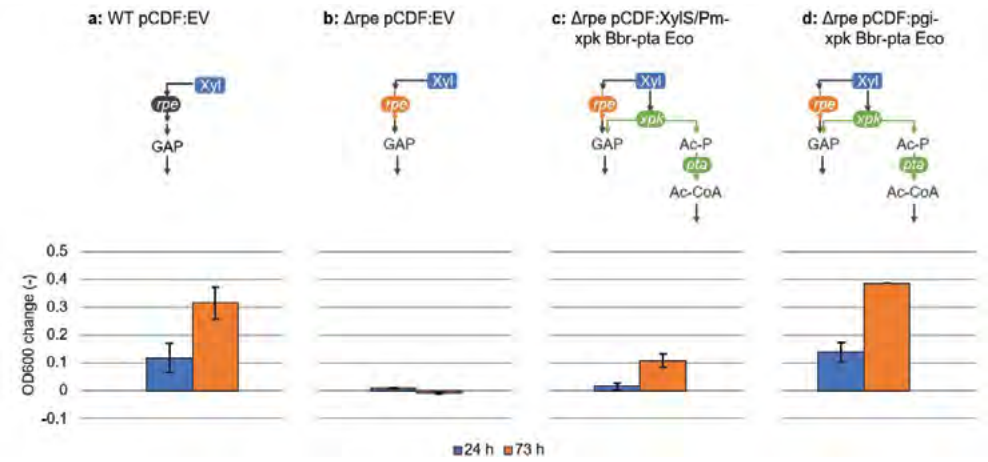


FIGURE 2: Growth restoration of *E. coli* BW25113 *Δrpe* on D-xylose by co-expression of the phosphoketolase of *Bacillus breve* and *E. coli's* phosphate acetyltransferase. The cultures were inoculated at an initial OD_{600} of 0.2 and OD_{600} increase after 24 h and 73 h is shown. WT BW25113 *(A)* and *E. coli* BW25113 *Δrpe* (B) were included as positive and negative controls. Performance of an inducible expression system (C), induced with 0.05 mM m-toluate, and a constitutive expression system (D) for heterologous *B. breve* phosphoketolase were evaluated under anaerobic conditions.

Considering growth on D-xylose *E. coli* BW25113 utilizes the rearrangement reactions of the pentose phosphate pathway to convert 3 mol xylose into 5 mol GAP which can be further converted into fermentation products or building blocks for biomass (Figure 3a). During the fermentation time of 96 h the WT control consumed 64 % of the provided D-xylose, grew until an OD_{600} of 0.65 and converted D-xylose into a mix of ethanol, acetate, lactate and formate (Figure 3a). According to expectations, to realize redox balance, similar concentrations of ethanol and acetate were obtained, about 25 mM respectively, whereas the lactate concentration reached 12 mM. As a consequence of PFL activity, co-producing formate with every ethanol and acetate, formate concentrations were highest among fermentation products, reaching a final titer of 55 mM.

As observed before, strain *BW25113 Δrpe* did not support growth on D-xylose. Accordingly, neither significant D-xylose consumption nor product formation could be observed (Appendix 1). Co-expression of *f/xpk* and *pta* enabled the utilization of the bifido shunt to convert Xu5P into acetyl-CoA and GAP (Figure 3b). Growth was restored and the final OD_{600} levelled around 0.65, similar to the WT strain, while about 10 % more D-xylose was consumed with respect to the WT (Figure 3a). Regarding the fermentation profile, acetate

titers were highest for *E. coli* BW25113 *Δrpe* f/xpk-pta, with 50 mM acetate (Figure 3b). This, as a consequence of the fixed split ratio of the phosphoketolase converting every mol of D-xylose into GAP and acetyl-P, the latter being easily converted into acetate. Lactate and ethanol concentrations reached similar levels around 20 mM, while formate concentrations dropped to around 30 mM compared to more than 50 mM for the WT control.

Introduction of the Pdc module

In order to conserve electrons from D-xylose to produce ethyl acetate or its precursors we chose to provide heterologous Pdc as alternative to the anaerobically active Pfl in a BW25113 *Δrpe* f/xpk-pta background strain (Figure 3c). Based on the fixed split ratio 1 mole acetate can be co-produced with either 1 mole lactate or 1 mole ethanol using the provided Pdc solution. The Pfl based co-production of 1 mole ethanol, together with 3 moles acetate and 2 moles formate, as described above, is also still possible. A severe growth impact was noted as BW25113 *ΔrpeΔfocA::pdc Zmob* f/xpk-pta only grew until an OD_{600} of 0.35 within 96 h consuming only 33 % of the D-xylose (Figure 3c). Acetate accounted for most of the products, but this time only reaching titers of 30 mM. A total of 10 mM ethanol was produced. Based on 100 % Pfl activity twice as much formate should be expected. A final concentration of 10 mM formate thus indicated that 5 mM ethanol was produced via Pfl. Consequently, Pdc was active and was the preferred conversion route in the production of the remaining 5 mM of ethanol.

Inactivation of *pfl*, thus making Pkt the sole provider of acetyl-P and subsequently acetyl-CoA, further reduced the amount of D-xylose taken up by strain BW25113 *ΔrpeΔfocA-pflAB::pdc Zmob* f/xpk-pta to 14 mM within 96 h (Figure 3d). Formate did not accumulate, showing that the *pfl* knockout was successful. After a lag phase of 48 h the OD_{600} did increase by 0.1 indicating that new biomass could be formed, based on the acetyl-CoA provided by f/xpk-pta. At 48 h cells co-produced equimolar amounts of acetate and lactate (Figure 3d). Only at later stages of the fermentation ethanol also accumulated reaching a final concentration of 5 mM. After 96 h of fermentation acetate again accounted for about 50 % of the products formed with a final concentration of 12 mM, followed by almost equimolar amounts of lactate and ethanol around 6 mM each, at the end of fermentation, indicating a shift from lactate to ethanol production via Pdc over time.

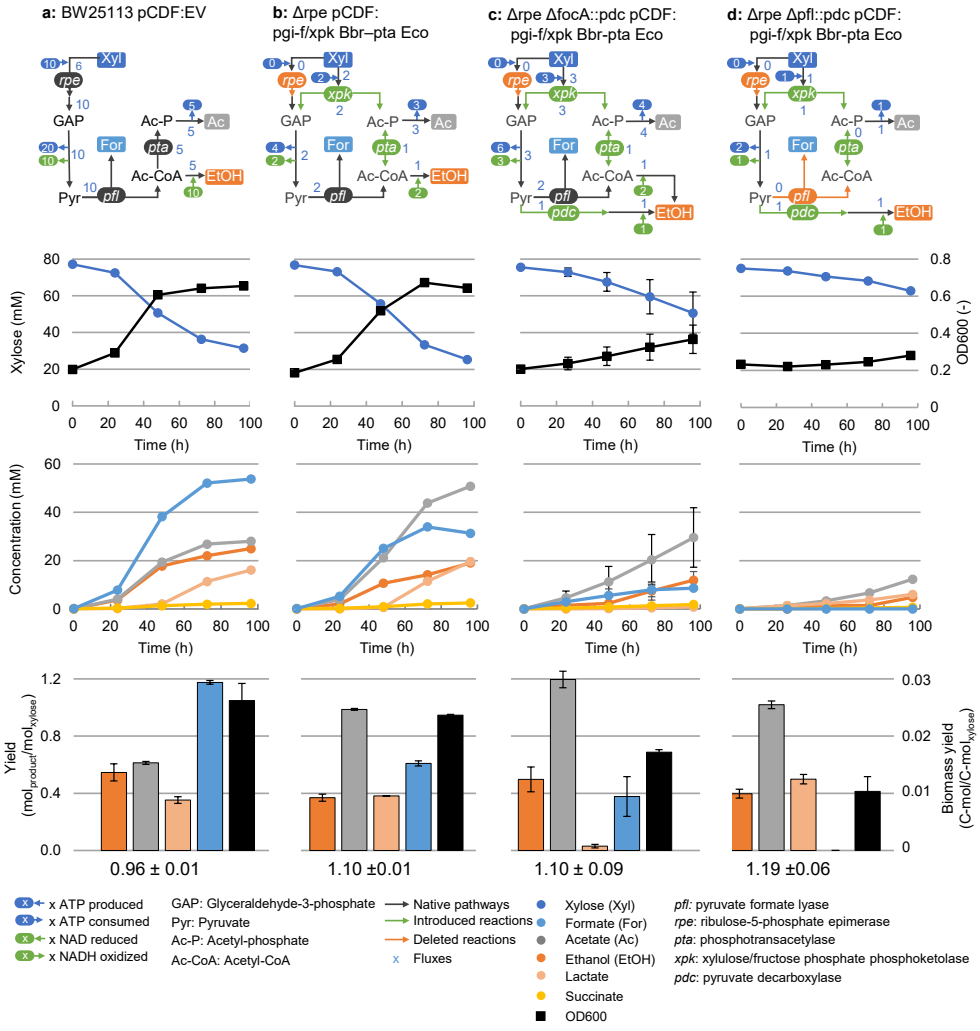


FIGURE 3: Effect of phosphoketolase and pyruvate decarboxylase by-pass in a *Δrpe* background strain. From A to D: WT, *Δrpe* f/xpk-pta, *ΔrpeΔfocA::pdc* f/xpk-pta *and ΔrpeΔfocA-pflAB::pdc* f/xpk-pta. Cultivation under anaerobic conditions with D-xylose as carbon source. From top to bottom: Schematic representation of the strain, biomass formation and glucose consumption over time, product formation over time, product yield (mol/mol) and biomass yield (C-mol/C-mol) on xylose after 96 h and the corresponding Y_{carbon} per strain. Data represent averages and standard deviations of biological duplicates.

Product yields supported the observations based on titers (Figure 3). The WT produced equal amounts of ethanol and acetate, and formate equaled the sum of both, as expected (Figure 3a). Inactivation of *rpe* and introduction of Pkt increased the amount of acetate formed and reduced the flux over Pfl, as witnessed by the decreased formate titers. Formate yield decreased by another 20 % once *pdc* was inserted to offer a by-pass to *pfl.* Ethanol yields for strain BW25113 *ΔrpeΔfocA::pdc Zmob* f/xpk-pta increased by 34 % with

respect to its parental strain BW25113 *Δrpe* f/xpk-pta, but not to a statistically significant extent. Lactate yields decreased from almost 0.4 $mol_{lactate}/mol_{xylose}$ by a factor ten to 0.03 $mol_{lactate}/mol_{xylose}$ indicating that most flux was directed towards ethanol. The last strain included inactivation of *pflAB*, thus an alternative to the heterologous Pdc was no longer available. No significant increase in ethanol yield could be observed. Instead, lactate yields increased with respect to the parental strain, to 0.5 ± 0.03 $mol_{lactate}/mol_{xylose}$ ($p<0.05$). This may be an indication for a bottleneck in Pdc. Increasing fluxes over Pdc results in lower fluxes to acetate and consequently reduce ATP formation. This could explain – at least for a part – why the expression of Pdc results in lower biomass yields.

The Eat1 module

Strain BW25113 *Δrpe* was used as a background strain to ensure the use of the employed phosphoketolase during ethyl acetate producing conditions. Expression of trEat1 was achieved via a plasmid with inducible LacI/*T7* promoter. Sequential modifications should lead to ethyl acetate production from D-xylose close to the maximum theoretical yield. A WT strain expressing pET26b:LacI/*T7-trEat1 Wan N-13* was used as control.

The WT control strain BW25113 expressing pET26b:LacI/*T7-trEat1 Wan N-13* and strain *BW25113 Δrpe pCDF:pgi-f/xpk Bbr-pta Eco pET26b:*LacI/*T7-trEat1 Wan N-13* were showing a similar growth profile. After about 70 h growth declined and the OD_{600} for both strains reached a plateau (Figure 4a). Slightly more than 50 % of D-xylose was consumed within the measured time frame without significant differences in the consumption rates of both strains (Figure 4a).

Significant ethyl acetate production was only observed for the WT strain, where a total of 9.01 ± 0.03 mM ethyl acetate accumulated (Figure 4a). According to the expected stoichiometry a mixture of ethanol, acetate, lactate and formate was co-produced. The resulting high flux through native Pfl led to significant amounts of formate being formed, accumulating up to 53.78 ± 1.04 mM respectively.

Despite the competitive growth and substrate consumption, strain BW25113 *Δrpe pCDF:pgi-f/xpk Bbr-pta Eco pET26b:*LacI/*T7-trEat1 Wan N-13* did not produce any ethyl acetate (Figure 4b). The main fermentation products were ethanol, acetate and lactate, with again formate as inevitable by-product. Nevertheless, catalytic activity of Pkt reduced the flux through Pfl leading to a reduced final titer for formate to 44.8 ± 0.7 mM, while increasing acetate levels to 32.2 ± 0.4 mM. Ethanol titers reached 22.6 ± 0.3 mM at the end of the fermentation (Figure 4b).

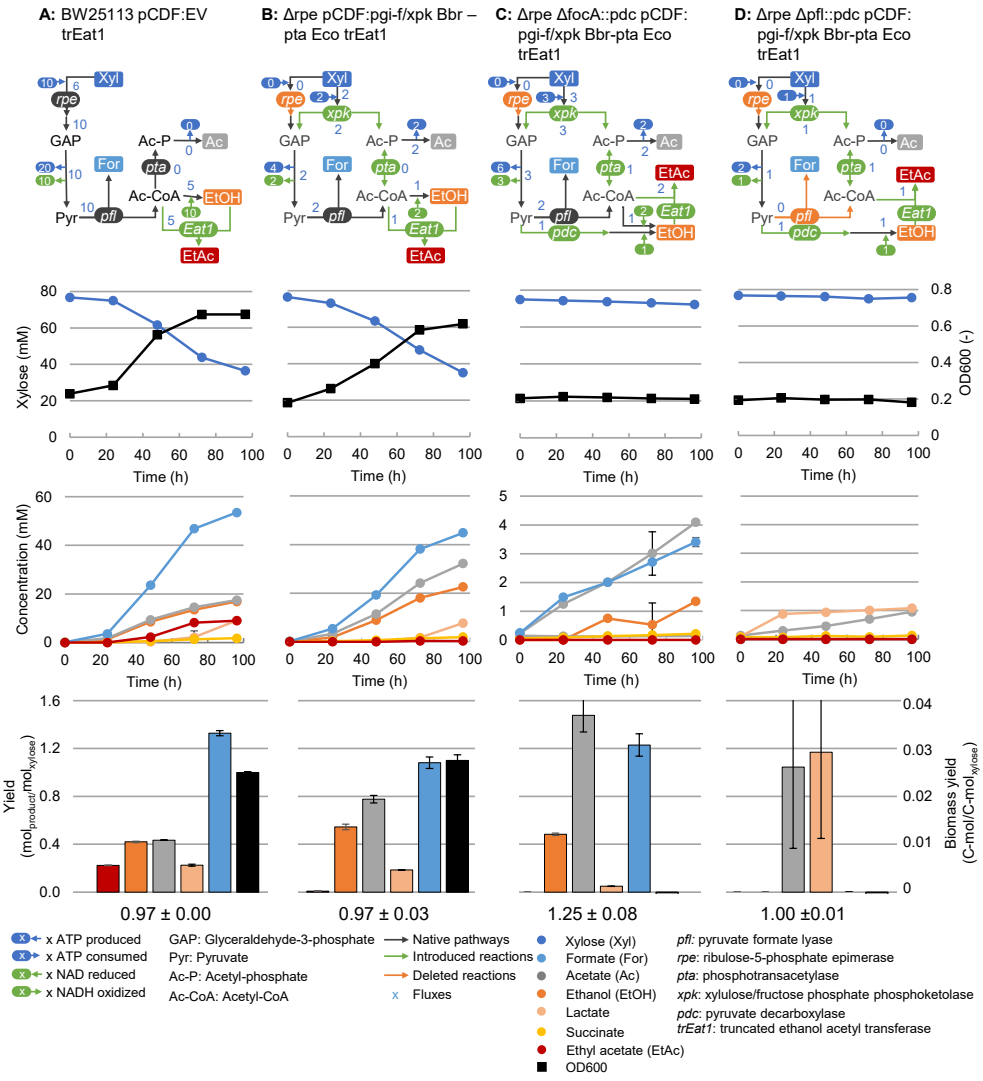


FIGURE 4: Effect of trEat1 expression in strains increasingly relying on phosphoketolase and pyruvate decarboxylase in a *Δrpe* background strain. From A to D: WT *trEat1*, *Δrpe* f/xpk-pta trEat1, *ΔrpeΔfocA::pdc Zmob* f/xpk-pta trEat1 and *ΔrpeΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1. From top to bottom: Schematic representation of the strains, biomass formation and glucose consumption over time, product formation over time, product yield and biomass yield (C-mol/C-mol) on xylose after 96 h and the corresponding Y_{carbon} per strain. Data represent averages and standard deviations of biological duplicates. Cultivation under anaerobic conditions with D-xylose as carbon source. Error bars represent standard deviations among duplicates.

