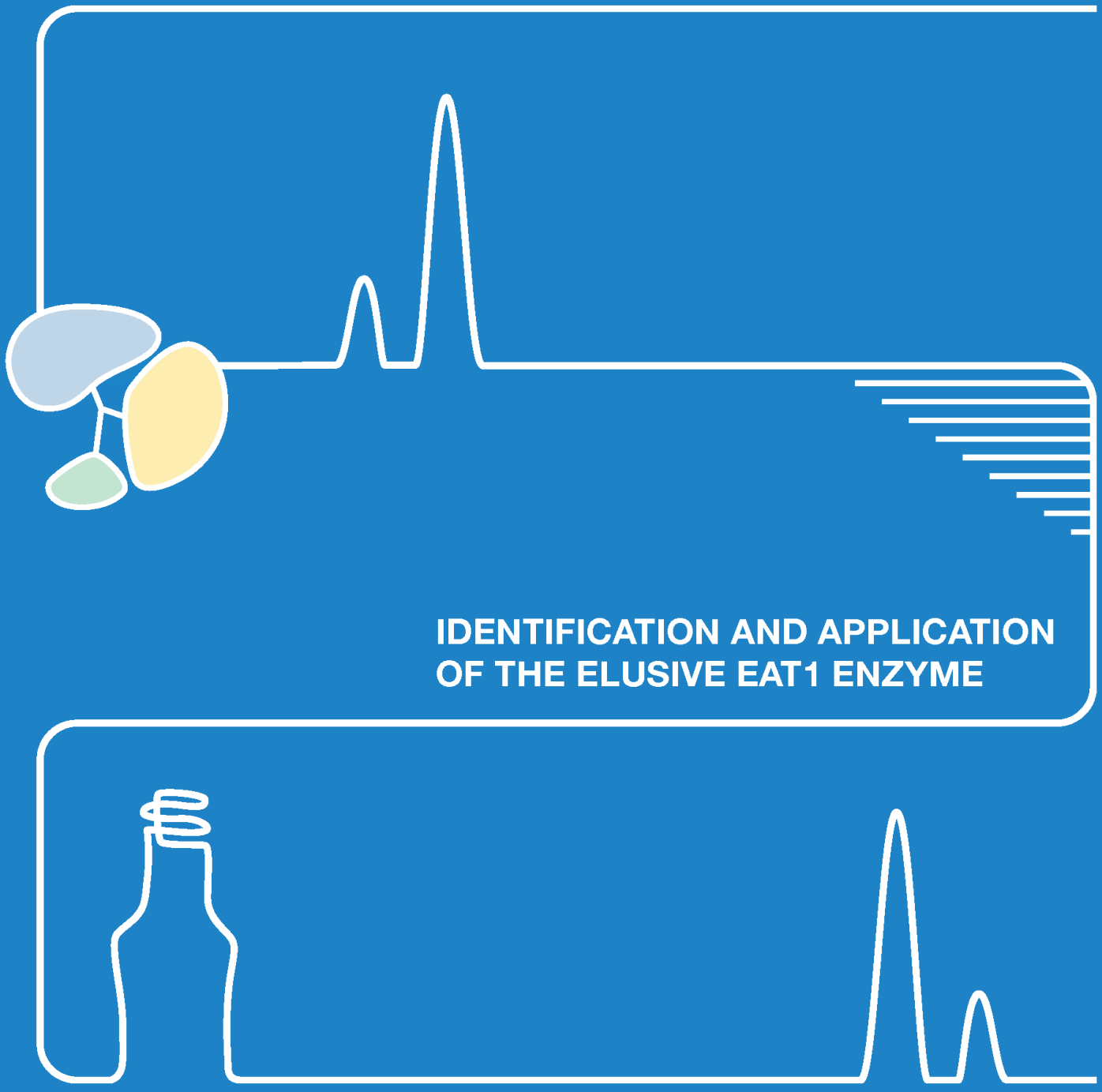
TOWARDS BIOBASED ETHYL ACETATE AND BEYOND



IDENTIFICATION AND APPLICATION OF THE ELUSIVE EAT1 ENZYME

Aleksander J. Kruis

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Thesis

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CHAPTER 1

General introduction and Thesis outline

General introduction

Volatile esters are abundant in nature and are mostly known as substances associated with plants, providing them specific aromas (Defilippi et al., 2009; Holland et al., 2005; Yan et al., 2018). However, microorganisms, especially yeasts, are also known as natural ester producers. In fact, ester levels play a vital role in the flavour and aroma profile of yeast fermented food products, such as beer and wine (Saerens et al., 2010). Esters are formed through an esterification reaction of an organic acid with an alcohol and it is the type of acid and alcohol that determines the ester's chemical and physical properties. Since many organic acids and alcohols are produced by organisms, many different esters have also been identified (Dzialo et al., 2017). Ethyl acetate is an example of such an ester that is the esterification product of ethanol and acetic acid. It is produced naturally by some plants, such as a species of fragrant pear during fruit ripening (Chen et al., 2006), as well as by several species of yeast (Löser et al., 2014; Saerens et al., 2010).

Industrially, ethyl acetate is used as a solvent in the chemical industry and is considered environmentally friendly due to its low toxicity and biodegradability (Chan and Su, 2008; Kam et al., 2005). Its global production reached an estimated 3.5 million tonnes at a total value of $3.7 billion (The Market Publishers, 2014). However, ethyl acetate is still produced from non-renewable resources, reducing its sustainability. In recent years, microbial conversion of biomass has received attention as an alternative for various petroleum and gas-derived chemicals. This includes biobased ethyl acetate. Several companies have been investigating the production of biobased ethyl acetate. Zeachem (US) has a patented pipeline process for the production of biobased ethyl acetate (de Jong et al., 2012). In the process, lignocellulose is converted to simpler carbohydrates which are then fermented by homoacetogens to acetate. In the next step, acetate is chemically esterified with ethanol to form ethyl acetate (Verser and Eggeman, 2013) Johnson Matthey Davy Technologies (UK) also developed a process to make biobased ethyl acetate, where biomass-derived ethanol is chemically converted to ethyl acetate in several steps using metal catalysts (Colley et al., 2004). In both examples, biobased ethyl acetate is produced from fermentation products, and as a consequence the production depends on the efficiency of acetate or ethanol

fermentation. Bio-ethanol fermentation is well established in the industrial world, but homoacetic acid fermentation can be problematic due to acetate toxicity (Sheu et al., 1975). Moreover, extra chemical steps are required to form ethyl acetate from ethanol or acetate, adding to the cost of the process. On the other hand, some yeast species produce ethyl acetate directly from sugars, without the need of a chemical esterification step (Table 1). They may provide a more efficient way of producing biobased ethyl acetate. Ethyl acetate is a volatile compound and evaporates during fermentation, facilitating *in situ* product removal, simplifying downstream processing and reducing product inhibition in the culture. The volatility of ethyl acetate has also been a pitfall of many studies. The continuous removal of the ester from the production medium was often neglected or not accounted for accurately (Löser et al., 2014). For this reason, ethyl acetate production by microorganisms is often underestimated (Table 1).

Bulk ethyl acetate production in yeast

Many yeasts produce esters, but they differ in the types and amounts of esters produced. For example, *Saccharomyces cerevisiae* produces several esters during wine and beer fermentation, of which ethyl acetate is the most abundant (Saerens et al., 2010). Compared to ethanol, which is the main fermentation product of *S. cerevisiae*, esters are produced in low amounts. Nevertheless, they have a pronounced impact on the flavour of fermented products. Ester production has been studied extensively in yeasts that are used in food production (Dzialo et al., 2017; Liu et al., 2004). Ethyl acetate is produced by *S. cerevisiae* in amounts less than 1% of the theoretical maximum (Table 1). However, other yeast species produce significantly higher amounts of ethyl acetate. *Kluyveromyces marxianus* (previously *Candida pseudotropicalis* and *Kluyveromyces fragilis*), *Wickerhamomyces anomalus* (previously *Hansenula anomala* and *Pichia anomala*) and *Cyberlindnera jadinii* (previously *Candida utilis*) have been studied the most in this respect. Different conditions have been described as the cause of ethyl acetate synthesis in these yeasts, but parallels between the species do exist. The yeasts produced large amounts of ethyl acetate only when ethanol was present in the medium. Moreover, they produced most of the ethyl acetate only after the sugars were depleted and ethanol was utilised for growth (Armstrong et al., 1984a; Löser et al., 2013; Tabachnick and Joslyn, 1953a). The need

for ethanol is not surprising since three of the four biological pathways that form ethyl acetate in yeast use ethanol as a substrate. All three species belong to the group of Crabtree-negative yeasts (Fredlund et al., 2004a; Postma et al., 1989; Wardrop et al., 2004), meaning that oxygen availability controls ethanol formation, and consequently also ethyl acetate formation. The effect of oxygen limitation on ethyl acetate production was demonstrated in *W. anomalus* (Fredlund et al., 2004a). Under fully aerobic conditions, this yeast produced only marginal amounts of ethanol and ethyl acetate. However, when oxygen became limiting, ethanol and ethyl acetate formation began rapidly. In contrast, Rojas *et al.* reported that *W. anomalus* produced ethyl acetate under aerobic conditions in a shake flask experiment (Rojas et al., 2001). However, it is possible that aeration of the flask cultures was not sufficient for fully aerobic conditions and that oxygen limitation occurred (Löser et al., 2014). Another way of inducing ethyl acetate synthesis in yeast is trace element limitation. Iron limitation specifically plays a major role, as was demonstrated in *K. marxianus* (Urit et al., 2012) and in *C. jadinii* (Armstrong and Yamazaki, 1984). Interestingly, this effect has not been observed yet in *W. anomalus.* To elucidate the mechanism, additional experiments are needed on the molecular level. However, research on ethyl acetate producing yeasts so far focused mostly on studying and optimising the environmental conditions under which ethyl acetate is produced. In that regard, *K. marxianus, W. anomalus,* and *C. jadinii* are the most studied.

K. marxianus is a natural ethyl acetate producer. Several strains were identified that form ethyl acetate from whey sugars (Kallelmhiri et al., 1993; Löser et al., 2011), glucose (Willetts, 1989), and cassava bagasse supplemented with glucose (Medeiros et al., 2001). The yeast has other remarkable physiological traits that add to its biotechnological potential (Fonseca et al., 2008). *K. marxianus* is naturally able to catabolise lactose and can utilise whey, a waste stream of the cheese industry to produce value-added products. It also grows at higher temperatures than most other yeasts. It grows above 40 °C (Anderson et al., 1986) and even at 52 °C (Banat et al., 1992). Cultivation at elevated temperatures helps facilitate *in situ* removal of ethyl acetate by evaporation, thus reducing product inhibition in the cultures. *K. marxianus* was successfully used to produce ethyl acetate at 42 °C at 56.2 % of the theoretical

pathway maximum (Urit et al., 2013a), which was higher than in a comparable fermentation at 32 °C (Table 1). Ethyl acetate production by *K. marxianus* was even scaled to pilot level at 32 °C where 51.4% of the theoretical pathway maximum was achieved (Löser et al., 2013). The studies by Löser *et al.* on ethyl acetate production in *K. marxianus* are some of the few that correctly account for ester evaporation.

Studies on ethyl acetate production in *W. anomalus* and *C. jadinii* did not accurately account for the loss of ethyl acetate. The reported titres and yields (Table 1) are therefore likely underestimated. *W. anomalus* was the first yeast for which bulk ethyl acetate was observed (Beijerinck, 1892). The yeast was cultured in standing flask cultures in glucose medium (Table 1). It produced ethyl acetate at 36.9 % of the theoretical pathway maximum (Tabachnick and Joslyn, 1953a). This yield of ethyl acetate is the highest reported for *W. anomalus* on glucose. When using ethanol as the sole source of carbon, it produced ethyl acetate at 44.8% of the theoretical pathway maximum. Interestingly, *W. anomalus* was also reported to degrade ethyl acetate, once the ethanol was consumed (Tabachnick and Joslyn, 1953a), most likely through an esterase reaction (Rojas et al., 2002; Schneider et al., 2012; Tabachnick and Joslyn, 1953b). *C. jadinii* needs iron limitation or oxygen limitation to produce ethyl acetate (Armstrong et al., 1984a; Armstrong and Yamazaki, 1984). On glucose, the yeast produced ethyl acetate with a similar yield observed in *W. anomalus* (Table 1). *C. jadinii* was grown on ethanol as a substrate in most of the ethyl acetate production studies. In one of the studies, it converted almost 50 % of the ethanol to ethyl acetate (Armstrong et al., 1984a).

K. marxianus, W. anomalus and *C. jadinii* were studied the most for ethyl acetate production. Other yeast species have been studied less but may still be interesting in terms of biobased ethyl acetate production. Examples of other yeasts include *Kluyveromyces lactis* (Löser et al., 2011), *Cyberlindnera fabianii* (Meersman et al., 2016; van Rijswijck et al., 2017), *Dipodascus aggregatus* (Norrman, 1969) and *Hanseniaspora guilliermondii* (Rojas et al., 2001). *D. aggregatus* studies failed to quantify the ethyl acetate, but still indicate that the yeast is an interesting potential producer. *H. guilliermondii* produced only 1.3 % of the maximum theoretical yield of

ethyl acetate, which is significantly less compared to the 21.4 % achieved by *W. anomalus* in the same experiment. However, it is possible that the fermentation conditions were not optimised for this yeast and that its potential is underestimated.

Table 1: Selected efforts of ethyl acetate production in microorganisms.

Strain	Substrate	Yield (g/g)	Fraction pathway maximum (%)	Notes	Reference
Saccharomyces cerevisiae					
T_{73}	40 g/L glucose	0.0005*	0.1		(Rojas et al., 2001)
Wickerhamomyces anomalus					
CECT 10590	40 g/L glucose	0.103*	21.4		(Rojas et al., 2001)
No. 317 in Berkley collection	52 g/L glucose	0.18*	36.9		(Tabachnick and Joslyn, 1953a)
No. 317 in Berkley collection	18 g/L ethanol	0.43*	44.8		(Tabachnick and Joslyn, 1953a)
No. 20 in Seagram collection	50g/L glucose	0.07*	13.3		(Gray, 1949)
Kluyveromyces marxianus					
DSM 5422	78 g/L whey sugars	0.25	48.7	Iron limited fermentation	(Urit et al., 2012)
DSM 5422	78 g/L whey sugars	0.04	8.6	Copper limited fermentation	(Urit et al., 2012)
DSM 5422	78 g/L whey sugars	0.23	45.0		(Urit et al., 2011)
DSM 5422	78 g/L whey sugars	0.26	49.9	Iron limited fermentation	(Löser et al., 2013)
DSM 5422	78 g/L whey sugars	0.29	56.2	Iron limited fermentation at 42 °C	(Urit et al., 2013a)
Cyberlindnera jadinii					
ATCC 9950	20 g/L glucose	0.23*	46.3	Iron limited fermentation, EDTA addition	(Armstrong and Yamazaki, 1984)
ATCC 9950	190 g ethanol	0.09*	9.8	Iron limited fermentation	(Corzo et al., 1995)
ICIDCA L/375-1	32 g/L ethanol	0.05*	4.9	Iron limited fermentation	(Christen et al., 1999)
ATCC 9950	30 g/L glucose	0.10*	19.9	Iron limited fermentation	(Armstrong, 1986)
Hanseniaspora guilliermondii					
CECT 11104	40 g/L glucose	0.01*	0.9		(Rojas et al., 2001)
Escherichia coli					
JCL 260	50 g/L glucose, 2.5 g/L ethanol	0.01	0.7	*S. cerevisiae* Atf1 as catalyst	(Rodriguez et al., 2014)
TmCHMO-MiADH	12.0 g/L tryptone, 24.0 g/L yeast extract, 5.0 g/L glycerol, 0.75 g/L 2-butanol	N/A (0.11 g/L/h)[1]	N/A	Conversion of 2-butanol to 2-butanone and further to ethyl acetate (BVMO)	(Pereira et al., 2018)

Yields marked with * are estimates due to inadequate quantification of ethyl acetate evaporation. Yields were calculated from total substrate added to the fermentation and total ethyl acetate produced. 1 – yield was not reported, productivity is given instead

Enzymatic reactions behind ethyl acetate formation

Four enzymatic activities have been associated with microbial ethyl acetate formation: alcohol acetyl transferases (AAT), esterases, hemiacetal dehydrogenation (HADH) and Baeyer-Villiger monooxygenases (BVMO) (Figure 1).

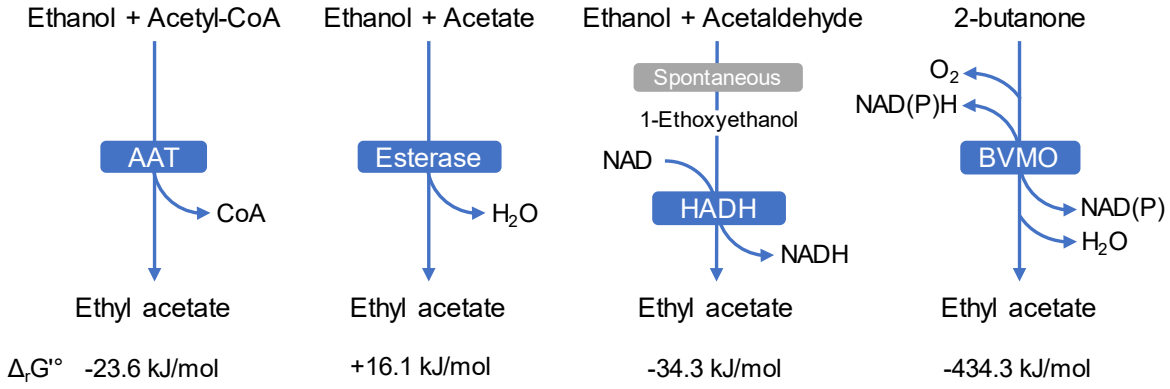

Figure 1: Four possible enzymatic mechanisms of ethyl acetate formation and their ΔrG'° values. ΔrG'° of ethyl acetate forming reaction under standard conditions at pH 7 were calculated with Equilibrator (Flamholz et al., 2014).

Under standard conditions, ethyl acetate formation by esterases is thermodynamically unfavourable (Figure 1). As a consequence, only small amounts of ester are formed from acetate and ethanol in aqueous environments. Despite this, several reports have found reverse esterase activity as the cause of ethyl acetate formation in microorganisms. Yoshioka *et al.* made such an observation in *W. anomalus* by incubating whole cells with acetate and ethanol (Yoshioka and Hashimoto, 1981). It is possible that *W. anomalus* metabolised the acetate or ethanol to ethyl acetate via any of the reactions shown in Figure 1. Other studies incubated purified protein or cell free extracts (CFE) at pH as low as 3, or at high initial ethanol concentrations (Costello et al., 2013; Kashima et al., 1998). Such conditions push the reaction equilibrium more towards ester formation (Flamholz et al., 2014). Nevertheless, the ethyl acetate that can be produced this way is still significantly lower than the amounts of ethyl acetate observed in yeasts such as *K. marxianus* and *W. anomalus* (Peel, 1951). It should be noted that esterases are able to synthesise esters in low-water systems (Kapoor and Gupta, 2012). Considering that biological reactions occur in aqueous systems makes esterases unlikely catalysts of bulk ethyl acetate production in yeast. On the other

hand, AATs, HADHs and BVMOs all catalyse reactions that are thermodynamically favourable under physiological conditions (Figure 1).

Alcohol acetyl transferases

Alcohol acetyl transferases (AATs) are a large and diverse group of enzymes and have been identified in plants (Aharoni et al., 2000; Holland et al., 2005; Shalit et al., 2001), bacteria (Costello et al., 2013), yeast (Fujii et al., 1994; Nagasawa et al., 1998; Saerens et al., 2006) and filamentous fungi (Yamauchi et al., 1989). AATs catalyse ester formation by transferring the acyl moiety from an acyl-CoA molecule to an alcohol, releasing free CoA in the reaction. Several AATs have been characterised and they greatly differ in their preference for alcohol and acyl-CoA substrates. Some AATs form short chain esters, such as ethyl acetate, whereas other AATs form long chain wax esters (Shi et al., 2012). AATs are used by *S. cerevisiae* to produce esters during wine and beer fermentation (Fujii et al., 1996; Saerens et al., 2010). Two main groups of esters are formed by *S. cerevisiae* during fermentation (Saerens et al., 2010). Medium chain fatty acids (MCFA) ethyl esters, such as ethyl hexanoate (pineapple aroma) and ethyl octanoate (sour apple aroma), are formed by Eht1 and Eeb1 (Dzialo et al., 2017; Knight et al., 2014; Saerens et al., 2006). The second and most abundant group are acetate esters. The most prominent examples include ethyl acetate (fruity, solvent-like aromas), isoamyl acetate (banana aroma) and phenylethyl acetate (rose, flowery aroma). In *S. cerevisiae*, acetate esters are formed by Atf1 (Minetoki et al., 1993) and its paralog Atf2 (Nagasawa et al., 1998). However, an Δ*atf1*Δ*atf2* strain was still able to produce roughly 50% of the original ethyl acetate amounts. This strongly indicated that other acetate ester-forming AATs are present in the yeast (Verstrepen et al., 2003b). Alternatively, *S. cerevisiae* may utilise other mechanisms to produce ethyl acetate. *S. cerevisiae* overexpressing *atf1* and *atf2* produced more ethyl acetate, while *atf1* showed a more significant effect on increasing ester content than *atf2*. However, both *atf1* and *atf2* overexpression led to a higher relative increase on isoamyl acetate production, compared to ethyl acetate (Verstrepen et al., 2003b). AAT activity has been associated to ethyl acetate formation is some bulk ethyl acetate-producing yeasts (Kusano et al., 1999; Thomas and Dawson, 1978), but the specific enzymes were not identified.

The catalytic mechanisms of AATs are also poorly understood. There are no known crystal structures of any AAT. All current knowledge is based on *in silico* models. They show that the yeast AATs belong to two groups. Atf1 and Atf2 share some characteristics of the BAHD O-acyltransferases. The abbreviation BAHD is derived from the first letters of the four members of this family that were originally characterised (D'Auria, 2006). BAHD O-acyltransferases are mainly present in plants and bacteria. Its members share the conserved HXXXD and DFGWG motifs (D'Auria, 2006; Galaz et al., 2013). Curiously, Atf1 and Atf2 lack the DFGWG motif (Nancolas et al., 2017), which is most likely not directly involved in the catalytic mechanism (Morales-Quintana et al., 2013). The second group of *S. cerevisiae* AATs, consisting of Eht1 and Eeb1, is characterised by the α/β-hydrolase fold (Knight et al., 2014). This fold is typical for esterases, a large group of hydrolytic enzymes that includes proteases, lipases and peroxidases (Rauwerdink and Kazlauskas, 2015). Esterases are mostly associated with ester hydrolysis. Nevertheless, Eht1 and Eeb1 are able to form esters (Saerens et al., 2006). The catalytic mechanism is unclear, although it seems to involve the canonical Ser-Asp-His catalytic triad (Knight et al., 2014). Despite their structural differences, most *S. cerevisiae* AATs display side activities. *In vitro*, Atf1 and Eht1 exhibit thioesterase, or both thioesterase and esterase activity, respectively (Knight et al., 2014; Nancolas et al., 2017). Whether these activities are also present *in vivo*, is unknown, but they should be considered when AATs are used in microbial ethyl acetate production.

S. cerevisiae Atf1 and Atf2 are the primary reference for identifying acetate ester-forming AATs in yeast. These AATs belong to the PF07247 alcohol acetyl transferase protein family model (Finn et al., 2016). This model was used to identify putative AATs in bulk ethyl acetate producing yeast. *Kluyveromyces lactis* contains a homolog of Atf1, but when it was expressed in *S. cerevisiae*, the effect on ethyl acetate production was low (Van Laere et al., 2008). Six putative AATs have been annotated in the *W. anomalus* genome (Schneider et al., 2012), but their impact on ethyl acetate production has not been investigated. At least one of the *W. anomalus* Atf1 homologs appears to localise to the cytosol (Zhu et al., 2015), which was also observed for the *S. cerevisiae* Atf1 (Lin and Wheeldon, 2014; Verstrepen et al., 2004). It should be

considered that *S. cerevisiae* produces only trace amounts of ethyl acetate compared to *K. lactis* or *W. anomalus*. This suggests that AATs, other that Atf1 and Atf2, or even other enzymatic reactions may be responsible for bulk ethyl acetate formation in non-*Saccharomyces* yeast. When Atf1 was used as the catalyst for ethyl acetate production, the achieved ester yields were low (Table 1). In comparison, Atf1 was able to catalyse efficient production of acetate esters with longer alcohol chains, such as isobutyl acetate (Rodriguez et al., 2014). To understand and engineer bulk ethyl acetate production, the responsible enzymes should be identified.

Hemiacetal dehydrogenation

HADHs convert spontaneously formed unstable adducts of aldehydes and alcohols, or hemiacetals, to esters. HADHs themselves are not a separate class of enzymes. They are alcohol dehydrogenases (ADH) that dehydrogenate hemiacetals as a side activity. HADH activity has mostly been observed in methylotrophic yeast, where it contributes to methyl formate production (Murdanoto et al., 1997a, 1997b; Sakai et al., 1997). HADHs may also contribute to ethyl acetate formation in yeasts such as *C. jadinii* that are able to form high concentrations of acetaldehyde from ethanol (Armstrong et al., 1984b). Ethyl acetate producing HADHs have been reported in *S. cerevisiae* (M Kusano et al., 1998) as well as *C. jadinii* (Kusano et al., 1999). AAT activity has also been reported in *C. utilis* (Thomas and Dawson, 1978). It is possible that both activities contribute to ethyl acetate synthesis in *C. jadinii.* Recently, an ADH from *K. marxianus* was identified that formed ethyl acetate through hemiacetal dehydrogenation (Löbs et al., 2017). However, disrupting the gene did not reduce ethyl acetate production in this yeast. The role of HADH activity in bulk ethyl acetate production in yeast thus remains unconfirmed.

Baeyer-Villiger monooxygenases

BVMOs convert ketones to esters. The metabolism of ketones and BVMOs have not been studied in yeast in much detail (Beier et al., 2014). The enzymes have not been associated with bulk ethyl acetate production, and do not seem to be common in yeast (Fraaije et al., 2002). Moreover, the substrate for ethyl acetate formation via BVMOs, 2-butanone, is not produced by yeast in significant amounts. In beer fermentations, *S.*

cerevisiae produced 12.4 µg/L 2-butanone (Saison et al., 2009). This additionally indicates that BVMOs are most likely not responsible for bulk ethyl acetate formation in yeast. Other organisms do possess ethyl acetate-forming BVMOs. *Gordonia* sp. has a BVMO that normally oxidises acetone to methyl acetate, but also showed activity for 2-butanone oxidation to ethyl acetate (Kotani et al., 2007). Recently, ethyl acetate and methyl propionate production from 2-butanone was engineered in *E. coli* using the *Thermocrispum municipal* cyclohexanone monooxygenase (Pereira et al., 2018).

Reverse esterases and BVMOs are probably not responsible for bulk ethyl acetate production in yeast. Their reaction is either thermodynamically unfavourable, or the enzymes are generally not observed in yeast. Hemiacetal dehydrogenation has been linked to ethyl acetate formation in yeast *in vitro*, but not *in vivo*. Only AATs, which are the most researched class of ester-producing enzymes, are known to contribute to ethyl acetate synthesis in yeast. However, the specific enzymes responsible for the bulk ethyl acetate production in non-*Saccharomyces* yeast are still unknown. Most likely an AAT is responsible because it is both thermodynamically feasible and uses common cellular metabolite, but this remains to be confirmed.

The biological role of ethyl acetate formation in yeast

The physiological role of ethyl acetate and ester synthesis in yeast has been under debate. In *S. cerevisiae* it has been linked to the dissemination of yeast in the environment by insects (Christiaens et al., 2014), and detoxification of certain metabolites (Saerens et al., 2010). In some non-*Saccharomyces* yeast, the production of ethyl acetate is much higher compared to *S. cerevisiae*. This production may therefore have other physiological roles. Bulk ethyl acetate may provide yeast with a competitive advantage. For example, ethyl acetate produced by *W. anomalus* in moist grain inhibited the growth of the spoilage mould *Penicillium roqueforti* (Fredlund et al., 2004c). However, the prevailing hypothesis suggests that bulk ethyl acetate production is a form of overflow metabolism, caused by trace element limitation (Löser et al., 2014). The hypothesis assumes that an AAT is the enzyme responsible for ethyl acetate production. During aerobic fermentation, glucose is oxidised to pyruvate, which is transported to the mitochondria. There it is metabolised to acetyl-CoA and oxidised

further in the TCA cycle. Some of the TCA cycle enzymes, like aconitase, require iron or other metals to function (Beinert et al., 1996; Levi and Rovida, 2009). Under iron-limiting conditions, the decreased activity of such enzymes impairs the TCA cycle, causes acetyl-CoA accumulation and depletes the free CoA pool. This low CoA concentration could limit other reactions in which CoA is required. To resolve the bottleneck, acetyl-CoA is then reacted with ethanol to from ethyl acetate and free CoA (Urit et al., 2012). This hypothesis has mostly been related to iron limitation, but similar bottlenecks in the TCA cycle appear under copper- or oxygen-limited conditions (Löser et al., 2014). Studies that have used inhibitors of central carbon metabolism enzymes to control ethyl acetate synthesis, support the hypothesis. Cyanide is an inhibitor of the electron transfer chain (ETC) and prevents efficient carbon oxidation in the TCA cycle. This condition may mimic the effect of iron limitation on ethyl acetate formation in yeast. Indeed, cultivation of *K. marxianus* in the presence of cyanide resulted in ethyl acetate accumulation (Löser et al., 2015). It should be noted that the current hypothesis explaining bulk ethyl acetate formation assumes the presence of a cytosolic AAT, such as a homologs of Atf1 and Atf2 (Löser et al., 2014, 2015). However, the majority of acetyl-CoA in Crabtree negative yeast is produced in the mitochondria. A translocation step is therefore needed to transport the acetyl-CoA from the mitochondria to the cytosol. No evidence of such transport has been presented in any of the bulk ethyl acetate producing yeasts. Alternatively, ethyl acetate could be formed in the mitochondrion directly by a mitochondrial AAT.

Clearly, there are still many unanswered questions with regard to bulk ethyl acetate formation in yeast such as *W. anomalus* and *K. marxianus*. The most obvious question is the identity of the elusive enzyme that is responsible for the ethyl acetate synthesis. There are strong indications that the elusive enzyme is an AAT, but this has yet to be confirmed. Identification of the responsible enzyme is the key step towards developing new processes for biobased ethyl acetate production.