Combined expression of *pdc* and *eat1*, led to growth impairment and an extremely slowed down metabolism (Figure 4c). Less than 3 mM D-xylose was consumed by strains expressing the heterologous Pdc and trEat1. In line with the limited D-xylose uptake, only little product formation could be observed. Acetate levels reached 4 mM, while almost

1.5 mM ethanol was formed in strain BW25113 *ΔrpeΔfocA::pdc Zmob* f/xpk-pta trEat1. Accumulation of formate to 3.4 ± 0.2 mM indicated the primary use of native PFL. Once this option was eliminated in BW25113 *ΔrpeΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 about 1 mM acetate formation was accompanied with equimolar concentrations of lactate (Figure 4d).

The control strain produced ethyl acetate at a product yield of 0.22 ± 0.01 $mol_{ethyl\ acetate}$/mol_{xylose} corresponding to about 30 % of the pathway maximum. A yield of 0.4 mol/mol_{xylose} for ethanol and acetate indicated where carbons are lost (Figure 4a). The highest product yield was obtained for formate, reaching 1.3 $mol_{formate}$/mol_{xylose}. In contrast to fermentations without trEat1, formate levels did not decrease as pronounced as observed before. Moreover, the co-expression of Pdc and Pfl in strain BW25113 *ΔrpeΔfocA::pdc Zmob* f/xpk-pta trEat1 did not seem to have much effect on product yields when compared to its parent BW25113 *Δrpe* f/xpk-pta trEat1. The comparable yields are likely a consequence of the slow metabolism and reduced xylose consumption, as also for BW25113 *ΔrpeΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 high standard deviations were obtained.

Using glucose as carbon source

During anaerobic growth on glucose *E. coli* performs mixed acid fermentation. Especially, the formation of lactate and acetate deviate carbons from our product of interest, ethyl acetate. Therefore, the effect of PDC to enable ethanol formation as main fermentation product was studied in *ΔldhAΔackA* background strains.

Insertion of the Pdc module

The streamlined strain BW25113 *ΔldhAΔackA* grew until an OD_{600} of almost 0.6 within 24 h. The strain settled around this value at the end of fermentation, likely due to product inhibition (Figure 5a). Similarly, glucose uptake slowed down and the glucose concentration approached 29 mM, with a total of 26.0 ± 0.7 mM glucose being consumed over the time course of 95 h. Ethanol and formate were co-produced reaching titers of 22.6 ± 2.3 mM and 27.7 ± 0.3 mM respectively. Due to the blocked acetate formation, pyruvate was secreted up to concentrations of 16.7 ± 0.2 mM, to balance NADH requirements.

Once Pdc was introduced as an additional way to convert pyruvate and enabling redox balanced ethanol production, glucose was fully depleted towards the end of the experiment (Figure 5b). Biomass was formed rapidly within the first 24 h and reached an OD_{600} of 1.1, 40 % higher than obtained by the background strain. Again the OD_{600} stabilized and remained unchanged for the remaining fermentation. Ethanol production increased almost 4-fold, by reaching a final concentration of 76.4 ± 2.4 mM. The insertion of *pdc* enabled redox balanced ethanol production from glucose by by-passing Pfl.

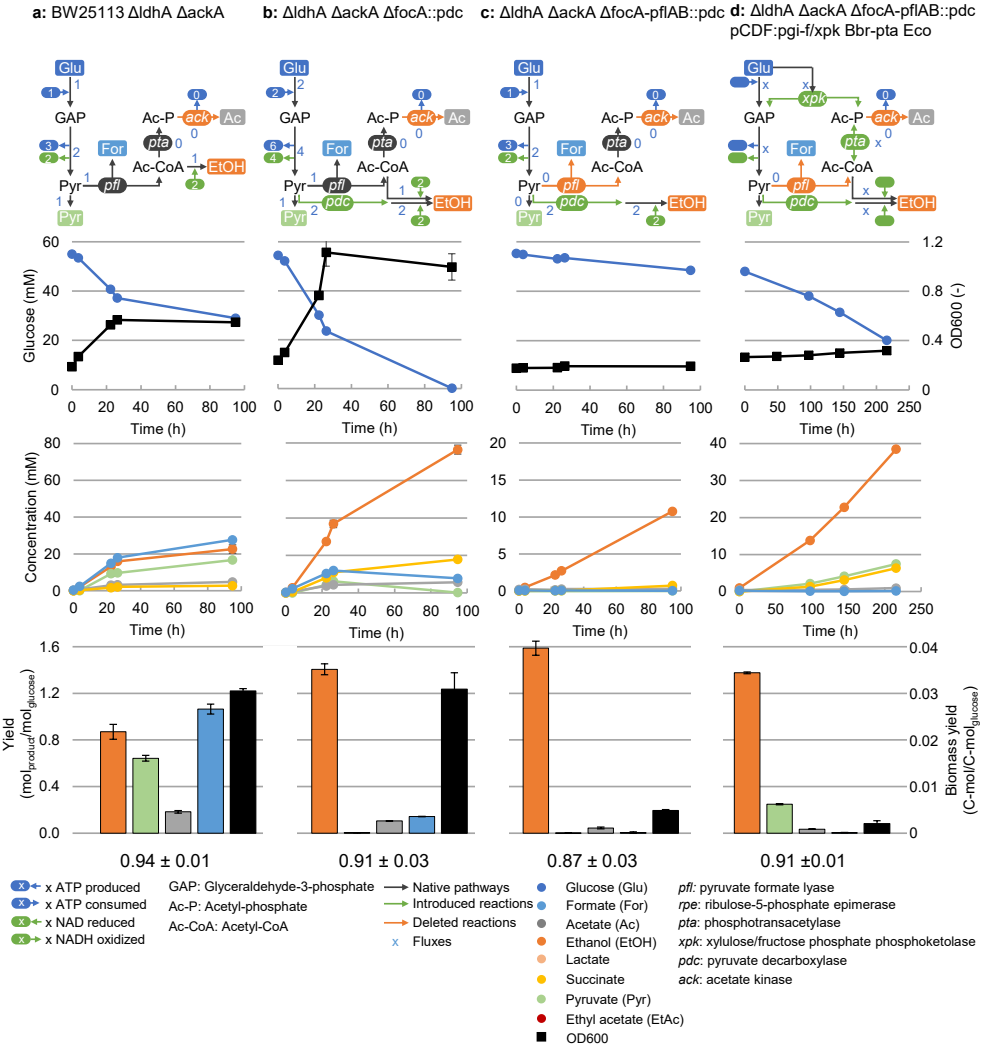


FIGURE 5: Effect of pyruvate decarboxylase in a *ΔldhAΔackA* background strain on ethanol production. Cultivation under anaerobic conditions with 55 mM D-glucose as carbon source. From A to D: *E. coli ΔldhAΔackA*, *E. coli ΔldhAΔackAΔfocA::pdc Zmob*, *E. coli ΔldhAΔackAΔfocA-pflAB::pdc Zmob* and *E. coli ΔldhAΔackAΔfocA-pflAB::pdc Zmob pCDF:pgi-f/xpk Bbr-pta Eco*. From top to bottom: Schematic representation of each strain, OD_{600} and glucose concentration over time, product formation over time, product yields (mol/mol) and biomass yield (C-mol/C-mol) on glucose after 96 h of cultivation and corresponding Y_{carbon} per strain. Error bars represent standard deviations among duplicates.

As a consequence, less formate was co-produced, due to the release of CO_2. Succinate accumulated to concentrations of 17.9 ± 0.1 mM, a concentration significantly higher compared to the other strains. This is most likely caused by the higher availability of CO_2, required in the pathway to succinate by the enzyme pyruvate carboxylase.

Insertion of Pdc with simultaneous inactivation of *pflAB*, however, severely impaired growth as observed for strain BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc* (Figure 5c). A non-significant change in OD_{600} was accompanied with a decrease of 6.79 ± 0.34 mM in glucose concentration during the entire fermentation time of 95 h. As observed during growth on D-xylose, inactivation of Pfl affects availability of acetyl-CoA, thus restricting the generation and use of building blocks for biomass formation. Nevertheless, the strain was metabolically active and produced almost solely ethanol as fermentation product, with final levels around 10.76 ± 0.13 mM respectively.

Consequently, highest ethanol yields on glucose were obtained by BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc,* followed by the intermediate strain expressing both heterologous *pdc* and native *pfl* genes, and the streamlined strain BW25113 *ΔldhAΔackA* (Figure 5). The latter produced ethanol at a product yield of 0.9 ± 0.1 $mol_{ethanol}/mol_{glucose}$, corresponding to 86 % of the maximum pathway yield of 1 $mol_{ethanol}/mol_{glucose}$. Flux through Pfl requires redox balancing by acetate, or in case of an *ackA* knockout, pyruvate secretion. Therefore, also the cumulative yields of pyruvate (0.64 $mol_{pyruvate}/mol_{glucose}$) and acetate (0.18 $mol_{acetate}/mol_{glucose}$) approached the value of 0.86. Availability of a redox balanced pathway towards ethanol as provided by Pdc decreased product yields of both, pyruvate and acetate, by 100 % and 50 % respectively. Ethanol yields exceeding the natural pathway yield indicated activity of Pdc, accompanied by a simultaneous decrease in formate yields. BW25113 *ΔldhAΔackAΔfocA::pdc,* had a 90 % reduction in formate yields and obtained an ethanol yield of 1.41 ± 0.05 $mol_{ethanol}/mol_{glucose}$. Despite poor growth, BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc* converted glucose into ethanol in the most efficient way. A product yield of 1.59 ± 0.06 $mol_{ethanol}/mol_{glucose}$ corresponded to 80 % of the maximum theoretical yield of 2 $mol_{ethanol}/mol_{glucose}$.

Insertion of the phosphoketolase module

The lack of growth was thought to be related to the lack of acetyl-CoA as building block. However, insertion of Pkt and the ability to generate acetyl-CoA from acetyl-P did only marginally improved growth for BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc* f/xpk-pta (Figure 5d).

The OD_{600} remained stable over time, whereas glucose uptake proceeded comparable to the Pkt negative strains lacking Pfl. Glucose uptake and product formation was rather linear over time with ethanol as main fermentation product. After 96 h about 14 mM ethanol accumulated in the medium and reached a concentration of 38.5 mM after 200 h of fermentation. After the initial 96 h also other products started to accumulate. Pyruvate and succinate were produced to concentrations of 7.5 + 0.16 mM and 6.3 + 0.14 mM, respectively. Excretion of pyruvate indicated the need to balance reducing equivalents which may be due to the insertion of Pkt.

An ethanol yield of 1.38 + 0.01 $mol_{ethanol}/mol_{glucose}$ corresponded to 69 % of the theoretical maximum yield, but was 10 % lower than for the Pkt negative precursor strain. The biomass yield dropped by about 50 % after inserting the plasmid carrying the *f/xpk-pta* operon.

The Eat1 module

The tested strains BW25113*ΔldhAΔackAΔfocA::pdc* and BW25113*ΔldhAΔackAΔfocApflAB::pdc* were equipped with a pDCF:*pgi-f/xpk Bbr-pta Eco* (f/xpk-pta) and the pET26b:LacI/*T7-trEat1 Wan N-13* (*trEat1*) plasmids and evaluated on their ethyl acetate production capacity. Strain BW25113 *ΔldhAΔackA trEat1* was included as a control.

Strain BW25113 *ΔldhAΔackA trEat1* was relying on the EMP pathway for generating all product precursors and biomass building blocks (Figure 6a). Glucose concentrations decreased rapidly within the first 24 h and levelled off to a stable 20 mM until 72 h of fermentation. Similarly, the OD_{600} increased to 0.6 within 24 h and fluctuated slightly around that value until the end of fermentation. Product formation continued until about 72 h of fermentation, with almost equimolar amounts of ethyl acetate and ethanol being produced, 17.5 ±0.8 mM and 20.2 ± 0.5 mM respectively. Formate by-production led to the accumulation of about 45.44 ± 1.30 mM. After 24 h pyruvate was secreted reaching a final concentration of 7.06 ± 0.07 mM indicating overflow metabolism. In order to balance NAD^+/NADH requirements and as a consequence of the *ackA* knockout, pyruvate is secreted during ethanol formation. Apparently, the conversion of ethanol and acetyl-CoA into ethyl acetate by trEat1 did not proceed fast enough, so that ethanol and pyruvate accumulation could not be fully avoided.

Again, introduction of Pdc benefitted fermentation performance and glucose was fully depleted within 72 h (Figure 6b). Biomass was formed rapidly within the first 24 h and stabilized around an OD_{600} of 0.6 for the remaining fermentation. Ethyl acetate titers reached levels similar to the control strain. In contrast, ethanol production almost tripled to concentrations of 64.9 ± 0.4 mM, whereas formate levels did not increase. Growth impairment due to inactivation of *pflAB*, could only slightly be relieved by Pkt activity as observed for strain BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc* f/xpk-pta trEat1 (Figure 6c). Changes in OD_{600} were minor, while about 14 mM glucose were consumed over a period of 126 h. The strain did not produce any detectable amount of ethyl acetate but accumulated ethanol as main fermentation product, about 22 mM respectively.