Metabolic engineering for improved ethyl acetate production

The highest ethyl acetate yields have been achieved by *K. marxianus* grown on whey under iron-limited conditions (Table 1). They have exceeded 50% of the theoretical

pathway maximum, which is potentially interesting in terms of bulk ethyl acetate production. However, yeast may not be the optimal ethyl acetate production hosts. The maximum theoretical yield of ethyl acetate on glucose is 1.2 $mol_{EA}/mol_{glucose}$. This conversion is thermodynamically feasible (Figure 2a). However, the native yeast carbon metabolism cannot achieve the maximum yield. Acetyl-CoA and ethanol are the substrates needed for ethyl acetate synthesis. They are both formed through the decarboxylation of pyruvate, which is formed from glucose via the Embden-Meyerhof-Parnas (EMP) pathway. As a consequence, two molecules of CO_2 are lost for every ethyl acetate produced. The maximum pathway yield in the native yeast metabolic network is thus 1 $mol_{EA}/mol_{glucose}$. The formation of ethyl acetate in this case is not redox balanced, and 2 mol excess NADH are produced (Figure 2b). The only way NADH can be recycled in the native yeast metabolism is through the ETC, using oxygen as the terminal electron acceptor (Figure 2c). This means that bulk ethyl acetate production in yeast is only possible under aerobic conditions. This has also been observed experimentally in *K. marxianus* and *W. anomalus* (Davies et al., 1951; Löser et al., 2014, 2015; Peel, 1951).

Large scale aerobic processes are energy intensive due to the low solubility of oxygen (Garcia-Ochoa and Gomez, 2009). The presence of oxygen also results in oxidation of glucose to CO_2 in the TCA cycle. This results in excess heat generation and loss of product yield. Anaerobic production processes can result in higher product yields and productivities (Weusthuis et al., 2011). However, the native yeast metabolism does not support anaerobic production of ethyl acetate due to the accumulation of excess reducing equivalents. Other microorganisms, mainly bacteria, decarboxylate pyruvate in a way that prevents the accumulation of excess reducing equivalents. Formate, or CO_2 and hydrogen are produced instead (Figures 2d and 2e, respectively). This enables the production of 1 $mol_{EA}/mol_{glucose}$ without the need for oxygen.

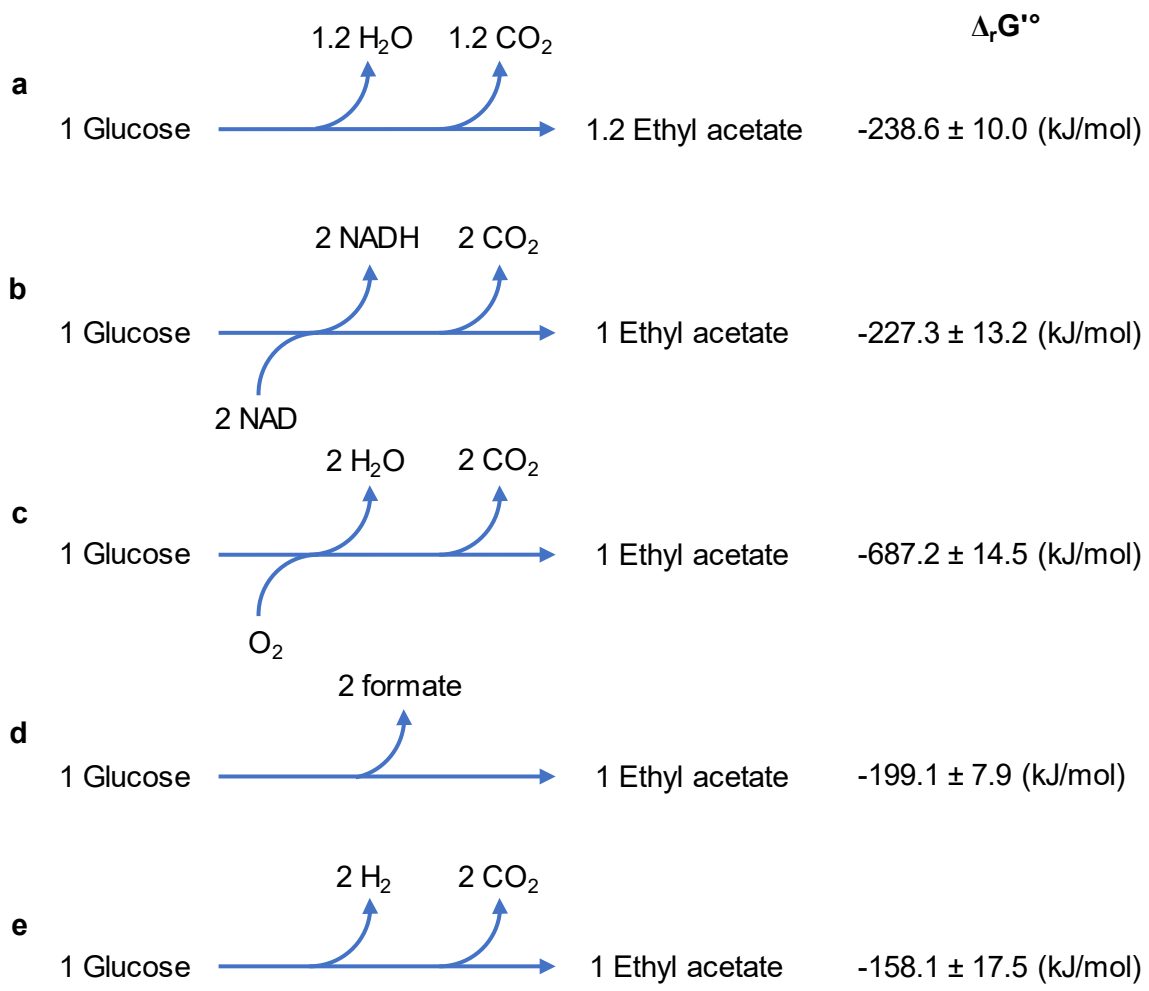

Figure 2: Stochiometric reactions of potential ethyl acetate production routes. Ethyl acetate production from glucose at the maximum theoretical yield (a). Ethyl acetate production limited by the native pathways present in most organisms (b). In the pathway, 2 mol excess NADH are produced, which can be regenerated by the reduction of oxygen (c). Alternatively, the reducing equivalents can be released by producing formate (d), or hydrogen gas and CO_2 (e).

Thesis outline

The goal of this thesis is to elucidate unknown ester producing enzymes in yeast as well as to explore their potential applications. The thesis focuses on ethyl acetate synthesis, but the production of other esters is considered as well. As was introduced in **Chapter 1**, ethyl acetate is a versatile commodity chemical that is currently produced in unsustainable processes. Several yeasts species are able to convert sugars to ethyl acetate at high yields and are considered as a biobased alternative to the established production processes. Rational strain design will be the key to maximising the efficiency of this process. However, the enzyme responsible for bulk ethyl acetate production in yeast has so far remained unknown.

Identifying the responsible enzyme is the key question that will be answered in **Chapter 2**. The discovery of a novel AAT enzyme in the yeast *Wickerhamomyces anomalus* is described. This new enzyme utilises ethanol and acetyl-CoA to make ethyl acetate and is therefore named Ethanol acetyltransferase 1 (Eat1). The basic kinetic parameters and catalytic properties of the *W. anomalus* Eat1 will be determined. Based on the identification of Eat1 homologs in other bulk ethyl acetate-producing yeasts, such as *Kluyveromyces marxianus* and *Kluyveromyces lactis*, Eat1 is proposed to form a novel family of yeast alcohol acetyltransferases.

The identification will enable characterisation of the Eat1 family of AATs in native yeasts. The current consensus in literature is that ethyl acetate synthesis occurs in the cytosol. This hypothesis will be challenged in **Chapter 3**. The Eat1 of *K. lactis* is tracked to yeast mitochondria. To examine if ethyl acetate may be produced in the mitochondria of other yeasts, comparative fermentation studies and *in silico* comparisons of various Eat1 homologs will be performed.

The metabolism of *Escherichia coli* can support the production of ethyl acetate under anaerobic conditions, which will be explored in **Chapter 4**. This could facilitate developing more economical production processes. The *E. coli* metabolism will be streamlined towards ethyl acetate production. The performance of three yeast AATs will be compared and optimised. Ways to improve the function of two Eat1 homologs

in *E. coli* will be explored. Finally, the effect of gas stripping on ethyl acetate yields and productivities in *E. coli* will be assessed.

In **Chapter 5** the potential of the Eat1 AAT family will be investigated beyond the production of bulk ethyl acetate. A total of 15 Eat1 homologs from 9 yeasts will be expressed in *Saccharomyces cerevisiae* to investigate their impact on ester production. The role of the *S. cerevisiae eat1* will then be studied by disrupting the gene in various combinations with other known *S. cerevisiae* AAT genes. The impact of these gene deletions on ester production will be investigated.

Chapter 6 provides and in-depth review on microbial ester synthesis and will outline future directions of the field. Biobased production of carboxylic esters as bulk chemicals will be discussed. The fundamental aspects of ester-producing mechanisms in microorganisms will be outlined, focusing on AATs. The established metabolic and process engineering strategies for improving ester production will be presented. The possibilities of using esters as a means of enabling economical production of other industrially relevant compounds will also be proposed.

A general discussion on the results described in this thesis will be provided in **Chapter 7**. The implications of the discovery of Eat1 on the fundamental understanding of ester production in yeast are discussed. Several open questions about ester-producing enzymes in yeast remain and will be addressed. The next critical steps towards scaling-up the production of ethyl acetate under anaerobic conditions will then be considered. The results obtained in this thesis are compared to those achieved by others. Finally, possibilities of using anaerobic ethyl acetate formation as a basis for producing other interesting compounds is discussed.

CHAPTER 2

Ethyl acetate production by the elusive alcohol acetyltransferase from yeast

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Abstract

Ethyl acetate is an industrially relevant ester that is currently produced exclusively through unsustainable processes. Many yeasts are able to produce ethyl acetate, but the main responsible enzyme has remained elusive, hampering the engineering of novel production strains. Here we describe the discovery of a new enzyme (Eat1) from the yeast *Wickerhamomyces anomalus* that resulted in high ethyl acetate production when expressed in *Saccharomyces cerevisiae* and *Escherichia coli*. Purified Eat1 showed alcohol acetyltransferase activity with ethanol and acetyl-CoA. Homologs of *eat1* are responsible for most ethyl acetate synthesis in known ethyl acetate-producing yeasts, including *S. cerevisiae*, and are only distantly related to known alcohol acetyltransferases. Eat1 is therefore proposed to compose a novel alcohol acetyltransferase family within the α/β-hydrolase superfamily. The discovery of this novel enzyme family is a crucial step towards the development of biobased ethyl acetate production and will also help in selecting improved *S. cerevisiae* brewing strains.

Keywords: Ethyl acetate; yeast; alcohol acetyltransferase; *Saccharomyces cerevisiae*; α/β−hydrolase; *Escherichia coli*

Introduction

Small volatile esters are important industrial compounds, and ethyl acetate stands out as one of the most important ones (Park et al., 2009). It is used as a chemical solvent and is applied in the synthesis of biodiesels, paints, adhesives, herbicides and resins (Alavijeh et al., 2015; Löser et al., 2014; Modi et al., 2007; Uthoff et al., 2009). Its annual production volume reached an estimated 3.5 million tonnes in 2015, which corresponds to a $ 3.7 billion global market (The Market Publishers, 2014). Apart from its broad application range, ethyl acetate is also popular because it is relatively non-toxic and fully biodegradable (Chan and Su, 2008; Kam et al., 2005). However, the sustainability of the ethyl acetate industry is severely hampered by the current, energy intensive production processes that are based exclusively on petrochemical resources (Löser et al., 2014). Efficient biobased alternatives are clearly needed.

Many yeasts are able to convert sugar into ethyl acetate (Löser et al., 2014). The model yeast *Saccharomyces cerevisiae*, however, produces only trace amounts of ethyl acetate. These traces help *S. cerevisiae* disseminate in the environment by attracting fruit flies (Christiaens et al., 2014), and are essential for the flavour of wine and beer (Rojas et al., 2001). However, the ethyl acetate yields in *S. cerevisiae* are not high enough to have an impact outside the fermented food industry. Other yeasts are significantly better ethyl acetate producers. *Wickerhamomyces anomalus* and *Kluyveromyces marxianus* are the most studied and produce 0.18 and 0.29 g ethyl acetate/g sugar, respectively (Tabachnick and Joslyn, 1953a; Urit et al., 2013a). The physiology of ethyl acetate production by these yeasts has been described in some detail. For instance, *W. anomalus* produces ethyl acetate under oxygen limitation (Fredlund et al., 2004b) and *K. marxianus* under iron limitation (Urit et al., 2012). However, the identity of the key enzyme is still a mystery, making rational strain design difficult.

Three types of enzymes have previously been associated with ethyl acetate formation in yeast: esterases, hemiacetal dehydrogenases (HADHs), and alcohol acetyl transferases (AATs) (Park et al., 2009) (Figure 1). The thermodynamic equilibrium of the esterase reaction strongly favours ethyl acetate hydrolysis in aqueous systems. Significant concentrations of ethyl acetate can only be formed by the reverse activity

if high acetic acid and ethanol concentrations are present, or if the water concentration is low (Stergiou et al., 2013). The HADH reaction is a side activity of some alcohol dehydrogenases, which reduce hemiacetals (spontaneously formed adducts of ethanol and acetaldehyde) to form ethyl acetate (Kusano et al., 1999). AATs catalyse the condensation of acetyl-CoA and an alcohol. The ΔG_r' of the latter two reactions are strongly negative and therefore more likely to be responsible for biological ethyl acetate production than esterases.

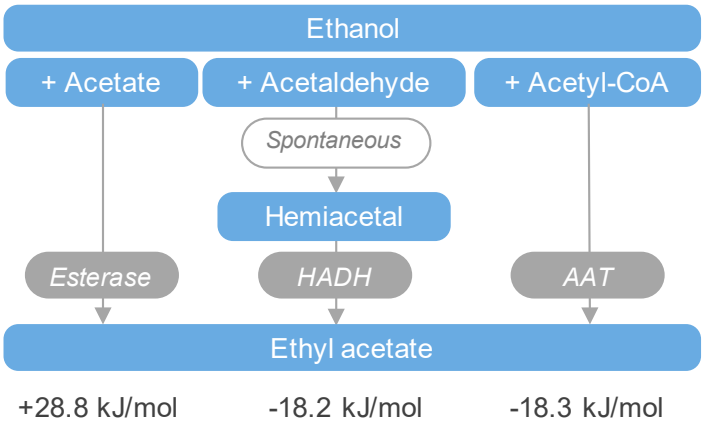

Figure 1. Possible enzymatic reactions associated with ethyl acetate formation in yeast. Numbers represent ΔGr'm values of the reactions and were calculated with the Equilibrator tool (Flamholz et al., 2014)

Our understanding of ester metabolism in yeast is mostly limited to *S. cerevisiae*, but even in this model yeast it is not fully understood. Two AATs, Atf1 and Atf2 are involved in synthesis of ethyl acetate and isoamyl acetate during wine and beer fermentation. However, a Δ*atf1*Δ*atf2* strain of *S. cerevisiae* still retained 50% of its ethyl acetate production, suggesting the presence of an additional ester producing enzyme (Verstrepen et al., 2003b). Eht1 and Eeb1 are also AATs, involved in the production of medium chain ethyl esters in *S. cerevisiae*. They do not resemble Atf1 and Atf2 at protein level and contain an α/β-hydrolase fold. The reaction typically associated with α/β-hydrolases is hydrolysis, but Eht1 and Eeb1 show AAT, as well as thioesterase and esterase activities (Knight et al., 2014; Saerens et al., 2006). It is unclear whether Eht1 or Eeb1 contribute to ethyl acetate synthesis in yeast. As ethyl acetate is a key flavour compound and the most abundant ester in wine and beer (Saerens et al., 2010), identification of the unidentified ethyl acetate-producing genes

in *S. cerevisiae* may therefore have a significant impact on the fermented foods and beverages industry.

The nature of the enzymes that are involved in ethyl acetate formation in other yeast species is even less clear. Reports on the enzymatic mechanism are inconclusive, as AATs, HADHs and esterases have all been suggested as the main catalysts of ethyl acetate synthesis, sometimes even in the same yeast species (Kallelmhiri and Miclo, 1993; Kurita, 2008; Kusano et al., 1999; Plata et al., 2003; Thomas and Dawson, 1978). However, the prevailing hypothesis is that an AAT is responsible, and that its function is to release free CoA under conditions where too much acetyl-CoA accumulates in the cell, thereby depleting the free CoA pool (Fredlund et al., 2004b; Löser et al., 2014; Thomas and Dawson, 1978). Despite the efforts, the specific ethyl acetate-producing enzyme has remained unidentified since the first report on ethyl acetate production by *W. anomalus* more than a century ago (Beijerinck, 1892; Gethins et al., 2015). Identification of the enzymes and their corresponding genes is clearly necessary to help develop biobased ethyl acetate production processes.

Here we describe the discovery of a novel enzyme family (Eat1) that is responsible for ethyl acetate production in yeasts and is only distantly related to known AATs. One member was characterized in detail by complementary *in vivo* analyses, *in vitro* enzyme assays and *in silico* modelling. The identification of this novel enzyme family opens new possibilities for the production of biobased ethyl acetate. We also identified a member of the Eat1 family in *S. cerevisiae* that is a key enzyme for ethyl acetate synthesis in this yeast. This discovery is relevant for the fermented foods industry, where ethyl acetate represents a key flavour compound.

Results

Identification of ethyl acetate-producing genes in W. anomalus

The genome of the ethyl acetate-producing *Wickerhamomyces anomalus* DSM 6766 (Fredlund et al., 2006; Schneider et al., 2012) contains five putative Atf1 or Atf2 homologs and one Eht1 homolog. To determine their capacity for ethyl acetate production, we expressed them in *S. cerevisiae* INVSc1. The transformants mainly produced ethanol and only 0.005 g/L ethyl acetate at most. However, the production

of isoamyl acetate from the endogenously produced isoamyl alcohol was 3 to 15-fold higher than the ethyl acetate production. The negative control strain did not produce esters (Figure 2a). Overexpression of the *S. cerevisiae atf1* gave comparable results as its *W. anomalus* homologs, while the *S. cerevisiae* INVSc1 transformants that overexpressed *atf2* and *eht1* showed poor growth and did not produce any esters.

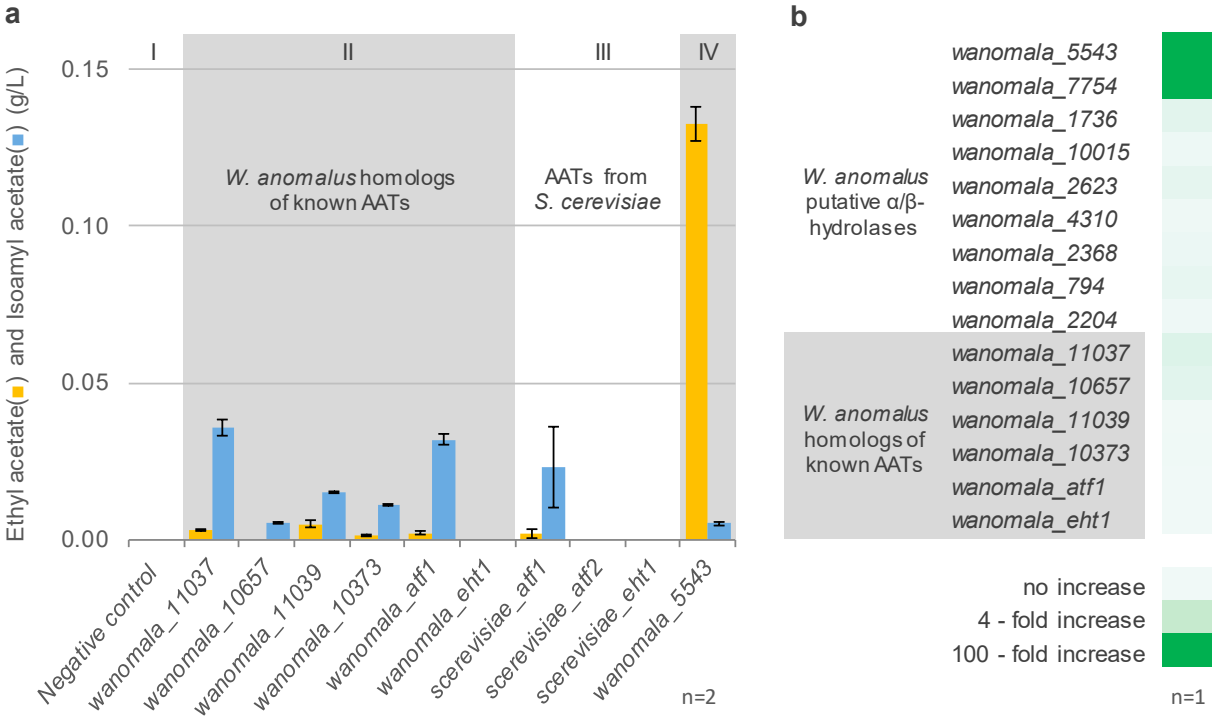

Figure 2. Putative involvement of *W. anomalus* DSM 6766 genes in ester production. (a) Ethyl acetate and isoamyl acetate production in shake flask cultures of *S. cerevisiae* INVSc1 harbouring (I) – the pYES2 empty vector, (II) – the *W. anomalus* homologs of the known *S. cerevisiae* AATs (*atf1, atf2, eht1*), (III) – the known AATs from *S. cerevisiae* (*atf1, atf2, eht1*), and (IV) – *wanomala_5543*, the most promising candidate for ethyl acetate production from the transcriptomics experiment. Cultures were grown in YS medium with 20 g/L galactose and 10 g/L raffinose. All genes were expressed from pYES2 under the GAL1 promoter. (b) Heatmap of the gene expression fold changes during ethyl acetate production in *W. anomalus* DSM 6766. The shown genes are α/β-hydrolases upregulated during ethyl acetate production, and all the *W. anomalus* homologs of the *S. cerevisiae atf1, atf2 and eht1*. The number of biological replicates is indicated in each panel. The error bars represent the standard deviation.

The poor ethyl acetate production of the known AAT homologs suggested that another enzyme is responsible for the greater part of ethyl acetate synthesis in *W. anomalus.* To search for new enzymes, we compared the transcriptomes of *W. anomalus* DSM 6766 under ethyl acetate-producing and non-producing conditions in glucose-limited continuous cultures. Ethyl acetate was not produced under fully aerobic conditions,

but oxygen limitation resulted in the production of fermentation products, including 0.64 ± 0.10 g/L ethyl acetate (13% of the maximum pathway yield). 168 genes were relevantly (>4-fold) upregulated under ethyl acetate-producing conditions. The known homologs of *atf1, atf2* or *eht1* did not show relevant changes in expression levels (Figure 2b). However, among the five most overexpressed genes were two that encoded hypothetical proteins with an α/β-hydrolase fold (*wanomala_5543* and *wanomala_7754*). This protein fold is common for a large variety of enzymes, including esterases and some AATs (Knight et al., 2014; Rauwerdink and Kazlauskas, 2015; Saerens et al., 2006). Both enzyme types are involved in ester metabolism in yeast, making *wanomala_5543* and *wanomala_7754* potential candidates for ethyl acetate formation in *W. anomalus*. Their protein products were 99% identical, indicating that the genes are most likely alleles in the diploid genome of *W. anomalus* DSM 6766. Therefore, only *wanomala_5543* was studied further.

The gene was expressed in *S. cerevisiae* INVSc1 (pYES2-Wanomala_5543), which produced 0.13 ± 0.01 g/L ethyl acetate. This is 26-fold higher than observed with the best *W. anomalus atf1*, *atf2* or *eht1* homolog (Figure 2a). Nevertheless, the achieved ethyl acetate yield was low (1.32 ± 0.10 % of the theoretical pathway maximum) compared to natural ethyl acetate-producing yeast. This might be because *S. cerevisiae* INVSc1 was grown on galactose, which also served as the inducer for gene expression under the GAL1 promoter. Protein production could therefore be reduced after the galactose was depleted. To study *wanomala_5543* in a system that does not depend on induction by the growth substrate we expressed the gene in *S. cerevisiae* CEN.PK2-1D (pCUP1:Wanomala_5543) under the copper-inducible CUP1 promoter. This led to the production of 0.12 ± 0.01 g/L ethyl acetate in pH-controlled batch bioreactors from 20 g/L glucose (Figure 3a). The new expression system did not improve the ethyl acetate titre or yield, indicating that *S. cerevisiae* may not be the optimal production host. Therefore, codon harmonised (Angov et al., 2008) *wanomala_5543* was expressed in *E. coli* BL21 (DE3) (pET26b:harmWanomala_5543-His). This strain produced 4.87 ± 0.02 g/L ethyl acetate by consuming 20 g/L glucose and 5.9 g/L ethanol (Figure 3b), which corresponds to 32.93 ± 0.11 % of the maximum pathway yield. The ethyl acetate yield in *E. coli* was 25-fold higher than in *S. cerevisiae*. The negative control strains

did not produce ethyl acetate. In both organisms the ethyl acetate concentration decreased after glucose was consumed, especially in the sparged *E. coli* fermentation (Figure 3b). This suggests stripping of the volatile ethyl acetate (Urit et al., 2011). However, the values shown in Figure 3b are the combined values of the ethyl acetate in the liquid and in the headspace, indicating that the ester was degraded and not stripped. Regardless, the results clearly show that Wanomala_5543 is a novel ethyl acetate-producing enzyme, although they did not reveal which catalytic mechanism was involved: AAT, esterase or HADH.

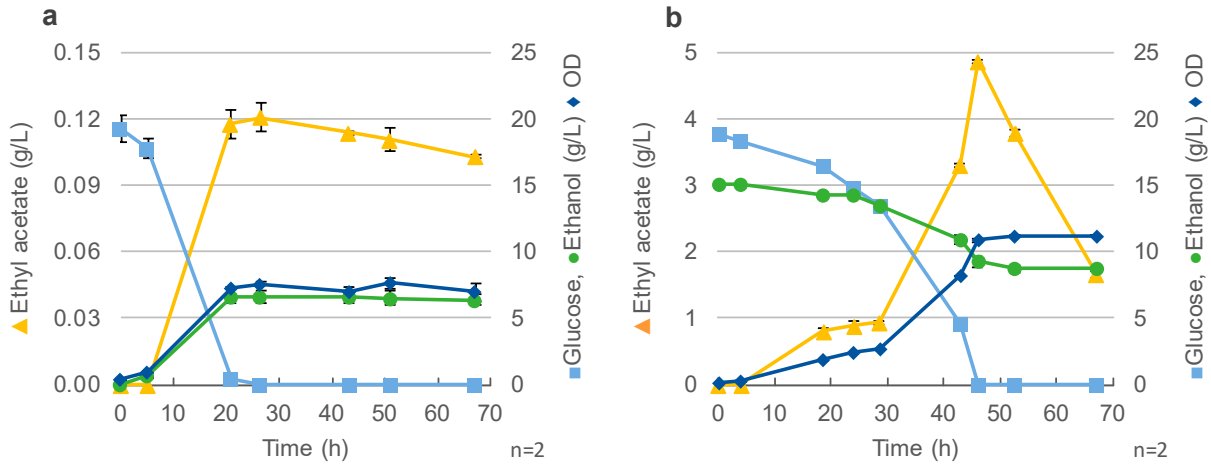

Figure 3. Ethyl acetate production by heterologous expression of *wanomala_5543* in pH-controlled bioreactors. (a) Production by *S. cerevisiae* CEN.PK2-1D (pCUP1:Wanomala_5543) from 20 g/L glucose. (b) Production by *E. coli* BL21 (DE3) (pET26b:harmWanomala_5543-His) from 20 g/L glucose and 15 g/L ethanol. Ethyl acetate values represent the combined amounts in the liquid and in the headspace. Ethanol was added to the *E. coli* cultures as *E. coli* does not produce ethanol aerobically. The number of biological replicates is indicated in the bottom right corner of each panel. Standard deviations are indicated by error bars.

Biochemical characterization of Wanomala_5543

Automatic annotation of the *W. anomalus* DSM 6766 proteome (Schneider et al., 2012) predicted an α/β-hydrolase fold in Wanomala_5543. Based on this we assumed that Wanomala_5543 makes ethyl acetate either as an AAT or as an esterase, and that HADH activity can be excluded. Cell-free extracts of *S. cerevisiae* INVSc1 (pCUP1:Wanomala_5543) did not show any AAT or esterase activity. Wanomala_5543 with a C-terminal His-tag was therefore produced in *E. coli* BL21 (DE3) (pET26b:harmWanomala_5543-His) and purified by Ni/NTA affinity

chromatography, followed by cation exchange chromatography. Through enzymatic assays we determined that Wanomala_5543 produced ethyl acetate in the presence of acetyl-CoA and ethanol, and therefore renamed it Eat1 (Ethanol Acetyltransferase 1).

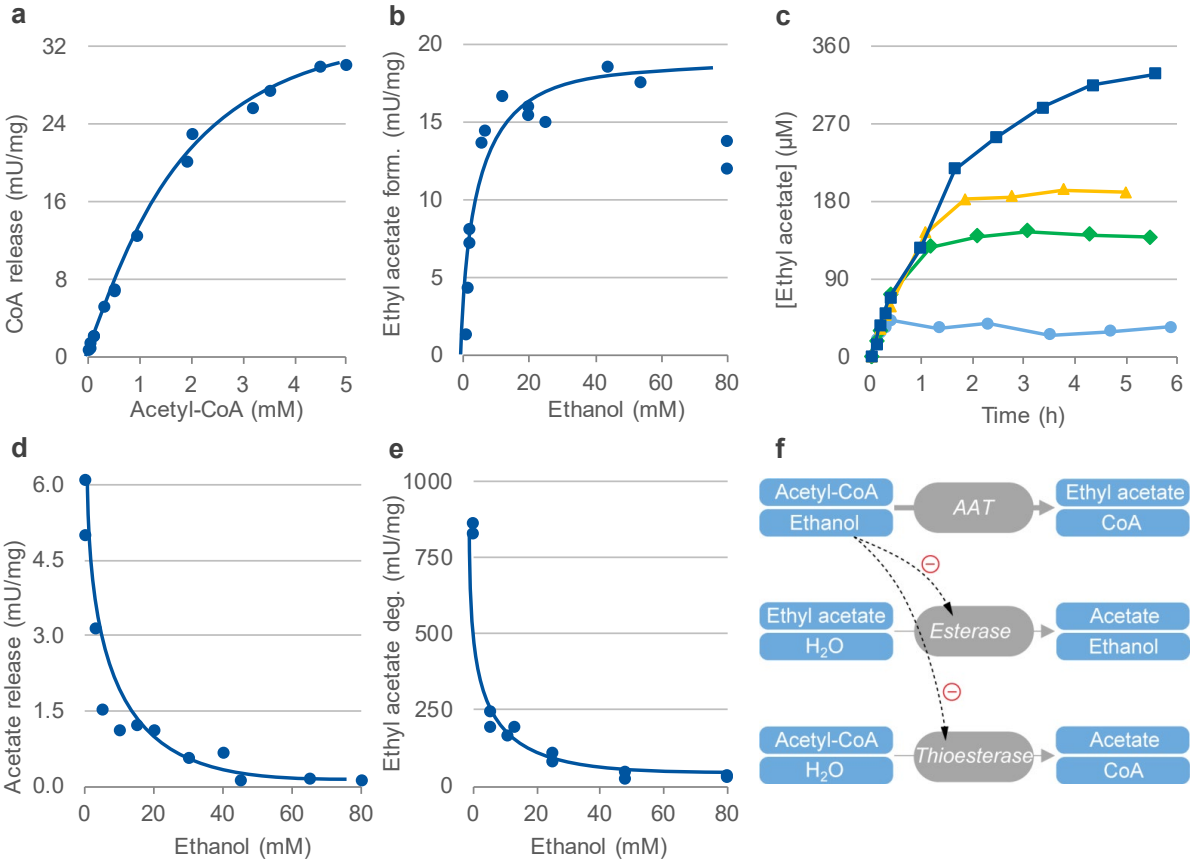

Figure 4. Enzymatic assays with Eat1. (a) Initial rates of the thioesterase reaction, measured colorimetrically as CoA release with DNTB at various acetyl-CoA concentrations. (b) Initial rates of the AAT reaction, assayed with 1 mM acetyl-CoA and various ethanol concentrations. The AAT rate at 80 mM ethanol was not included in the calculation of the K_m due to unspecific inhibition by ethanol. (c) Formation of ethyl acetate via the AAT reaction in the presence of 1 mM acetyl-CoA and 2 (light blue), 10 (green), 20 (yellow) or 40 (dark blue) mM ethanol. (d) Initial rates of the thioesterase reaction, monitored by measuring acetate release from 1 mM acetyl CoA at various ethanol concentrations. (e) Initial rates of the esterase reaction, assayed with 2 mM ethyl acetate and various ethanol concentrations. 1 mU=1 nmol/min (f) Schematic representation of the AAT, thioesterase and esterase reactions of Eat1. Dashed arrows indicate inhibition of the esterase and thioesterase activities of Eat1 by ethanol.

When we measured AAT activity by monitoring CoA release with DTNB, we observed that CoA was released both in the presence and absence of ethanol. This suggested that Eat1 can also function as a thioesterase by hydrolysing acetyl-CoA. The rate of CoA release in the absence of ethanol depended on the acetyl-CoA concentration and followed Michaelis-Menten kinetics, with a K_m of 2.43 ± 0.24 mM (Figure 4a). To

determine specifically the AAT activity of Eat1, we measured ethyl acetate production, instead of CoA release, by analysing the assay mixture by GC. The AAT assays showed that the initial rate of ethyl acetate formation was dependent on the ethanol concentration with an apparent K_m of 3.12 mM ± 0.63 mM (Figure 4b). However, higher ethanol concentrations also led to substantially higher final concentrations of ethyl acetate (Figure 4c). The results showed that the AAT reaction did not run to completion and that it depended highly on the ethanol concentration. This indicated that competing reactions were in play. We already showed that Eat1 hydrolyses acetyl-CoA in the absence of ethanol. Eht1, another AAT with an α/β-hydrolase fold, showed such thioesterase activity (Knight et al., 2014), as well as esterase activity (Saerens et al., 2006). Since the DTNB assay cannot differentiate between AAT and thioesterase activities, we measured thioesterase activity by monitoring the release of acetate from acetyl-CoA (Figure 4d). We also showed that Eat1 can function as an esterase by incubating it with ethyl acetate and following ester degradation by GC (Figure 4e). Since ethanol increased the rate of the AAT reaction, we determined its effect on thioesterase and esterase reactions as well. Both activities were at least 90% repressed when 30 mM ethanol was present in the reaction. At 80 mM ethanol, Eat1 also showed reduced AAT activity (Figure 4b), presumably due to the high ethanol concentration, but this inhibition was much smaller than the reduction of the thioesterase and esterase activities, which were repressed more than 95 %.

The three activities exhibited by Eat1 have all been observed in α/β-hydrolases containing a Ser-Asp-His catalytic triad (Rauwerdink and Kazlauskas, 2015). This suggested that a Ser-Asp-His catalytic triad may also be present in Eat1. To identify the triad, we aligned Eat1 with 15 homologs from related ascomycete yeasts. The alignment (Figure 5a) revealed highly conserved amino acids that potentially constitute the catalytic triad (Ser121, Asp145 or Asp178 and His295). Ser121 is located in a pentapeptide consensus sequence, Gly-Xaa-Ser-Xaa-Gly, typical for serine α/β-hydrolases. The catalytic site was visualized with a 3D model of Eat1, which revealed that Ser121, Asp145 and His295 were located in close proximity, most likely representing the actual active site (Figure 5b). To confirm their function, the three residues were individually substituted by alanine and the genes were expressed in *S.*

cerevisiae CEN.PK2-1D. None of the Eat1 variants formed ethyl acetate, supporting the identity of the residues composing the Ser-Asp-His catalytic triad.

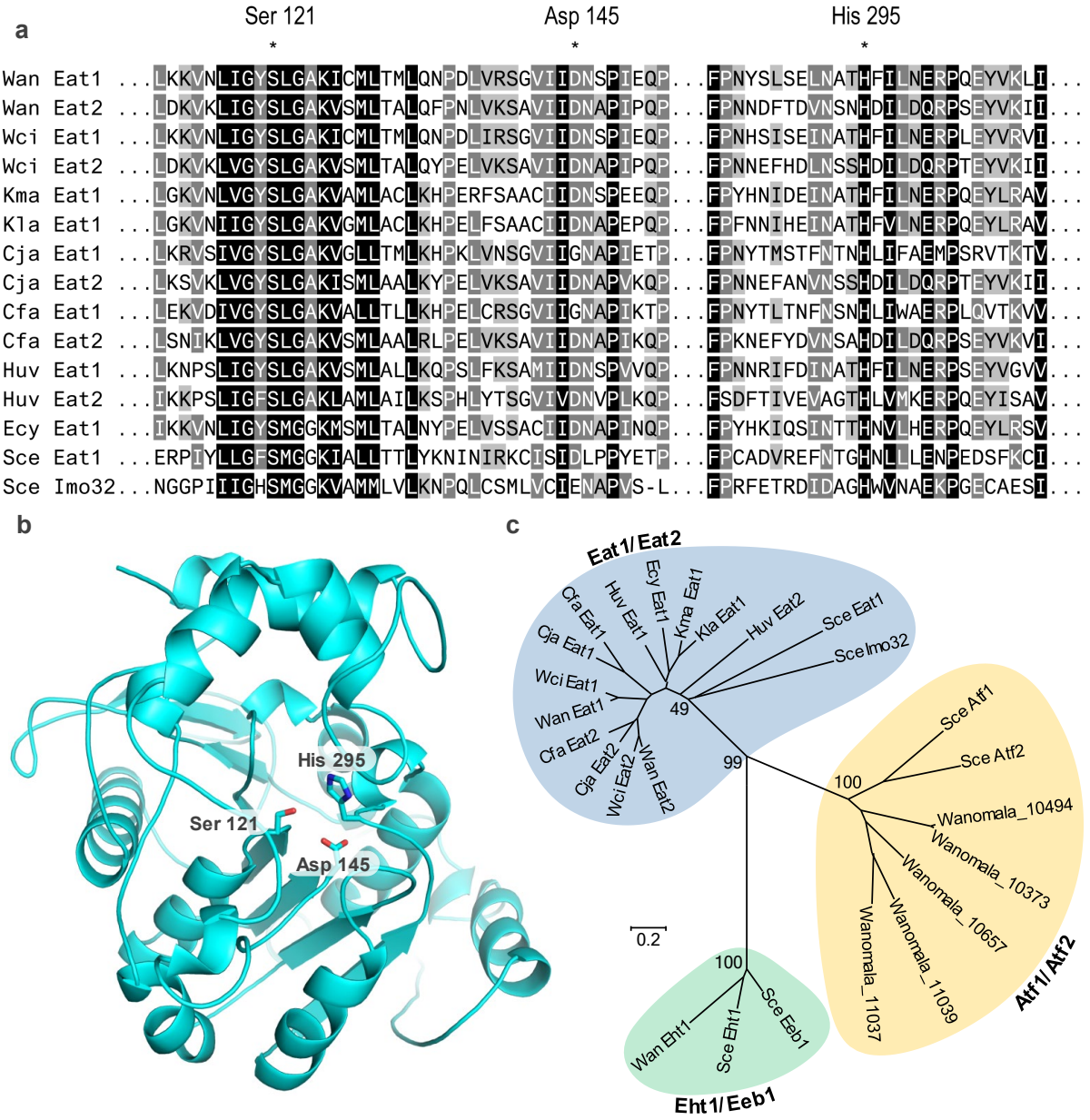

Figure 5. *In silico* analyses of Eat1 variants. (a) Multiple sequence alignment of Eat1 with its most related homologs. Asterisks indicate the conserved Ser-Asp-His catalytic triad. Ser and His are present in all the homologs. Asp is present in all but the Cja Eat1 and Cfa Eat1 homologs. The full alignment is available in Supplementary Figure 1. (b) Structural model of Wan Eat1, showing the Ser-Asp-His catalytic triad. (c) Phylogenetic tree of AATs studied in this paper. Blue colour indicates the new Eat1 family of AATs. Yellow and green colors indicate the Atf1, Atf2 family and the Eht1, Eeb1 family of AATs, respectively. Numbers represent bootstrap values. Imo32 is the Sce Eat2 homolog. 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*

Prevalence of the new Eat1 AAT family in other ethyl acetate-producing yeasts

The presence of an α/β−hydrolase domain and the interplay of AAT, thioesterase and esterase activities was previously described in Eht1 (Knight et al., 2014; Saerens et al., 2006). This suggests some level of relatedness of Eat1 and Eht1. However, Eat1 did not show significant sequence similarity to Eht1 or Eeb1. It also did not show significant similarity to Atf1 or Atf2, but this was expected since these AATs do not belong to the α/β-hydrolase superfamily. We therefore propose that Eat1 represents a new family of AATs (Figure 5c), composed of homologs from other ethyl acetate-producing yeast species, such as *K. marxianus* (Löser et al., 2013), *K. lactis* (Löser et al., 2011), *C. jadinii* (Armstrong and Yamazaki, 1984) and *C. fabianii* (Meersman et al., 2016; van Rijswijck et al., 2014). We expressed all 15 homologs (Figure 5a) from various yeasts in *S. cerevisiae* CEN.PK2-1D and found 10 that were able to catalyse ethyl acetate production (Figure 6a). At least one ethyl acetate-producing homolog of *eat1* was present in each yeast species. Furthermore, Eat1 homologs were also present in *Wickerhamomyces ciferrii* CBS 111, a yeast species that had never been studied for ethyl acetate production before. Its production capacity was tested in YM medium, where it produced 6.48 ± 0.32 g/L ethyl acetate from 50 g/L glucose (Figure 6b. To further prove that the Eat1 family of enzymes is responsible for ethyl acetate synthesis in yeast, we tried to disrupt *eat1* in *W. anomalus*, but were unable to transform it. Instead, we knocked out the *eat1* homolog in *K. lactis* CBS2359 Δ*ku80* (Kooistra et al., 2004), which resulted in an 80 % decrease in ethyl acetate production (Figure 6c). Interestingly, *S. cerevisiae* also has an ethyl acetate-producing homolog of Eat1 (Figure 6a), which may be responsible for the unexplained ethyl acetate production in this yeast. When the homolog (*ygr015c*) was disrupted in *S. cerevisiae* IMX585 (Mans et al., 2015) with CRISPR-Cas9, ethyl acetate production was reduced at least 50% (p-value < 0.014) compared to the parental strain (Figure 6d).

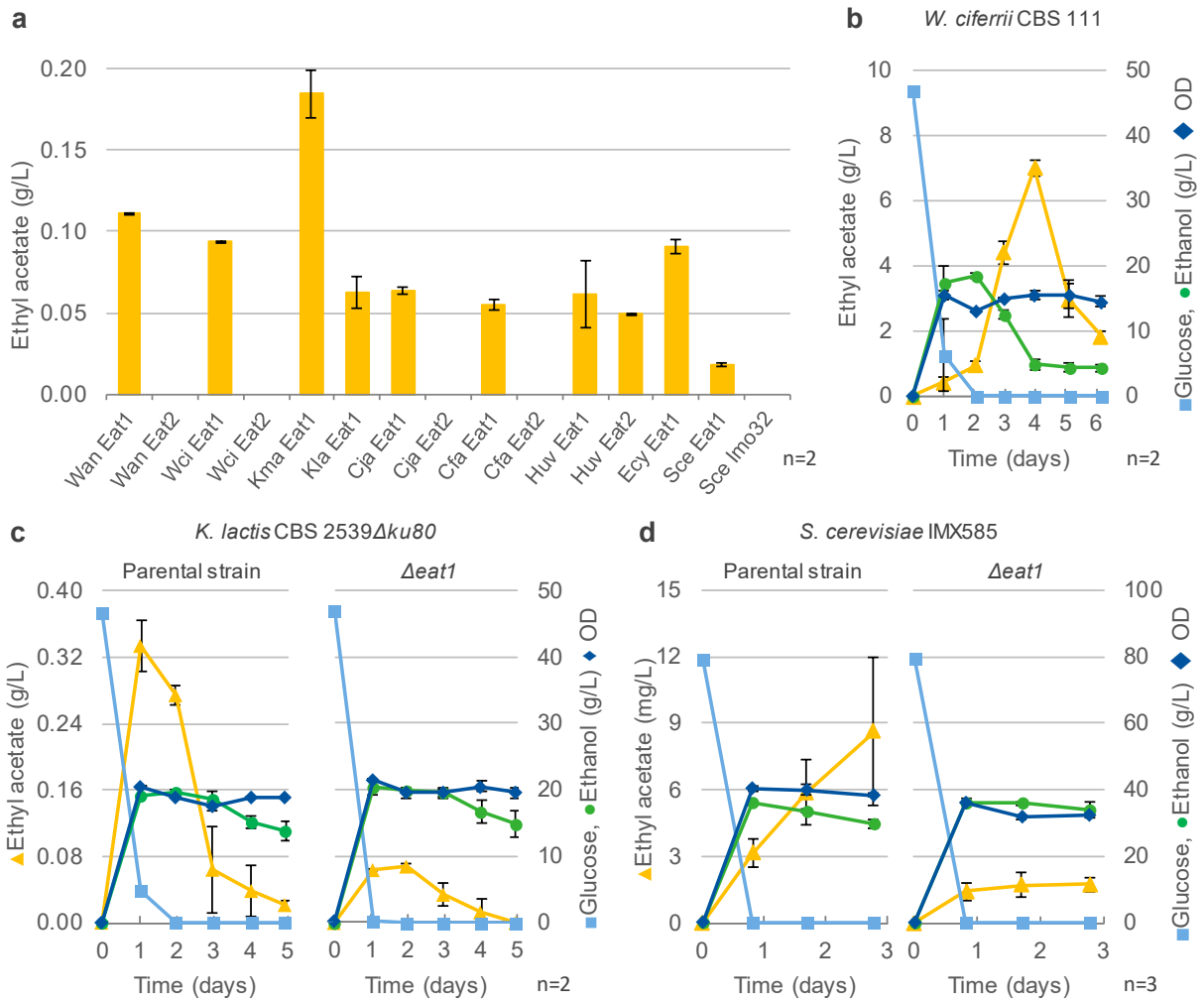

Figure 6. Role of the Eat1 AAT family in ethyl acetate production in yeast. (a) Screening of the ethyl acetate-producing capability of Eat1 homologs from yeast. Proteins were produced in *S. cerevisiae* CEN.PK2-1D from the pCUP1 plasmid and studied in shake flasks in 50 mL YS medium with 20 g/L glucose. 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.* (b) Ethyl acetate production by *W. ciferrii* CBS 111 in YM medium. (c) Effect of *eat1* disruption on ethyl acetate production in *K. lactis* CBS2359 Δ*ku80*. Ethyl acetate production was assessed in YM medium. (d) Effect of *S. cerevisiae eat1* (*ygr015c*) disruption on ethyl acetate production in *S. cerevisiae* IMX585. Ethyl acetate production was assessed in YM medium. The number of biological replicates for each experiment is indicated in the bottom right corner of each panel. The error bars represent standard deviations.

Discussion

The results presented here clearly show that Eat1 and its homologs form the elusive AAT family that is responsible for the bulk ethyl acetate synthesis in some yeast species. We have shown that *eat1* is strongly upregulated during ethyl acetate-production in *W. anomalus* and that a disruption of the gene in *K. lactis* reduced ethyl acetate production by 80 %. The enzyme is also present in all ethyl acetate producing yeasts with a sequenced genome. We were even able to identify a new ethyl acetate-producing yeast species based on the presence of Eat1 homologs. These combined results strongly support the hypothesis that Eat1 plays a central role in ethyl acetate production in yeast, and also show that yeasts mainly produce ethyl acetate with AATs, and not HADHs or reversed esterases. The identification of Eat1 and its homologs is therefore a substantial leap forward in the understanding of ester production in yeast.

A key aspect of Eat1 is its triple catalytic activity (Figure 4f). At first, the AAT, thioesterase and esterase activities are counterintuitive. However, the activities can be explained by considering that Eat1 is a versatile α/β-hydrolase and that all three reactions use the same general catalytic mechanism involving the Ser-Asp-His catalytic triad. The nucleophile (Ser) is activated by an acid (Asp) and a base (His). This allows the Ser to attack the acetyl-CoA substrate (acetyl donor), and form an acetyl-enzyme intermediate. The enzyme is then regenerated by coupling the acetyl moiety with either ethanol or water; the outcome is, respectively, ethyl acetate formation (AAT) or acetyl-CoA hydrolysis (thioesterase). Ethyl acetate hydrolysis (esterase) follows the same catalytic mechanism, but in this case, ethyl acetate becomes the acyl donor and water is used to regenerate the acetyl-enzyme intermediate. The hydrolysis reaction is typical for α/β-hydrolases, but Eat1 is remarkable in its ability to use ethanol instead of water and form ethyl acetate as an AAT. Only a small number of α/β-hydrolases are able regenerate the acetyl enzyme intermediate with compounds other than water (in aqueous environments) (Rauwerdink and Kazlauskas, 2015). The experimental data confirm that ethanol is a critical factor in controlling the type of reaction catalysed by Eat1 (Figure 4b, 4d and 4e). Higher ethanol concentrations resulted in higher AAT reaction rates and lower thioesterase and esterase rates. A likely explanation is that

the enzyme creates a hydrophobic environment around the catalytic site and thereby reduces the rates of the reactions that require water as a substrate (Rauwerdink and Kazlauskas, 2015). Increasing ethanol concentrations thus turn Eat1 from a thioesterase or esterase into an AAT. The affinity of Eat1 for ethanol is relatively high (apparent $K_m = 3.12 \pm 0.63$ mM), given that it can outcompete water even in the low mM range. On the other hand, the affinity of Eat1 for acetyl-CoA ($K_m = 2.43 \pm 0.24$ mM) is relatively low, compared to other yeast enzymes that use acetyl-CoA as a substrate (Bulfer et al., 2010; Middleton, 1972; Parvin and Atkinson, 1968). This crucial metabolite is involved in many reactions, and the low affinity of Eat1 may prevent draining of the acetyl-CoA pool. The observation also supports the proposed function of Eat1 in the original yeast: the enzyme acts as an overflow valve under conditions in which too much acetyl-CoA accumulates in the cell, limiting the availability of free-CoA (Löser et al., 2014). Arguably, the same function could be performed by a thioesterase, but forming ethyl acetate instead of acetate gives yeasts like *W. anomalus* a competitive advantage by inhibiting growth of other organisms (Fredlund et al., 2004c). Moreover, the acetate released during the thioesterase reaction might negatively impact cellular metabolism and would require energy to export, while ethyl acetate can freely diffuse over the cell membrane.

The complex interplay of the activities of Eat1 could potentially affect ethyl acetate yields in a production process. However, our *in vitro* results showed that the thioesterase and esterase activities of Eat1 are more than 90 % reduced at 30 mM ethanol. *In vivo* ethanol concentrations can exceed 30 mM by far, as was also reflected in the batch *S. cerevisiae* and *E. coli* fermentations, where ethanol levels were 140 mM and 330 mM, respectively. Both organisms produced the highest titre of ethyl acetate ever reported in these species when expressing *eat1*. The ethyl acetate production in *S. cerevisiae* was significantly lower compared to natural ethyl acetate producers, which could be the result of low expression, compartmentalization or other unknown factors. On the other hand, *E. coli* produced 4.87 ± 0.02 g/L ethyl acetate by consuming 20 g/L glucose and 5.86 g/L ethanol. This corresponds to 32.93 % ± 0.11 % of the maximum pathway yield, putting *E. coli* on a par with natural ethyl acetate-producing yeasts (Löser et al., 2014). The achieved ethyl acetate yield is also 24.4 fold higher than

the highest yield in *E. coli* reported to date (Rodriguez et al., 2014). It should be noted that the peak in ethyl acetate production (Figure 3b) is followed by a sharp decline, indicating ester degradation. This might be due to residual esterase activity of Eat1, or due to native *E. coli* esterases. It is tempting to speculate that reducing these activities by rational protein and strain engineering will increase the ethyl acetate production yields in the future.

The discovery of the Eat1 family of ethyl acetate-producing enzymes will open the door for developing efficient ethyl acetate-producing strains, and as such help the transition of ethyl acetate production into the biobased economy. Additionally, it holds important implications for the fermented food and beverages industry. Ethyl acetate is produced by *S. cerevisiae* during fermentation but only half of its production has been explained thus far (Verstrepen et al., 2003b). Our results show that the Eat1 homolog present in this yeast might account for the remaining ethyl acetate production in this model yeast. This knowledge will help select new *S. cerevisiae* strains with improved flavour production profiles.

Materials and methods

Strains and plasmids construction

The strains and plasmids that were used in this study are given in Table 1 and Table 2, respectively. Gene sequences are available in Supplementary Table 1. pYES2-derived plasmids were constructed by inserting genes into the multiple cloning site, either by using appropriate restriction enzymes, or by *in vivo* yeast recombination (Finnigan and Thorner, 2015). pCUP1-plasmids were constructed by replacing the GAL1 promoter of pYES2 with the *S. cerevisiae* NCYC 2629 CUP1 promoter (Mascorro-Gallardo et al., 1996), and inserting the gene of interest with *in vivo* yeast recombination, using *S. cerevisiae* CEN.PK2-1D. *S. cerevisiae* transformations were performed as described previously (Gietz and Woods, 2002). The pYES2 and pCUP1 plasmids were characterized in *S. cerevisiae* INVSc1 and CEN.PK2-1D, respectively. pET26b:harmWanomala_5543-His was constructed by cloning the *E. coli* codon-harmonised (Angov et al., 2008) *wanomala_5543* gene between the NdeI and XhoI sites of pET26b in frame with the 6X His tag. All plasmids were propagated in *E. coli*

NEB® 5-alpha. Site-directed mutagenesis in pCUP1:Wanomala_5543 was performed with Quickchange (Agilent). The *K. lactis* CBS 2359 Δ*ku80* (Kooistra et al., 2004) homolog of *wanomala_5543* (KLLA0_E24421g) was disrupted by homologous recombination with a disruption cassette containing the TEF1 promotor-KanMX-TEF1 terminator sequence from pUG6 (Güldener et al., 1996). The marker sequence was flanked by 1000 bp upstream and 1000 bp downstream sequence of the KLLA0_E24421g locus. The cassette was constructed by fusion PCR of the three parts. The linear PCR product was purified and transformed to *K. lactis* CBS 2359 Δ*ku80* (Gietz and Woods, 2002). Transformants were selected on selective plates containing 200 µg/mL geneticin (G418) and confirmed by PCR. Gene disruption in *S. cerevisiae* IMX585 was performed as described previously (Mans et al., 2015), using the plasmid pMEL12.

Table 1. Strains used and produced in this study.

Strain	Genotype	Source
Wickerhamomyces anomalus DSM 6766	Wild type	DSMZ
Wickerhamomyces ciferrii CBS 111	Wild type	CBS
Kluyveromyces marxianus DSM 5422	Wild type	DSMZ
Kluyveromyces lactis CBS 2359	Wild type	CBS
Kluyveromyces lactis CBS 2359 Δ*ku80*	Δ*ku80*	Kooistra *et. al.*(Kooistra et al., 2004)
Kluyveromyces lactis CBS 2359 Δ*ku80*Δ*klaEat1*	Δ*ku80*Δ*eat1*	This study
Cyberlindnera jadinii DSM 2361	Wild type	DSMZ
Cyberlindnera fabianii CBS 5640	Wild type	CBS
Hanseniaspora uvarum CECT 11105	Wild type	CECT
Eremothecium cymbalariae CBS 270.75	Wild type	CBS
Saccharomyces cerevisiae NCYC 2629	Wild type	NCYC
Saccharomyces cerevisiae INVSc1	MATa, his3D1, leu2, trp1-289, ura3-52, MATalpha, his3D1, leu2, trp1-289, ura3-52	Invitrogen
Saccharomyces cerevisiae CEN.PK2-1D	MATalpha, his3D1, leu2-3_112, ura3-52, trp1-289, MAL2-8c, SUC2	(Entian and Kötter, 2007)
Saccharomyces cerevisiae IMX585	MATa can1D::cas9-natNT2 URA3 TRP1 LEU2 HIS3	(Mans et al., 2015)
Saccharomyces cerevisiae IMX585 Δ*ygr015C*	MATa can1D::cas9-natNT2 URA3 TRP1 LEU2 HIS3 Δ*ygr015C*	This study
Escherichia coli BL21 (DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] Δ hsdS λ DE3 = λ sBamHIo Δ EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δ nin5	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 and produced in this study.

Plasmid	Host organism, promoter	Gene/locus (locus tag on original host genome)	Source
pYES2	*S. cerevisiae,* GAL1		Invitrogen
pYES2:Wanomala_11039	*S. cerevisiae,* GAL1	*W. anomalus* DSM 6766 *wanomala_11039*	This study
pYES2:Wanomala_11037	*S. cerevisiae,* GAL1	*W. anomalus* DSM 6766 *wanomala_11037*	This study
pYES2:Wanomala_10657	*S. cerevisiae,* GAL1	*W. anomalus* DSM 6766 *wanomala_10657*	This study
pYES2:Wanomala_10373	*S. cerevisiae,* GAL1	*W. anomalus* DSM 6766 *wanomala_10373*	This study
pYES2:WanATF1	*S. cerevisiae,* GAL1	*W. anomalus* DSM 6766 *atf1* (*wanomala_10494*)	This study
pYES2:WanEHT1B	*S. cerevisiae,* GAL1	*W. anomalus* DSM 6766 *eht1b* (*wanomala_6862*)	This study
pYES2:Atf1	*S. cerevisiae,* GAL1	*S. cerevisiae* NCYC 2629 *atf1* (YOR377W)	This study
pYES2:Atf2	*S. cerevisiae,* GAL1	*S. cerevisiae* NCYC 2629 *atf2* (YGR177C)	This study
pYES2:Eht1	*S. cerevisiae,* GAL1	*S. cerevisiae* NCYC 2629 *eht1* (YBR177C)	This study
pYES2:Wanomala_5543	*S. cerevisiae,* GAL1	*W. anomalus* DSM 6766 *wanomala_5543*	This study
pCUP1:Wanomala_5543 or pCUP1:Wan Eat1	*S. cerevisiae,* CUP1	*W. anomalus* DSM 6766 *wanomala_5543*	This study
pCUP1:Wanomala_5543 Ser121Ala	*S. cerevisiae,* CUP1	*W. anomalus* DSM 6766 *wanomala_5543*, Ser 121 substituted for Ala	This study
pCUP1: Wanomala_5543 Asp145Ala	*S. cerevisiae,* CUP1	*W. anomalus* DSM 6766 *wanomala_5543*, Asp 145 substituted for Ala	This study
pCUP1: Wanomala_5543 His295Ala	*S. cerevisiae,* CUP1	*W. anomalus* DSM 6766 *wanomala_5543*, His 295 substituted for Ala	This study
pCUP1:Wan Eat2	*S. cerevisiae,* CUP1	*W. anomalus* DSM 6766 *wanomala_5545*	This study
pCUP1:Wci Eat1	*S. cerevisiae,* CUP1	*W. ciferrii* CBS 111 homolog of *wanomala_5543* (XP_011273049.1)	This study
pCUP1:Wci Eat2	*S. cerevisiae,* CUP1	*W. ciferrii* CBS 111 homolog of *wanomala_5543* (XP_011273050.1)	This study
pCUP1:Kma Eat1	*S. cerevisiae,* CUP1	*K. marxianus* DSM 5422 homolog of *wanomala_5543* (KMAR_10772)	This study
pCUP1:Kla Eat1	*S. cerevisiae,* CUP1	*K. lactis* CBS 2359 homolog of *wanomala_5543* (KLLA0_E24421g)	This study
pCUP1:Cja Eat1	*S. cerevisiae,* CUP1	*C. jadinii* DSM 2361 homolog of *wanomala_5543* (CEP25158.1)	This study
pCUP1:Cja Eat2	*S. cerevisiae,* CUP1	*C. jadinii* DSM 2361 homolog of *wanomala_5543* (CEP25159.1)	This study

Table 2 (continued). Plasmids used and produced in this study.