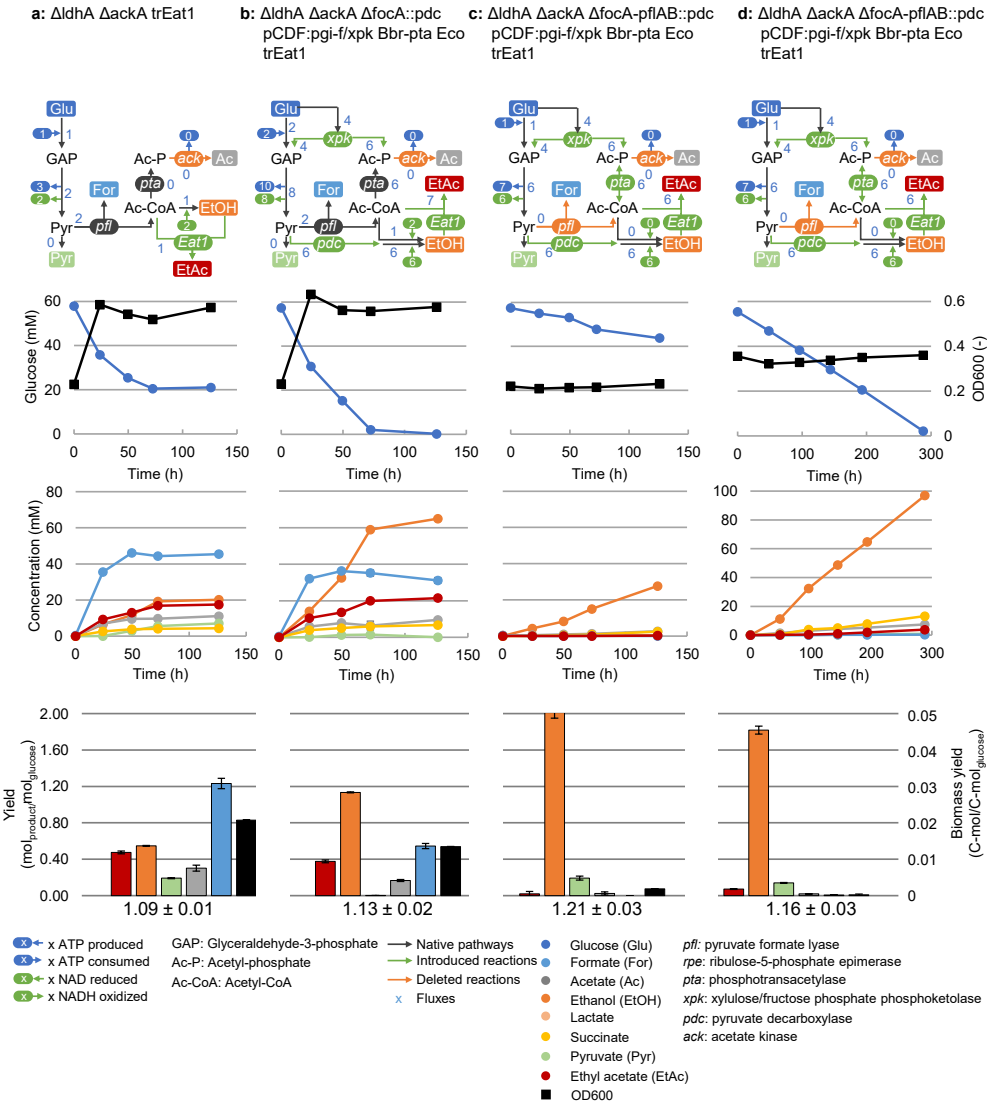


FIGURE 6: Effect of trEat1 and Pkt in *ΔldhAΔackA* background strains expressing *pdc* on ethyl acetate production. Strains expressing *trEat1* were induced with 0.01 mM IPTG and cultivated under anaerobic conditions in M9 medium containing 55 mM D-glucose as carbon source. From A to C *ΔldhAΔackA* trEat1, *ΔldhAΔackAΔfocA::pdc Zmob* f/xpk-pta trEat1, *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1. D Strain , *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 inoculated at initial OD_{600} of 0.35 and cultivated for 288 h. From top to bottom: Schematic representation of each strain, OD_{600} and glucose concentration over time, product formation over time, product yields (mol/mol) and biomass yield (C-mol/C-mol) on glucose after 120 h or 288 h of cultivation and corresponding Y_{carbon} per strain. Error bars represent standard deviations among duplicates.

Comparing product yields on glucose, highest ethyl acetate yields were obtained by the control strain utilizing only the EMP pathway and trEat1 reaching 0.47 $mol_{ethyl\ acetate}$/$mol_{glucose}$ (Figure 6). Despite accumulation of similar ethyl acetate levels, ethyl acetate yield for strain BW25113 *ΔldhAΔackAΔfocA::pdc Zmob* f/xpk-pta trEat1 was about 20 % reduced because metabolism shifted towards ethanol production. Consequently, ethanol yields doubled, with a reduction in formate yields by approximately half due to Pdc activity. The ethanol yield obtained by BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 is almost equivalent with the theoretical maximum yield of 2 $mol_{ethanol}$/$mol_{glucose}$. However, the intended increased ethyl acetate yield on glucose could not be achieved as no ethyl acetate was detected within the fermentation time. A pyruvate yield of 0.19 $mol_{pyruvate}$/$mol_{glucose}$ in contrast to the absence of any pyruvate in the intermediate strain BW25113 *ΔldhAΔackAΔfocA::pdc Zmob* f/xpk-pta trEat1 suggest redox imbalances.

Effect of induction levels on ethyl acetate production

The proposed pathway may have shifted the priority away from utilizing acetyl-CoA and ethanol for ethyl acetate production. Strain BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 relied on functional Pkt for both, acetyl-CoA for biomass and for ethyl acetate generation. However, no ethyl acetate could be detected in a fermentation time of 122 h while overall metabolism was slow. Therefore a longer fermentation period was considered and different IPTG concentrations were applied, in order to increase the availability of trEat1 to condense acetyl-CoA and ethanol. Moreover, fermentation was inoculated to an initial OD_{600} of 0.35. The extended fermentation time resulted in almost linear glucose uptake and ethanol production as shown for cells induced with 0.05 mM IPTG (Figure 7d). Other products started to accumulate after approximately 100 h of fermentation.

Strain BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 was barely changing in OD_{600} while due to the extended fermentation time, glucose was depleted in most cases by the end of fermentation (Figure 7 ab). After more than 100 h ethyl acetate could be detected in all strains, independent of the chosen induction level. Final concentrations varied between 3.9 + 0.22 mM and 2.4 + 0.16 mM ethyl acetate, where induction levels higher than 0.05 mM IPTG had a negative effect on final titers (Figure 7c). However, ethanol remained the main fermentation product and accumulated up to concentrations around 100 mM (Figure 7d). Also pyruvate secretion was affected by induction levels, as more pyruvate was secreted in fermentation where higher the induction level were applied, reaching 9.3 + 0.07 mM during induction with 0.5 mM IPTG (Appendix). Comparison of product yields show little variations among the different conditions (Figure 7e). Ethyl acetate yields on glucose approached values of 0.05 $mol_{ethyl\ acetate}$/$mol_{glucose}$ while ethanol yields of around 0.64 $mol_{ethanol}$/$mol_{glucose}$ are close to the theoretical maximum. Despite poor ethyl acetate production, the results suggest that acetyl-P from catalytic activity of Pkt was converted into acetyl-CoA and subsequently used by Eat1 for ethyl acetate production.

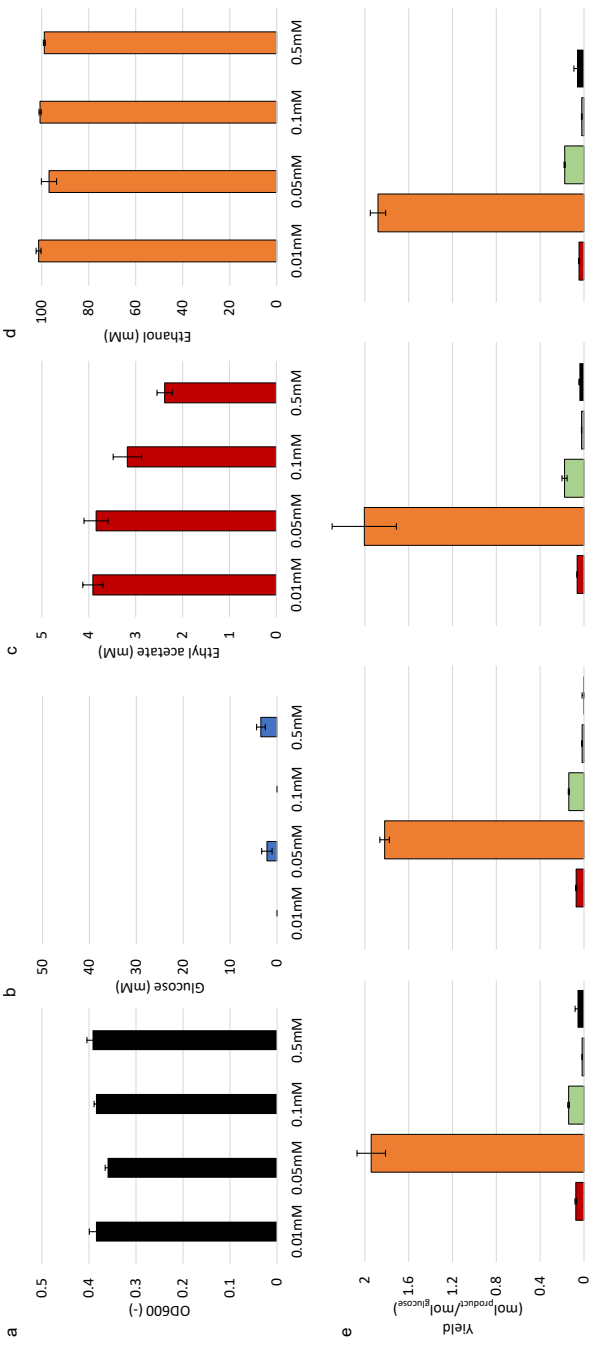


FIGURE 7: Effect of IPTG levels on ethyl acetate production by BW25113 (DE3) *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* pCDF:*pgi-f/xpk-pta* pET26:LacI/*T7-Eat1 Wan* N13. Strains expressing trEat1 were induced with different IPTG concentrations (0.01 – 1mM) and cultivated under anaerobic conditions on M9 medium containing 55 mM glucose (a) OD_{600}, (b) remaining glucose concentration, (c) ethyl acetate, and (d) ethanol produced after 288 h of fermentation and (e) product yields for ethyl acetate, ethanol, pyruvate, acetate (mol/mol) and biomass yield (C-mol/C-mol) on glucose after 288 h of cultivation Data represent averages and standard deviations of biological duplicates.

Discussion

In order to approach maximum theoretical yields carbons need to be transferred from substrate to product in the most carbon efficient way. Here we proposed a pathway relying on three key enzymes for ethyl acetate production near the maximum theoretical yield, pyruvate decarboxylase for redox balanced ethanol production, dual-activity phosphoketolase for carbon conserving generation of acetyl-CoA and ethanol acetyltransferase for ethyl acetate synthesis, respectively.

During growth on xylose catalytic activity of Pkt was directly related to growth and product formation in a *Δrpe* background strain that is otherwise incapable of growing on D-xylose (Lyngstadaas, Sprenger and Boye, 1998). Strain *BW25113 Δrpe* pCDF:*pgi–f/xpk Bbr–pta* Eco exhibited an almost similar growth profile to the WT control strain, and reached a higher final optical density when cultivated under anaerobic conditions. These findings match other, predominantly aerobic, screenings on growth deficient strains and *in vitro* tests on phosphoketolase activities and demonstrate the suitability of the concept (Bogorad, Lin and Liao, 2013; Krüsemann *et al.*, 2018). While there are also several benefits regarding the use of an inducible system including tunability, here delayed onset of expression, or an inhibitory effect of the reagent m-toluate may have caused the poorer performance of strain BW25113 *Δrpe* pCDF:XylS/Pm – *f/xpk Bbr – pta Eco* (Diaz Ricci and Hernández, 2000; Malakar and Venkatesh, 2012; Balzer *et al.*, 2013).

While strain BW25113 *Δrpe* f/xpk-pta trEat1 showed a similar growth and D-xylose uptake pattern as the WT control, only traces of ethyl acetate were found. Additionally, any further modification led to severely impaired strains with respect growth and product formation. As a result of growth restoration and the fixed split ratio of converting Xu5P into Acetyl-P and GAP, effects of Pt were pronounced. Most acetyl-P was converted into acetate, thereby generating valuable ATP, while GAP was further converted into a mix of ethanol, lactate or acetate, and formate for strains with native Pfl expression. The cells preferences to secrete acetate, even despite the co-expression of *f/xpk* and *pta* has also been observed in other studies, but mainly relates to higher ATP yields for secretion compared to conversion into acetyl-CoA (Sonderegger, Schümperli and Sauer, 2004; van Rossum, Kozak, Pronk, *et al.*, 2016; Bergman *et al.*, 2019). Similar conclusions were drawn for lactic acid bacteria that natively express phosphoketolase, where growth and ATP availability is tightly connected with acetate production (Zaunmüller *et al.*, 2006). Conversion of acetyl-P into acetate generates one ATP, while conversion of acetyl-P into acetyl-CoA for ethyl acetate formation does not. This bottleneck was also observed during growth on D-xylose. Pkt enabled growth, but any further modification led to less flexibility in the pathway, less options to produce acetate and consequently less options to generate ATP. Insertion and expression of trEat1 from another multi-copy plasmid worsened the case by increasing the metabolic load (Glick, 1995; Diaz Ricci and Hernández, 2000). More acetyl-P was directed towards

product formation. Moreover, ethyl acetate production requires the parallel generation of ethanol and acetyl-CoA, causing a very rigid flux demand that the strains may not be able to meet, once native acetyl-CoA generation via Pfl is inactivated.

Fermentation on glucose lifted the stiffness of the designed pathway, but introduced a greater degree of uncertainty, as native EMP remained an attractive first choice. Inactivation of Pfl should have again forced the use of Pkt to generate acetyl-P to replenish the acetyl-CoA pool but, again, significantly impacted fermentation performance. In contrast to D-xylose fermentation, strain BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 did consume glucose and produced ethyl acetate, but product yields stayed well below expectations. In other studies product yields of acetyl-CoA derived products were increased after introducing heterologous Pkt, but most cultivations were performed under aerobic conditions (Chinen *et al.*, 2007; Yang *et al.*, 2016; Dele-Osibanjo *et al.*, 2019; Wang *et al.*, 2019). While high acetate yields were obtained during anaerobic fermentation of an *E. coli* strain fully relying on the NOG pathway, the strain was unable to grow due to a lack of reducing equivalents and concentrated to an OD_{600} about 200-times higher than used in our experiments (OD 20) (Lin *et al.*, 2018).

Here, reducing equivalents are playing a crucial role not only to support maintenance but also to support ethanol formation. Ethanol usually requires the investment of 2 molecules of NADH for the formation of one molecule ethanol, that is usually achieved by secretion of one molecule of acetate. This co-production benefitted anaerobic ethyl acetate production and enabled a redox balanced pathway for ethyl acetate leading to high product yields during batch fermentations (Bohnenkamp *et al.*, 2020). Carbon conserved generation of acetyl-CoA via the NOG pathway, however, required also the carbon efficient production of ethanol. Moreover, during fermentations on glucose, formate accumulation may lead to acidification of the medium and cell inhibition. Most organic acids and fermentation products impose inhibitory effects on the cell at rather low concentrations hampering performance of the fermentation (Trinh *et al.*, 2010; Vázquez *et al.*, 2011). This bottleneck could be lifted during conversion of formate into hydrogen and CO_2 (Bohnenkamp et al submitted). In the present study, electrons should be redirected to product formation, instead of secretion of H_2.

Pyruvate decarboxylase from *Zymomonas mobilis* has been successfully used in the development of ethanologenic *E. coli* throughout the years (Ingram *et al.*, 1987; Ingram and Conway, 1988; Ohta *et al.*, 1991; Jarboe *et al.*, 2007). Insertion of Pdc reduced the overall NADH requirements of the process by converting pyruvate directly into acetaldehyde with the release of one molecule of CO_2. Native acetaldehyde-alcohol dehydrogenase (*adhE*) was responsible for the subsequent conversion acetaldehyde into ethanol. Previous research has shown that *adhE* is able to convert free acetaldehyde produced in Pdc mediated conversion leading to ethanol yields close to the theoretical maximum (Bräu and

Sahm, 1986). AdhE usually catalyzes the conversion of acetyl-CoA via acetaldehyde into ethanol. During this two-step conversion acetaldehyde is channeled through the enzyme and directly converted into ethanol, thus limiting exposure of the toxic intermediate (Pony *et al.*, 2020). Identification of *adhE* mutants that lost activity for one of the two sub-conversions suggest that acetaldehyde can be converted independently and can access the channel (Cunningham and Clark, 1986). Nevertheless, physical separation of the two conversions likely increased the exposure time of toxic acetaldehyde in the cells. This may have added to the poor performance of the engineered strains solely relying on Pdc activity. In this context flux through Pdc was highest, which may have led to temporary accumulation of acetaldehyde. BW25113 *ΔldhAΔackAΔfocA::pdc Zmob* f/xpk-pta trEat1 on the other hand, depleted all glucose within 72 h and efficiently produced ethanol at levels exceeding the native maximum pathway yield. Formate levels stayed below the stoichiometrically expected concentrations and indicated flux through Pdc.

Therefore, poor growth of *E. coli* BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc*, is likely a consequence of the *pflAB* knockout and consequently the lack of acetyl-CoA, rather than acetaldehyde accumulation. To avoid the loss of Pfl but still benefit from reduced formate levels, insertion of a NAD^+-dependent formate dehydrogenase such as *fdh* from *Candida boidinii* or *Mycobacterium vaccae* may offer an elegant alternative to balance redox requirements for high yield ethanol production (Berríos-Rivera, Bennett and San, 2002; Ernst *et al.*, 2005).