Plasmid	Host organism, promoter	Gene/locus (locus tag on original host genome)	Source
pCUP1:Cfa Eat1	*S. cerevisiae,* CUP1	*C. fabianii* CBS 5640 homolog of *wanomala_5543* (CDR40574.1)	This study
pCUP1:Cfa Eat2	*S. cerevisiae,* CUP1	*C. fabianii* CBS 5640 homolog of *wanomala_5543* (CDR40570.1)	This study
pCUP1:Huv Eat1	*S. cerevisiae,* CUP1	*H. uvarum* CECT 11105 homolog of *wanomala_5543* (D499_0A01740)	This study
pCUP1:Huv Eat2	*S. cerevisiae,* CUP1	*H. uvarum* CECT 11105 homolog of *wanomala_5543* (D499_0F00170)	This study
pCUP1:Ecy Eat1	*S. cerevisiae,* CUP1	*E. cymbalariae* CBS 270.75 homolog of *wanomala_5543* (Ecym_7076)	This study
pCUP1: Sce Eat1	*S. cerevisiae,* CUP1	*S. cerevisiae* NCYC 2629 homolog of *wanomala_5543* (YGR015C)	This study
pCUP1: Sce Imo32	*S. cerevisiae,* CUP1	*S. cerevisiae* NCYC 2629 homolog of *wanomala_5543* (YGR031W)	This study
pET26b	*E. coli,* T7	/	Novagen
pET26b:harmWanomala_5543-His	*E. coli,* T7	Codon harmonised(Angov et al., 2011) *wanomala_5543*	This study
pMEL12	-	-	(Mans et al., 2015)
pUG6	-	-	Güldener *et al.*, 1996

Cultivation conditions

Wild type yeast strains were routinely cultured and propagated in YPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract). *S. cerevisiae* strains carrying pYES2 derived plasmids were routinely cultured and propagated in YS (yeast synthetic) medium (6.7 g/L yeast nitrogen base without amino acids, 1.92 g/L Drop-out Medium Supplements without uracil) with 20 g/L glucose. *E. coli* strains were routinely cultured and propagated in LB or M9 medium supplemented with 50 µg/mL ampicillin or kanamycin. Yeast and *E. coli* strains were grown at 30 °C and 37 °C, respectively, unless stated otherwise. *S. cerevisiae* INVSc1 strains carrying pYES2-derived plasmids were characterized in 250-mL Erlenmeyers containing 50 mL YS medium with 20 g/L galactose and 10 g/L raffinose. Erlenmeyers were inoculated with 1 mL overnight pre-culture and cultivated at 250 rpm for 20 hours. *K. lactis* CBS 2359 Δ*ku80, K. lactis* CBS 2359 Δ*ku80*Δ*eat1,* and *W. ciferrii* CBS 111 were tested in 1-L Erlenmeyers containing 250 mL YM (yeast minimal) medium (Thomas and Dawson, 1978). Iron was omitted from the medium. 1 mL 1000x vitamins (Verduyn et al., 1992) was added

to the medium. Flasks were inoculated with 2 mL overnight YPD culture that was washed in 10 mL sterile water. The flasks were shaken at 250 rpm. *S. cerevisiae* IMX585 strains were assayed for ester production in 250 mL Erlenmeyer flasks containing 50 mL YPD medium with 80 g/L glucose (Verstrepen et al., 2003b). Cultures were inoculated to an OD_{600} of 0.20 from an overnight YPD culture and cultured at 30 °C and 150 rpm. The amount of ethyl acetate in the headspace was not determined in shake flask experiments due to minimal evaporation. Shake flask experiments were performed as biological replicates in duplicate or in triplicate.

Batch bioreactors

Batch fermentations with *S. cerevisiae* and *E. coli* were performed in 1-L DasGip bioreactors (Eppendorf) in 0.5 L defined medium (Verduyn et al., 1992). 1 mM $CuSO_4$ was added to the medium in *S. cerevisiae* fermentations and 10 g/L NaCl and 15 g/L ethanol in *E. coli* fermentations. The pH was kept at 7.0 by automatic addition of 3M KOH. *S. cerevisiae* fermentations were not sparged and were stirred at 600 rpm. Since the *S. cerevisiae* fermentations were not sparged, the ethyl acetate evaporation was minimal and was not quantified. *E. coli* fermentations were sparged with 2 L/h air and were kept aerobic (DO>25%) by gradually increasing the stirring speed from 200 to 800 rpm and increasing the fraction of O_2 in the sparging gas. All batch fermentations were operated at 30 °C. The *S. cerevisiae* and *E. coli* pre-cultures were made by inoculating 50 mL YS medium with 20 g/L glucose or 50 mL M9 medium, respectively in a 250-mL Erlenmeyer flask. *S. cerevisiae* cultures were grown at 250 rpm and 30°C until an OD_{600} of 3. *E. coli* cultures were grown to an OD_{600} of 0.8. The cells were centrifuged at 4816 *x g* for 5 min, resuspended in 50 mL sterile water and transferred to the fermentor. *E. coli* cultures were induced with 0.2 mM IPTG after 4 hours of growth. The ethyl acetate in the headspace of the *E. coli* fermentations was quantified by analysing 250 µL of the headspace by GC-FID, as described below. The total mass of ethyl acetate produced was calculated as described before (Urit et al., 2011) and expressed per L fermentation broth. All fermentations were performed as biological duplicates.

Continuous bioreactors

Continuous culturing of *W. anomalus* was performed in a 3-L Labfors bioreactor (InforsHT) with a working volume of 2 L. The pH was controlled at 5.0 by addition of 8M KOH. The culture was stirred at 800 rpm and the temperature was controlled at 30°C. The culture was kept glucose-limited by adding medium that contained 10 g/L glucose, 5 g/L $(NH_4)_2SO_4$, 2.5 g/L KH_2PO_4, trace elements without iron (Thomas and Dawson, 1978), 50 µM $FeCl_3$, 0.4 mM $CaCl_2$, 2 mM $MgSO_4$, 15 mg/L ethylenediaminetetraacetic acid (EDTA) and 0.15 mL/L Antifoam 204 (Sigma). The pH of the medium vessel was lowered to 2.0 by adding 37% HCl. The dilution rate was 0.1 h^{-1}. Ethyl acetate production was controlled by aeration. Non-producing (aerobic) and producing (oxygen-limited) conditions were achieved by sparging the fermentor with 0.3 L/min air, or with a mixture of 0.03 L/min air and 0.27 L/min N2, respectively. The amount of ethyl acetate in the headspace was quantified by analysing 250 µL of the headspace by GC-FID, and added to the amount in the broth. Steady state was achieved after 50 h. The reported results are an average of four steady states. A 50-ml culture sample was withdrawn from the steady state, centrifuged and the pellet frozen at -80°C until RNA isolation.

RNA isolation and RNAseq analysis

The pellets from the continuous cultures were kept on ice as much as possible during the protocol. The pellets were resuspended in 0.5 mL cold TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and divided into two 2-mL screw-capped tubes containing 0.5 g zirconium beads, 30 µL of 10 % sodium dodecyl sulphate, 30 µL of 3 M sodium acetate (pH 5.2) and 500 µL of Roti-Phenol (pH 4.5-5.0, Roth). The cells were disrupted with a FastPrep apparatus (MP biomedicals) at speed 6 for 40 s and centrifuged at 4 °C and 18,400 x *g* for 5 min. 400 µL of the combined aqueous phase from both tubes was mixed with 400 µL of chloroform-isoamyl alcohol (Roth) and centrifuged at 4 °C at 18,400 *x g* for 6 min. 300 µL of the aqueous phase was mixed with 300 µL of the lysis buffer from the High Pure RNA Isolation kit (Roche). All subsequent steps were performed according to the kit instructions, except the DNAse treatment which was performed for 45 min. The RNAseq library preparation, sequencing and analysis were performed by BaseClear B.V. (Leiden). mRNA was first purified by polyA capture and

prepared with the TruSeq Stranded Total RNA Library Prep Kit (Illumina). Subsequently, the library was fragmented and converted to double-stranded cDNA fragments (100-400 bp). DNA adapters were ligated to the fragments and subjected to PCR amplification. The resultant library was sequenced on the HiSeq 2500 platform (Illumina) using a 50 cycles single-read protocol. The reads were mapped to the annotated genome of *W. anomalus* DSM 6766 (Schneider et al., 2012). A threshold of at least 4-fold change in gene expression was considered as relevant. RNAseq analysis was performed once.

Bioinformatics

Homologs of Wanomala_5543 were identified by performing standard BLASTP against the non-redundant protein sequences database. Phylogenetic analysis was performed by making a structural alignment of Wanomala_5543 and close homologues using the Clustal X2.1 multiple sequence alignment program (Larkin et al., 2007). Phylogenetic trees were calculated and visualized using Mega 6. A three dimensional structure of Wanomala_5543 was modelled using the PHYRE2 Protein Fold Recognition Server (http://www.sbg.bio.ic.ac.uk/phyre2) on intensive mode (Kelley et al., 2015). Six templates (pdb: 2y6v, 4d9j, 1cr6, 3i1i, 3i28, & 2vav) were selected based on heuristics and 52 residues were modelled by *ab initio*. In the final model 87% of Wanomala_5543 was modelled at >90% confidence. The structure was analysed and visualized using PyMol (Schrödinger, n.d.).

Wanomala_5543 purification

His-tagged Wanomala_5543 was purified from *E. coli* BL21 (DE3) (pET26b:harmWanomala_5543-His). Three 1-L Erlenmeyers with 250 mL M9 medium were inoculated with 2 mL overnight pre-culture and cultivated at 37 °C and 200 rpm. After 4 hours the cultures were chilled on ice for 15 min and induced with 0.2 mM IPTG. The cultures were then incubated at 20 °C and 200 rpm. After 18 hours, the cells were combined, harvested by centrifugation at 2350 *x g* for 5 min, washed with 50 mL of 50 mM potassium phosphate buffer (KPi, pH 7.5) and stored at -20 °C. To extract the protein, the cells were resuspended in 20 mL buffer HA (50 mM KPi, 300 mM NaCl, pH 8.0) and passed twice through a chilled French Press Cell (Thermo

Scientific) at 20,000 psi. 25 mg of DNAseI was added to the lysate, after which it was centrifuged at 4 °C and 41650 *x g* for 20 min. The supernatant was filtered through a 0.45 µm filter and was used for protein purification. An AKTA Purifier system was used to purify Wanomala_5543. The cell-free extract was loaded on a 1-mL HisTrap HP column (GE Healthcare Life Sciences) that was equilibrated with buffer HA. The protein was eluted with a gradient of buffer HB (50 mM KPi, 300 mM NaCl, 500 mM imidazole, pH 8.0). The fractions containing the protein were desalted over three connected 5-mL HiTrap Desalting columns (GE Healthcare Life Sciences), equilibrated with buffer CA (50 mM KPi, pH 7.0). The desalted protein fractions were loaded on a 1-mL HiTrap SP HP column (GE Healthcare Life Sciences). Wanomala_5543 was eluted with NaCl by a concentration gradient of buffer CB (50 mM KPi, pH 7.0, 1 M NaCl). The fractions with the highest content of protein were combined and used for further analyses.

Analytical

Glucose and organic acids were analysed by HPLC using an ICS5000 HPLC system (Thermo Scientific) equipped with a Dionex DP pump, Dionex AS-AP autosampler, Dionex VWD UV detector operated at 210 nm and Shodex RI detector operated at 35°C. An Aminex HPX-87H cation-exchange column was used with a mobile phase of 0.16 N H_2SO_4 and was operated at 0.8 mL/min and 60°C. 10 mM dimethylsulfoxide in 0.04 N H_2SO_4 was used as internal standard. Volatile compounds were analysed on a Shimadzu 2010 gas chromatograph equipped with a temperature controlled 20i-s autosampler. 0.5 µL of liquid sample or 250 µL headspace were injected on a Stabilwax column (30 m x 0.25 mm, 0.5 µm coating, Restek). The column temperature was held at 60°C for 1 minute and increased to 120°C at a rate of 20°C/minute. The split ratio was 20. 2 mM 1-butanol was used as internal standard.

Enzyme assays

All enzyme reactions were performed at 30°C in the presence 0.164 mg of purified Wanomala_5543/mL assay reaction. K_m values were estimated by a computer-aided direct fit to the Michaelis-Menten curve (Tablecurve 2d, version 5.0). Alcohol acetyl transferase (AAT) and esterase activity assays were performed in 50 mM potassium

phosphate buffer (pH 7.5) with 150 mM NaCl. AAT activity was assayed at 1 mM acetyl-CoA and varying concentrations of ethanol (1-80 mM). Esterase activity was measured with 2 mM ethyl acetate and varying concentrations of ethanol (0-80 mM). The AAT and esterase reactions were measured by analysing ethyl acetate directly on a Shimadzu GC 2010 equipped with a temperature controlled 20i+s autosampler. 0.5 µL of the liquid sample was injected and analysed approximately every 4 minutes, as described above. The final temperature of the column was adjusted to 85 °C and the split ratio to 5.

Thioesterase activity was measured in 50 mM TRIS-HCl buffer (pH 7.5) with 150 mM NaCl by following acetate release from 1 mM acetyl-CoA. The reactions were performed at various ethanol concentrations (0-80 mM) and measured in clear, flat-bottom 96 well-plates (Greiner). Acetate formation was coupled to the decrease of NADH with the Acetic Acid Assay Kit (Acetate Kinase Manual Format, Megazyme), according to the manufacturer instructions. The reagent amounts were adjusted for a final volume of 133 µL. The reaction was initiated by addition of acetyl-CoA. Reactions were incubated at 30°C and measured at 340 nm in a BioTek Synergy MX microplate reader. The initial reaction rates were calculated from the slope by using 6220 $M^{-1}cm^{-1}$ as the extinction coefficient and 0.294 cm as the light path length.

The K_m of Wanomala_5543 for acetyl-CoA was estimated in 50 mM TRIS-HCl buffer (pH 7.5) with 150 mM NaCl by following CoA release at various acetyl-CoA concentrations (0.02-5 mM). The reaction was performed in clear, flat-bottom 96 well-plates (Greiner) in a final volume of 300 µL. 40 µL of the enzyme reaction was transferred every 5 min to a measuring well. The final volume of the measuring well was 200 µL, which contained a final 0.25 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in 50 mM TRIS-HCl buffer (pH 7.5) with 150 mM NaCl. The reactions were measured immediately at 412 nm. The initial reaction rate was calculated from the slope, using 14150 $M^{-1}cm^{-1}$ as the extinction coefficient and 0.588 cm as the light path length.

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Supplementary material

Supplementary Table1 and Supplementary Figure 1 are available in the online version of the manuscript:

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CHAPTER 3

The alcohol acetyltransferase Eat1 is located in yeast mitochondria

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Abstract

Eat1 is a recently discovered alcohol acetyltransferase responsible for bulk ethyl acetate production in yeasts such as *Wickerhamomyces anomalus* and *Kluyveromyces lactis*. These yeasts have the potential to become efficient biobased ethyl acetate producers. However, some fundamental features of Eat1 are still not understood, which hampers the rational engineering of efficient production strains. The cellular location of Eat1 in yeast is one of these features. To reveal its location, Eat1 was fused with yEGFP to allow intracellular tracking. Despite the current assumption that bulk ethyl acetate production occurs in the yeast cytosol, most of Eat1 localised to the mitochondria of *K. lactis* CBS 2359 Δ*ku80*. We then compared five bulk ethyl acetate-producing yeasts in iron-limited chemostats with glucose as carbon source. All yeasts produced ethyl acetate under these conditions. This strongly suggests that the mechanism and location of bulk ethyl acetate synthesis are similar in these yeast strains. Furthermore, an *in silico* analysis showed that Eat1 proteins from various yeasts were mostly predicted as mitochondrial. Altogether, it is concluded that Eat1-catalyzed ethyl acetate production occurs in yeast mitochondria. This study has added new insights to bulk ethyl acetate synthesis in yeast, which is relevant for developing efficient production strains.

Introduction

Ethyl acetate is a valuable bulk chemical and an important aroma compound in fermented foods (Park et al., 2009). Industrially, ethyl acetate is produced from petrochemical resources, but biological production routes have been explored in recent years. Yeasts are prominent natural ethyl acetate producers. Ester production is well known in *Saccharomyces cerevisiae*, which typically produces between 8 and 32 mg/L ethyl acetate in beer fermentations (Saerens et al., 2010). Several non-*Saccharomyces* yeast species produce ethyl acetate from carbohydrates at a much higher yield than *S. cerevisiae* (Löser et al., 2014). Ethyl acetate yields up to 51.4 % of the theoretical pathway maximum have been reported in *Kluyveromyces marxianus* (Löser et al., 2013). Other bulk ethyl acetate producing yeasts include *Wickerhamomyces anomalus* (Fredlund et al., 2006; Passoth et al., 2011), *Cyberlindnera fabianii* (Meersman et al., 2016) and *Kluyveromyces lactis* (Löser et al., 2011).

Alcohol acetyl transferases (AATs) are the main ethyl acetate producing enzymes which use acetyl-CoA and ethanol as substrate. Most research on ethyl acetate producing AATs in yeast is based on Atf1 and Atf2 from *S. cerevisiae* (Minetoki et al., 1993; Yoshimoto et al., 1999). A *S. cerevisiae* strain lacking *atf1* and *atf2* produced 50 % less ethyl acetate compared to the parental strain (Verstrepen et al., 2003b). Homologs of Atf1 and Atf2 are present in bulk ethyl acetate producing yeasts (Schneider et al., 2012; Van Laere et al., 2008).

The prevailing hypothesis on the physiological function of bulk ethyl acetate production suggests that it is produced as an overflow metabolite under conditions where the TCA cycle does not function optimally (Armstrong and Yamazaki, 1984; Löser et al., 2014). Yeasts that naturally produce bulk amounts of ethyl acetate are Crabtree-negative. They oxidise glucose and other carbohydrates to pyruvate in the cytosol. Under aerobic conditions, Crabtree-negative yeasts preferentially transport the pyruvate to the mitochondria. There, it is further oxidised via pyruvate dehydrogenase to acetyl-CoA (Figure 1, Reaction I) and subsequently oxidized in the TCA cycle (Pfeiffer and Morley, 2014). Ethyl acetate is formed under conditions where the efficiency of the TCA cycle is impaired by e.g. iron or oxygen limitation (Fredlund et al., 2004b; Urit et al., 2012).

As a consequence, acetyl-CoA cannot enter the TCA cycle and accumulates in the mitochondria. It is assumed that yeasts use an AAT-catalysed reaction to relieve the acetyl-CoA accumulation and regenerate free CoA (Löser et al., 2014; Thomas and Dawson, 1978). Ethyl acetate is formed in the process. This hypothesis would imply that mitochondrial acetyl-CoA accumulation causes ethyl acetate production (Löser et al., 2015).

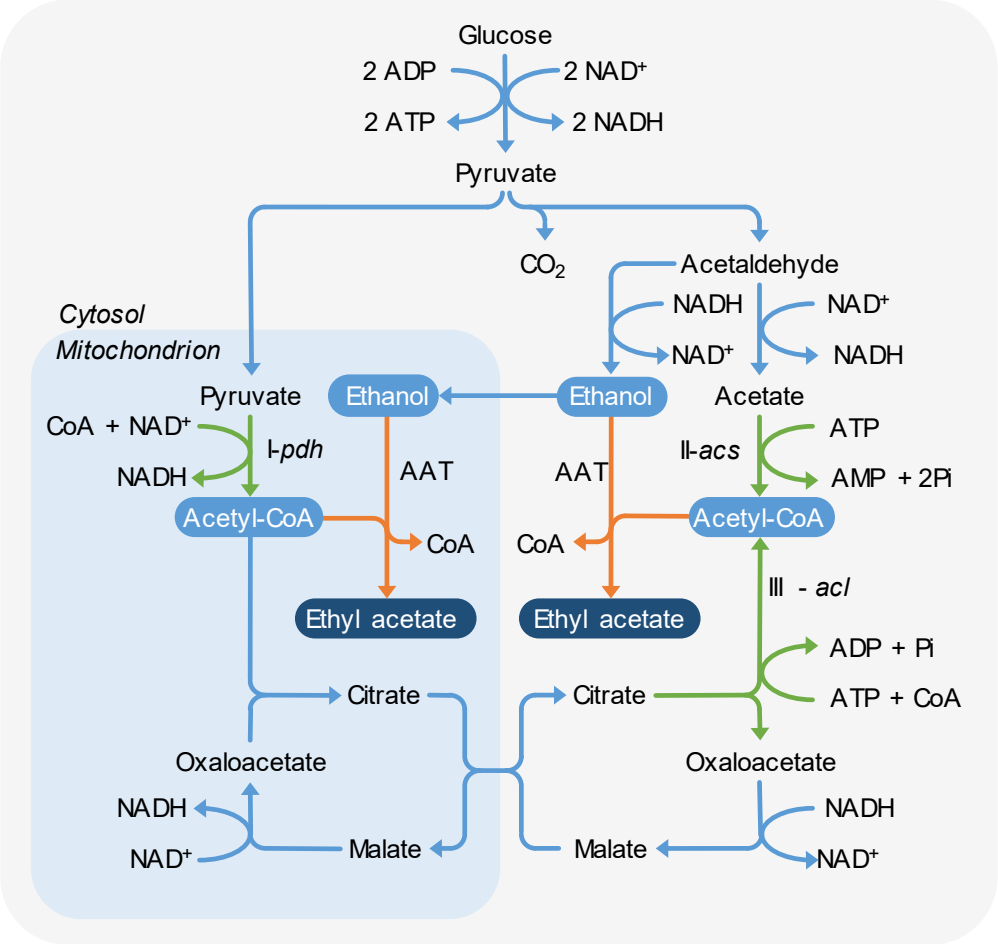

Figure 1: Potential pathways of ethyl acetate production via an AAT in yeast. The AAT catalysed reaction is indicated in orange. The three reactions forming acetyl-CoA during glucose catabolism are shown in green. Reaction I: pyruvate dehydrogenase (*pdh*), Reaction II: acetyl-CoA synthetase (*acs*), Reaction III: ATP-citrate lyase (*acl*).

Ethanol is the second substrate needed for ethyl acetate synthesis by AATs. Crabtree-negative yeasts typically do not form ethanol under aerobic conditions. However, unfavourable conditions, such as iron limitation, lead to ethanol formation in *K. marxianus* even in the presence of oxygen (Urit et al., 2012; van Dijken et al., 1993). Ethanol is produced from pyruvate via acetaldehyde in the cytosol (Figure 1). The

acetaldehyde may also be converted to acetate and further to cytosolic acetyl-CoA via acetyl-CoA synthetase (Figure 1, Reaction II). This reaction is essential in most yeasts as it supplies acetyl-CoA for fatty acid synthesis (Van den Berg and Steensma, 1995). However, during aerobic growth on sugars, the acetyl-CoA flux in the cytosol is much lower compared to the mitochondria (van Rossum et al., 2016b). It is therefore unlikely that it contributes significantly to bulk ethyl acetate synthesis. Moreover, bulk ethyl acetate synthesis in yeast does not occur in the absence of oxygen (Löser et al., 2015). Under anaerobic conditions, carbohydrate catabolism occurs in the cytosol, and mitochondrial acetyl-CoA cannot accumulate. These observations strongly suggest that acetyl-CoA used to synthesise ethyl acetate is derived from the mitochondria.

Atf1, Atf2 and their homologs appear to be cytosolic, or located in the endoplasmic reticulum (Lin and Wheeldon, 2014; Verstrepen et al., 2004; Zhu et al., 2015). A translocation step would therefore be required to transfer acetyl-CoA from the mitochondria to the cytosol. Some yeasts are able to translocate acetyl-CoA to the cytosol in the form of citrate. This shunt relies on the presence of ATP-citrate lyase, which converts citrate to acetyl-CoA and oxaloacetate at the expense of one ATP (Figure 1, Reaction III). The reaction is typically present in oleaginous yeasts, such as *Yarrowia lipolytica* or *Rhodosporidium toruloides* (Boulton and Ratledge, 1981; Shashi et al., 1990). It is not known if ATP-citrate lyase is present in any of the yeasts that produce high amounts of ethyl acetate. Without this enzyme, transport of acetyl-CoA from the mitochondria to the cytosol is unlikely.

The hypothetical function of bulk ethyl acetate production is the release of excess mitochondrial acetyl-CoA. However, the previously assumed ethyl acetate producing enzymes are located either in the cytosol or in the endoplasmic reticulum. These locations do not match with the mitochondrial function of ethyl acetate formation. Recently, a new family of AATs was discovered, designated Eat1. This family catalyses ethyl acetate synthesis in *S. cerevisiae, K. marxianus*, *W. anomalus, K. lactis* and other yeasts (Kruis et al., 2017). It was shown that Eat1 is responsible for 80% and 50% of ethyl acetate production in *K. lactis* and *S. cerevisiae*, respectively. In this study, we show that Eat1 of *K. lactis* is located in the mitochondria. In addition, we used *in silico*

analyses and fermentations of bulk ethyl acetate producing yeasts to support this view for the location of Eat1 in other yeasts as well.

Results

Localisation of Eat1 in yeast

Huh *et al.* (2003) performed a global protein localisation study in *S. cerevisiae* (Huh et al., 2003). This included the hypothetical protein YGR015C, which was later identified as the *S. cerevisiae* homolog of Eat1 (Kruis et al., 2017). The *S. cerevisiae* Eat1 was tracked to the mitochondria (Huh et al., 2003), which suggests that Eat1 may be located in the mitochondria of bulk ethyl acetate producing yeast as well. We initially tested the hypothesis by overexpressing the *W. anomalus eat1* fused with *mCherry* at the C-terminus from a multi-copy plasmid in *S. cerevisiae*. The fusion protein was generally localised to the mitochondria of *S. cerevisiae*. However, we also observed a number of artefacts associated with heterologous overexpression of fluorescent protein fusions, such as variations of the fluorescence within the cell population or protein aggregation (unpublished result). To obtain more conclusive results, we fused Eat1 with yEGFP (yeast-enhanced green fluorescent protein) at the C-terminus in *K. lactis* CBS 2359 Δ*ku80. K. lactis* CBS 2359 was chosen because it was previously demonstrated that Eat1 is the main enzyme responsible for bulk ethyl acetate production in this yeast (Kruis et al., 2017).

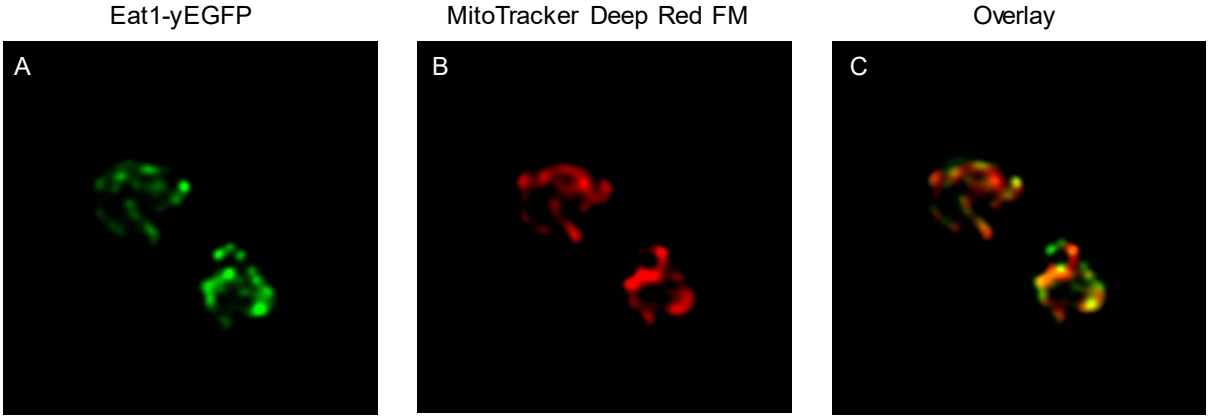

Figure 2. Visualisation of Eat1 in the mitochondria of *K. lactis* CBS 2359 Δ*ku80 eat1-yegfp*. (A) Visualisation of Eat1-yEGFP. (B) Mitochondria visualised by MitoTracker Deep Red FM. (C) Overlay of the two signals. The images shown are representative of the entire cell population.

The location of Eat1-yEGFP in living cells of *K. lactis* CBS 2359 Δ*ku80 eat1-yegfp* was visualised using confocal microscopy. Eat1-yEGFP was clearly concentrated in structures within the cell (Figure 2A). The mitochondria of these cells were stained with MitoTracker Deep Red FM (Figure 2B). The overlay of the two images showed that the signals of yEGFP and the mitochondrial marker overlap almost completely (Figure 2C). However, there are some areas where Eat1 fluorescence did not overlap with MitoTracker. This may be an artefact of the stain but could also indicate that Eat1 is located in multiple organelles. Nevertheless, the *in vivo* experiments showed that Eat1 is mostly located in the mitochondria of *K. lactis* CBS 2359 Δ*ku80*. To exclude that the Eat1-yEGFP fusion affected the function of Eat1, we compared the ethyl acetate production of *K. lactis* CBS 2359 Δ*ku80 eat1-yegfp* and its parental strain. The strains were cultivated in 50 mL YM medium without iron supplementation. The strain producing the Eat1-yEGFP fusion was still able to synthesise ethyl acetate (Figure 3). Surprisingly, the ethyl acetate titre achieved by *K. lactis* CBS 2359 Δku80 *eat1-yEGFP* was 3.75-fold higher compared to *K. lactis* CBS 2359 Δ*ku80*. The reason for this increase is not clear, but it demonstrated that the Eat1-yEGFP fusion is still functional. These results show that ethyl acetate itself is primarily a mitochondrial product of *K. lactis* CBS 2359 Δ*ku80*.

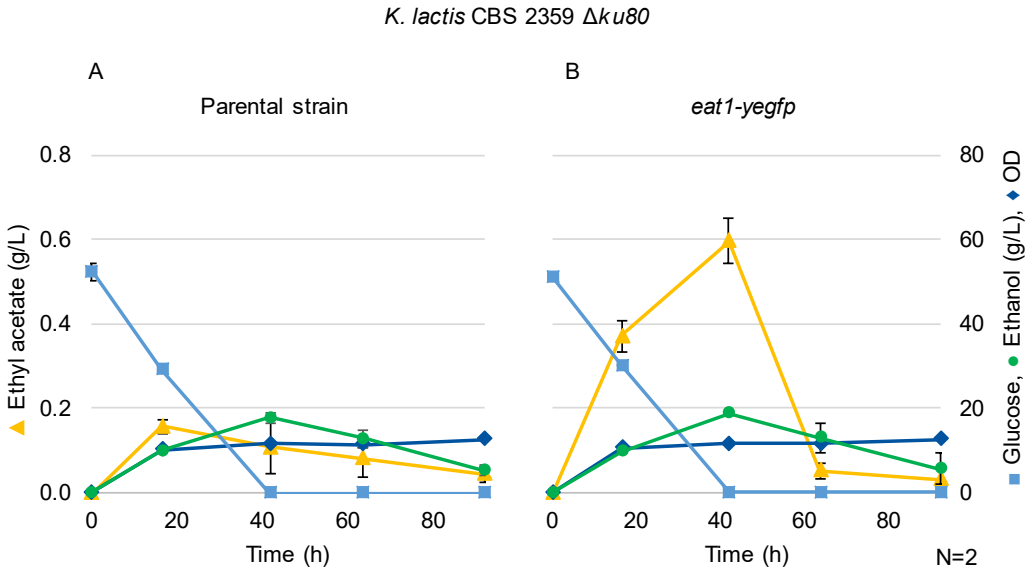

Figure 3. Fermentation profiles of (A) *K. lactis* CBS 2359 *Δku80* and (B) *K. lactis* CBS 2359 Δku80 *eat1-yEGFP* growing in shake flasks in 50 ml YM medium without iron supplementation. The numbers shown are the averages of biological duplicates. Error bars represent the standard deviation. Ethyl acetate evaporation was not measured.

Continuous fermentations indicate a common mechanism of ethyl acetate synthesis in yeast

As Eat1 is located in the mitochondria of *S. cerevisiae* and *K. lactis* CBS 2359 Δ*ku80,* we were wondering whether it is located in the mitochondria of other bulk ethyl acetate producing yeasts as well. The expression of GFP-fused proteins in these yeasts is cumbersome because of their poor genetic accessibility. We attempted to transform *W. anomalus* DSM 6766 and *K. marxianus* DSM 5422, but were not successful. To gain further insight on the location of ethyl acetate synthesis in other yeast strains, we compared the natural producers *in vivo* instead. We reasoned that if the conditions that trigger bulk ethyl acetate formation are similar, the underlying pathways are likely shared as well, including the cellular location of Eat1. However, there are no studies that accurately compare ethyl acetate production by multiple yeasts under the same conditions. Moreover, many studies on bulk ethyl acetate synthesis in yeast often did not control or measure parameters such as oxygen levels or ethyl acetate evaporation (Löser et al., 2014). This makes metabolic comparisons between different yeast species impossible. To resolve the issue, we examined bulk ethyl acetate production in five yeast species under the same controlled conditions. We used aerobic, iron-limited chemostats to induce ethyl acetate production in *W. anomalus* DSM 6766, *C. fabianii* CBS 5640, *C. jadinii CECT* 1946, *K. marxianus* DSM 5422, and *K. lactis* CBS 2359. When 1 mM $FeSO_4$ was added to the medium, all five yeasts fully consumed the glucose, and virtually no ethyl acetate or other fermentation products were formed (Figure 4). To induce ethyl acetate production, iron was omitted from the medium. Sufficient iron impurities were present to stably support between 3.4 ± 0.2 and 8.3 ± 0.0 g_{DW}/L biomass (Figure 4A). Under iron-limited conditions, the yeast strains consumed between 54.5 ± 0.0 and 79.3 ± 0.0 g/L glucose (Figure 4B). Iron limitation induced ethyl acetate production in the five-yeast species (Figure 4C). The amount of ethyl acetate removed through gas stripping was added to the concentrations measured in the liquid. The headspace contained 25.9 ± 0.0 % of the total ethyl acetate produced. The highest ethyl acetate titres were obtained with *W. anomalus* DSM 6766 and *K. marxianus* DSM 5422 (11.6 ± 0.2 and 10.7 ± 0.3 g/L, respectively). However, *K. marxianus* DSM 5422 consumed more sugar, resulting in a lower ethyl acetate yield (Figure 4D). *W. anomalus* DSM 6766 and *C. fabianii* CBS 5640 were the best ethyl

acetate producers in terms of yield. They produced 0.17 ± 0.00 $g_{EA}/g_{glucose}$ and 0.16 ± 0.01 $g_{EA}/g_{glucose}$, respectively. *K. lactis* CBS 2359 produced the least ethyl acetate per glucose (0.04 ± 0.01 $g_{EA}/g_{glucose}$). The maximum theoretical pathway yield of ethyl acetate on glucose is 0.49 $g_{EA}/g_{glucose}$.

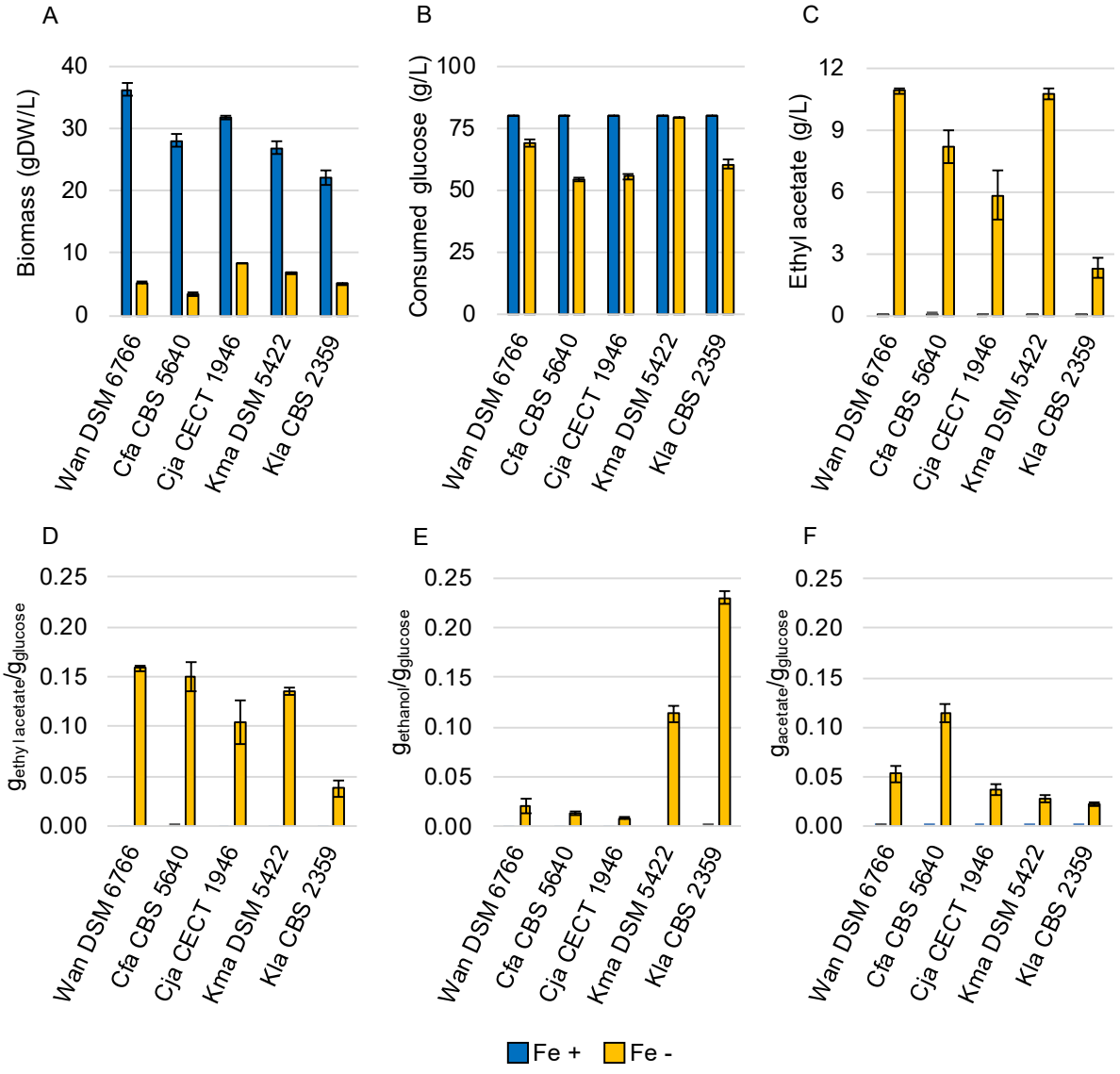

Figure 4. Cultivation parameters of five different yeast species that were grown in aerobic, pH-controlled chemostats in the presence or absence of 1 mM of FeSO4. The numbers shown are averages of three steady states. Error bars indicate standard deviation. The amount of ethyl acetate that was removed by gas stripping was determined by headspace measurements and was added to the amount that was measured in the liquid phase. (A) Biomass concentration. (B) Glucose consumption. (C) Ethyl acetate titre. (D, E, F) The yields of ethyl acetate, ethanol and acetate on glucose consumed, respectively.

Besides ethyl acetate, the yeasts also formed significant amounts of ethanol or acetate as by-products (Figure 4E and 4F, respectively). Crabtree-negative yeasts generally do not produce ethanol under aerobic conditions like the ones used in this study. However, iron limitation results in a metabolic deregulation which leads to ethanol production (Urit et al., 2012; van Dijken et al., 1993). *W. anomalus* DSM 6766, *C. fabianii* CBS 5640 and *C. jadinii CECT* 1946 produced between 0.02 ± 0.01, and 0.01 ± 0.00 $g_{ethanol}/g_{glucose}$. This was significantly lower compared to *K. marxianus* DSM 5422 and *K. lactis* CBS 2359, which produced 0.11 ± 0.01, and 0.23 ± 0.01 (Figure 4E). The yeasts also produced between 0.02 ± 0.00, and 0.12 ± 0.01 $g_{acetate}/g_{glucose}$ (Figure 4F).

The five tested yeasts produced ethyl acetate under aerobic and iron-limited conditions. However, there were significant levels of ethanol and acetate produced. These products indicate that glucose is catabolised in the cytosol as well, despite the aerobic conditions. The effect was most pronounced in *K. lactis* CBS 2359, which produced 6.1-fold more ethanol than ethyl acetate. Most of this ethyl acetate was produced by the mitochondrial Eat1 (Figure 2), despite the high carbon flux in the cytosol (Figure 4EF). The remaining four yeasts produced ethyl acetate under the same iron-limited conditions as *K. lactis* CBS 2359, which suggests that the ethyl acetate produced by these yeasts is of mitochondrial origin as well.

In silico *indications of Eat1 localisation in yeast*

Acetyl-CoA used for bulk ethyl acetate synthesis is produced in the mitochondria. We investigated whether the five yeast strains are able to transport acetyl-CoA from the mitochondria to the cytosol via citrate. The enzyme needed for the realisation of this pathway is the cytosolic ATP-citrate lyase (Figure 1). We used BlastP to search for homologs of three fungal ATP-citrate lyase proteins in *W. anomalus*, *C. jadinii*, *C. fabianii*, *K. marxianus* and *K. lactis.* The ATP-citrate lyase homologs originated from *Yarrowia lipolytica* CLIB 122, *Aspergillus nidulans* FGSC A4 and the *Rhodosporidium toruloides* IFO 0880. None of the bulk ethyl acetate producing yeasts contained apparent ATP-citrate lyase homologs. The absence of the ATP-citrate lyase suggests that ethyl acetate-producing yeasts cannot transport acetyl-CoA to the cytosol

(Boulton and Ratledge, 1981; van Rossum et al., 2016b). Bulk ethyl acetate synthesis by Eat1 is therefore more likely to be located in the mitochondria.

The sub-cellular location of proteins can also be predicted *in silico* based on their primary sequence. We predicted the location of nine Eat1 homologs originating from nine bulk ethyl acetate producing yeast species (Kruis et al., 2017). The mitochondrial citrate synthase (Cit1) and the cytosolic Atf1 and Atf2 from *S. cerevisiae* were included in the analysis as controls. Seven tools were used in the analysis: MitoProt II (Claros and Vincens, 1996), MultiLoc 2 (Blum et al., 2009), Yloc (Briesemeister et al., 2010), WoLF PSORT (Horton et al., 2007), Protein Prowler (Boden and Hawkins, 2005) and BacCelLo (Pierleoni et al., 2006) and MitoFates (Fukasawa et al., 2015). The Eat1 homologs were generally predicted as mitochondrial (Figure 5). The Eat1 homologs of *K. lactis, K. marxianus* and *Wickerhamomyces ciferri* were predicted as mitochondrial by all seven tools. The least mitochondrial localisation predictions were given to the *W. anomalus* and *C. fabianii* Eat1 homologs. Most of the non-mitochondrial predictions were assigned by Protein Prowler, Mito Prot II and MitoFates. These tools also did not identify the *S. cerevisiae* Eat1 protein as mitochondrial. This indicates that the predictions made by these tools may not be reliable for the Eat1 homologs. On the other hand, MultiLoc2 and WoLF PSORT predicted all the proteins as mitochondrial, including the *S. cerevisiae* and *K. lactis* Eat1 homologs. These two tools also performed better compared to Protein Prowler (Blum et al., 2009; Casadio et al., 2008).

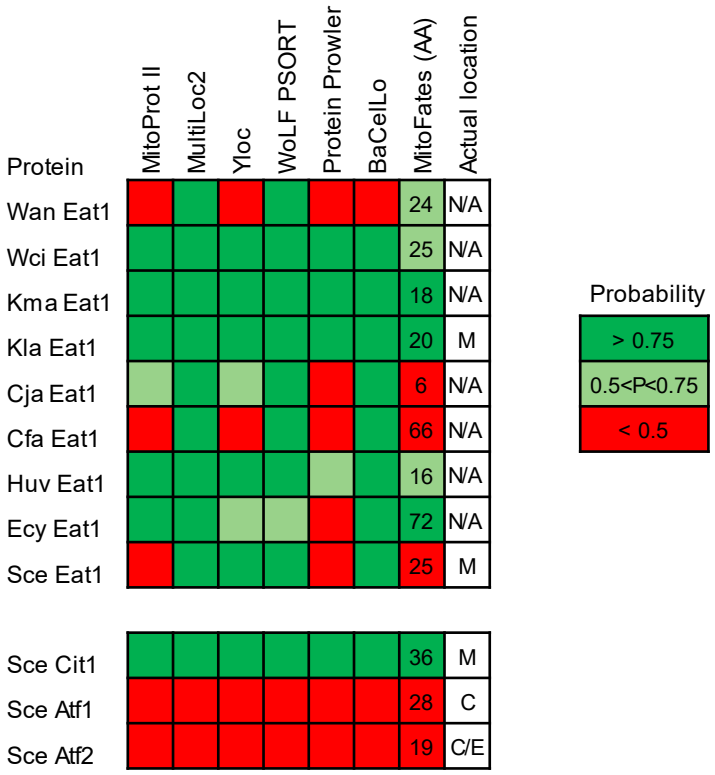

Figure 5. Predicted probabilities of mitochondrial localisation of the Eat1 proteins. Seven tools were used to predict the mitochondrial localisation of nine Eat1 homologs from 9 yeast species. The numbers in the column represent the length of the pre-sequence, predicted by MitoFates. Abbreviations: Wan - *Wickerhamomyces anomalus*, Wci - *Wickerhamomyces ciferrii*, Kma – *Kluyveromyces marxianus*, Kla – *Kluyveromyces lactis*, Cja – *Cyberlindnera jadinii*, Cfa – *Cyberlindnera fabianii*, Huv – *Hanseniaspora uvarum*, Ecy – *Eremothecium cymbalarie,* Sce – *Saccharomyces cerevisiae*, M – mitochondria, C – cytosol, E – endoplasmic reticulum, N/A – not available

Discussion

In this study, we included the cellular location of Eat1 into the hypothetical model of bulk ethyl acetate production in yeast. This AAT was previously linked to 80 % and 50 % of ethyl acetate synthesis in *K. lactis* and *S. cerevisiae*, respectively (Kruis et al., 2017). Results presented here showed that Eat1 is a mitochondrial protein in both yeasts. However, the current model of bulk ethyl acetate production assumes that cytosolic AATs are responsible for ethyl acetate formation (Löser et al., 2015). The mitochondrial location of Eat1 in *K. lactis* CBS 2359 described in this study disagrees with this assumption. It is also likely that bulk ethyl acetate by other yeast is of mitochondrial origin as well. Ideally, confocal microscopy could be used to confirm this. However, this is hampered by the lack of genetic tools needed to perform gene fusions

in other bulk ethyl acetate producing yeast. It is also not possible to discriminate between the mitochondrial and cytosolic acetyl-CoA flux using ^{13}C tracking experiments as the same carbon atoms are removed during the cleavage of pyruvate in both compartments. The acetyl-CoA produced in the mitochondria is therefore identical to the one produced in the cytosol. The *in silico* tools used in this study generally predicted that all known Eat1 homologs are mitochondrial. The apparent lack of ATP-citrate lyase homologs also seems to indicate that acetyl-CoA cannot be transported to the cytosol. Mitochondrial acetyl-CoA may play a role in ethyl acetate synthesis in *S. cerevisiae* as well. This yeast contains a functional Eat1 homolog, but produces only traces of ethyl acetate (Saerens et al., 2010). This is likely caused by the Crabtree-positive nature of *S. cerevisiae*. When glucose is present in excess, the main carbon flux in *S. cerevisiae* bypasses the mitochondria in favour of cytosolic ethanol formation (De Deken, 1966; Pfeiffer and Morley, 2014). It is possible that the low ethyl acetate production in *S. cerevisiae* is caused by the low mitochondrial acetyl-CoA flux.

W. anomalus DSM 6766, *C. fabianii* CBS 5640, *C. jadinii CECT* 1946, *K. marxianus* DSM 5422 and *K. lactis* CBS 2359 are all Crabtree-negative yeasts. Under aerobic conditions, such yeasts convert glucose to cytosolic pyruvate and further to mitochondrial acetyl-CoA. Conditions such as iron limitation repress the synthesis of enzymes in the TCA cycle and respiratory chain (Puig et al., 2005; Shakoury-Elizeh et al., 2004). As a consequence, ethanol is produced in the cytosol even under aerobic conditions (Löser et al., 2012; van Dijken et al., 1993). In the mitochondria, iron limitation leads to the accumulation of acetyl-CoA. The accumulation may be resolved by Eat1 in the mitochondria by forming ethyl acetate (Armstrong and Yamazaki, 1984; Löser et al., 2012; Thomas and Dawson, 1978). The mitochondrial localisation of Eat1 thus agrees with its proposed physiological function of preventing the accumulation of acetyl-CoA in yeast. However, more research is needed to confirm this hypothesis. It has been shown that Eat1 can function as a thioesterase *in vitro* (Kruis et al., 2017). This activity could relieve acetyl-CoA accumulation by releasing acetate instead of ethyl acetate. However, it is possible that ethyl acetate formation provides ancillary benefits to the yeasts. It has been shown that ethyl acetate inhibits the growth of

competitive microorganisms (Fredlund et al., 2004c) and helps yeast disperse in the environment by attracting fruit flies (Christiaens et al., 2014).

The demonstration that Eat1 is a mitochondrial enzyme is critical for improving ethyl acetate production by microorganisms. Yeasts like *K. marxianus* and *W. anomalus* are naturally able to produce ethyl acetate at high yields. However, they also produce considerable amounts of ethanol and acetate as by-products. The acetyl-CoA and ethanol used for ethyl acetate synthesis are produced in the mitochondria and cytosol, respectively. Cytosolic pyruvate is the precursor of both substrates. Efficient ethyl acetate production would therefore require precise control over the pyruvate flux so that acetyl-CoA and ethanol production are stoichiometrically balanced. Alternatively, Eat1 can be used to produce ethyl acetate in heterologous hosts. In such cases, consideration should be given to identifying the localisation pre-sequence. In eukaryotes, N-terminal pre-sequences are cleaved from the nascent protein during translocation to the mitochondria, giving rise to the mature protein (Wiedemann et al., 2001). Unrelated hosts may not be able to perform this cleavage. The presence of the N-terminal localisation sequence has resulted in lower protein activity and stability in some cases (Veling et al., 2017; Vögtle et al., 2011). Proper N-terminal processing of the Eat1 proteins may therefore improve the activity of the protein in heterologous hosts.

Until now it was assumed that yeasts such as *K. lactis, K. marxianus* and *W. anomalus* produce ethyl acetate in the cytosol. The present study has established that the synthesis occurs in mitochondria instead. This finding agrees with the proposed biological function of bulk ethyl acetate synthesis. Our understanding of ester synthesis in yeast is hereby expanded, which will enable the design of more efficient processes for the production of biobased ethyl acetate.

Materials and Methods

Strain and plasmid construction

Strains and plasmids that were used in this study are listed in Table 1. Wild type yeast strains were obtained from culture collections. *Kluyveromyces lactis* CBS 2359 Δ*ku80* (Kooistra et al., 2004) was a gift from Paul Hooykaas (Leiden University). Plasmid

pCY 3040-01 was a gift from Anne Robinson (Addgene plasmid # 36217). Plasmid pUG75 (Hegemann and Heick, 2011) was obtained from Euroscarf (Plasmid #P30671). *K. lactis* CBS 2359 Δ*ku80 eat1-yEGFP* was constructed by integrating the *yegfp* gene (Young et al., 2012) in frame at the 3' end of *eat1*. A $(GGTGGTAGTGGT)_2$ linker was inserted between *eat1* and *yegfp*. The native *eat1* stop codon was removed. The linear integration cassette contained the linker, *yegfp* and pAgTEF1-*hphMX*-tAgTEF1, flanked by 1000 bp sequence upstream and downstream of the integration site. The flanking regions, *yegfp* and pAgTEF1-*hphMX*-tAgTEF1 were amplified from the *K. lactis* CBS 2359 Δ*ku80* genome, pCY-3040-01 and pUG75, respectively. The parts were assembled to yield the plasmid pYES2-KlaEat1-yEGFP-hphMX-1000 with the HiFi assembly kit (NEB), according to manufacturer instructions. pYES2 (Invitrogen) was used as the backbone. The linear integration cassette was PCR amplified from pYES2-KlaEat1-yEGFP-hphMX-1000. 1 µg of the cassette was transformed into *K. lactis* CBS 2359 Δ*ku80* with the Li-acetate method (Gietz and Woods, 2002). Transformants were selected on plates containing 100 µg/mL hygromycin B. Correct clones were confirmed by PCR and sequencing.

Cultivation conditions

Yeast and *E. coli* cultures were routinely cultivated in YPD (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone) or LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) medium, respectively. Bacteriological agar (15 g/L) was added to make plates. Ampicillin (50 µg/mL) and hygromycin B (100 µg/mL) were added to the media when appropriate. Yeast and *E. coli* were grown at 30 °C and 37 °C, respectively, unless stated otherwise. All strains were stored as glycerol stocks at -80 °C. Yeast and *E. coli* strains were revived by streaking frozen cultures on agar plates and cultivating until colonies appeared. Single colonies were used to inoculate liquid precultures used in further experiments.

The ethyl acetate production of *K. lactis* CBS 2359 Δ*ku80* and *K. lactis* CBS 2359 Δ*ku80 eat1-yEGFP* was assessed as reported previously (Kruis et al., 2017). The cells were grown in 250-mL Erlenmeyer flasks containing 50 mL YM (yeast minimal) medium adapted from Thomas and Dawson, 1978 (Thomas and Dawson, 1978). YM

medium contained glucose (50 g/L), $(NH_4)_2SO_4$ (2.5 g/L), KH_2PO_4 (2.5 g/L), 3-(N-morpholino) propanesulfonic acid (MOPS, 23.1 g/L), $MgSO_4$ (60 mg/L), $ZnSO_4$ *7 H_2O (25.0 mg/L), $MnCl_2$ *4H_2O (4.0 mg/L), $CuSO_4$ *5 H2O (2.5 mg/L), $CaCl_2$ *2 H_2O (1.5 mg/L), H_3BO_3 (1.5 mg/L) Na_2MoO_4 *2 H_2O (0.4 mg/L), $CoCl_2$ (0.2 mg/L) and KI (0.3 mg/L). The pH of the medium was set to 6.0 with 3 M NaOH. Iron was omitted from the medium. The medium was supplemented with 1 mL 1000x vitamins mix according to Verduyn *et al.* 1992 (Verduyn et al., 1992). The vitamins mix contained Biotin (0.05 mg/L), Ca-panthothenate (1 mg/L), Nicotinic acid (1 mg/L), Inositol (25 mg/L), Thiamine-HCl (1 mg/L), Pyridoxine-HCl (1 mg/L), 4-amino benzoic acid (0.2 mg/L). Erlenmeyer flasks (neck width 34 mm) were inoculated with 0.5 mL preculture grown overnight in liquid YPD medium to an initial OD_{600} of 0.03. The flasks were closed with aluminium foil and shaken at 250 rpm. Experiments were performed as biological duplicates. Ethyl acetate evaporation was not measured in shake flasks.

Continuous fermentations

The ethyl acetate production of *W. anomalus* DSM 6766, *C. fabianii* CBS 5640, *C. jadinii CECT* 1946, *K. marxianus* DSM 5422, and *K. lactis* CBS 2359 was studied in aerobic continuous fermentations. The culturing was performed in 1-L DasGip bioreactors (Eppendorf). The working volume was 0.5 L. The pH was controlled at 5.0 (± 0.05) by automatic addition of 3 M KOH. The temperature was controlled at 30°C. Cultures were kept aerobic by controlling the DO at 40%. Sufficient oxygen transfer was achieved by stirring the fermenter at 1200 rpm and automatically varying the oxygen fraction from 21% to 100%. The sparging was kept constant at 6 L/h. The defined feed medium was designed to emulate the mineral composition of concentrated whey permeate augmented with ammonium sulphate (Urit et al., 2011) as closely as possible. Distilled water (dH_2O) was used to prepare the medium. The medium contained $(NH_4)_2SO_4$ (13.16 g/L), Na_2HPO_4*2 H_2O (2.08 g/L), NaCl (1.39 g/L), KCl (1.89 g/L), $MgSO_4$ *7 H_2O (0.81 g/L), $CaCl_2$ * H_2O (0.139 g/L), $ZnSO_4$ * 7 H_2O (50 mg/L), $CuCl_2$ * 2H_2O (6.6 mg/L), Na_2MoO_4 * 2 H_2O (1 mg/L), H_3BO_3 (2 mg/L), $MnSO_4$ * 1 H_2O (1.51 mg/L), $CoSO_4$ * 7 H_2O (2 mg/L), $NiSO_4$ * 6 H_2O (1 mg/L) and 4 mL/L of a 1000x vitamin mix (Verduyn et al., 1992). Glucose (80 g/L) was used as carbon source. 20 mL 37% HCl was added to the 20 L medium vessel to lower the pH

of the medium. The dilution rate was 0.1 h^{-1}. Ethyl acetate production was controlled by iron limitation. The producing (iron-limited) condition was achieved by omitting all sources of iron from the medium. The non-producing (iron-rich/glucose-limited) condition was achieved by adding 1 mM $FeSO_4$ to the medium vessel. Steady state was achieved after five culture volumes were exchanged during which physiological parameters remained stable. The reported numbers are an average of three sequential steady states achieved in one bioreactor. The amount of ethyl acetate in the headspace was quantified by extracting 250 µL of the bioreactor headspace through a septum with a syringe and analysing the ethyl acetate content immediately by gas chromatography (GC). The concentration of ethyl acetate in the headspace was used to calculate the mass flow of ethyl acetate continuously discharged from the fermenters. The mass flow of ethyl acetate was related to the liquid flow of 0.05 L/h and added to the ethyl acetate concentration in the liquid phase. Dry cell weight was determined in 50 mL fermentation broth.

Confocal microscopy

Confocal microscopy was carried out on a LeicaTCS SP8 X system equipped with a 63x/1.20 numeric aperture water-immersion objective. Excitation of EGFP and MitoTracker Deep Red FM (Thermo Scientific) was performed using a white light laser selecting the lasers lines 488 nm and 633 nm, respectively. Confocal imaging was executed using internal filter-free spectral Hybrid detectors. For EGFP detection, a spectral window of 495 to 545 nm was selected, whereas MitoTracker Deep Red FM was detected using 640 to 670 nm. Images with 1024x1024 pixels were acquired using the HyVolution software interface of Leica operating in a sequential imaging configuration. The HyVolution software includes deconvolution of the confocal images by Huijgens deconvolution software (Borlinghaus and Kappel, 2016).

Bioinformatics

The subcellular locations of proteins was predicted with six tools: MitoProt II (Claros and Vincens, 1996), MultiLoc 2 (Blum et al., 2009), Yloc (Briesemeister et al., 2010), WoLF PSORT (Horton et al., 2007), Protein Prowler (Boden and Hawkins, 2005) and BacCelLo (Pierleoni et al., 2006). Where applicable, the prediction settings were set to

fungal. MitoFates (Fukasawa et al., 2015) was used to predict mitochondrial pre-sequences under fungal prediction settings. BlastP under default settings was used to look for homologs of ATP citrate lyase in *C. fabianii* CBS 5640, *C. jadinii CECT* 1946, *K. marxianus* DSM 5422, *W. anomalus* DSM 6766 and *K. lactis* CBS 2359. The ATP-citrate lyase from *Yarrowia lipolytica* CLIB122 (XP_503231.1), the ATP citrate lyase subunit 1 (XP_660040.1) from *Aspergillus nidulans* FGSC A4 and the ATP-citrate synthase from *Rhodosporidium toruloides* IFO 0880 (PRQ71611.1) were used as query (Coradetti et al., 2018; Dujon et al., 2004; Galagan et al., 2005).

Analytical

Glucose and organic acids were analysed by HPLC on a Thermo Scientific ICS5000 HPLC system, equipped with a Dionex DP pump, Dionex AS-AP autosampler, Dionex VWD UV detector operated at 210 nm and a Shodex RI detector operated at 35 °C. An Aminex HPX-87H cation-exchange column (Bio-Rad) was used with a mobile phase of 0.008 M H_2SO_4 and was operated at 0.8 mL/min and 60 °C. 10 mM dimethylsulfoxide or 125 mM propionic acid were used as internal standard.

Volatile compounds were analysed on two gas chromatography systems equipped with a flame ionization detector (GC-FID). In both cases, 0.5 µL of liquid or 250 µL headspace sample were analysed. For liquid samples, 2 mM 1-butanol was used as internal standard. The first system used a Shimadzu 2010 gas chromatograph equipped with a 20i-s autosampler. Samples were analysed on a Stabilwax column (30 m x 0.53 mm, 0.5 µm coating, Restek). The column temperature was held at 60 °C for 1 minute and increased to 120 °C at a rate of 20 °C/minute. The split ratio was 20. The second system used an Agilent 7890B gas chromatograph equipped with an Agilent 7693 autosampler. The compounds were separated on a Nukol™ column (30 m x 0.53 mm, 1.0 µm coating, Supelco). The column temperature was maintained at 50 °C for 2 minutes and increased to 200°C at a rate of 50 °C/minute. The split ratio was 10.

Dry cell weight in continuous fermentations was measured by centrifuging 50 mL culture for 5 min at 4000 *x g*. The pellet was then washed with 50 mL ultrapure MilliQ water (MQ), resuspended in 3 mL MQ and dried overnight at 120 °C in a pre-weighted aluminium tray before weighing.

Acknowledgements

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CHAPTER 4

Enhanced ethyl acetate production in *Escherichia coli* under anaerobic conditions

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Abstract

Ethyl acetate is a widely used industrial solvent that is currently produced in unsustainable chemical conversions. Several yeast species are able to convert sugars to ethyl acetate. However, this conversion only proceeds under aerobic conditions, resulting in low bioprocess efficiencies. Performing ethyl acetate synthesis anaerobically, can make the process economically more viable. We here describe the development of an *E. coli* strain that is able to convert glucose to ethyl acetate as the main fermentative product under anaerobic conditions. The key enzyme of the pathway is an alcohol acetyltransferase (AAT) that catalyses the generation of ethyl acetate. To achieve efficient ethyl acetate formation, the metabolic pathway was streamlined and a suitable AAT was selected. The ethyl acetate-forming capacities of Atf1 from *Saccharomyces cerevisiae,* as well as Eat1 from *Kluyveromyces marxianus* and *Wickerhamomyces anomalus* were compared. Heterologous expression of the AAT-encoding genes under control of the inducible LacI/*T7* and XylS/*Pm* promoters allowed for optimisation of their expression levels, and for improved ethyl acetate formation. The two Eat1 homologs were further optimised by removing their N-terminal mitochondrial localisation sequences. Several truncated Eat1 (trEat1) variants formed ethyl acetate more efficiently compared to unprocessed Eat1. A characteristic of *E. coli* strains producing either *W. anomalus* or *K. marxianus* Eat1 was the hydrolysis of ethyl acetate when ethanol concentrations were low. To prevent degradation, ethyl acetate was continuously removed by gas stripping in batch fermentors. The combined effect of the optimisation efforts resulted in an *E. coli* strain that catabolised glucose to ethyl acetate at 63.4 % of the pathway maximum under anaerobic conditions.