Ultimately, also ethyl acetate generation needs to proceed at high efficiency. Previously, expression levels and *in vivo* performance of eat1 in *E. coli* were improved by artificially truncating the mitochondrial enzyme (Kruis *et al.*, 2020). However, the various catalytic activities of *Eat1* impose also certain risks to the process as ethyl acetate may be degraded, or thiolysis and alcoholysis activities may dominate the desired AAT activity (Kruis *et al.*, 2017; Patinios *et al.*, 2020). Esterase activity has been reported to be inhibited by ethanol concentrations around 20 mM and above, which is in line with the trace amounts of ethyl acetate formed in strain BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 after more than 100 h of cultivation and ethanol concentrations clearly exceeding 20 mM.

However, the predominant ethanol production in modified strains suggests that also flux distribution needs to be further optimized to fine-tune and balance equimolar supply of the ethyl acetate precursors ethanol and acetyl-CoA to approach higher yields. Next to Pkt, also transaldolase (Tal) and transketolase (Tkt) are key enzymes of the NOG pathway and have been exchanged with heterologous versions for faster conversion before (Lin *et al.*, 2018). Downregulation of glucose-6-phosphate isomerase (Pgi) could further benefit higher fluxes through the NOG (Qin *et al.*, 2020).

While all three components work independently and seemingly with sufficient flux capacities the combined effect with the aim to produce ethyl acetate at maximum yields

is not reached. In a final strain containing all three building blocks the yield of ethyl acetate did not exceed the pathway maximum. Production of ethyl acetate in strain BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc Zmob* f/xpk-pta trEat1 suggests that all elements of the designed pathway are utilized which could not be validated in the intermediate strain co-expressing Pdc and Pfl. Therefore, carbon labelling will be an inevitable next step to identify pathway usage and targets for further modifications to improve overall performance with regard to ethyl acetate production.

Conclusions

The present study evaluated the effect of carbon conservation on acetyl-CoA derived product yields under anaerobic conditions. After assessing basic functionality of *B. breve* phosphoketolase in *E. coli*, its capacity to provide acetyl-CoA for increasing ethyl acetate yields during anaerobic fermentation is tested on two industrially relevant carbon sources, glucose and D-xylose. In a stepwise approach, the bifido shunt or the synthetic NOG pathway was introduced and pyruvate decarboxylase for redox balanced ethanol formation was provided. To ensure the use of the intended pathway, native Pfl was bypassed and later inactivated, thereby restricting acetyl-CoA generation under anaerobic conditions.

Consequently, on both carbon sources, biomass formation was significantly reduced but OD_{600} increased slightly over the fermentation times once Pkt was introduced. Ethyl acetate production could be achieved on both substrates, but the developed high yield pathway only led to ethyl acetate production on glucose. Moreover, highest yields were still obtained by the control strains, whereas modifications led to a shift from ethyl acetate to ethanol production.

Material and methods

Strain and plasmid construction

All strains and plasmids generated and used in this study are listed in Tables 1 and 2, respectively. Genomic knock outs and gene insertions were performed using CRISPR-Cas9 (Jiang *et al.*, 2015). Homologous regions of 50 bp immediately upstream the ATG and downstream the stop codon were used for all modifications. Various pTarget and pCDF-Duet plasmids were assembled using 2´ HiFi assembly master mix (NEB) according to supplier instructions. Synthetic gBlocks (IDT) were ordered containing homologous regions, the gRNA and/or a heterologous gene for the corresponding pTarget plasmid. PCR amplifications were performed with Q5 polymerase (NEB) according to instructions of the supplier.

TABLE 1: Strains used in this study.

Strain	Characteristics	Source
Escherichia coli BW25113		
Escherichia coli BW25113 *Δrpe*	disruption of ribulose-5-phosphate 3-epimerase	This study
Escherichia coli BW25113 *ΔrpeΔfocA::pdc Zmob*	disruption of ribulose-5-phosphate 3-epimerase and replacement of focA with pyruvate decarboxylase from *Zymomonas mobilis*	This study
Escherichia coli BW25113 *ΔrpeΔfocA-pfl::pdc Zmob*	disruption of ribulose-5-phosphate 3-epimerase and replacement of focA-pflAB with pyruvate decarboxylase from *Zymomonas mobilis*	This study
Escherichia coli BW25113 *ΔldhAΔackA*	Disruption of lactate and acetate production (via ackA)	(Kruis *et al.*, 2020)
Escherichia coli BW25113 *ΔldhAΔackAΔfocA::pdc Zmob*	Modifications in 1 and replacement of formate transporter by pyruvate decarboxylase from *Zymomonas mobilis*	This study
Escherichia coli BW25113 *ΔldhAΔackAΔfocA-pflAB::pdc Zmob*	Modifications in 1 and replacement of formate transporter and pyruvate formate lyase complex by pyruvate decarboxylase from *Zymomonas mobilis*	This study
Escherichia NEB® 5-alpha	*fhuA2 D(argF-lacZ)U169 phoA glnV44 Æ80 D(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB

TABLE 2: Plasmids used in this study.

Plasmid	Promoter	Gene/Protein	Source
pCas9	/		(Jiang *et al.*, 2015)
pTarget	/		(Jiang *et al.*, 2015)
pTarget-rpe	/	-	This study
pTarget-focA::pdc	/	Codon harmonized pyruvate decarboxylase from *Zymomonas mobilis*	This study
pTarget-focA-pflAB::pdc	/	Codon harmonized pyruvate decarboxylase from *Zymomonas mobilis*	This study
pDCF:XylS/Pm - EV	XylS/Pm	-	This study
pCDF: XylS/Pm - hBbr f/xpk- Eco pta	XylS/Pm	Codon harmonized phosphoketolase from *Bifidobacterium breve* and phosphate acetyltransferase *Escherichia coli*	This study
pDCF: pgi – hBbr f/xpk – Eco pta	pgi	Codon harmonized phosphoketolase from *Bifidobacterium breve* and phosphate acetyltransferase *Escherichia coli*	This study
pET26b:LacI/*T7*-hWan-trEat1-N13	LacI/*T7*	Codon harmonized eat1 from *Wickerhamomyces anomalus DSM 6766* truncated at N-13	(Kruis *et al.*, 2020)

Cultivation

Strains of *E. coli* were routinely cultivated in LB medium with the addition of kanamycin (50 ug/mL) and/or spectinomycin (50ug/mL) if required. Anaerobic cultivations were performed in sterile 250-mL serum bottles with a 50mL working volume, capped and flushed with N_2 gas. Modified M9 medium, consisting of M9 salts (Difco 1´), $MgSO_4$ (2 mM), $CaCl_2$ ´ 2 H_2O (0.1 mM), MOPS (100 mM) and trace elements (1´) and vitamins (1´) solutions according to Verduyn, 1992, was used for cultivations. As carbon source either D-glucose monohydrate (11 g/L) or D-xylose (11 g/L) was added. Precultures were inoculated from single colonies for an overnight culture in LB medium and subsequently pre-cultured in 50 mL M9 medium as described earlier (Kruis *et al.*, 2020). Cultivations were started with an initial OD_{600} of 0.2 unless noted otherwise. Precultures were centrifuged 10 min at 4255 *g* and resuspended in modified M9 medium with the respected carbon source prior to inoculation. Aerobic cultivation of precultures were routinely performed at 30°C and 250 rpm whereas anaerobic fermentations proceeded at 150 rpm. For induction of gene expression isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.01-0.5 mM) or m-toluate (0.05 mM) was added to the medium at the beginning of the experiment. All experiments were performed as biological duplicates.

Analytical

For quantification of D-glucose, D-xylose and organic acids HPLC analyses were performed as described previously using 50 mM propionic acid as internal standard (Bohnenkamp *et al.*, 2020). The HPLC used was an Agilent 1290 LC II system, equipped with an Agilent 1290 Infinity Binary Pump, Agilent 1290 Infinity Autosampler, Agilent 1290 Infinity diode array detector operated at 210 nm, and an Agilent 1260 Infinity RI detector operated at 45°C. An Aminex HPX-87H (Bio-Rad) column was used with a mobile phase of 0.008 mM H_2SO_4 at a flow rate of 0.8 mL/min.

Ethyl acetate and ethanol in the liquid fraction were measured on an Agilent 7890B gas chromatograph equipped with a flame ionization detector (GC-FID) and Agilent 7693 autosampler. Samples were analyzed by injecting 0.5 µL of liquid sample onto a NukolTM column (30 m × 0.53 mm, 1.0 µm coating, Supelco). The column temperature was maintained at 50 °C for 2 min and increased to 200 °C at a rate of 50 °C/min. The split ratio was 10. 1-Butanol (2 mM) was used as an internal standard.

Calculations

In order to calculate carbon yields (Y_{Carbon}) ethyl acetate, ethanol, pyruvate, acetate, lactate, formate, succinate, CO_2 and biomass formation were taken into account. The latter was calculated assuming a composition of $CH_2O_{0.5}N_{0.2}$ and using a dry weight conversion factor

of 0.3232 from OD_{600} to g/L (Bohnenkamp *et al.*, 2020). CO_2 concentrations were calculated based on expected stoichiometries resulting from Pdc activity, according to Equation 5 but accounting for CO_2 incorporation for succinate formation:

$$C_{CO_2} = \left(2 * C_{C_4H_8O_2} + C_{C_2H_5OH} + C_{CH_3COOH}\right) - C_{CH_2O_2} - C_{C_4H_6O_4} \quad \text{(Eq. 5)}$$

For calculations during growth on D-xylose, the equation was rephrased into Equation 6, as a substantial fraction of acetate is directly derived from acetyl-P by Pkt activity.

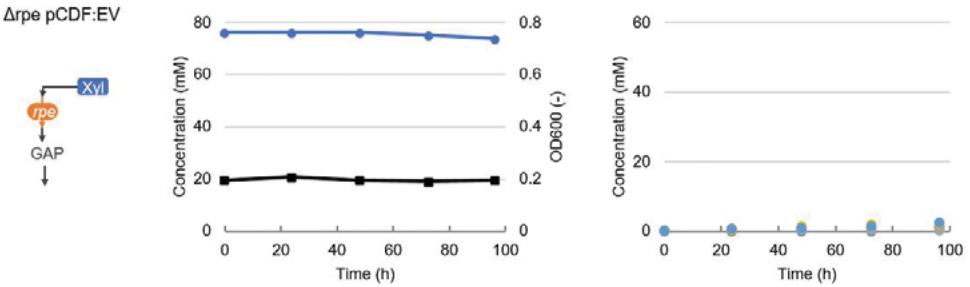
$$C_{CO_2} = \left(2 * C_{C_4H_8O_2} + 2 * C_{C_2H_5OH}\right) - C_{CH_2O_2} - C_{C_4H_6O_4} \quad \text{(Eq. 6)}$$

It should be noted that this calculation only considers product formation via the EMP route, thus leading to an overestimation of CO_2 once Pkt is functionally expressed and contributes to replenishing the acetyl-CoA pool.

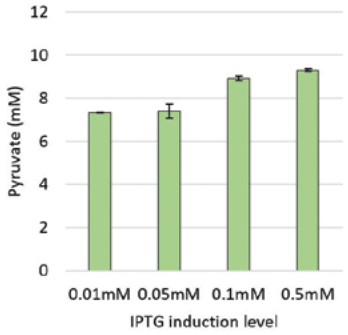
After compounds were measured or calculated, Y_{Carbon} was generated according to Equation 7:

$$Y_{Carbon} = \frac{\text{C} - \text{mol products formed}}{\text{C} - \text{mol glucose consumed}} \quad \text{(Eq. 7)}$$

Supplementary Material



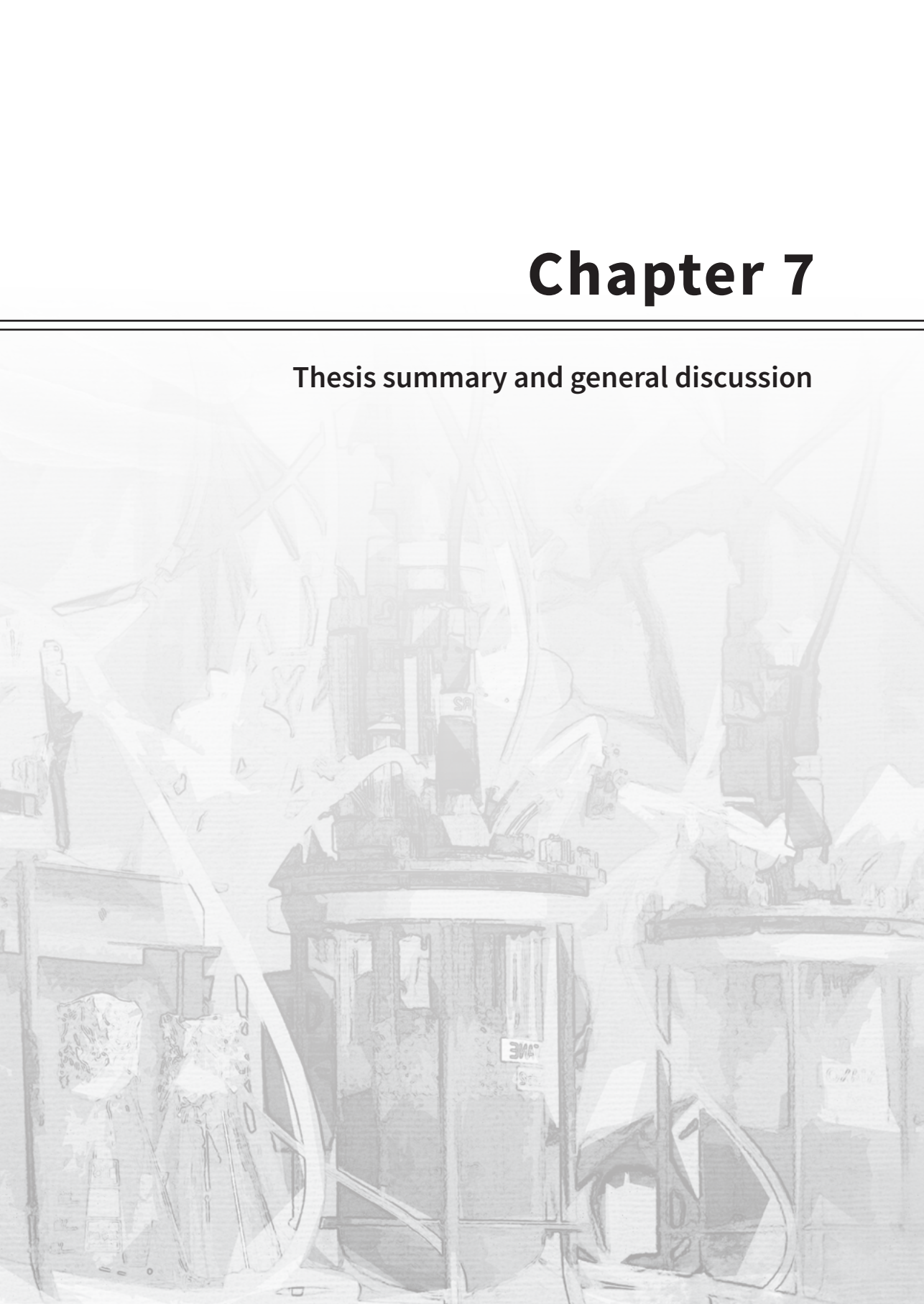
SUPPLEMENTARY FIGURE 1: Change in OD_{600}, D-Xylose consumption and product formation over time in *E. coli* BW25113 *Δrpe* during anaerobic cultivation at 30 °C. Data represent averages and standard deviations of biological duplicates.



SUPPLEMENTARY FIGURE 2: Pyruvate concentration after 288 h of anaerobic fermentation on 55 mM glucose. Strain BW25113 (DE3) *ΔldhAΔackAΔfocA-pflAB::pdc* pCDF:*pgi-f/xpk Bbr–pta Eco* pET26:LacI/*T7*-trEat1 *Wan* N13 was induced with different levels of IPTG at the beginning of the fermentation. Values represent averages with standard deviations among duplicates.

Chapter 7

Thesis summary and general discussion



Thesis summary

The technical advances of the last decades have boosted developments in the field of biotechnology and led to the implementation of several commercialized bio-based production processes. Microbial cell factories were developed at a higher speed and efficiency and the increased availability of genetic engineering tools expanded the host and product range massively. However, still only a few examples reached the scale-up phase and were developed into a commercialized bio-based process. **Chapter 1** covers the general requirements of a competitive bio-based process and highlights the challenges within the key parameters titers, rates, yields (TRY) and overall costs. Using ester, and more specifically ethyl acetate, as an example these parameters are used to support choices within the design strategy. Three strategies to efficiently transfer electrons and carbons towards product formation are elaborated, and gas stripping is proposed as an in situ product removal (ISPR) strategy.

A roadmap to engineering strategies for efficient ester production has been outlined in **Chapter 2**. First, the natural production of short and medium chain esters is discussed, and the different enzyme classes catalyzing ester formation are introduced. The group of alcohol acyltransferases (AATs) is responsible for most natural ester production and their characteristics are summarized in more detail. Various efforts towards engineering ester production are revised and processed into an overview for efficient generation of metabolic precursors and ester production strategies. Finally, an outlook is provided on how ester production could contribute to the production of other industrially relevant compounds, such as alcohols, carboxylic acids or diols.

Anaerobic processes can conserve electrons and carbons, which would otherwise be lost during aerobic processes. However, the native yeast metabolism cannot support ethyl acetate under fully anaerobic conditions. Therefore, the bulk ethyl acetate producing enzyme ethanol acetyltransferase 1 (Eat1) was evaluated on its suitability to catalyze ethyl acetate production in *E. coli,* where anaerobic ethyl acetate production is redox balanced. As Eat1 is located in the yeast mitochondria, it is undergoing mitochondrial processing events before folding into its stable and active form. Therefore, heterologous Eat1 expression may not be optimal unless the destabilizing pre-sequence is removed. In **Chapter 3**, the N-terminal sequences of Eat1 originating from *Wickerhamomyces anomalus* and *Kluyveromyces marxianus* were synthetically removed and the optimal cleavage sites were determined. The resulting truncated Eat1 variants (trEat1) showed improved *in vivo* stability and ethyl acetate production in *E. coli* cells.

Chapter 4 focuses on improving anaerobic ethyl acetate production in *E. coli*. Next to the decrease in by-product formation by inactivating lactate dehydrogenase and acetate kinase genes, the expression levels for *eat1* were optimized. The use of trEat1 further improved ethyl acetate production but medium acidification and hydrolytic side-activities of Eat1 imposed new bottlenecks to the process. Fermentations in 1.5-L pH-controlled bioreactors positively affected glucose uptake and fermentation time. In addition, gas-stripping was validated as suitable ISPR strategy and simultaneously limited ethyl acetate degradation in the fermentation broth. A product yield of 72 % of the pathway maximum indicated that an anaerobic process using *E. coli* as expression host can compete with natural producers.

During anaerobic ethyl acetate production in *E. coli* a pathway yield of 1 $mol_{ethyl\ acetate}$/ $mol_{glucose}$ can be obtained. The anaerobic use of pyruvate formate lyase results in the loss of carbons and electrons in the form of formate. **Chapter 5** exploits the natural ability of *E. coli* to convert formate into hydrogen and carbon dioxide. By applying several genetic modifications related to hydrogen uptake and regulation of formate hydrogen lyase, ethyl acetate and hydrogen co-production could be achieved. Both products were produced at a 70 % product yield regarding the pathway maximum and ethyl acetate production rates increased 2-fold.

Chapter 6 describes the engineering efforts towards anaerobic ethyl acetate production near the theoretical maximum yield. In this scenario electrons and carbons are channeled towards product formation in the most efficient way. Next to glucose, also xylose as potential feedstock for bio-based ethyl acetate production is explored. In a modular approach, modifications and their effect on central *E. coli* metabolism and product formation were evaluated. Remaining challenges and possible solutions are discussed.

Summarizing, an integrated process for bio-based ethyl acetate was developed. Establishment of an anaerobic process relying on heterologous expression of Eat1 in *E. coli* led to high product yields. Different strategies to utilize carbons and electrons efficiently were explored and gas stripping demonstrated the potential of *in situ* product removal.

General Discussion

With increased awareness about the planet and the climate, sustainable solutions and alternatives to carbon intense petrochemical processes are getting more public attention. Still, there is a big gap between proof-of-concept studies and upscaled processes. Often the economics of a potential bioprocess are not sufficiently considered or uneconomical choices on small scale make linear upscaling impossible. Major bottlenecks in bio-process development for bulk chemicals are substrate and downstream processing (DSP) costs.

Good bioprocess design considers all factors and carefully outweighs the effect of each choice on the overall setup including DSP. Consequently, the guidelines for benchmarks around titers, rates, yield (TRY) have evolved. Regarding substrate costs, cheap and abundant feedstock should be considered while conversion into the desired product needs to proceed at high yield. Titers obtained determine the DSP options. Often the product is dissolved in the aqueous phase and needs to be separated via distillation, or precipitation steps. With respect to economic feasibility, the product should be as concentrated as possible. However, often product toxicity prevents high titers to be reached. *In situ* applications for product isolation exploiting natural driving forces, such as gravity or evaporation provide an elegant solution to this problem. Increased interest in *in situ* product removal (ISPR) has showcased some alternative approaches to obtain a concentrated product stream without exceeding critical thresholds regarding product toxicity. It has been applied to several existing processes leading to improvements in yields and production rates (Ezeji *et al.*, 2005; Pereira, van der Wielen and Straathof, 2018). Higher rates, in turn, benefit the fermentation volume or fermentation time. These factors ultimately determine the process dimensions and the requirements for DSP.

In research often strain design, fermentation and DSP are considered individual units and strategies are developed and expanded rather independently. More emphasis should be laid on integrated process design which is already being practiced in most industrial process developments.

Integrated bio-process design for ethyl acetate

In the presented research bio-process design for ethyl acetate was aiming at a holistic approach including a clear DSP strategy within process developments.

Bio-based ethyl acetate production has long been centered around natural bulk producing yeasts. However, yeast-based ethyl acetate production requires the addition of oxygen as external electron acceptor. Consequently, electrons are lost that should ideally be transferred to the product of interest. While the processes were optimized to an extent where high yields were obtained, the supply of oxygen, transfer limitations and cooling requirements remain limiting factors (Löser *et al.*, 2013). Moreover, conditions such as

iron limitations need to be applied to stimulate ethyl acetate formation that is naturally regulated in native producers.

Unraveling of AAT families opened the door to rational strain design. The absence of oxygen in the overall reaction equation indicated the general applicability of an anaerobic process and gave a promising outlook on reachable product yields. Considering the chemical properties of the product an integrated DSP strategy was considered. Due to its volatile character, ethyl acetate can readily be stripped and extracted via the gas phase. In different approaches carbon and electron conservation were further investigated and their effect on ethyl acetate production evaluated.

The feasibility of anaerobic ethyl acetate production coupled to gas stripping as ISPR approach of choice was successfully demonstrated. In pH-controlled bioreactor fermentations, produced ethyl acetate was efficiently stripped from the fermentation broth using a nitrogen as inert gas However, the operating conditions within this study were not yet resembling industrial settings and further upscaling and validations need to be performed. Still, the results give valuable insights to current and future challenges and opportunities.

Conserving electrons

Heterologous expression of enzymes is typically first explored under aerobic conditions. As ethyl acetate production requires the supply of acetyl-CoA and ethanol, ethanol needs to be provided externally. While initial characterizations may be performed this way, supply of oxygen and ethanol render the process unfeasible because of the high costs connected to the second substrate ethanol and aeration. Availability of oxygen enables complete carbon oxidation to CO_2 and electrons are used for biomass formation and metabolic energy generation. Different ways on how to conserve and (re-) direct electrons and carbons to product formation were explored (Figure 1).

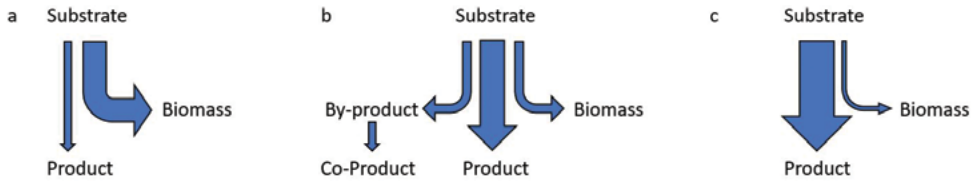


FIGURE 1: Schematic overview of possible electron and carbon fluxes. (a) Aerobic process with oxygen as external electron acceptor and release of CO_2 and H_2O. Most electrons are used for metabolic energy generation and biomass production. (b) Anerobic process in which electrons are directed towards product formation. Redox requirements need to be balanced by formation of by-products. During co-production the by-product is or can easily be converted into a product of economic value. (c) Anaerobic process with product formation at the theoretical maximum yield. All electrons are converted into the product of interest in the most efficient way and biomass formation is minimized.

Conserving electrons by anaerobic product formation

To limit the loss of electrons and carbon towards biomass formation and to enable *de novo* ethyl acetate production, anaerobic conditions were applied. With the absence of oxygen as external electron acceptor, electron and carbon loss by complete oxidation of substrate is restricted. Consequently, in a redox balanced metabolism all electrons are directed towards other products than oxygen. However, this may not always lead to an increased formation of the desired product. In fact, in the current research several rounds of optimization were required to channel the electrons as much as possible towards ethyl acetate formation.

Naturally *E. coli* performs mixed acid fermentation resulting in a mix of ethanol, acetate, lactate, formate and succinate. Therefore, initial ethyl acetate formation was low and electrons were dispersed across several by-products. In our case mainly lactate was produced, followed by ethanol and acetate. Elimination of enzymes leading to those undesired products redirected the electron flow but secretion of the intermediate pyruvate indicated insufficient activity of Eat1, downstream of pyruvate (Chapter 3). Truncation of the N-terminus of Eat1 to simulate the mitochondrial cleavage event improved protein stability and activity *in vivo*. Due to the higher flux towards ethyl acetate, pyruvate accumulation was significantly reduced but medium acidification and ethyl acetate degradation in serum bottles prevented higher yields to be obtained. Consequently, fermentation in pH-controlled bioreactors improved overall fermentation performance and ethyl acetate production exceeded 70 % of the pathway yield of 1 $mol_{ethyl\ acetate}/mol_{glucose}$ (Chapter 4).

In the current approach not all electrons could be directed towards product formation but some were inevitable lost due to formate by-production. This also resulted in the accumulation of formate to inhibitory levels and as such slowed down the fermentation process. Formate can be a cheap carbon source for C1 fermentation and carbon upcycling (Cotton *et al.*, 2020), but it can neither be efficiently removed from the fermentation broth nor does it have a big economic value, justifying the connected DSP efforts. Therefore, an alternative solution had to be found.

Conserving electrons by co-production

By enhancing the natural ability of *E. coli* to convert formate into CO_2 and H_2, not only the inhibition by formate is lifted but also an interesting new product is generated. Green hydrogen is difficult to obtain as sole product with economic feasibility as overall yields are usually low (Balat and Kirtay, 2010). Coupling H_2 production to the co-production of another industrial relevant product offered an elegant solution to make better use of the feedstock.

Ethyl acetate production was not negatively affected by converting formate into hydrogen but yields remained stable around 70 % of the pathway yield. On the contrary, reduction

of formate in the medium prevented the acidification and consequently reduced the formate inhibition stress on the cells leading to improved production rates (Chapter 5). Likely due to higher CO_2 availability as a result of the conversion of formate into CO_2 and H_2, increased succinate formation was noticed. This prevented ethyl acetate yields to become even higher and thus should be targeted in future strains.

Regarding H_2 production, 70 % of the pathway yield of 2 $mol_{hydrogen}/mol_{glucose}$ was achieved in this study, with further room for improvements with respect to engineering targets (Maeda, Sanchez-Torres and Wood, 2012). Whereas the maximum yield for hydrogen from fermentation is 4 $mol_{hydrogen}/mol_{glucose}$, most yields obtained lie in the range of 2-3 mol/mol (Wang, Cao and Liu, 2011), which is also the case for ethyl acetate and hydrogen co-production.

Conveniently, ethyl acetate as well as the newly generated hydrogen can readily be extracted from the fermentation broth using ISPR. As hydrogen production depends on formate production which is linearly correlated to ethyl acetate production, hydrogen accumulation can be estimated and accounted for during further process optimization. Similar to most products, hydrogen accumulation can cause inhibitory effects on the cells, which can be prevented by ISPR. Therefore, co-production of hydrogen and ethyl acetate, combined with gas stripping results in a robust process with only limited accumulation of potentially inhibiting products and a significant improvement of production rates.

Hydrogen co-production is also connected to certain risks. Accumulation of gasses may lead to a pressure build-up and an increased risks of explosion. Hydrogen has been a sensitive topic in the past but slowly re-gains value and recognition as future biofuel. Technical advancements led to improved vessels, storage and also a better overall framework for microbial fermentation. Especially in the field of syn-gas fermentation a lot of experience on handling gaseous flows and reactor setups has been gained (Kundiyana, Huhnke and Wilkins, 2010; Munasinghe and Khanal, 2010). This knowledge should be transferable to the proposed co-production process in order to minimize risks.

Conserving electrons by approaching maximum theoretical yields

The third approach in our attempt to design a carbon and electron efficient process aimed at a further reduction of the loss of electrons by directing them to ethyl acetate itself in the most efficient way. Unfortunately, ethyl acetate production on glucose as well as on xylose was accompanied with several pitfalls and the strain performances stayed well below our expectations.

Summarizing, strain development towards the maximum theoretical yield was based on the interplay of three modules: i) the phosphoketolase module to conserve carbons and electrons, ii) the redox module to balance reducing equivalents and to prevent the dissipation of electrons to by-products, and iii) the AAT module that finally catalyzed the formation of ethyl acetate (Chapter 6).

Phosphoketolase (Pkt) played a central role in approaching the maximum theoretical yields, as it technically enables complete carbon conservation. Several studies have exploited Pkt to increase yields of acetyl-CoA-derived products but mostly under aerobic conditions. Anaerobic carbon conservation using Pkt has been showcased by the Liao group, engineering a synthetic non-oxidative glycolysis (NOG) into *E. coli*, which enabled acetate production close to the theoretical maximum yield (Lin *et al.*, 2018). Many industrially relevant compounds are, however, more reduced than acetate, including ethyl acetate. Consequently, additional reducing equivalents are needed to enable ethyl acetate formation, either by providing these externally or by (re-) generating these in another pathway. Here, we chose the expression of pyruvate decarboxylase to ensure a redox balanced process, an approach that has been used before for generating ethanologenic bacteria (Jarboe *et al.*, 2007).

Functional Pkt expression was validated by growth restoration in a *Δrpe* background strain on D-xylose, but its activity on glucose-grown cells remained elusive. Insertion of Eat1 and induced ethyl acetate production further had aversive effects on substrate uptake, growth and product formation. Possible shortcomings in the strains may be a non-ideal flux distribution, accumulation of intracellular acetaldehyde, or energy limitations.

During growth on glucose, Pkt did not directly utilize the substrate but was relying on the efficient distribution of F6P among the EMP pathway and the PPP, as well as the subsequent conversion and carbon reshuffling reactions within the PPP. Other research has shown that especially transaldolase (Tal) and transketolase (Tkt) may not be able to cope with the high fluxes desired and required for use of the NOG. Addition of heterologous *tal* from *Klebsiella pneumoniae* and *tkt* from *Methylomicrobium buryatense* removed this carbon reshuffling bottleneck and improved flux through the NOG (Lin *et al.*, 2018). In this study, additional expression of heterologous *tal* and *tkt* on a plasmid has been attempted but resulted in even longer lag phases and no significant effect compared to strains just carrying the Pkt.