Introduction

Ethyl acetate is used on a large scale as an industrial solvent for the production of paints, coatings and resins (Löser et al., 2014), as well as in the flavours and fragrances industry (Dzialo et al., 2017; Park et al., 2009). The global production of the ester was predicted at 3.5 million tonnes in 2015 (The Market Publishers, 2014). Currently, ethyl acetate is produced from petrochemicals in energy intensive and unsustainable processes. Biobased alternatives have focused on yeast that are naturally able to produce ethyl acetate from sugars or ethanol at high yields (Armstrong et al., 1984a; Fredlund et al., 2004b; Löser et al., 2012). The most prominent and well-studied yeast is *Kluyveromyces marxianus* which produced ethyl acetate from whey sugars at more than 50% of the maximum pathway yield (Löser et al., 2013; Urit et al., 2013a). Other examples include *Wickerhamomyces anomalus* and *Kluyveromyces lactis* (Kruis et al., 2017).

Ethyl acetate production in yeast is catalysed by alcohol acetyltransferases (AATs). They synthesise ethyl acetate from acetyl-CoA and ethanol, releasing free CoA in the reaction (Park et al., 2009). The first described ethyl acetate-producing AATs was the *Saccharomyces cerevisiae* Alcohol acetyltransferase 1 (Atf1) (Minetoki et al., 1993). This enzyme contributes to 50 % of the ethyl acetate synthesis in *S. cerevisiae* (Verstrepen et al., 2003b). However, its homologs have only a minor role in bulk ethyl acetate production in yeasts such as *W. anomalus* and *K. marxianus*. Instead, they use the recently identified ethanol acetyltransferase 1 (Eat1) to produce ethyl acetate (Kruis et al., 2017). An undesirable characteristic of yeast AATs is the presence of esterase or thioesterase side activities, which may hydrolyse esters and acetyl-CoA, respectively. In Eat1, however, the side activities could be subdued by the presence of ethanol (Kruis et al., 2017; Nancolas et al., 2017).

Organisms that oxidise glucose via the Emden-Meyerhof-Parnas (EMP) pathway can produce 1 $mol_{ethyl\ acetate}/mol_{glucose}$, or 0.67 C-$mol_{ethyl\ acetate}$/C-$mol_{glucose}$. In the pathway, 1 mol glucose is converted to 2 mol pyruvate and 2 mol NADH. The pyruvate is then decarboxylated and further converted to ethanol and acetyl-CoA, which are needed for ethyl acetate synthesis (Figure 1). During these reactions, 2 single-carbon compounds

are released. The stoichiometry of the reaction reveals that excess redox equivalents are formed in the process. The pyruvate decarboxylation step determines the form of the redox equivalents. If pyruvate is decarboxylated via pyruvate decarboxylase (Pdc, Figure 1a) or pyruvate dehydrogenase (Pdh, Figure 1b), a net surplus of 2 NADH is formed in the subsequent generation of ethyl acetate (Figure 1ab). To maintain the redox balance of the cell, excess NADH must be regenerated in the electron transport chain (ETC). In yeast, oxygen serves as the terminal electron acceptor in the ETC. As a consequence, yeasts are only able to produce ethyl acetate under aerobic conditions. This agrees with experimental observations in *K. marxianus* and *W. anomalus* (Davies et al., 1951; Löser et al., 2014, 2015; Peel, 1951).

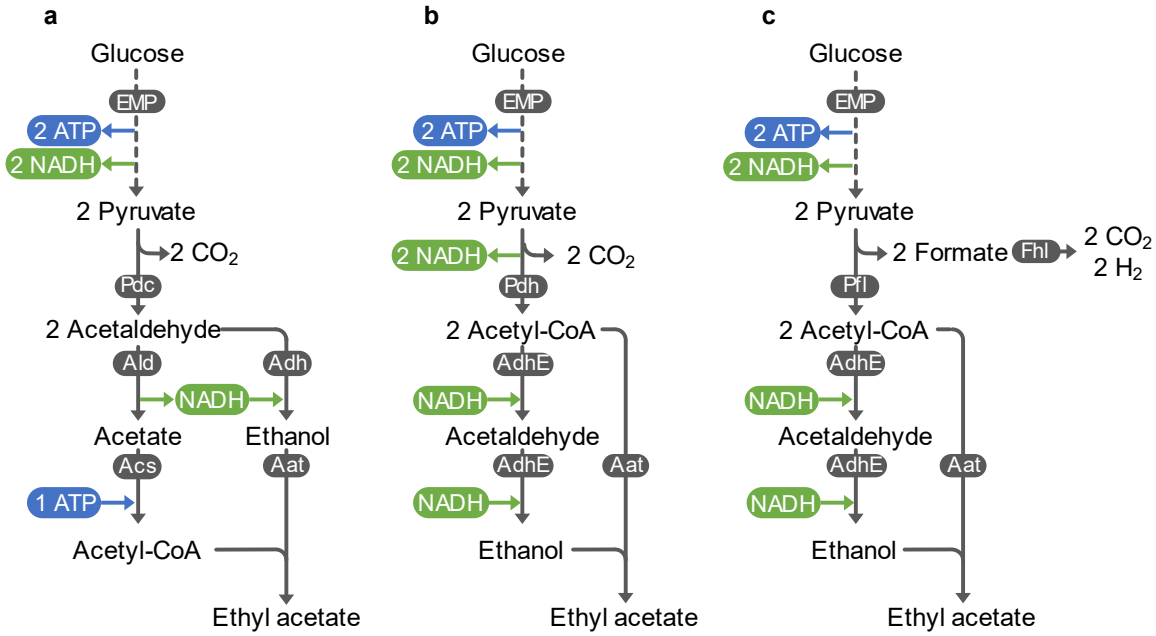

Figure 1: Three variants of ethyl acetate production from glucose via the Embden-Meyerhof-Parnas (EMP) pathway. An alcohol acetyltransferase (Aat) catalyses the production of ethyl acetate from acetyl-CoA and ethanol. The pathways differ in the conversion of pyruvate to acetyl-CoA and ethanol. a – Acetyl-CoA 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 – Acetyl-CoA is formed directly from pyruvate via pyruvate dehydrogenase (Pdh). Ethanol is formed from acetyl-CoA via the bifunctional alcohol-aldehyde dehydrogenase (AdhE). CO_2 is produced as a by-product. c – 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).

Large scale aerobic cultivations are expensive because of the low solubility of oxygen (Garcia-Ochoa and Gomez, 2009). Furthermore, a significant part of glucose is oxidised in the TCA cycle under aerobic conditions, leading to lower product yields (Weusthuis et al., 2011). Both problems can be avoided anaerobically, when oxygen or other electron acceptors are absent. To achieve the 0.67 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$ yield anaerobically, the redox balance of the reaction must be maintained. *E. coli* and other bacteria can achieve this by converting pyruvate into acetyl-CoA with pyruvate formate lyase (Pfl, Figure 1c). 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. The 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 the glycolysis are regenerated by converting 1 mol acetyl-CoA to ethanol via the bifunctional alcohol-aldehyde dehydrogenase (AdhE). A heterologous AAT then reacts the remaining acetyl-CoA with ethanol to form ethyl acetate (Figure 1c). This allows redox-neutral production of 0.67 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$ under anaerobic conditions. The only by-product of the pathway is formate which can be further converted to CO_2 and hydrogen by formate-hydrogen lyase (Fhl, Figure 1c). The latter can also be considered a valuable product that has potential as a biobased fuel (Stephen et al., 2017).

The 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. The yield of ethyl acetate achieved by *E. coli* producing the *W. anomalus* Eat1 was 24.4-fold higher compared to the *S. cerevisiae* Atf1 (Kruis et al., 2017; Rodriguez et al., 2014): However, the enzymes have not been compared in the same metabolic background yet. A key difference between Atf1 and Eat1 is their cellular location in yeast. Atf1 localises to lipid particles in the cytosol (Lin and Wheeldon, 2014; Verstrepen et al., 2004) while Eat1 homologs are located in yeast mitochondria (Kruis et al., 2018) . Most mitochondrial proteins contain a positively-charged N-terminal localisation tag, which is prone to form amphipathic α-helices (Mossmann et al., 2012). A characteristic of such α-helices is an exposed hydrophobic patch that serves as a signal for mitochondrial import. In the native hosts, this patch

is protected to prevent protein aggregation (Young et al., 2004). The localisation tag is cleaved after translocation to the mitochondria by the mitochondrial processing peptidase (MPP) and other peptidases, such as Icp55 or Oct1, releasing the mature protein (Gakh et al., 2002; Mossmann et al., 2012). The mature protein forms are often more stable than their precursor. This effect has been demonstrated in Imo32, a *S. cerevisiae* homolog of Eat1 (Kruis et al., 2017), which needs to be processed by MPP and Oct1 (Vögtle et al., 2011). Heterologous hosts, particularly bacteria are unable to perform this cleavage and may therefore express less-stable versions of mitochondrial proteins. The efficiency of ethyl acetate production with un-optimised Eat1 variants may therefore be impaired.

In this study we established anaerobic production of ethyl acetate in *Escherichia coli.* We compared and evaluated ethyl acetate production of three AATs from different yeasts. To maximise ethyl acetate production, we optimised the gene expression levels and the N-termini of the mitochondrial AATs. Finally, we performed a series of anaerobic, pH-controlled fermentation experiments where ethyl acetate was produced at high yield.

Results

Anaerobic ethyl acetate production in E. coli

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 *E. coli* BW25113 (DE3) (pET26b:hKmaEat1) produced 2.7 ± 0.1 mM ethyl acetate (Figure 1a). This demonstrated that ethyl acetate can be produced in *E. coli* BW25113 (DE3) under anaerobic conditions. Due to the formation of by-products, particularly lactate and acetate, the ethyl acetate titre was low. To maximise 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 titre to 9.1 ± 0.3 mM in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) (pET26b:hKmaEat1) (Figure 2b). The final ethyl acetate yield increased from 0.03 ± 0.00 to 0.13 ± 0.00 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$, or 21.4 % of the maximum pathway yield (Figure 2c). Lactate production in *E. coli*

BW25113 Δ*ackA*Δ*ldhA* (DE3) was almost completely abolished, but contrary to our expectations, acetate yields did not decrease significantly despite the *ackA* disruption (Figure 1c). It is possible that the effect of the disruption was masked by the increased carbon flux via Kma Eat1. This AAT can function as an esterase and thioesterase *in vitro* when ethanol is absent (Kruis et al., 2017), which may have contributed to *in vivo* acetate production.

Disruption of lactate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) (pET26b:hKmaEat1) removed a major NADH regeneration route. To compensate, pyruvate was instead converted to acetyl-CoA via Pfl and further to ethanol, which became the main route of NADH regeneration. The increased yields of formate, ethanol and ethyl acetate agree with this observation (Figures 1c, 2c). The disruption strain also produced 0.14 ± 0.01 C-mol$_{pyruvate}$/C-mol$_{glucose}$, while the parental strain produced only traces.

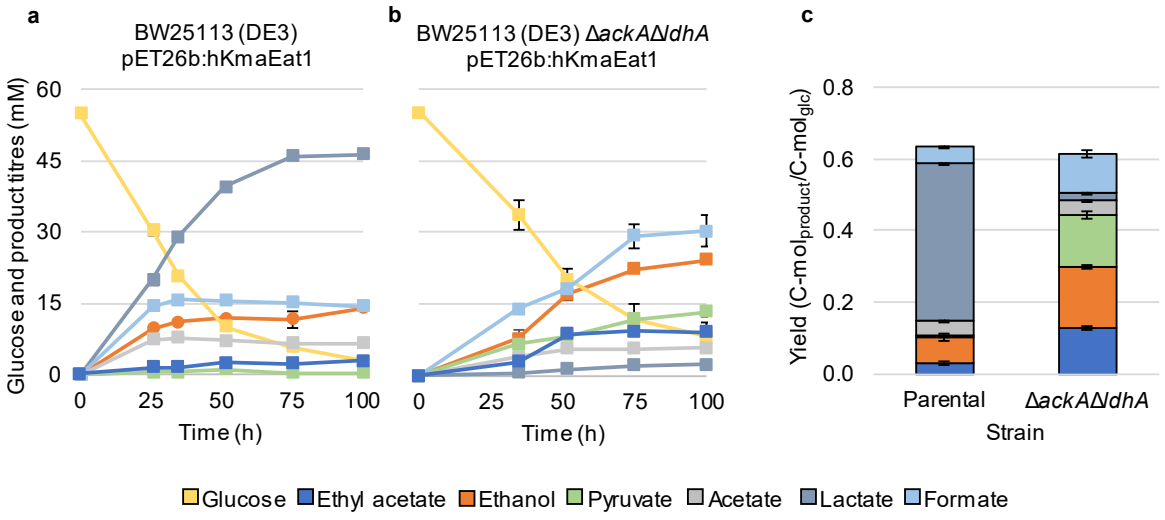

Figure 2: Anaerobic ethyl acetate production in *E. coli* BW25113 (DE3) and *E. coli* BW25113 Δ*ackA*Δ*ldhA*. a – Fermentation profile of *E. coli* BW251,13 (DE3) (pET26b:hKmaEat1). b – Fermentation profile of *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) (pET26b:hKmaEat1). c – Product yields during anaerobic ethyl acetate production. Experiments were performed as biological duplicates. Strains were grown in modified M9 medium under anaerobic conditions. Cultures were expressing the Kma *eat1* from pET26b:hKmaEat1. Gene expression was induced with 0.05 mM IPTG. Error bars represent the standard deviation. Ethyl acetate concentration in the headspace, CO_2 and other gases were not measured.

Pyruvate accumulation indicates that the carbon flux leading to pyruvate was faster than the carbon flux leading to ethyl acetate production. Such imbalances in the

metabolic pathways occur when one or more enzymatic steps are slower compared to the rest of the pathway. In *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) (pET26b:hKmaEat1), the flux from pyruvate towards ethyl acetate can be limited by Pfl, AdhE, Eat1, or a combination thereof (Figure 1c). The increased pyruvate yields were accompanied by increased ethanol yields. This was expected as ethanol had become the main route of maintaining the cellular redox balance. Since pyruvate was excreted instead of converted to acetyl-CoA, the ethanol could not be used to form ethyl acetate. Instead, ethanol was secreted. The accumulation of ethanol indicates that AdhE is not the limiting step in ethyl acetate formation. On the other hand, the activities of Pfl or Kma Eat1 may have limited ethyl acetate synthesis in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3). The ethyl acetate titre reached its maximum after 50 hours, whereas ethanol and pyruvate concentrations continued to rise until the end of the fermentation. Since ethanol production depends on the activity of Pfl, the activity of this enzyme likely did not limit ethyl acetate production either. This indicated that Eat1 was the bottleneck in the pathway. Suboptimal function of heterologous genes within a novel metabolic network is a common problem of metabolic engineering. We therefore focused on optimising the activity of the AAT step, which forms ethyl acetate in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3).

Selection of ethyl acetate-producing AAT and gene expression optimisation

A simple and effective way of relieving a metabolic bottleneck is by selecting the best catalyst and optimising its gene expression levels. We compared the ethyl acetate-production capacity of the *S. cerevisiae atf1* (Sce atf1), the Kma *eat1* and the *W. anomalus eat1* (Wan *eat1*) genes in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) under anaerobic conditions. The genes were placed under the control of the inducible LacI/*T7* (Figure 3abc) or XylS/*Pm* promoter (Figure 3def) to allow modulation of their expression levels. To induce gene expression, IPTG or m-toluate, respectively were added at various concentrations.

The genes, promoters and induction levels strongly influenced the final yield of ethyl acetate in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3). Strains expressing Wan *eat1* showed the highest ethyl acetate yields compared to the other AAT genes controlled by the

same promoter (Figure 3be). High yields of ethyl acetate were also reached by strains expressing Kma *eat1* under control of the lac-*T7* promoter (Figure 3a). Surprisingly, strains expressing Kma *eat1* under the XylS/*Pm* promoter produced only traces of ethyl acetate under all induction levels (Figure 3d). The Sce *atf1* also evoked ethyl acetate production, although the yields were significantly lower compared to the two *eat1* genes (Figure 3cf). The strains expressing *atf1* instead reached high yields of acetate (Figure 3cf), which was unexpected. This indicated that Eat1 homologs are better catalysts than the Sce Atf1 for *in vivo* ethyl acetate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) under the tested conditions.

The expression of the 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 lower IPTG concentrations to reach the same or higher ethyl acetate yield than Kma *eat1* (Figure 3ab). 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 3e), while Kma *eat1* produced almost no ethyl acetate (Figure 3d). 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 indicate that the Wan Eat1 may be more active than its *K. marxianus* homolog under these cultivation conditions. To test this, we purified the two Eat1 proteins and tested their activity *in vitro*. It was previously shown that Eat1 can function as an esterase when no ethanol is present. I*n vitro* AAT assays are laborious and require high concentrations of purified protein (Kruis et al., 2017). In contrast, *in vitro* esterase activity can readily be measured spectrophotometrically by following the hydrolysis of 1-naphthylacetate (1-NA) (He, 2003). At 30 °C, the specific 1-NA hydrolysis activity of the Wan Eat1 was 2-fold higher compared to the Kma Eat1. This may partially explain why strains producing Wan Eat1 achieved higher ethyl acetate yields *in vivo*.

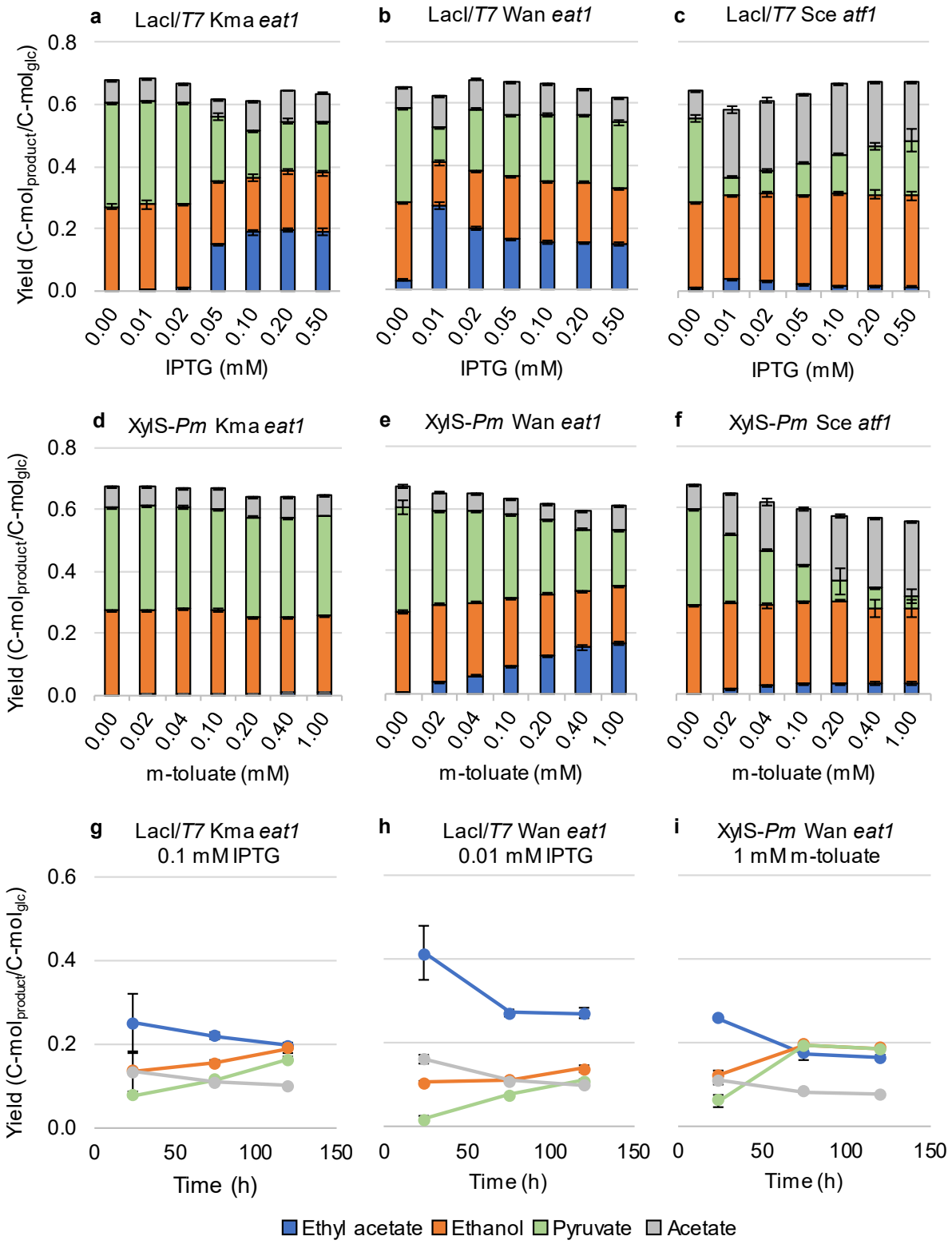

Figure 3: Comparison of three ethyl acetate producing AAT genes under various gene expression levels. (a, b, c) – Fermentation product yields of strains expressing Kma *eat1,* the Wan *eat1* and the Sce *atf1*, respectively, under the control of the LacI/*T7* promoter after 120 hours of cultivation. 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 hours of cultivation.

(g, h, i) – The changes in product yields for three selected conditions. Gene expression was induced with 0.02-1 mM m-toluate. Strains were grown under anaerobic conditions in modified M9 medium. Genes were expressed in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) from a series of pET26b plasmids. Succinate and formate were detected, but are not shown in the figure. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: Kma – *K. marxianus*, Wan – *W. anomalus,* Sce – *S. cerevisiae*

Fine-tuning the gene expression-levels increased the ethyl acetate yield, although the effect of inducer concentration depended on the gene-promoter combination. The ethyl acetate yields either increased with rising inducer concentrations (Figure 3ef), reached a plateau (Figure 3a) or even began to decline after an optimal inducer concentration (Figure 3bc). Determining the optimal inducer concentrations resulted in significantly improved ethyl acetate yields. For example, optimising IPTG concentrations used for gene induction in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) (pET26b:hKma Eat1) increased the ethyl acetate yield from 0.13 ± 0.00 (Figure 2c) to 0.19 ± 0.00 C-$mol_{ethyl\ acetate}$/C-$mol_{glucose}$ (Figure 3d). However, the highest ethyl acetate yield was achieved in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) (pET26b:hWan Eat1) 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.

Selecting the optimal AAT gene and optimising its expression level diminished the metabolic bottleneck present in ethyl acetate-producing *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3). This was reflected in increased ethyl acetate yields as well as decreased ethanol and pyruvate yields (Figure 3a-f). However, even the best tested combination of AAT gene, promoter and expression level only lessened the effects of the bottleneck. The maximum pathway yield of ethyl acetate was not reached, while pyruvate and ethanol continued to accumulate. This indicated that the activity of Eat1 may still limit ethyl acetate production. However, the ethyl acetate yield decreased by as much as 34.6 % over time, while the ethanol and pyruvate yields increased (Figure 3ghi). This means that ester synthesis stopped after a certain ethyl acetate titre was reached (Figure 2b), but glucose still continued to be metabolised to ethanol and pyruvate. This suggest that the activity of Eat1 may not have been the only factor limiting ethyl acetate synthesis.

Optimisation of the Eat1 enzyme

We recently showed that Eat1 is a mitochondrial protein in *Kluyveromyces lactis* and other bulk ethyl acetate producing yeast (Kruis et al., 2018). This indicates that a destabilising N-terminal localisation sequence may be present and may interfere with Eat1 activity in *E. coli*. To evaluate the impact of this pre-sequence on ethyl acetate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3), we focused on engineering the N-terminus of Eat1. Tools such as MitoFates (Fukasawa et al., 2015) predict the presence of such pre-sequences and the amino acid (AA) positions where they are cleaved in the native hosts. We analysed the N-termini of the Wan Eat1 and Kma Eat1 proteins. The latter contained a clear pre-sequence (probability = 0.86) and recognition sites for two mitochondrial peptidases, MPP and Icp55 at AA positions 19 and 20, respectively (Figure 4a). The shorter N-terminus of Wan Eat1 still showed the characteristics of a pre-sequence, although the prediction was not as reliable (probability = 0.50). Moreover, no clear mitochondrial peptidase motifs were detected. We therefore initially focused on optimising 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. The truncated variants are denoted by the first AA appearing after the cleavage position. However, in reality, all Kma trEat1 variants begin with methionine (M). We assessed the ethyl acetate production capacity of the Kma trEat1 versions in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) grown under anaerobic conditions. The *eat1* genes were under the control of the lacI/*T7* promoter. The optimal IPTG concentration for inducing expression of Kma *eat1* in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) was 0.1 mM (Figure 3a). However, introducing the N-terminal truncations likely affected the ethyl acetate-producing capacity of the Kma trEat1 variants. This may have also affected the optimal inducer concentrations. We therefore induced gene expression with a suboptimal 0.01 mM IPTG. At this concentration, the ethyl acetate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) can only be 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.

E. coli BW25113 Δ*ackA*Δ*ldhA* (DE3) producing Kma trEat1 Y-19 or S-20 produced the same levels of ethyl acetate as the unprocessed Kma1 (Figure 4bc). These truncated

variants lie at the predicted MPP/Icp55 cleavage sites of the Kma N-terminus. This suggests that cleaving the Kma Eat1 only at the predicted MPP/Icp55 site was not sufficient to produce the mature form of the protein. The *S. cerevisiae* Imo32, which shows some similarity to Eat1 proteins (Kruis et al., 2017) is additionally processed by the secondary mitochondrial peptidase Oct1 at a non-canonical cleavage site (Vögtle et al., 2011). Oct1 typically cleaves the N-terminus 8 AA after the MPP or Icp55 cleavage site. It is possible that Eat1 proteins contain such a cleavage site as well, although it was not predicted by MitoFates. We designed four Kma trEat1 variants, F-26, N-27, Q-28 and K-30 with cleavage sites 7-11 AA residues after the predicted MPP cleavage site (Figure 4a). All four variants significantly increased ethyl acetate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) in the presence of 0.01 mM IPTG (Figure 4bc). The strains producing the four variants all formed ethyl acetate 24 hours of cultivation, whereas no ethyl acetate was detected in the strains producing the unprocessed Kma Eat1 or most other Kma trEat1 variants (Figure 4bc). After 144 hours, most strains produced some ethyl acetate, but the performance of Kma trEat1 F-26, N-27, Q-28 and K-30 remained significantly better than the rest. The best performer was *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing Kma trEat1 K-30, which formed 11.8-fold more ethyl acetate than the unprocessed variant (Figure 4c).

Most trEat1 variants either increased ethyl acetate production, or did not affect it significantly (Figure 4bc). The exceptions were 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 versions were located in the proximity of the first conserved region that is present in all Eat1 homologs from various yeasts (Kruis et al., 2017). The loss of AAT activity in Kma trEat1 P-36 and I-37 indicated that this conserved region is critical for ethyl acetate formation by Eat1. However, it was unexpected that ethyl acetate formation was reduced in the strain producing Kma trEat1 T-15 (Figure 4bc). The reason for this is unclear, but it indicates that the N-terminus indeed influences the activity of Kma Eat1.

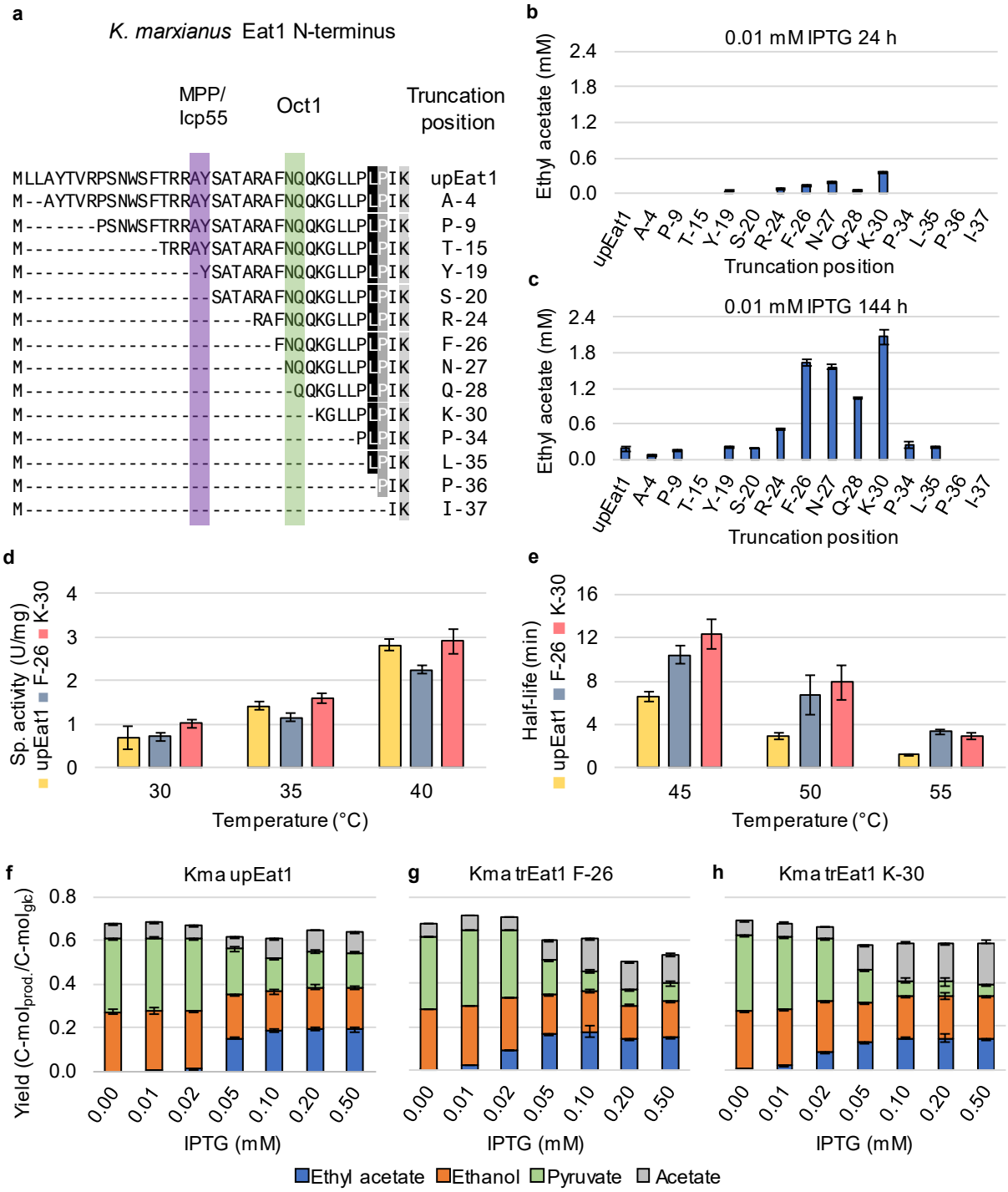

Figure 4: Improved ethyl acetate production by N-terminal truncated versions of Kma Eat1. (a) – truncated variants of the Kma Eat1 N-terminus. Predicted mitochondrial peptidase cleavage sites of MPP/Icp55 and Oct1 are indicated in purple and green, respectively. The highlighted AA residues (LP) indicate the first region that is conserved in all known Eat1 proteins. (b, c) – Ethyl acetate titres reached by cultures producing Kma trEat1 variants at low induction levels (0.01 mM IPTG) after (b) 24 hours and (c) 144 hours. (d, e) – *In vitro* specific activity and half-life, respectively 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. (f, g, h) Fermentation product yields

achieved by *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing unprocessed Kma, Kma trEat1 F-26 and Kma trEat1 K-30, respectively. Strains were grown under anaerobic conditions in modified M9 medium. Genes were expressed from a series of pET26b plasmids. Gene expression was induced with 0.01-0.50 mM IPTG. Succinate and formate were detected, but are not shown in the figure. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: Kma – *K. marxianus*, upEat1 – unprocessed Eat1, trEat1 – truncated Eat1.