Pdc was used to redirect electrons towards ethanol formation. Potential accumulation of acetaldehyde and the reduction of the acetyl-CoA pool were potential drawbacks of this solution. The use of NAD^+-dependent *fdh* from *Mycobacterium vaccae* (Ernst *et al.*, 2005) or *fdh1* from *Candida boidinii* (Berríos-Rivera, Bennett and San, 2002) would make additional NADH available for a redox balanced two-step conversion of acetyl-CoA to ethanol by native AdhE. The applicability of *fdh* from *M. vaccae* has already been tested in initial screening experiments (Figure 2). Overexpression with a strong promoter was necessary to obtain improvements in ethanol yields and to reduce accumulation of pyruvate and formate. Still, formate accumulated in the liquid and indicated that conversion did not proceed immediately, which may lead to shifts in NADH formation and disbalance in reducing equivalents within the desired pathway.

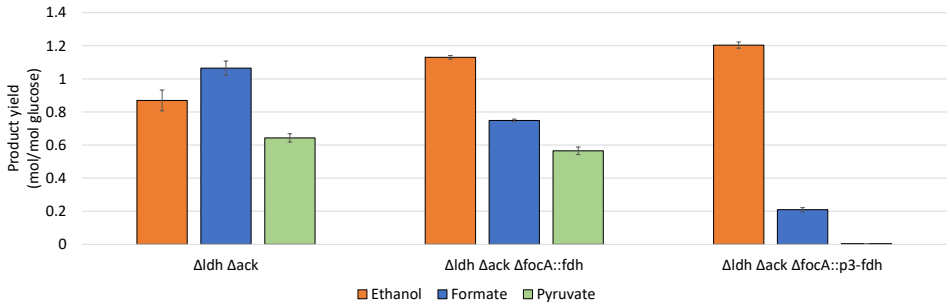


FIGURE 2: Ethanol, formate and pyruvate yields on glucose show the effect of heterologous *fdh* expression in an *E. coli BW25113 ΔldhAΔackA* background strain cultivated under anaerobic conditions at 30°C. *M. vaccae fdh* was inserted at the formate transporter (focA) locus and expression was enhanced by replacing the native promoter and RBS site with the p3-promoter.

Another option for electron recovery is the pyruvate dehydrogenase (Pdh) complex, the aerobic counterpart of Pfl catalyzing the conversion of pyruvate into acetyl-CoA. During the pyruvate dehydrogenase step CO_2 is released and electrons are transferred to NADH. Tight regulation of the Pdh complex and their corresponding subunits made it difficult to achieve anaerobic expression. Nevertheless, engineering efforts towards anaerobic expression of the Pdh complex have already identified some crucial factors such as a point mutation in the *lpd* gene (Kim, Ingram and Shanmugam, 2007, 2008), or the importance of the PdhR regulator (Zhou, Iverson and Grayburn, 2008; Maeda *et al.*, 2017). With increasing knowledge and first successes, this strategy may provide another interesting solution in future approaches.

In addition to these remaining bottlenecks within the designed pathway, the importance of the energetics of the metabolism may have been addressed too little. Energy is required to support growth and maintenance requirements of the cells, which includes protein synthesis and processing, RNA synthesis and processing, DNA synthesis and processing and various membrane processes (Neidhardt, Ingraham and Schaechter, 1990; Pramanik and Keasling, 1997). During strain engineering a host organism often acquires new genes, expresses new proteins or is exposed to new conditions. A shift in metabolism usually occurs which adds a metabolic load to the cells (Glick, 1995). Expression of additional genes, carrying of (multi-copy) plasmids, or (induced) production of secondary metabolites increases the metabolic load and therefore, adds to the overall maintenance requirements.

Especially during growth on xylose, growth was heavily dependent on acetate production. This is in accordance with other micro-organisms that naturally possess Pkt. *Bifidobacteria* and lactic acid bacteria, express phosphoketolase and automatically conserve carbons more efficiently than, for instance *E. coli*, relying on the EMP pathway. Whereas their energy conservation capacity is low they are more efficient in extracting energy from

pentose sugars than from hexose sugars (Zaunmüller *et al.*, 2006). The shift towards redox balanced ethanol and acetate production reduced overall acetate formation and associated ATP production (Table 1). As a consequence of ethyl acetate formation, the ATP yield was reduced by up to 50 %, as acetyl-P is converted to acetyl-CoA and subsequently to ethyl acetate instead of acetate.

TABLE 1: Product yield and ATP yield on xylose for strains using different pathways.

	Pathway characteristic	Product yield	ATP yield
E. coli WT	Mixed acid fermentation	0.8 $mol_{ethanol}/mol_{xylose}$	2.17 mol_{ATP}/mol_{xylose}
E. coli Pkt	Bifido shunt	0.5 $mol_{ethanol}/mol_{xylose}$	2.5 mol_{ATP}/mol_{xylose}
E. coli Pkt Pdc	Ethanologenic fermentation	1.0 $mol_{ethanol}/mol_{xylose}$	2.0 mol_{ATP}/mol_{xylose}

Here, expression of two multi-copy plasmids increased the metabolic load. For a strain that is already limited in ATP generation, this may have detrimental consequences. Also increased lag phases after insertion of a plasmid carrying Tal and Tkt, support this theory. Therefore, strategies to reduce the metabolic load should be explored and genomic integration of desired heterologous genes should be anticipated. It avoids the expense of carrying a plasmid and concomitantly avoids the need of using antibiotics or expressing antibiotic resistance cassettes. This again decreases the metabolic load of the cells. With the availability of more knowledge on regulatory mechanisms and simulation tools, inclusion of metabolic load into strain design and model validations should be encouraged (Wu *et al.*, 2016).

Expression of heterologous genes from the genome likely needs to be (re-)optimized but can also be coupled to laboratory evolution strategies, to finetune the metabolic flux network. Laboratory evolution is widely applied to increase fitness of a desired cell factory towards the bioprocess. Formulation of a clear objective establishing a robust and easy screening method are key to successful laboratory evolution. For ethyl acetate production no direct laboratory evolution strategy can be applied as ethyl acetate is neither directly growth coupled nor easily measurable in a high throughput way. Nevertheless, laboratory evolution may help in obtaining a high yield ethyl acetate producing strain. More specifically it can help fine tuning the flux distributions and the availability of acetyl-CoA via Pkt and Pta activity.

Ultimately, carbon labelling with ^{13}C-labelled substrates offers an elegant way to validate the general functionality of the designed pathway. In case of the NOG, bottlenecks have been identified within the carbon reshuffling reactions of the PPP but also the flux distribution at the F6P node may limit functionality of the envisioned pathway. Based on the outcome of ^{13}C-labelling more insight into the used fluxes can be gained and potential bottlenecks can be identified and tackled.

Uncoupling biomass and product formation

Optimizing yields aims at minimizing biomass formation, as electrons and carbons of the substrate should be directed to product formation rather than to biomass and growth. The more constraints are imposed on the cell, the more difficult it becomes to deviate fluxes. Moreover, additional ATP needs to be generated to cover not only the metabolic load and maintenance requirements but also the synthesis of new building blocks and biomass.

It has been shown that the central metabolism of *E. coli* can be re-engineered. By combining structural knockouts, overexpression and laboratory evolution an *E. coli* strain relying solely on the synthetic NOG has been established (Lin *et al.*, 2018). As a proof-of-concept acetate was produced at high yields. However, it should be noted that a low ATP yield was observed, too. Thus, product formation under anaerobic conditions was only possible after concentrating biomass that was produced during an aerobic growth phase.

Whereas this study only relies on partial use of the NOG, the ATP yield during ethyl acetate production remains limited. Therefore, a growth uncoupled system may also be a legitimate approach to achieve high yield conversion for ethyl acetate. Retentostat cultivations may be required which are mainly exploited for studying cell physiology and aging at near zero growth conditions (Chesbro, Evans and Eifert, 1979; Boender *et al.*, 2009; Liu *et al.*, 2021). Additionally, uncoupling production from biomass demands a tight and well tunable expression system. Because the use of inducing agents like IPTG, adds another cost factor to the process, the use of other induction systems should be explored before moving to larger scales.

***In situ* product removal**

For the production of bio-based bulk chemicals the overall costs of the process are crucial for economic feasibility. Next to the efficient conversion of substrate into product, expressed as yield, the titers obtained affect the DSP approach and influence the second major cost factor. Often toxicity prevents high titers from being reached and thus extensive downstream processing is required to purify the product of interest, which increases the costs. An alternative approach is *in situ* product removal, in which the product is continuously extracted from the fermentation broth during the fermentation. In this way the product can already be pre-concentrated and product accumulation in the broth is reduced.

Gas stripping was chosen to enable continuous product removal and to prevent accumulation of ethyl acetate to toxic concentrations and the degradation of ethyl acetate into ethanol and acetate. While several improvements were made regarding ethyl acetate production the process conditions were set in such a way that inhibitory conditions could not be reached. Gas stripping was primarily benefiting the process by reducing product

degradation (Chapter 4,5). Consequently, more knowledge should be gained about the trade-offs of higher productivities versus higher stripping.

Gas stripping to investigate ethyl acetate toxicity

A continuous process would offer the ideal platform for investigating different liquid ethyl acetate concentrations and stripping rates. Additionally, information can be gained on toxicity effects and production rates in different scenarios. Thus, a continuous setup will be a valuable contribution to evaluating the next steps in process development.

In order to ensure a robust cultivation, genomic integration of Eat1 should be considered. Currently, Eat1 is expressed from a plasmid carrying a resistance cassette and requiring induction via IPTG. Both features limit the applicability of current strains in larger systems and in continuous setups. Attempts to establish steady state failed even with very low dilution rates. Reasons for this are likely plasmid loss or inconsistent Eat1 activity. Nevertheless, also the effect of different inert gases or mixes of N_2 and CO_2, formate accumulation in the fermentation broth and conversion rate into H_2, as well as potential pressure build up should be evaluated in addition to ethyl acetate production and stripping.

While integration of Eat1 on the genome may improve robustness of the strain, it may also lead to a drop in performance when compared to expression from a multi-copy plasmid. Therefore, re-evaluation and re-optimization of ethyl acetate production will be inevitable.

Effect of temperature on ethyl acetate production and gas stripping

All fermentations in this research were performed at 30 °C, which was the ideal fermentation temperature for Eat1. Temperature and stripping rate can be used to modulate and tune liquid concentrations of ethyl acetate. On a large scale, especially the provision of inert gas will add to the overall costs of the process, and thus should be limited. Therefore, for economic feasibility higher temperatures and a high partial concentration in the liquid phase is desired. However, higher concentrations in the liquid broth increases the risk of reduced production rates due to inhibitory effects.

An increase in temperature from 30 °C to 37 °C should have benefitted productivities and gas stripping behavior, but ethyl acetate production dropped in serum bottle as well as in bio-reactor experiments (Figure 3). With Eat1 *in-vitro* activities measured up to 45 °C, protein stability should not have been affected at this temperature range (Chapter 3). More likely, increase in temperature enhanced inclusion body formation reducing the amount of functional enzyme for ethyl acetate production. Better solubility of the protein in heterologous hosts should therefore be targeted in the future.

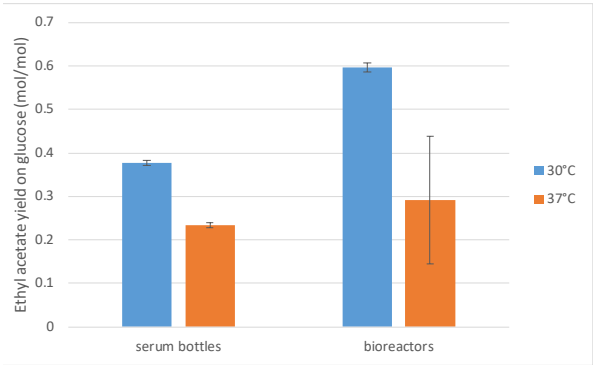


FIGURE 3: Product yields for ethyl acetate on glucose (mol/mol) after anaerobic fermentation of streamlined *E. coli ΔldhAΔackA* expressing Eat1 at 30 and 37 °C. For serum bottles the yield was calculated after 72 h whereas the product yield in bioreactor fermentation was upon glucose depletion after 45 h.

Ethyl acetate production at elevated temperatures

To be able to increase fermentation temperature even further, other expression hosts need to be found. The use of thermophiles, growing in temperatures ranging from 40 – 60 °C may be considered. Especially one-pot conversion of cellulosic material into fermentation products has led to regained interest in thermophiles in biorefinery approaches (Turner, Mamo and Karlsson, 2007). Efforts in understanding and modifying central metabolism of thermophiles have increased so that basic genetic engineering tools for expression are available for some species (Taylor *et al.*, 2011; Olson, Sparling and Lynd, 2015; Mougiakos *et al.*, 2017). Still those are more restricted and less tunable than for *E. coli* or yeast.

TABLE 2: Thermophilic organisms that were considered for ethyl acetate production at elevated temperatures. Selection based on temperature range, availability of genetic tools and tolerance towards ethyl acetate.

Strain	T optimum	Ethyl acetate tolerance[a]	Key reference
Geobacillus thermoglucosidasius	60 °C	Low	(Cripps *et al.*, 2009)
Caldicellulosiruptor bescii	75 °C	NA	(Chung *et al.*, 2012)
Clostridium thermocellum	55 °C	NA	(Biswas *et al.*, 2015)
Thermoanaerobacter mathranii	70 °C	Moderate	(Yao and Mikkelsen, 2010)
Thermoanaerobacter italicus	66 °C	Moderate	(Andersen, Jensen and Mikkelsen, 2015)
Thermoanaerobacterium saccharolyticum	55 °C	NA	(Shaw *et al.*, 2008)
Thermoanaerobacter ethanolicus	55 °C	High	(Shaw, Hogsett and Lynd, 2010)

[a] – ethyl acetate tolerance determined during screening in universal medium with exposure of cells to different concentrations of ethyl acetate. Classification based on growth limitations upon exposure levels of ethyl acetate below 100 mM – low, below 250 mM – moderate, and above 250 mM – high.

A few thermophiles were explored for their suitability for ethyl acetate production based on the following criteria: substrate range, genetic accessibility and tolerance towards ethyl acetate (Table 2). *Thermoanaerobacterium saccharolyticum* gained attention due to its natural competence and has been engineered for improved ethanol production (Shaw

et al., 2008; Shaw, Hogsett and Lynd, 2010). These characteristics provide a good starting point for genetically engineering *T. saccharolyticum* towards ethyl acetate production. Whereas genomic knockouts and plasmid insertions succeeded, no ethyl acetate producing strain was generated. Formation of inclusion bodies of Eat1 and decreasing half-lives at temperatures higher than 45 °C may have been limiting factors (Chapter 3).

ISPR of other esters

Whereas ethyl acetate is a volatile compound and gas stripping was proposed as ISPR strategy, volatility decreases for longer chain length esters. At the same time, the solubility in aqueous phases decreases, giving rise to phase separation as ISPR approach. By providing an organic phase, the product can transfer from the water into the organic phase (Bar and Gainer, 1987; Malinowski, 2001; Oudshoorn, Van Der Wielen and Straathof, 2009). Similar to the transition into a gas phase, the compound is no longer interfering with the cells and limitations based on solubility or toxicity of the product can be avoided. However, it should be considered that the organic phase itself will cause some inhibitory interaction with the microbial host, and needs to be selected based on toxic effect and partition coefficient of the product (Straathof, 2003). ISPR strategies in general, receive more and more attention and technical advances enable easier implementations (López-Garzón and Straathof, 2014; Murali, Srinivas and Ahring, 2017; Atasoy *et al.*, 2018).

Eat1 as AAT for ethyl acetate production

The enzyme ethanol acetyltransferase 1 (Eat1) has been identified to play a major role in ethyl acetate bulk production by yeasts. Upon first characterization of Eat1 three activities were identified; AAT, esterase and thioesterase activity (Kruis *et al.*, 2017). Later, even more activities have been found with high affinity such as thiolysis and alcoholysis activity (Patinios *et al.*, 2020). It becomes apparent that Eat1 is a multitasker and its activities are highly affected by the environmental conditions. Still, under the tested anaerobic conditions, Eat1 readily competes with other AATs with respect to ethyl acetate production. For instance, *E. coli* strains expressing Eat1 homologs outperformed strains relying on the expression of Atf1, a well-known cytosolic AAT with high preference for acetate esters (Chapter 4). This strengthens the position of Eat1 as main biocatalyst for bulk ethyl acetate production. However, during this project also several bottlenecks and risks were identified correlated to the use of Eat1 as AAT of choice.