The improved ethyl acetate production of e.g. Kma trEat1 F-26 and K-30 was presumably caused by changes to Eat1 on a protein level. To test this, we purified the unprocessed Kma Eat1, Kma trEat1 F-26, and Kma trEat1 K-30, and measured their initial 1-NA hydrolysis rates at 30 °C, 35 °C and 40° C. (Figure 4d). The specific esterase activities of the three proteins increased with the temperature. However, there were only small differences in their specific activities at any of the tested temperatures. 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. The half-lives of Kma trEat1 F-26 and K-30 were significantly higher at all tested temperatures compared to the unprocessed Kma Eat1 (Figure 4e, Supplementary Figure 1). Kma trEat1 F-26 and K-30 were thus more stable. This observation agrees with the biological function of the N-terminus. It facilitates mitochondrial protein import by exposing the hydrophobic AA patches of the amphipathic α-helices. These hydrophobic patches can lead to protein aggregation. Once the pre-sequence is removed, the stability and folding of the protein improves (Mossmann et al., 2012). This enhanced protein folding is likely the reason for the improved *in vivo* performance of Kma trEat1 F-26 and K-30. 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 et al., 2010). We compared the translation initiation rates with the ethyl acetate titres achieved by *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing the Kma trEat1 variants with 0.01 Mm IPTG (Figure 4c) and found no 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 a protein level.

The ethyl acetate titres and yields achieved by *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing improved Kma trEat1 variants were low due to the suboptimal IPTG concentrations. To boost ethyl acetate production, we focused on the strains producing two promising Kma trEat1 variants, F-26 and K-30 and optimised their gene expression levels (Figure 4 fgh). The optimal inducer concentration in strains producing Kma trEat1 F-26 and K-30 was 0.05 mM IPTG, which was 50% lower compared to the strain producing the unprocessed Kma Eat1. The same strains also produced more ethyl acetate at 0.01 and 0.02 mM IPTG (Figure 4gh). This indicates that less Kma trEat1 F-26 or K-30 protein is required to induce ethyl acetate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3), relative to unprocessed Kma Eat1. This again reflected the beneficial effect of removing the N-terminus from the mitochondrial Eat1 on ethyl acetate production. However, despite the improvements to the *in vivo* ethyl acetate-forming capacity of Eat1, the final ethyl acetate yields did not increase, but were in fact 10-25 % lower compared to unprocessed Kma Eat1 (Figure 4fgh). At the same time, the acetate yields increased, while the pyruvate yields decreased. Since the catalytic capacity of Eat1 was increased, this was unexpected and indicated that another bottleneck limited ethyl acetate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3).

We examined if the function of Wan Eat1 could also be improved by truncating its N-terminus and whether the same bottlenecks in ethyl acetate production would appear. Predicting the structure of the N-terminal localisation sequence of Wan Eat1 using MitoFates did not result in clearly defined protease cleavage positions. We therefore used Kma trEat1 as a guide to design two WantrEat1 variants. The truncation positions were chosen relative to the conserved region at AA positions 36 and 37 within the Kma N-terminus (Figure 4a). The modified N-terminus of Wan trEat1 contained the same number of AA before the conserved region was reached as in the Kma trEat1Q-28 and K-30 (Figure 5a). The Q-28 truncation is located at the putative Oct1 cleavage site of the Kma Eat1 N-terminus, while strains producing Kma trEat1 K-30 showed improved ethyl acetate formation capacities (Figure 4). The resulting Wan trEat1 V-11 and N-13 were produced in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3). Gene expression was induced with 0.001, 0.005 and 0.01 mM IPTG so ethyl acetate

production could be compared at optimal and suboptimal inducer concentrations. At suboptimal IPTG concentrations, the production of ethyl acetate should only be limited by activity of Wan Eat1 and its truncated variants.

The ethyl acetate formation trends in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing Wan trEat1 N-13 were similar to those observed in strains producing the Kma trEat F-26 and K-30 (Figures 4gh and 5d). At the lowest IPTG concentration, the strains producing Wan trEat1 N-13 formed 5.9-fold more ethyl acetate relative to the unprocessed Wan Eat1 (Figure 5bd). However, at higher IPTG concentrations the strains produced ethyl acetate with the same yield. Both observations reflect the behaviour of the strains producing the Kma trEat1 F-26 and K-30 variants (Figure 4gh). The acetate yield in the strain producing Wan trEat1 N-13 also increased relative to the strain producing unprocessed Wan Eat1. However, the increase in acetate production was not as pronounced as with the Kma trEat1 F-26 and K-30 (Figure 4 fgh). Producing Wan trEat1 V-11 did not affect ethyl acetate formation (Figure 5c).

Truncating the N-terminus improved the ethyl acetate-producing capacity of Kma Eat1 and Wan Eat1 proteins *in vivo*. The best performing trEat1 variants were the Kma trEat1 F-26 and K-30, and the Wan trEat1 N-13 relative to their respective unprocessed versions. Once the N-terminal sequence of the was removed, the trEat1 variants were conceivably less prone to misfolding, which was reflected in their increased *in vitro* half-lives (Figure 4de). As a result, the strains producing the beneficial trEat1 variants could achieve similar ethyl acetate yields at lower gene expression levels (Figures 4fgh, 5bcd). However, improving the *in vivo* capacity of Eat1 did not lead to higher ethyl acetate titres. The disadvantageous effect of the intact N-terminus on Eat1 could be compensated by increased gene expression levels (Figure 3). The ethyl acetate formation by Eat1 thus became catalytically more efficient, but did not improve ethyl acetate yields in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3). This strongly indicates that the AAT activity of Eat1 was optimised to the point where it no longer limited *in vivo* ethyl acetate production.

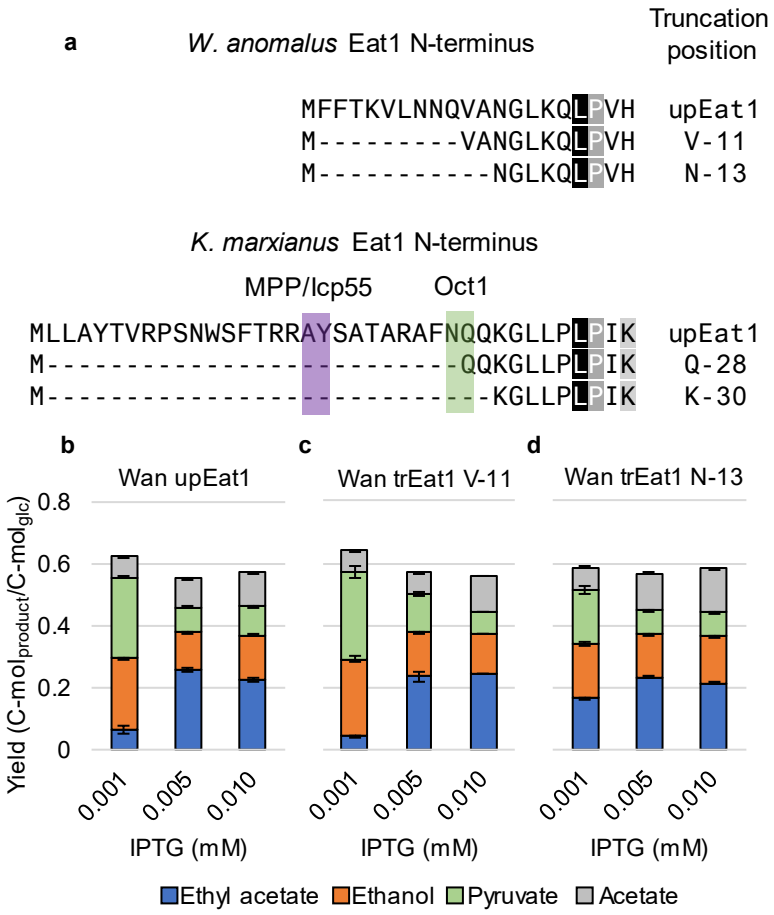

Figure 5: Improved ethyl acetate production by N-terminal truncated versions of Wan Eat1. (a) – truncated variants of the Wan Eat1 N-terminus based on the Kma trEat1 Q-28 and K-30. Predicted mitochondrial peptidase cleavage sites of MPP/Icp55 and Oct1 are indicated in purple and green, respectively. The highlighted AA residues (LP) indicate the first region that is conserved in all known Eat1 proteins. (b, c, d) – Fermentation product yields achieved by cultures producing unprocessed Wan Eat1, trEat1 V-11 and trEat1 N-13 at various induction levels after 120 hours. Strains were grown under anaerobic conditions in modified M9 medium. Genes were expressed in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) from a series of pET26b plasmids. Succinate and formate were detected, but are not shown in the figure. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: Wan – *W. anomalus*, upEat1 – unprocessed Eat1, trEat1 – truncated Eat1

E. coli BW25113 Δ*ackA*Δ*ldhA* (DE3) converts 1 mol glucose to 2 mol pyruvate. To maintain the redox balance, 1 mol pyruvate must be converted to ethanol (Figure 1c). In absence of ethyl acetate synthesis, the final products of the pathway should be 1 mol ethanol and 1 mol pyruvate. In reality, a part of the pyruvate is converted to acetate despite the disruption of *ackA* (Figures 2, 3). Introduction of Eat1 enables ethyl acetate synthesis from ethanol and acetyl-CoA. The latter is derived directly from pyruvate. Increased ethyl acetate production therefore resulted in a proportional

decrease of ethanol and pyruvate, while acetate yields were not affected significantly (Figure 3). These trends were only apparent when Eat1 activity limited ethyl acetate formation in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3). Implementing the improved trEat1 variants and optimising their gene expression levels reduced the bottleneck imposed by low Eat1 activity. As a result, the pyruvate yields decreased. Contrary to our expectations, this did not increase ethyl acetate yields. Instead it increased acetate yields while ethanol yields remained unchanged (Figures 4fgh, 5bcd). The additional flux of pyruvate to acetate may be explained by the esterase and thioesterase side activity of Eat1. It is possible that truncating the N-terminus improved not only the AAT activity of Eat1, but also its esterase and thioesterase activity.

We previously showed that ethanol concentrations control whether Wan Eat1 functions as an AAT or a hydrolase *in vitro* (Kruis et al., 2017). Here we used whole-cell assays to test whether this activity was also present *in vivo*. Washed *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) cells were incubated in in buffer containing 10 mM ethyl acetate and various ethanol concentrations. Six strains were tested that produced three Kma Eat1 and three Wan Eat1 variants (Figure 6a and 6b, respectively). A control without cells showed that approximately 50-60% of ethyl acetate was lost due to evaporation during the incubation. The actual concentration of ethyl acetate in the liquid was therefore ~4.5 mM (Kma variants, Figure 6a) and ~4 mM (Wan variants, Figure 6b).

Ethyl acetate was degraded at low ethanol concentrations in all cases. When no ethanol was added, the Wan Eat1 variants (Figure 6b) degraded more ethyl acetate compared to the Kma Eat1 variants. There was no difference between the unprocessed Wan Eat1 and its truncated versions. However, the Kma trEat1 F-26 and K-30 degraded ethyl acetate more efficiently than their unprocessed version. This may explain why acetate yields increased in the cultures producing the Kma trEat1 variants compared to the unprocessed enzyme (Figure 4 fgh). Once ethanol concentrations exceeded 15-20 mM, the ethyl acetate degradation was almost entirely repressed for both Kma and Wan Eat1 (Figure 6).

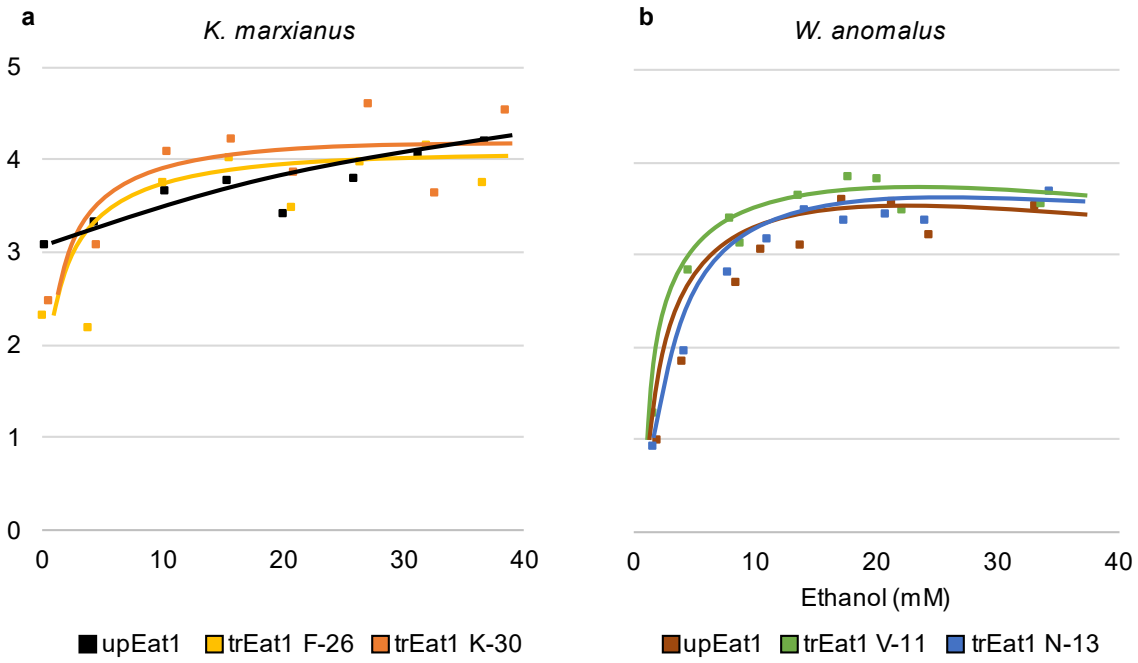

Figure 6: Inhibition of ethyl acetate hydrolysis by ethanol in cells producing Eat1 variants. The assays were performed by incubating whole cells with 4.5 mM ethyl acetate in the presence of various ethanol concentrations. (a) – *E. coli* T7 Express producing unprocessed Kma Eat1, trEat1 F-26 and trEat1 K-30. (b) – *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing the unprocessed Wan Eat1, trEat1 V-11 and trEat1 N-13. Solid lines represent the trend through the data points. Negative controls where *eat1* expression was not induced showed minimal ester degradation (not shown). Abbreviations: upEat1 – unprocessed Eat1, trEat1 – truncated Eat1.

The whole cell assays confirmed that between 15 to 20 mM ethanol must be present to prevent ester hydrolysis *in vivo* (Figure 6). The final ethanol concentrations in most batch anaerobic serum bottles were typically between 10 and 15 mM. These ethanol concentrations may not have been sufficient to fully prevent ester hydrolysis. This indicates that the esterase and thioesterase side activities of Eat1 limited ethyl acetate yields of *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing the optimal trEat1 variants in anaerobic serum bottles.

Improving ethyl acetate production by in situ product removal

Preventing ethyl acetate degradation is critical for developing efficient production processes. Ethyl acetate is a volatile ester which facilitates *in situ* product removal (ISPR) through gas stripping (Urit et al., 2011). Removing the ethyl acetate from the broth prevents it from being hydrolysed and provides a convenient way of product recovery (Löser et al., 2014). Controlled gas stripping can be achieved in fermentors. We cultivated *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing several Eat1 variants

in pH-controlled fermentors under anaerobic conditions. Ethyl acetate was stripped by a constant flow of nitrogen and measured in the off-gas. The mass flow of ethyl acetate was then calculated and related to the concentration in the liquid. Ethanol was also stripped from the fermentor. However, this process was much slower due to the lower volatility of ethanol compared to ethyl acetate at 30 °C.

The growth of *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) was dramatically improved in fermentors. This was a consequence of pH control which was insufficient when the strains were cultivated in serum bottles, despite the addition of buffering compounds. Strains cultivated in serum bottles generally never consumed more than 75 % of the glucose (Figure 2b). In fermentors, glucose consumption was both complete and faster, leading to higher product titres (Figure 7). Two of the best producers tested in batch fermentors were *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing Kma trEat1 K-30 and Wan trEat N-13. They produced 27.6 ± 2.1 and 38.2 ± 3.4 mM ethyl acetate from 55 ± 2.5 mM glucose, respectively (Figure 7). The variation in glucose concentrations were due to the glucose transferred from the M9 precultures. Gene expression was induced with the optimal IPTG concentration of each strain. Other main products of the fermentation were ethanol, acetate, pyruvate, formate, and succinate.

E. coli BW25113 Δ*ackA*Δ*ldhA* (DE3) also converted formate to CO_2 and H_2 during the anaerobic fermentation, although this conversion by Fhl (Figure 1c) was inconsistent. To promote Fhl activity, we added 0.3 µg/L Na_2SeO_3 to the medium (McDowall et al., 2014). However, the strains converted anywhere between 5 % and 100% of the formate to CO_2 and H_2. There was no correlation between the conversion efficiency, the strain, or the fermentor vessel. The reason for this inconsistency is not clear, but it did not allow accurate estimations of H_2-production rates. However, since all metabolites were measured and quantified, the carbon balances of the fermentations could still be calculated. We observed that between 87± 4% and 99 ± 2 % of the carbon could be recovered, although this calculation does not include biomass formation (Figure 8ab).

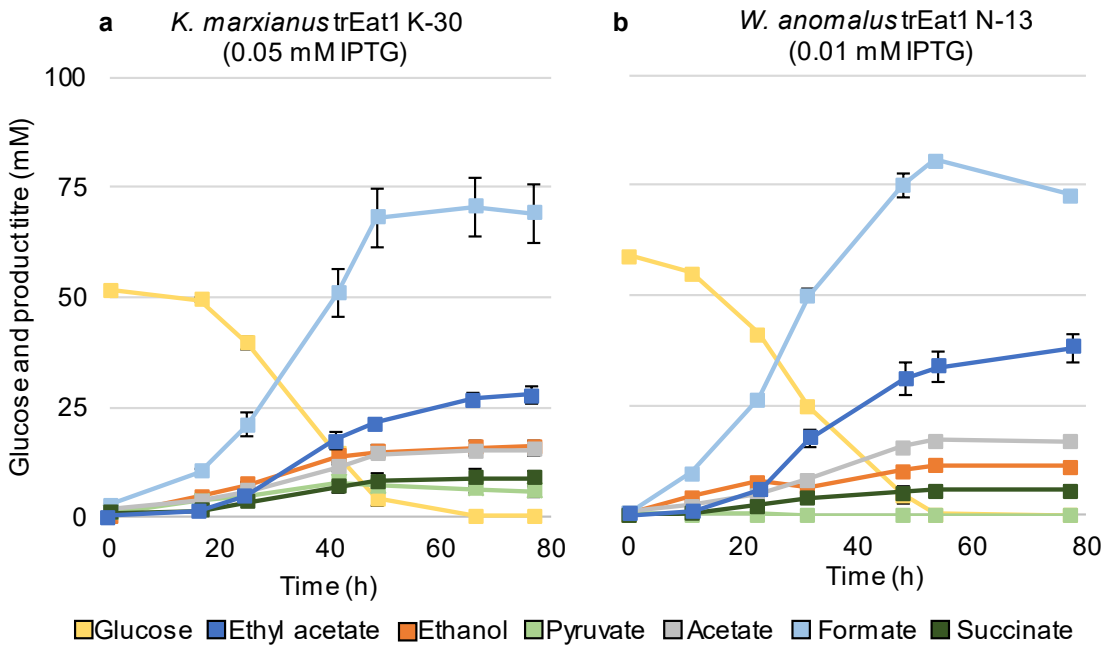

Figure 7. Ethyl acetate production in pH-controlled fermentors with continuous gas stripping. Two examples of controlled batch fermentations are shown. (a) – Fermentation profile of the strain producing Kma trEat1 K-30 in the presence of 0.05 mM IPTG. (b) Fermentation profile of the strain producing 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 Δ*ackA*Δ*ldhA* (DE3) from a series of pET26b plasmids. The mass flow of ethyl acetate removed by gas stripping was calculated and related to the ethyl acetate concentrations in the liquid. Experiments were performed as biological duplicates; error bars represent the standard deviation. Abbreviations: trEat1 – truncated Eat1.

Continuous removal prevented ester hydrolysis and increased ethyl acetate yields 1.6- to 2.8-fold compared to cultivations in serum bottles (Figures 3ab, 8ab). It also ensured that ethyl acetate was synthesised until the glucose was fully consumed. This was an improvement over cultivation in serum bottles, where ethyl acetate titres reached a plateau and yields began to decline even when glucose was still present and consumed (Figures 2b, 3ghi). *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing Wan trEat N-13 variant in the presence of 0.01 mM IPTG reached the highest ethyl acetate yield (Figures 7b, 8b). It formed 0.42 ± 0.02 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$, or 63.4 ± 3.4 % of the theoretical pathway maximum. This was a 1.6-fold improvement compared to cultivation in closed serum bottles, where ethyl acetate was not stripped (Figure 3b).

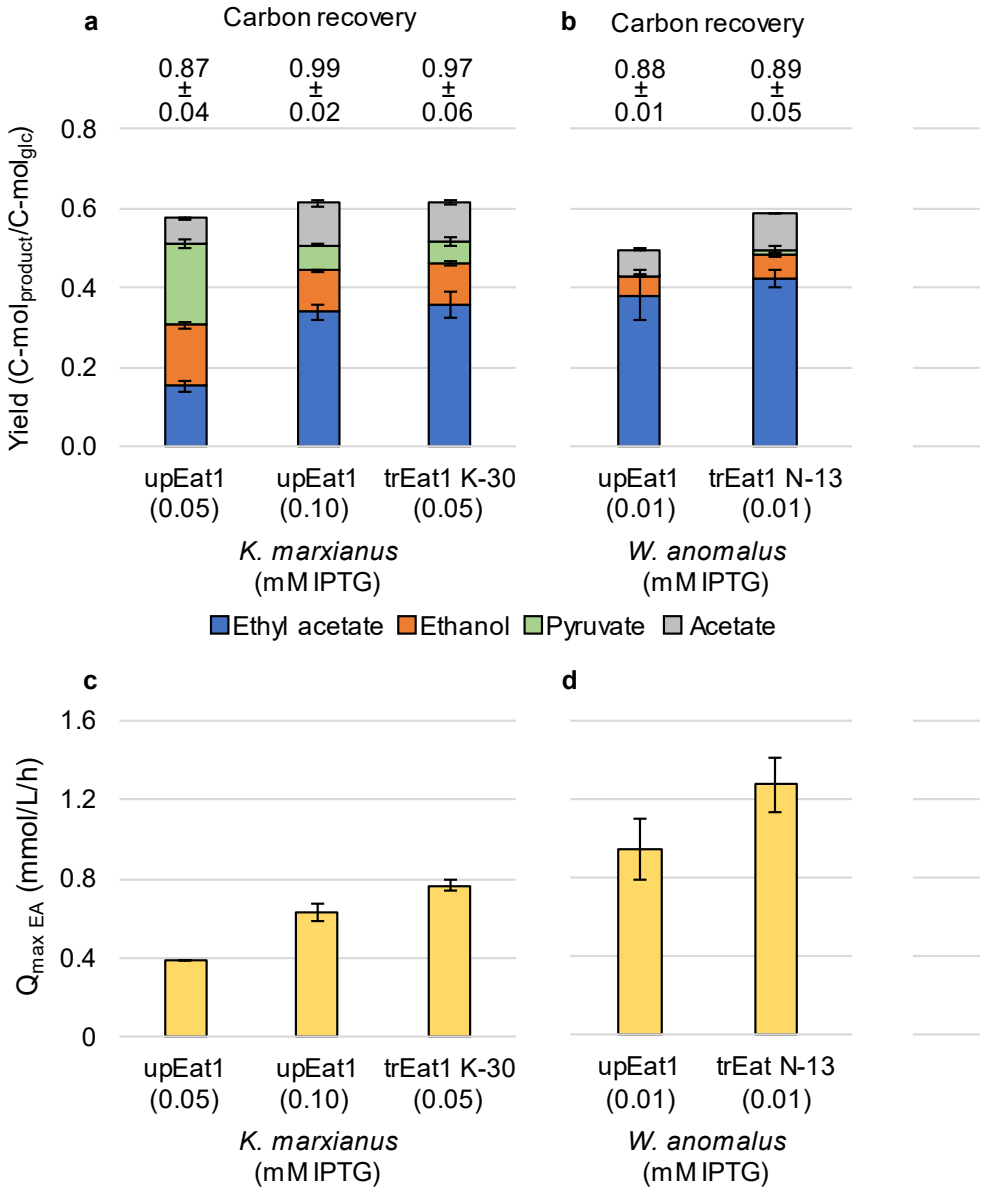

Figure 8: Effect of continuous ethyl acetate stripping on product yields and maximum productivities. (a) – Final product yields achieved by cultures producing unprocessed Kma Eat1 and trEat1 K-30 in the presence of 0.05 or 0.1 mM IPTG. The numbers above the columns represent the carbon recovery of the 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 columns represent the carbon recovery of the fermentations. (c, d) – The maximum volumetric productivities of ethyl acetate ($Q_{max\ EA}$) of 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 Δ*ackA*Δ*ldhA* (DE3) from a series of pET26b plasmids. The mass flow of ethyl acetate removed by gas stripping was calculated and related to the ethyl acetate concentrations in the liquid. Succinate and formate were detected, but are not shown in the figure. Experiments were performed as biological duplicates or triplicates; error bars represent the standard deviation. Abbreviations: upEat1 – unprocessed Eat1, trEat1 – truncated Eat1.

Other strains that were cultivated in the pH-controlled fermentors showed similar levels of improvement (Figure 8ab). The exception was *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing the unprocessed Kma Eat1 when 0.05 mM IPTG was used to induce gene expression (Figure 7a). This strain reached 0.15 ± 0.01 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$ in fermentors as well as in closed serum bottles (Figures 3a, 8a). Ethanol and pyruvate yields remained the same as well. At a concentration of 0.05 mM IPTG, the activity of the unprocessed Kma Eat1 limited ethyl acetate production in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3), and not ester hydrolysis (Figures 3a, 4f). Stripping therefore had almost no effect on the ethyl acetate yield. Sufficient IPTG concentrations were needed to boost Eat1 activity. In *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing the unprocessed Kma Eat1, this concentration was 0.1 mM IPTG. Under these conditions, the yield was again limited by ethyl acetate degradation, and in serum bottles, acetate accumulated as a result (Figure 3a). In fermentors, the continuous removal of ethyl acetate prevented this degradation. As a result, the ethyl acetate yield increased 1.9-fold from 0.19 ± 0.01 to 0.34 ± 0.02 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$. This corresponds to 51.0 ± 3.0 % of the theoretical pathway maximum.

The same yield could be achieved in strains producing Kma trEat1 K-30 in the presence of 0.05 mM IPTG (Figure 8a). Truncating the N-terminus of Kma Eat1 allowed similar ethyl acetate yields to be reached at lower gene expression levels. This reflected the trend observed in serum bottles (Figure 4). Since suboptimal functioning of unprocessed Eat1 could be compensated by increased gene expression levels, it is difficult to estimate the benefit of truncating the N-terminus of Eat1 on ethyl acetate production in *E. coli*. We observed that the strains producing trEat1 variants generally grew faster and reached the end of the fermentations sooner when cultivated in serum bottles and fermentors (data not shown). As a result, the maximum volumetric productivities of ethyl acetate ($Q_{\text{max EA}}$) were increased. The $Q_{\text{max EA}}$ of the strain producing Kma trEat K-30 (0.05 mM IPTG) was 18 % higher (p-value=0.02) compared to the strain producing unprocessed Kma Eat1 (0.1 mM IPTG) (Figure 8c). A similar trend was present in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing unprocessed Wan Eat1 and trEat1 N-13 in the presence of 0.01 mM IPTG. The $Q_{\text{max EA}}$ of the latter strain was 26

% higher (p-value=0.03) compared to the strain producing the unprocessed Wan Eat1 (Figure 8d).

The yields of ethanol and pyruvate decreased with increasing ethyl acetate yields (Figure 8ab). This effect was similar to the one observed in serum bottles (Figure 3). Strains producing the unprocessed Kma Eat1 and Kma trEat1 K-30 in the presence of optimal IPTG concentrations accumulated 66% less pyruvate (Figure 8a) compared to cultivations in serum bottles (Figure 4 gi). This could be the result of higher ethyl acetate yields, as well as an overall improvement in strain performance in pH-controlled fermentors. The accumulation of pyruvate suggests that the activity of the Kma Eat1 may still be insufficient and that ethyl acetate production could have been higher. The pyruvate yields in the strains producing unprocessed Wan Eat1 and Wan trEat1 N-13 returned to the levels observed in the parental *E. coli* BW25113 (DE3) strain (Figures 3c, 7b, 8b). The ethyl acetate yield 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=0.04) was only found for the strain producing Wan trEat1 N-13 (Figure 8ab). This strain converted approximately 75% of pyruvate to ethyl acetate, while the remainder was distributed between ethanol and acetate. The lack of pyruvate in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing unprocessed Wan Eat1 and Wan trEat1 N-13 indicates that the AAT activity did not limit ethyl acetate formation in these strains.