During gene expression and protein stability optimizations, pyruvate accumulation indicated that upEat1 could not catalyze ethyl acetate formation fast enough, resulting in ethanol and pyruvate secretion in an *ackA* knockout strain. Whereas the final yield of ethyl acetate on glucose did not significantly improved upon synthetic cleavage of the N-terminal pre-sequence, pyruvate accumulation was prevented (Chapter 3, 4). Increase

in acetate and ethanol yields suggested hydrolysis of ethyl acetate by the esterase side activity. Gas stripping in pH-controlled bioreactors was applied to reduce the residence time of ethyl acetate and to reduce product degradation (Chapter 4). Product yields for ethyl acetate exceeded 70 % of the maximum pathway yield but ethanol and acetate accumulation could never be fully avoided.

Characterization of Eat1 has shown that sufficiently high levels of ethanol can inhibit the esterase and thioesterase side-activities (Kruis *et al.*, 2017). Consequently, ethanol will always be co-produced to levels of at least 20 mM, using the current Eat1. This phenomenon was also observed during development of a high yield strain (Chapter 6). Due to slow metabolic activity, little to no ethyl acetate was measured in strain *BW25113 ΔldhAΔackAΔfocA-pflAB::pdc* f/xpk-pta trEat1. After around 200 h ethanol levels passed the threshold of 20 mM and ethyl acetate could be detected. Addition of ethanol to the fermentation medium was tested in various contexts but did not improve overall ethyl acetate production.

During heterologous expression of Eat1 in *E. coli* formation of inclusion bodies were observed. For upEat1 but also for trEat1 proteins were detected in the insoluble fraction during protein isolation processes (Chapter 3). Different rounds of optimization did improve the *in-vivo* performance of Eat1 but inclusion bodies indicated that the true potential of the system has yet to be unlocked. Non-optimal folding and expression of Eat1 may be the reason for the poor performance, as *in vitro* assays show activity and half-lives up to 55 °C (Chapter 3). Enzyme engineering may therefore already benefit ethyl acetate production in an *E. coli* expression system but will also play a crucial part once a higher temperature expression system is used.

Protein engineering of Eat1

Protein engineering is an increasingly employed technique to improve performance of an enzyme catalyst. In case of Eat1 protein engineering three bottlenecks can be targeted. Firstly, by preventing the formation of inclusion bodies a higher functional expression level of the native or truncated enzyme in heterologous expression hosts such as *E. coli* could be achieved. Secondly, engineering towards a higher thermotolerance is required to enable the process to be transferred to elevated temperatures, which is needed for the envisioned ISPR approach. Lastly, protein engineering could also be used for decreasing the undesirable side activities of Eat1, such as the esterase- and thioesterase activity.

One major bottleneck with respect to protein engineering of Eat1 is the lack of a 3D model. Several attempts to elucidate such a model by obtaining Eat1 crystals and performing x-ray diffraction have so far not been successful. This limits protein engineering strategies for Eat1 to the use of random or semi-rational approaches.

Consensus-based engineering relies on multiple sequence alignments (MSA) of a protein family rather than making use of a crystal structure. It is hypothesized that in the course of evolution, conserved amino acids have a positive effect on enzyme stability (Steipe *et al.*, 1994; Lehmann *et al.*, 2000). A few successful examples of proteins with increased thermo-stability and retained activity have been generated, thereby demonstrating general feasibility of this semi-rational approach (Lehmann *et al.*, 2000; Vazquez-Figueroa *et al.*, 2008).

Alignment of 19 homologs of the Eat1 / Eat2 family resulted in the generation of four consensus-based Eat1 sequences (Table 4). Based on the relative high temperature range for *Kluyveromyces marxianus*, its respective Eat1 was used as reference sequence including its N- and C-termini (Urit, Li, *et al.*, 2013). Manual processing of the N-terminus to remove the mitochondrial pre-sequence led to the generation of truncated variants, that previously improved enzyme stability (Chapter 3).

TABLE 3: Generation of four consensus based Eat1 sequences. Different Eat1 homologs and selection biases were used in the multiple sequence alignments (MSA) leading to different extents of AA changes.

Consensus sequence	MSA	Number of AA changes with respect to *K. marxianus* Eat1
IB0242	13 Eat1 homologous sequences using a 60% cut-off value below which *K. marxianus* AA was kept	71
IB0243	13 Eat1 homologous sequences with 5 extra copies of *K. marxianus* Eat1	22
IB0244	13 Eat1 homologous sequences	84
IB0245	13 Eat1 and 5 Eat2 homologous sequences	88

Different truncated versions of consensus sequences were generated, of which not all could be successfully cloned into an expression host. Streamlined *E. coli* BW25113 *ΔldhAΔackA* was chosen as expression host to test *in vivo* AAT and esterase activity. Interestingly, it appeared that consensus engineering also affected the AAT and esterase activity. One out of four consensus sequences appeared to have lost AAT activity, and showed esterase activity as the main activity. Here, information about the three-dimensional structure could give insights in the reasons for the activity changes. Exploiting this knowledge could subsequently help develop an Eat1 variant expressing only AAT activity. Sequential modifications of this variant may also improve the general understanding of the individual as well as combined effect of the AA changes with respect to activity, stability and affinity.

Based on further *in vitro* assays three promising consensus Eat1 sequences with retained AAT activity were characterized and compared to native Eat1 from *K. marxianus*. For variant IB245 a 2-fold improvement of the half-life at 55 °C was measured and the melting temperature of the protein, determined by measuring the intrinsic tryptophan

fluorescence, also improved slightly by approximately 2 °C. This indicated that consensus engineering can indeed offer a good approach to semi-rational protein engineering for thermostability.

There is still a significant knowledge gap in understanding the different activities of Eat1 and multiple other acetyl transferases and their physiological role. The ping-pong-bi-bi mechanism forms the basis of acyltransferase and esterase reactions (Rauwerdink and Kazlauskas, 2015). The preference of the enzyme towards the acyl donor and the acyl acceptor, are crucial in determining which conversion will take place. Here, the three-dimensional structure of the fully folded protein plays a central role. The oxyanion hole and its surrounding chain interactions determine overall hydrophobicity of the pocket (Jiang *et al.*, 2011). A trend has been observed by relating the hydrophobicity score of the cap domain to acetyltransferase activity of hydrolases (Müller *et al.*, 2020). With an increasing number of computational tools and protein engineering concepts becoming available, more options for improving AAT activity and thermo-stability of Eat1 by semi-rational approaches should be explored.

Ultimately, obtaining crystals of Eat1 is essential to perform the x-ray diffraction required to elucidate the 3D-structure. Only then, the folding, reaction mechanisms and activity preferences of Eat1 can be fully understood. To date, two laboratories have already attempted to obtain Eat1 crystals but did not yet succeed.

Use of alternative AATs

Eat1 was and remained a promising catalyst for bio-based ethyl acetate production. However, as elaborated earlier, there are several pitfalls connected to the use of Eat1. Use of Atf1 as AAT was tested within this project but resulted in mainly acetate formation. While Atf1 has been the most prominent yeast AAT for acetate ester production, the availability of AATs is not limited to the yeast phylae. Several plant AATs have been isolated and successfully expressed in *E. coli*. Especially VAAT and SAAT from strawberries have been shown to exhibit good ethyl acetate producing capacities in *E. coli* (Layton and Trinh, 2014, 2016a). However, data on temperature stability and *de novo* ethyl acetate producing capacities is lacking. Recently also chloramphenicol acetyltransferase has been explored and engineered for the synthesis of short chain esters (Seo *et al.*, 2019). Its high thermostability enabled ester production at 50 °C and makes it an interesting candidate for bio-based ethyl acetate production at elevated temperatures. Ideally, characterization and evaluation of AATs should be further standardized to simplify comparisons among different AATs and to enable fast selection of an AAT for microbial ethyl acetate or other ester production (Chapter 2). As the general principle of supplying an alcohol and an acyl-CoA moiety stays valid, ester production can be modulated by choosing an AAT with desired substrate preferences. The most challenging part will be to ensure provision of

equimolar amounts of both pre-cursors and to restrict production to just the desired chain lengths or acyl-CoA groups.

Outlook and remaining challenges

This research focused on evaluating different strategies for bio-based ethyl acetate production. One crucial aspect was the integration of a DSP vision during process and strain development. Moreover, some remaining bottlenecks were discussed and potential solutions to them were addressed. These insights and considerations are not limited to process design for ethyl acetate but may also be applied in a broader context.

T-R-Y – an outlook on microbial ethyl acetate production

Ethyl acetate is a relevant industrial compound, for which bio-based production should be stimulated and emphasized. However, in contrast to food products or cosmetics, bulk production has to compete with traditional petrochemical processes in terms of quality and price (Van Dien, 2013). The TRY parameters function as guidelines to estimate general feasibility of the envisioned process.

Product toxicity will a remain a crucial bottleneck in bio-process design as most industrially relevant compounds are antimicrobial and thus cannot be produced at high titers. During the design phase, physical properties of the product as well as closely related compounds were explored. By applying gas stripping as ISPR strategy, ethyl acetate concentrations in the fermentation broth can be controlled and product inhibition as well as product degradation can be avoided (Chapter 4). *In situ* product removal based on chemical characteristics of the compounds, such as density, volatility and solubility further initiates a pre-concentration of the product that benefits subsequent DSP steps, as lower volumes have to be processed.

Production rates set the process dimensions once a production goal is set. Whereas under aerobic conditions especially growth rates are high, anaerobic processes characteristically have high product formation rates. However, more fermentation products and specifically organic acids are secreted during anaerobic conditions, leading to acidification of the medium. Several improvements in enzyme expression faded out in serum bottles and improvements by truncating the N-terminus of Eat1 only became significant in bioreactor fermentations (Chapter 3, 4). Both yields and production rates were improved by running pH-controlled bioreactors. Even beyond the acidification and pH effect, the accumulated formate itself may hamper production (Warnecke and Gill, 2005; Vázquez *et al.*, 2011). Once formate levels were reduced by hydrogen co-production, production rates for ethyl acetate doubled while succinate accumulation prevented a concomitant increase in product yield as well (Chapter 5). Consequently, accumulation of all remaining by-products should be carefully analyzed regarding their impacts on overall fermentation performance,

even though pH-controlled systems may be used. In the course of this study, volumetric productivities increased from 0.3 mmol/L/h to 1.6 mmol/L/h, or from 26.4 to 140.8 mg/L/h respectively. Currently, fermentations were initiated with a starting OD_{600} of 0.4, which does not resemble larger scale or commercial fermentations. By increasing the inoculation density volumetric productivities of 2 g/L/h should be possible without much effort.

In order to minimize substrate costs, product yields on substrate needed to be maximized. This can be achieved by conserving electrons and carbons for product formation. The easiest way to conserve carbons are anaerobic processes, as complete oxidation is prevented and the electron transport chain is not active (Weusthuis *et al.*, 2011). Removing competing pathways has become standard procedure, but during anaerobic fermentation some by-products may be inevitable due to redox balance requirements. We presented two approaches to compensate the loss of electrons, first by co-producing another relevant product, and second by metabolically engineering *E. coli* so that ethyl acetate remained the sole fermentation product (Chapter 5, 6). Despite accounting for the recovery of redox cofactors and a positive energy balance, especially fermentation on xylose suggested that there was a discrepancy between energy generated and energy required for maintenance and metabolic load. Further engineering and optimization may lead to a high yield strain for ethyl acetate production but will likely remain very limited in growth.

TABLE 4: Comparison and considerations for different approaches of microbial ethyl acetate production.

Co-production	High yield production
Max pathway yield 1 mol/mol (83 %)	Max. pathway yield 1.2 mol/mol (100 %)
At least two products	Ethyl acetate as sole product
Growth coupled product formation	Growth-uncoupled product formation
Anaerobic process	Aerobic growth phase required
Fed batch or continuous setup	Retentostat setup
Continuous Eat1 expression	Tunable Eat1 expression

Ultimately, one has to consider the pros and cons of engineering towards the maximum theoretical yield, as it will affect further process considerations (Table 5). In the present case a robust pathway was developed with limited by-products, reaching 72 % of the maximum pathway yield of 1 $mol_{ethyl\ acetate}/mol_{glucose}$. With some final optimization such as elimination of succinate production, this maximum pathway yield can be further approached. Engineering towards the theoretical maximum yield, will increases the maximum yield by another 17 %. However, overall metabolism was heavily impacted by the modifications. It remains questionable, if productivities of a strain producing near the maximum theoretical yield can keep up with those obtained by co-production. Ultimately, a conversion yield of 80 % substrate into product is considered potentially feasible for bio-processes. Both explored strategies have the ability to meet this threshold after further optimization.

A comparative outlook on microbial ethyl acetate production

Based on the obtained results and some general assumptions a more economic evaluation can be made regarding economic feasibility of microbial ethyl acetate production. As mentioned before, substrate costs and DSP costs will be the most crucial factors determining feasibility of a bioprocess. Both factors have been addressed thoroughly within this study by including ISPR and engineering towards high product yields. By applying an anaerobic setup, costs for cooling are reduced and transfer limitations are prevented. Gas stripping ensures consistently high production rates and reduced DSP costs due to pre-concentration. Recovery of the product from the gas stream needs to be evaluated in more detail, but has been considered promising in the case of ethanol or butanol before (Groot *et al.*, 1992; Groot, van der Lans and Luyben, 1992; Ezeji *et al.*, 2005). In comparison to these two examples, ISPR of ethyl acetate is even more favorable due to its higher volatility. Consequently, production costs for bio-based ethyl acetate, should be estimated in a similar range as for bio-ethanol.

Bio-ethanol is among the most prominent example of production by bio-based conversion of a cheap product and may therefore serve as a good reference point. It was concluded that about one third of the production costs relate to the feedstock and volatility in the market imposes a big threat for existing production plants (Balat, Balat and Öz, 2008). Therefore, reduction of costs by using cheaper feedstocks, such as lignocellulosic biomass will remain key not only for competitive bio-based ethanol production but for any future bio-based bulk chemical (Keasling *et al.*, 2021).

A few bio-based esters have already entered the market, mainly focusing on esterification of bio-based derived acids and alcohols (Datta and Tsai, 1995; Corbion, 2019). As mentioned before, this approach is limited by product toxicity and inefficient DSP options, especially for acetate in the case of ethyl acetate. Moreover, an additional conversion step needs to generate the ester from the bio-based substrates. Consequently, two bio-processes need to proceed at high yields to be ultimately combined and condensed into the desired ester in an additional operational unit. The proposed one step conversion of ethyl acetate from carbohydrates by microbial conversion only requires final purification of the pre-concentrated product and should therefore be more efficient and economical. Co-production of H_2 may provide an additional sales opportunity enabling additional revenue options.

The decent extractability of ethyl acetate and its reduced toxicity in direct comparison to its precursors, make direct microbial ethyl acetate production not only interesting for the compound itself (Chapter 2). By applying a hydrolysis step after ethyl acetate extraction, bio-based ethanol and acetate can be obtained in a concentrated stream. Therefore, esters offer great potential towards a bio-based production platform that can operate at high yields and rates, while being coupled to an ISPR strategy. Based on demands and

process capacities, a flexible pipeline for ester, alcohol and acid production can be set up making the process more robust towards changing market demands (Noorman and Heijnen, 2017).

Bio-process engineering means dealing with compromises

Whereas yeasts are natural ethyl acetate producers, we chose the development of a microbial cell factory for heterologous ethyl acetate production. A benefit of heterologous enzymes is the lack of regulatory mechanisms in the expression host. The expression can be tuned and regulated by promoter and ribosome binding site (RBS) couples of choice, without further interference by native regulation. This is an advantageous feature when compared to natural producers such as yeast where native regulation and mechanisms of ester production are still not fully understood (Kruis, Gallone, *et al.*, 2018). Nevertheless, ethyl acetate production by natural producers has been optimized to a great extent and may be especially interesting for applications with considerable overlap to the food industry (Hoffmann *et al.*, 2021). The use of thermophiles as microbial cell factory has been proposed as well, due to beneficial effects of elevated temperatures on the ISPR strategy. Despite the ability to use a wide range of substrates and being relatively robust, tools for genetic modifications and optimizations are rather limited (Taylor *et al.*, 2011). Considering the vast engineering efforts in *E. coli* to enable maximum theoretical yields, even more resources will have to be invested to engineer an efficient thermophilic production strain.

It needs to be considered that every (micro) organism is occupying a specific niche within its ecosystem (Baquero *et al.*, 2021). High tolerances towards toxic compounds, metabolic pathways to capture and utilize scarce nutrients and carbon sources, or the ability to survive in extreme environments mark a few characteristics. However, seldomly all features are found in one single organism. Within the concept of microbial cell factories positive attributes are collected and merged into a highly specific and optimized manner (Lee, Mattanovich and Villaverde, 2012; Nielsen and Keasling, 2016). Every choice is connected to certain consequences within the process, either during strain development, fermentation or downstream. In order to design a robust and competitive bio-process, the analysis of pros and cons of each choice should be outweighed and a clear goal including DSP options should be formulated.

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Back matter

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Of course a big thank you to office E2.133! We set the bar high, at least one of us was sitting in the office working hard, no matter the time. But still there was always room for a chat, to share problems or ideas, to talk about the weekend or vacation plans or just to take a break or to discuss the latest impact factors of some journals. **Pauline**, you were my supervisor during my MSc Thesis, but once I started my PhD we were directly more than colleagues. Having our morning coffee at the department, having a break watching videos or cute animal pictures, working out at the gym or kicking the weekend off with a Friday borrel; we had quite a routine at and around work! **Enrico**, with your presence and stories you could always cheer up everyone. No matter if in the lab, in the office, at a borrel or dinner, there are so many good memories thanks to you! **August**, you are incredible. I have never seen anyone else so hard working, focussed and on the other hand so sneaky. Almost every time we felt guilty for disturbing your workflow, you just turned around smiling and overhearing the conversation all along. It was great having you in the office and I am sure that at one point you will become a chair! **Chunzhe**, you were the latest addition to the office. At first we thought of nicknaming you October, but you made it quite clear that we will all learn your real name earlier or later and Enrico surely did his part of the job as well. I admire your strength and will to learn, languages, habits, and

skills and to stay positive. Keep up the good work and good luck for the last part of your thesis. Eventually, my second office was located in E2.129 to discuss latest results or new ideas, to vent about another failed experiment or to just procrastinate at the end of a long working day. Thanks to **Edgar** and later **Pedro** for tolerating my presence and bearing with me all those times.

Thanks to the Dinner/Beers group for keeping a good work/life balance or for at least relocating work to the Bongerd, the Spot or a nice terrasse. **Calvin**, my recruit and partner in crime in the VLAG Council. I enjoyed your company throughout the years, your calmness and politeness. It was great catching up on things in the lab and to look after each other in this last year, especially. You are also close to the finish line and I know you can make it! **Sabine**, we probably interacted more on a VLAG level than on a BPE level, but it was nice getting to know you better. Thanks for the cookies, treats and sweets in the office and your always positive attitude. **Narcis**, I am happy to look back at all our trips starting with our Coffee corner MSc group and continuing as PhDs. Trips to Texel, the Christmas market or our California trip, it was great having you on board, including your ability to take naps almost instantly and anywhere. Sometimes you are just too nice for the world and with your overplanning and crazy working ours, we often needed to drag you out of the lab to relax a bit. You were our team captain at the Bravo FC and motivated quite some colleagues and students to join. Thank you for keeping everyone fit and allowing me to be coach. ;) **Kylie**, thanks for the good memories during girls nights, short trips or during organizing borrels or labuitjes. We could always count on some interesting topics for thorough discussions and got quite some insights into the vegan cuisine when enjoying a drink or two. **Fabian**, you are the most Italian person I know and your cocktail mixing skills are dangerous. So many good memories at your house, from BBQ to a pasta workshop or just chill evenings on your roof terrasse. Thanks for being a fantastic host! **Pieter**, thanks for your happy face and a great trip along the West Coast. While at times your orange algae drove me crazy, especially when the whole lab was smelling like dead fish, it was always nice to look at. **Barbara**, thank you for your enthusiasm and joy you bring to the department. **Camilo,** thanks for the nice chats about photography, cameras and lenses, stocks or just work. **Robin**, thanks for the fun times at your birthdays and the nice chats in the hallway about working loads, students and Council issues. **Jin,** thanks for hosting a great dumpling and BBQ dinner at your place and for introducing us to the world of Chinese liquor.

Some people may have only stayed in BPE for a limited time, were located outside BPE or left for new challenges after finishing their PhD. Still you are part of all the good memories I have, for nice chats, fun activities and great reunions throughout the years. Thank you to my colleagues **Lukas**, **Renske**, **Rocca**, **Mihris** and **Sebastian;** to my short-term colleagues **Rafa, Filippo, Sofia, Azar, Ana, Ivar** and **Aamna** for the good times and conversations in- and outside the lab and the coffee corner. And of course thank you to the old guard;

Youri, Edgar, Catalina, Elisa, Aziz, Stephanie it was a pleasure meeting you and seeing you finish. After four years of work I am happy to follow your footsteps and to take off to new adventures.

Unfortunately, the Corona pandemic limited the options to get to know the new generation. **Jort, Pedro, Nuran, Nicola**, and **Carlos**, I am sure there will be plenty of time to go wild and to compensate for the missed opportunities. It was great meeting you and getting to know you and your projects a bit for the limited time everybody was working in the labs. Good luck with your PhDs and don't forget that there is also a life outside the lab!

Also outside BPE, there were plenty of people to count on and I am happy to have met and worked with so many smart, interesting and kind people.

A toast to the Coffee corner - group, **Gisela, Narcis, Anastasia, Adil, Fons, Rob, Marina**, **Timo.** Even though our golden times were during working on our MSc theses, you all set the fundament to my good memories at BPE. And even after we managed to see and support each other somewhat from the distance. I am happy to have shared those 6 months with you guys. We worked hard and played hard, experienced our first struggles in the lab, but also our first sweet successes.

Thanks to the colleagues at MIB, **Wen, Nico, Jeroen, Costas, Ioannis, Melvin, Joyshree, Max, Belen, Prarthna, Eric,** and **Martijn** for nice chats, useful advice and mental support whenever we met.

Cheers to the VLAG PhD Council for all the extra hours we pulled next to our PhD. I enjoyed our meetings and the coaching trajectory and I am sure you are up to something great in the future. **Koen** and **Katharina**, thanks for the nice and easy transition, leading to a fun dinner where we all got to know each other better. At this point I would also like to thank the VLAG office, especially **Anouk** and **Vesna**, for their commitment and the nice collaboration, as well as the VLAG Board and **Karin**, for the insightful meetings and good discussions.

Linde, I am so proud of us that we managed to keep the contact throughout the years. Whereas our studies went into different directions, every time we met we could directly continue with where we had left. Especially in the last months you were a great asset checking on me, and telling me that stressing out is normal. Thanks for keeping me on track! And even though your positive vibes easily spread across the Atlantic, I am happy you are located in Germany for the future.

Marie, we have crossed paths multiple times but somehow we only really met once I joined the VLAG PhD council. When I look back at the last 1.5 years I can only vaguely imagine what could have been if we met earlier and before Corona. We were directly on the same page, found a great way of getting our VLAG things organized, and could rely on each other. But most importantly we managed to combine work with pleasure and I am happily looking back at our dinners, drinks and audio messages.

Thanks to the Stickers, and particularly **Bram, Nico, Carrie, Anne, Stijn, Maketa, David** and **Martine.** Despite the fact that I didn't play floorball during my PhD, we met there and still crossed paths every now and then in Wageningen. It was always great to catch up and to hear what is going on outside the lab. You are still part of some good Wageningen memories and I am thankful for that.

Thank you **Kevin, Linn, Tom, Kitty, Lukas** and **Pim** and all the people I met at Stal Doorwerth. I enjoyed working with you and the horses a lot and those days I definitely returned home with a big smile on my face. Thanks for being so flexible with my varying availability, for your trust and warm welcoming, and for the cozy coffee breaks.

I would like to thank the Dutch Studentriders, for welcoming me with open arms and adopting me as one of their Dutchies. Over the years we had great fun, crazy weekends, BBQs and memorable (road)trips. It was always a nice contrast to the Wageningen bubble and yet you never complained when I talked about bacteria, goldfish, genes, reactors or m&m's. But luckily you also made sure I stayed up to date on Dutch music, typical phrases and how to pronounce 'onvoldoende'! Thank you **Marieke, Floor, Rosan, Ans, Renske, Milou, Martenique, Jos, Michael, Nancy** and **Gaetan, Max, Louise** and **Dennis, Sophie, Marije, Annelous** and **Geert, Marjolein, Alex, Alex, Elise, Marlies, Joanne, Nicky** and **Jaco, Noortje, Rosemarijn, Joyce, Maaike, Fleur, Leo, Niels,** and my OC and board colleagues, **Marlieze, Daphne, Mark, Rik, Lisanne,** and **Shannon** for all the memories. It was also nice to have some more German support via **Hanna** and **Mariell,** and great to get more in touch with the German student riders. Big thanks to the entire AIEC family. It is amazing to meet so many great and crazy people from all across the world. Those trips and competitions were retreats and you could just blend out the real world for a while. They also led to great friendships across borders, and a network beyond compare. I am thankful for some great activities, such as vacation in Spain, attending Ascot race day or a hunts ball, or just meeting fellow student riders at Military Boekelo, Zangersheide or Amsterdam. Thank you **Sophie, Lauren, Sophie, Tatiana, Katrin, Annette, Luana, Lauren, Sarah, Andi, Beatrice, Cecilia, Frank, Rosie, Jen, Joel, Kaeye, Dries, Jolien, Nina, Robin, Ian, Maria**, **Yasemin**, **Thomas, Yannick, Gro, Shane, Elena, Max, Philip, Ines, Anke, Joey** and so many more! I am also looking back to two amazing vacations in Croatia with special thanks to **Bronson,** for being a great skipper and **Moritz, Julia, Sarah, Moritz** for being part of a fun crew.

Julia, wir haben uns auf dem Boot kennengelernt und den Kontakt gehalten. In der kurzen Zeit hat sich eine unglaubliche Freundschaft entwickelt. Wir haben die Feststelltaste für Sprachnachrichten gefeiert, Rekorde aufgestellt und Distanz-Fernsehen quasi erfunden. Trotz der Entfernung von schlappen 400km warst du immer da, hattest ein offenes Ohr wenn ich mich mal wieder sortieren musste, hast mich bestätigt und unterstützt. Es war manchmal schon unheimlich wie einig wir uns immer waren und wie ähnlich unsere

Sichtweisen und Ansprüche sind. Vielen Dank, das du da bist und auf viele weitere Filmabende, Wellness-Wochenenden, Urlaube und Eintreffabende!

Natürlich auch ein großes Dankeschön an meine Familie. **Dirk**, auch wenn wir in vielen Dingen ziemlich unterschiedlich sind, können wir immer auf einander zählen. Unsere Trips zum SuperCar Sunday oder zur Messe haben wir immer gut genutzt um uns wieder auf den neusten Stand zu bringen und deine Sprüche haben so manches Gespräch erheitert. **Mama, Papa**; Danke für all die Jahre der Unterstützung. Ihr habt immer an mich geglaubt, euch mit mir gefreut und mir Mut zugesprochen wenn es mal nicht so lief. Nach Hause zu kommen war und ist immer wie ein Kurzurlaub für mich, bei dem ich neue Kraft und Motivation tanken konnte, vor allem durch euch! Danke, dass ihr immer für uns da seid!

About the author

Anna Christina Bohnenkamp was born on May 7th 1992, in Osnabrück (Germany). After high school she moved to the Netherlands and pursued her BSc degree in Biotechnology at Wageningen University. During an exchange semester at Cornell University (US) she completed her minor courses with focus on biological and environmental engineering and passed her degree with distinction.

In 2014, Anna started her MSc in Biotechnology with specialization in process technologies. Her MSc thesis on additional metabolic energy generation in *E. coli* was performed at the bioprocess engineering group of Wageningen UR. During an internship at Chr. Hansen in Denmark in the fermentation department she studied the effects of trace elements and stresses on lactic acid bacteria and their shelf lives. Anna concluded her MSc career in which she also participated in summer schools at Aarhus University (DEN) and Imperial College (UK) and graduated in 2017 with distinction.

After her graduation, Anna stayed at Wageningen UR and pursued her PhD in the group of Bioprocess Engineering starting in April 2017. As part of the Microbial Biotechnology cluster, she evaluated different approaches towards efficient microbial production of ethyl acetate for bulk applications. Optimizing the fermentation process and ensuring a feasible downstream processing strategy played a central role in her project.

List of publications

Bohnenkamp, A.C., Wijffels, R.H., Kengen, S.W.M., Weusthuis, R.A., 2021. Co-production of hydrogen and ethyl acetate in *Escherichia coli*. Biotechnology for Biofuels.

Bohnenkamp, A.C.*, Kruis, A.J.*, Mars, A.E., Wijffels, R.H., van der Oost, J., Kengen, S.W.M., Weusthuis, R.A., 2020. Multilevel optimisation of anaerobic ethyl acetate production in engineered *Escherichia coli*. Biotechnology for Biofuels. 13.10.1186

Kruis, A.J.*, Bohnenkamp, A.C.*, Nap, B. Nielsen, J., Mars, A.E., Wijffels, R.H., van der Oost, J., Kengen, S.W.M., Weusthuis, R.A., 2020. From Eat1 to trEat1: Engineering the mitochondrial Eat1 enzyme for enhanced ethyl acetate production in *Escherichia coli*. Biotechnology for Biofuels. 13. 10.1186

Kruis, A.J.*, Bohnenkamp, A.C.*, Patinios, C.*, van Nuland, Y.M., Levisson, M., Mars, A.E., van den Berg, C., Kengen, S.W.M., Weusthuis, R.A., 2019. Microbial production of short and medium chain esters: Enzymes, pathways, and applications. Biotechnology Advances. 37. 107407.10.1016

Overview of completed training activities

Discipline specific activities

Conferences

NBC Biotechnology Conference, NBV, (Wageningen, The Netherlands)	2017
Microbial Centennial Symposium, WUR (Wageningen, The Netherlands)	2017
Industrial Synthetic Biology Congress (Munich, Germany)*	2018
NBC Biotechnology Conference, NBV (Ede, The Netherlands)**	2019
Emerging Applications in Microbes Conference, VIB (Leuven, Belgium)*	2019
ECCE/ECAB, AIDIC (Florence, Italy)**	2019
NBC Biotechnology Conference, NBV (online)	2020

* Poster presentation, ** oral presentation

Courses

Advanced Course Microbial Physiology and Fermentation Technology, BDSL (Delft, The Netherlands)	2018
Scientific Computing for Life Scientists and Metabolic Engineering for Cell Factory Design, DTU (Lyngby, Denmark)	2018

General courses

VLAG PhD Week, VLAG (Baarlo, The Netherlands)	2017
PhD Carousel, WGS (Wageningen, The Netherlands)	2017/18
Competence Assessment, WGS (Wageningen, The Netherlands)	2017
Adobe InDesign, WGS (Wageningen, The Netherlands)	2019
Scientific Artwork, WGS (Wageningen, The Netherlands)	2020
Introduction to R, WGS (Wageningen, The Netherlands)	2020
Career Perspectives, WGS (Wageningen, The Netherlands)	2020
Critical Thinking and Argumentation, WGS (Wageningen, The Netherlands)	2020
Coaching VLAG PhD Council, VLAG (Wageningen, The Netherlands)	2021

Other activities

Preparation of research proposal	2017
Group Day BPE	2017-21
Microbial Biotech Theme Meeting	2017-21
PhD study tour	2018
VLAG PhD Council	2018-21

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