Discussion

We engineered *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) to produce ethyl acetate as the main fermentative product under anaerobic conditions. The maximum theoretical pathway yield of ethyl acetate in this strain is 0.67 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$. The initial introduction of the Kma *eat1* gene in *E. coli* BW25113 (DE3) resulted in only 0.03 ± 0.00 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$ or 4.5 ± 0.0 % of the pathway maximum. This yield was then increased in four optimisation rounds. The metabolic pathway towards ethyl acetate was streamlined by disrupting lactate and acetate production. This did result in increased ester production, but pyruvate and ethanol accumulated at the cost of ethyl acetate formation. It was anticipated that accumulation of these by-products

was caused by insufficient AAT activity. To remove this bottleneck, we compared the *in vivo* catalytic capabilities of three AAT enzymes after optimisation of the AAT gene expression levels. This partially relieved the bottleneck and resulted in a 9-fold increased product yield to 0.27 ± 0.01 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$. To further improve the function of the enzyme, we removed the destabilising N-terminus from the two mitochondrial Eat1 proteins (Kruis et al., 2018). The resulting trEat1 variants were as much as 11.8-fold more efficient at forming ethyl acetate *in vivo.* However, they were not able to further increase the *in vivo* ethyl acetate yields. Ethanol concentrations in serum bottles were too low to fully repress the hydrolytic activities of Eat1, which also contributed to limited ethyl acetate production. To prevent this unwanted hydrolysis, we removed the ethyl acetate from the fermentation broth by continuous gas stripping. This prevented the degradation of ethyl acetate and led to improved product yields. The combined effect of the optimisation efforts resulted in a 14.3-fold increase in ethyl acetate yield. The highest ethyl acetate yield was achieved by *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing Wan trEat1 N-13 in the presence of 0.01 mM IPTG. This strain formed 0.42 ± 0.02 C-mol$_{\text{ethyl acetate}}$/C-mol$_{\text{glucose}}$, which corresponds to ~ 63 % of the theoretical pathway maximum. This was more than the highest ethyl acetate yield reported in *K. marxianus.* This yeast converted whey sugars (predominantly lactose) to ethyl acetate under aerobic conditions and reached 56% of the theoretical pathway maximum (Urit et al., 2013a).

Formate was an obligatory by-product of the fermentation. It was needed to maintain the cellular redox balance during the anaerobic conversion of 1 mol glucose to 1 mol ethyl acetate. A build-up of high formate levels could be detrimental to cell growth and function (Warnecke and Gill, 2005). This accumulation can be prevented by converting formate to CO_2 and H_2 via the formate-hydrogen lyase (Fhl) complex. This would allow the co-production of ethyl acetate and H_2, which is a valuable biofuel (Brentner et al., 2010). In our study, the formate conversion varied between 5% and 100%, with no correlation with the strain or fermentor vessel. The reason for this not clear, but it may be due to the complex transcriptional regulation of the 15 genes required to form an active Fhl complex (Birkmann et al., 1987; Rossmann et al., 1991; Zinoni et al., 1984). The issue can be prevented in the future by constitutively

overexpressing *fhlA*, the transcriptional activator of Fhl system (Rossmann et al., 1991; Yukawa et al., 2007).

Other dissimilatory products of the here described fermentations included succinate, ethanol, acetate, and in some cases pyruvate. To increase ethyl acetate yields, their formation should be minimised. Succinate production can be prevented by disrupting the fumarate reductase gene (*frd*). Residual ethanol and acetate at the end of the batch fermentations were likely the result of the aforementioned hydrolytic side activities of Eat1. This AAT can only produce ethyl acetate efficiently above a critical ethanol concentration. Until then, there was no net ethyl acetate synthesis and ethanol and acetate were produced instead. Once the ethyl acetate synthesis started, it continued until the glucose was consumed. Since the fermentations were performed under anaerobic conditions, the ethanol and acetate could not be assimilated for additional ethyl acetate formation. Instead, they remained as by-products of the fermentation. The acetate could also be a product of the native *E. coli* metabolism. 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 *ackA*. When we disrupted *ackA*, the acetate formation was reduced, but not eliminated. It cannot be ruled out that other enzymes with similar catalytic activities, such as propionate kinase are able to perform the same reaction (Heßlinger et al., 1998). Another explanation could be that the gas stripping rate in fermentors was not sufficient to completely prevent ethyl acetate hydrolysis. In some cases, pyruvate accumulated as well, which suggests that the activity of Eat1 could still be improved. Further optimisation of the pathway and process will likely lead to higher ethyl acetate yields.

The three tested AATs varied significantly in their capacity to produce ethyl acetate under anaerobic conditions. Strains producing Sce Atf1 formed the least ethyl acetate. The expression of Sce *atf1* unexpectedly evoked acetate production, which may be related to its thioesterase activity (Nancolas et al., 2017). This activity of Sce Atf1 has not been investigated *in vivo*. It is not known if ethanol inhibits the hydrolytic activity of Sce Atf1 in the same way as was demonstrated for Eat1 (Kruis et al., 2017). The thioesterase activity may explain the low ethyl acetate titres in *E. coli* producing Sce

Atf1. On the other hand, Sce Atf1 enabled isobutyl acetate production at 80 % of the pathway maximum (Rodriguez et al., 2014). This indicates that it can be an effective AAT in *E. coli*. The inefficient ethyl acetate production by Sce Atf1 may therefore be caused by differences in substrate specificities. Of the two tested Eat1 enzymes, Wan Eat1 and its truncated variants were most efficient. The strains producing Wan Eat1 variants consistently formed 10-15% more ethyl acetate *in vivo* compared to strains producing Kma Eat1. The higher specific activity of Wan Eat1 at 30 °C may explain why it performed better *in vivo*. The yeast *K. marxianus* produced ethyl acetate more efficiently at 42 °C compared to lower temperatures (Urit et al., 2013a). It is therefore possible that Kma Eat1 was less efficient in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) due to suboptimal cultivation temperatures.

The *in vivo* function of the mitochondrial Eat1 proteins could be improved by removing the destabilising N-termini of the proteins. In yeast they 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). In *E. coli* the truncations of Eat1 were introduced manually since bacteria cannot perform the required cleavage. Predicting the processing events and cleavage sites *in silico* can be challenging as the primary sequences and lengths of mitochondrial localisation tags are poorly conserved (Mossmann et al., 2012). Cleavage events were only predicted within the N-terminus of Kma Eat1. The strains producing Kma trEat1 variants truncated at those positions (Kma trEat1 Y-19 and S-20) did not show improved ethyl acetate production relative to the unprocessed Kma Eat1. The ethyl acetate production improved only 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 (Kruis et al., 2017). This may suggest that similar events occur during the processing of Eat1 in *K. marxianus*. However, it could also be a coincidence. It is likely that Eat1 homologs from other yeasts undergo different processing events. For example, the N-terminus of Wan Eat1 had no predicted cleavage sites and is roughly half the length of its *K. marxianus* counterpart. Confirming the true final forms of Eat1 proteins is only possible in the native yeasts. While this may

be laborious, it is tempting to speculate that it would help identify the most functional trEat1 variants.

The improved activity of the trEat1 variants enabled ethyl acetate synthesis at lower IPTG concentrations. This was presumably linked to lower gene expression and protein production levels. The suboptimal performance of the unprocessed Eat1 enzymes could be compensated by higher IPTG concentrations. As a result, the strains in *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing the unprocessed Eat1 or the truncated trEat1 variants reached the same ethyl acetate yields at their respective optimal IPTG concentrations. However, the $Q_{max\ EA}$ was 18 % - 26 % higher in strains producing the trEat1 versions. The improvement was likely the result of a reduced metabolic burden due to lower IPTG concentrations (Malakar and Venkatesh, 2012). It is also possible that the native Eat1 variants aggregate more easily due to the presence of the destabilising N-terminal sequence. This may negatively impact the growth of *E. coli,* resulting in lower $Q_{max\ EA}$. The improved activity of the trEat1 variants can help to develop ethyl acetate production on a large scale. In this study, the *eat1* genes and their variants were expressed from multi-copy plasmids under the control of inducible promoters. The system therefore relied on the addition of antibiotics and external inducers for ethyl acetate synthesis. The addition of these compounds is too expensive on a large scale. Moreover, plasmid maintenance presents an additional metabolic burden to the cell (Diaz Ricci and Hernández, 2000). In an industrial production strain, the *eat1* gene would ideally be integrated at a suitable site on the host genome. As the gene copy number would consequently be much lower compared to plasmid-borne expression systems, the production of improved trEat1 variants would make it more likely that sufficient amounts of active enzyme are present to efficiently catalyse ethyl acetate production.

In this study we demonstrated that *E. coli* can be engineered to efficiently convert glucose to ethyl acetate as the primary fermentative product. The high yields were achieved after several rounds of metabolic, protein and process engineering. These results may serve as a point of reference for the future development of biobased ethyl acetate-production processes in which Eat1 serves as the AAT catalyst.

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. The pET26b-XylS/*Pm* plasmids were obtained by replacing the lacI/*T7* promoter with the XylS/*Pm* promoter (Balzer et al., 2013). 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 circularised 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 Δ*ackA*Δ*ldhA*	Disruption of lactate and acetate production (via *ackA*)	This study
Escherichia coli T7 Express	fhuA2 [lon] ompT gal (λ DE3) [dcm] Δ hsdS λ DE3 = λ sBamHIo Δ EcoRI-B int::(LacI::PlacUV5::T7 gene1) i21 Δ nin5	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 harmonised *eat1* from *Wickerhamomyces anomalus* DSM 6766	Kruis
pET26b:hKmaEat1	LacI/*T7*	Codon harmonised *eat1* from *Kluyveromyces marxianus* DSM 5422	This study
pET26b:opSceAtf1	LacI/*T7*	Codon harmonised atf1 from *Saccharomyces cerevisiae* (Rodriguez et al., 2014)	This study
pET26b: XylS/*Pm* -hWanEat1	XylS/*Pm*	Codon harmonised *eat1* from *Wickerhamomyces anomalus* DSM 6766	This study
pET26b: XylS/*Pm* hKmaEat1	XylS/*Pm*	Codon harmonised *eat1* from *Kluyveromyces marxianus* DSM 5422	This study
pET26b: XylS/*Pm* opSceAtf1	XylS/*Pm*	Codon optimised atf1 from *Saccharomyces cerevisiae*	This study
pET26b:hKma-trEat1-A4	LacI/*T7*	Kma Eat1 truncated at A-4	This study
pET26b:hKma-trEat1-P9	LacI/*T7*	Kma Eat1 truncated at P-9	This study
pET26b:hKma-trEat1-T15	LacI/*T7*	Kma Eat1 truncated at T-15	This study
pET26b:hKma-trEat1-Y19	LacI/*T7*	Kma Eat1 truncated at Y-19	This study
pET26b:hKma-trEat1-S20	LacI/*T7*	Kma Eat1 truncated at S-20	This study
pET26b:hKma-trEat1-R24	LacI/*T7*	Kma Eat1 truncated at R-24	This study
pET26b:hKma-trEat1-F26	LacI/*T7*	Kma Eat1 truncated at F-26	This study
pET26b:hKma-trEat1-N27	LacI/*T7*	Kma Eat1 truncated at -N27	This study
pET26b:hKma-trEat1-Q28	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 V-11	LacI/*T7*	Wan Eat1 truncated at V-11	
pET26b:hWan-trEat1 N-13	LacI/*T7*	Wan Eat1 truncated at N-13	
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, 1992. The serum bottles were made anaerobic by flushing with nitrogen. 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) or m-toluate (0.02-1 mM) were added to induce gene expression. 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 (300 μg/L). 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 3M KOH and 0.5 M H2SO4. 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 fermentors were inoculated to an initial OD_{600} of 0.4. Metabolites in the liquid phase were measured by high pressure liquid chromatography (HPLC) and gas chromatography coupled to

a flame ionisation 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™).

In vivo *ethyl acetate degradation by cells producing Eat1*

E. coli cells producing various *W. anomalus* and *K. marxianus* Eat1 variants were incubated in sealed 10 mL glass vials containing 2 mL potassium phosphate buffer (50 mM, pH 7.5), NaCl (150 mM), ethyl acetate (10 mM) and various ethanol concentrations (0-40 mM). Washed cells were added to a final concentration of 1 OD unit/mL (strains producing *W. anomalus* Eat1 variants) or 2 OD units/mL (strains producing *K. marxianus* Eat1 variants). The mixture was incubated for 1.5 h at 30 °C. The residual ethyl acetate was measured in the liquid. The cell suspensions were prepared by inoculating 0.5 mL of a fresh LB preculture to 50 mL modified M9 medium in a 250 mL Erlenmeyer flask. The culture was grown overnight aerobically at 20°C and 250 rpm. The next day, the cells were washed once with potassium phosphate buffer (50 mM, pH 7.5) containing NaCl (150 mM) and used immediately in the assay.

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 $\mu g mL^{-1}$ 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 hours 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 30s 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 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 minutes. 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 was measured spectrophotometrically by monitoring the release of 1-naphtol 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 minute. Measurements were performed as technical triplicates.

Calculations

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

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

With Pr representing the products (ethyl acetate, CO_2 and H_2) $X_{Pr,\,gas}$ the volumetric fraction of product in the gas (-), P_{atm} the atmospheric pressure (Pa), R the ideal gas

constant (m^3 Pa mol^{-1} K^{-1}) and T the temperature (K). The amount of product ($m_{Pr,gas}$, mol) stripped up to each time point (t_n, h) was calculated using Eq. 2

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

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

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

The mass flow of ethyl acetate in the off-gas was related to liquid concentrations and added to the ethyl acetate concentrations in the liquid at corresponding time points.

Enzyme half-life was defined as the time required for the fraction of active enzyme to halve at a given temperature and was determined according to Eq. 4.

$$t_{0.5} = \frac{ln(0.5)}{k_i} \quad \text{(Eq. 4)}$$

Where $t_{0.5}$ represents the enzyme half-life and k_i the rate constant of inactivation. The k_i was determined as the slope obtained by plotting the natural log of residual activity against the time of heat exposure (Supplementary Figure 1).

Carbon balance calculation

Carbon balances were calculated according to Eq. 5.

$$C_{balance} = \frac{\text{C-mol products formed}}{\text{C-mol glucose consumed}} \quad \text{(Eq. 5)}$$

The compounds included in the calculation were glucose, ethyl acetate, ethanol, acetate, succinate, pyruvate, formate and CO_2. Biomass formation was not included in the calculation.

Bioinformatics

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 analysed by high-pressure 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 210nm, and an Agilent 1260 Infinity RI detector operated at 45 °C. Either an Aminex HPX-87H (Bio-Rad) or a 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 0.8mL/min and 60 °C. A final conentration of 50 mM propionic acid 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 analysed 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 minutes and increased to 200°C at a rate of 50 °C/minute. The split ratio was 10. A final concentration of 2 mM 1-butanol was used as internal standard.

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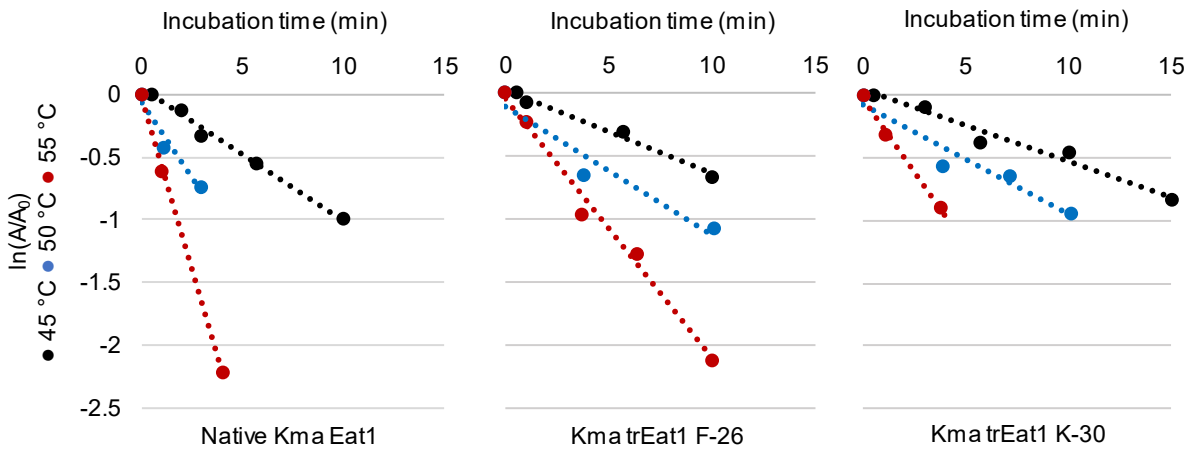
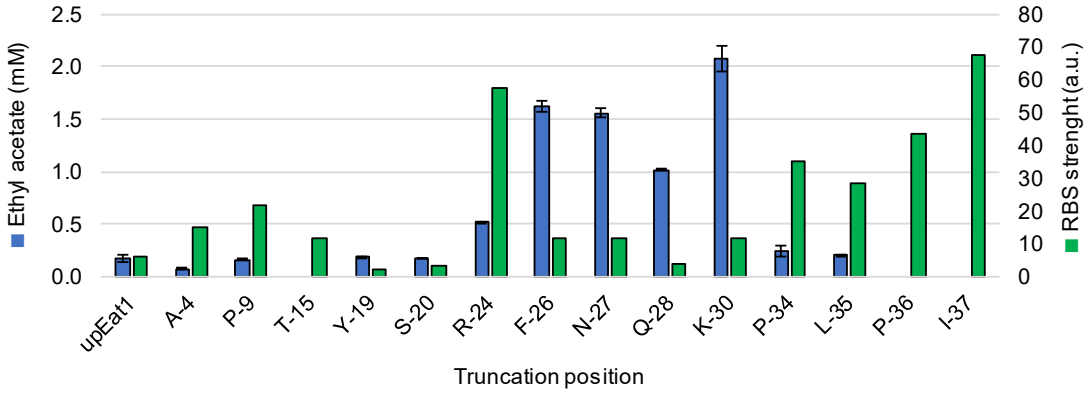
Supplementary Material



Supplementary Figure 1: Thermal inactivation kinetics used to determine the inactivation constants (k_i) of three *K. marxianus* Eat1 variants at 45 °C, 50 °C and 55 °C.



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 titres shown were obtained from *E. coli* BW25113 Δ*ackA*Δ*ldhA* (DE3) producing *K. marxianus* trEat1 variants at 0.01 mM IPTG concentration (Figure 4c). The translation initiation rates of the RBS were predicted with the RBS calculator (Salis et al., 2010).

CHAPTER 5

Contribution of Eat1 and other alcohol acyltransferases to ester production in *Saccharomyces cerevisiae*

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Abstract

Esters are essential for the flavour and aroma of fermented products, and are mainly produced by alcohol acyl transferases (AATs). A recently discovered AAT family named Eat (Ethanol acetyltransferase) contributes to ethyl acetate synthesis in yeast. However, its effect on the synthesis of other esters is unknown. In this study, the role of the Eat family in ester synthesis was compared to that of other *S. cerevisiae* AATs (Atf1, Atf2, Eht1, and Eeb1) *in silico* and *in vivo*. A genomic study in a collection of industrial *S. cerevisiae* strains showed that variation of the primary sequence of the AATs did not correlate with ester production. Fifteen members of the *eat* family from nine yeast species were overexpressed in *S. cerevisiae* CEN.PK2-1D and were able to increase the production of acetate and propanoate esters. The role of Eat1 was then studied in more detail in *S. cerevisiae* CEN.PK2-1D by deleting *eat1* in various combinations with other known *S. cerevisiae* AATs. Between 6 and 11 esters were produced under three cultivation conditions. Contrary to our expectations, a strain where all known AATs were disrupted could still produce e.g. ethyl acetate and isoamyl acetate. This study has expanded our understanding of ester synthesis in yeast but also showed that some unknown ester-producing mechanisms still exist.

Keywords: Eat1; alcohol acyltransferase; AAT; ester; yeast; *Saccharomyces cerevisiae*

Introduction

Volatile esters are produced by yeasts during fermentation and are key contributors to the flavour of fermented food products (Swiegers et al., 2005; Verstrepen et al., 2003a). Esters are present at concentrations around the human detection limit. Consequently, even small changes in ester concentrations can lead to profound changes in the beer and wine bouquets (Saerens et al., 2010). Esters generally add a fresh fruity aroma to beverages. However, in excessive amounts they can also lead to undesirable off-flavours (Liu et al., 2004). A well-balanced volatile ester profile is therefore key for palatable fermented products. The amounts and variety of esters produced depends highly on the strain of *S. cerevisiae* used for fermentation (Gallone et al., 2016). The main contributors to product flavour are acetate esters, including ethyl acetate (sweet aroma), isoamyl acetate (banana) and phenylethyl acetate (rose, flowery aroma) (Dzialo et al., 2017; Verstrepen et al., 2003b). The second major group of esters are medium chain fatty acid (MCFA) ethyl esters, like ethyl octanoate (sour apple) and ethyl hexanoate (apple, anise) (Knight et al., 2014).

Esters in *Saccharomyces cerevisiae* are produced by alcohol acyltransferases (AATs), which couple an acyl-CoA with an alcohol, producing esters and free CoA. MCFA ethyl esters are produced by the paralog pair Eht1 and Eeb1, which couple an MCFA-CoA with ethanol. A Δ*eeb1* strain produced less ethyl butanoate, ethyl hexanoate, ethyl octanoate and ethyl decanoate. The deletion of *eht1* only reduced ethyl hexanoate and ethyl octanoate levels. Overexpression of *eht1* or *eeb1* did not increase ester production (Saerens et al., 2006). Acetate esters in *S. cerevisiae* are produced by Atf1 and its paralog Atf2 (Fujii et al., 1996; Nagasawa et al., 1998). Overexpression of *atf1* and *atf2* resulted in increased acetate ester production (Lilly et al., 2006; Verstrepen et al., 2003b). A Δ*atf1*Δ*atf2* deletion strain produced approximately 50% less ethyl acetate and 80% less isoamyl acetate, as well as significantly lower amounts of other acetate esters (Verstrepen et al., 2003b). However, the production of acetate esters was not entirely abolished. MCFA ethyl esters were not significantly affected by the deletion of *atf1* and *atf2.*

Recently, a new family of ethyl acetate producing AATs was discovered in yeasts which was named Eat (Kruis et al., 2017). These AATs are the main enzymes responsible for bulk ethyl acetate production in yeasts such as *Wickerhamomyces anomalus, Kluyveromyces marxianus* and *Cyberlindnera fabianii*. Two putative homologs of *eat* were identified in *S. cerevisiae*, *ygr015c* (now renamed *eat1*) and *ygr031w*. The latter was named *imo32* in a previous study (Vögtle et al., 2011). Overexpression of *eat1* increased ethyl acetate production in *S. cerevisiae*, while overexpression of *imo32* had no apparent effect (Kruis et al., 2017). Previous studies showed that the activities of Atf1, Atf2, Eht1 and Eeb1 did not account for all the ester production observed in *S. cerevisiae* (Dzialo et al., 2017). When *eat1* was deleted in *S. cerevisiae*, ethyl acetate production was reduced by approximately 50 % (Kruis et al., 2017). However, the effect of Eat1 on the production of esters other than ethyl acetate, and the effect of the combined deletions of all ester-forming AATs have not been reported yet.

This study focused on the effect of Eat1 and other AATs on ester production in *S. cerevisiae*. We performed a genomic analysis of all six AATs in a collection of industrial *S. cerevisiae* strains with known ester production profiles. We then studied the ester-forming capacities of 15 members of the *eat* family by overexpressing them in *S. cerevisiae*. Next, we determined the contribution of *atf1, atf2, eht1, eeb1, imo32* and *eat1* on ester production in *S. cerevisiae* using a series of overexpression and deletion strains, including a strain lacking all six AAT-encoding genes.

Results

Genomic analysis of AATs in industrial S. cerevisiae strains

Ester production profiles of diverse *S. cerevisiae* strains used in beer brewing, wine making and other industrial processes vary significantly. Gallone *et al.* (2016) previously reported the detection of ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate production by 157 industrial yeast strains with known genome sequences. The total amount of esters produced by the strains varied from 7 mg/L to 57 mg/L. Ethyl acetate was the most abundant ester (88.0-98.5 % of the esters measured), followed by isoamyl acetate (0.9-10.0 % of the esters measured). Ethyl hexanoate and ethyl octanoate constituted between 0.1 and 3.0 % of the total ester

produced by the strain collection. We investigated whether this large variability can be explained by differences at the genomic level. We compared the sequence diversity and copy number variation (CNV) of *atf1, atf2, eht1, eeb1, imo32* and *eat1.* All six genes were present in all strains of the yeast collection. The conservation of the translated nucleotide sequences was measured using Shannon entropy (Shannon, 1948). This method calculates the variability of each amino acid position in the sequence alignment. The average Shannon entropy of the protein sequences showed that Atf2 was the most variable while Atf1 was the most conserved AAT (Figure 1), although all AATs were generally highly conserved. We sorted the ester production profiles of the yeasts according to the phylogenetic distribution of each individual AAT. Even upon visual inspection it became clear that there was little to no correlation between the primary sequence variation of the AATs and the ester production profiles (Supplementary Figure 1).

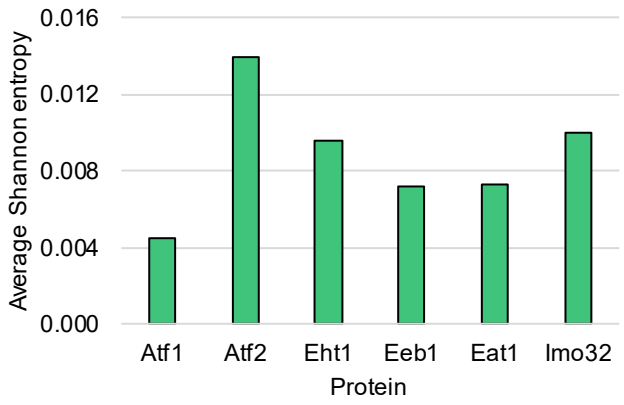


Figure 1: Conservation of the six AAT protein sequences. The average Shannon entropy was calculated as the mean of Shannon entropies at individual amino acid positions. Higher Shannon entropy indicates higher conservation.

A typical characteristic of industrial *S. cerevisiae* strains is their high variability in chromosome content (ploidy). These strains often exhibit abnormal numbers of chromosomes relative to their expected ploidy (aneuploidy), or several small and local changes in CNV, such as duplications and deletions (Gallone et al., 2016). Based on the estimated ploidy levels and copy number profiles reported by Gallone *et al.* (2016), we examined the copy number levels of each AAT gene. Generally, copy number changes of the AAT genes were associated with duplications and deletions involving

full chromosomes or large chromosomal fragments, relative to the rest of the genome. The comparison of the copy number changes of each AAT with their ester production profiles again showed no correlation with either the total ester amount produced, or with the ratios between the four measured esters (data not shown). The results of the *in silico* analysis suggest that the dramatic differences in ester production in the yeast collection cannot be explained by the variation of the coding regions of the AATs on a genomic level.

Overexpression of the eat family increases acetate and propanoate ester production

We determined the effect of *atf1, atf2, eht1, eeb1, imo32* and *eat1* overexpression on ester production in *S. cerevisiae* CEN.PK2-1D. The parental strain produced five different acetate esters (ethyl-, propyl-, isobutyl-, isoamyl-, and phenylethyl acetate), and two propanoate esters (ethyl- and isoamyl propanoate). These esters were produced from acetyl-CoA or propionyl-CoA, and a range of (fusel) alcohols formed innately by *S. cerevisiae* CEN.PK2-1D. The strain also produced four ethyl-MCFA esters (ethyl -hexanoate, -octanoate, -decenoate and -decanoate). Overexpression of *atf1* resulted in a broad increase in acetate ester production. In contrast, overexpression of *atf2*, *eht1* and *eeb1* did not show a significant effect on ester production (Figure 2A). These results agree with previous observations (Saerens et al., 2008; Verstrepen et al., 2003b). The strain overexpressing *eat1* produced more acetate esters and ethyl propanoate but had no effect on MCFA production. The overexpression of *imo32* had no effect on ester production (Figure 2A).

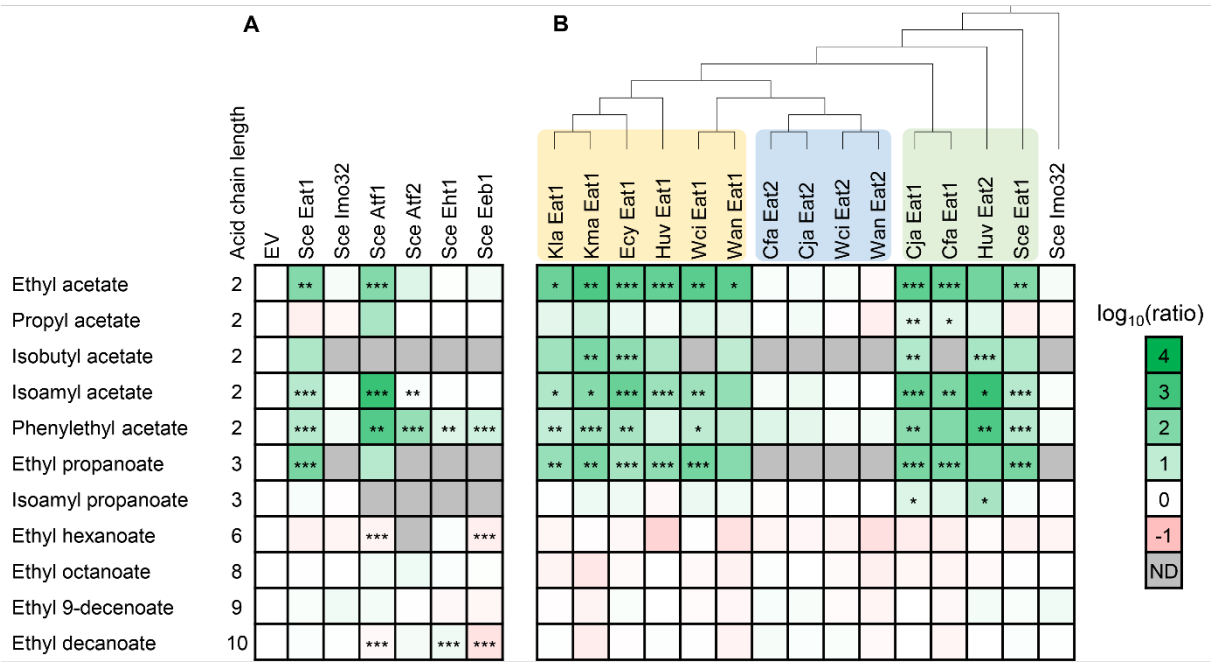


Figure 2: Overexpression of yeast AATs in *S. cerevisiae* CEN.PK2-1D. A – Overexpression of the known *S. cerevisiae* AATs. B – Ester production profiles of *S. cerevisiae* CEN.PK2-1D expressing 15 *eat* homologs from nine yeast species. Each square represents the $\log_{10}$ of the ratio between the ester peak area of an overexpression strain relative to the area detected in the empty vector (EV) strain. Values represent the average of two biological replicates. Stars indicate statistical significance: * α=0.1, ** α=0.05, *** α=0.01. The p(critical) values after the Bonferroni adjustment were 0.0067, 0.0033 and 0.0007, respectively. Abbreviations: Wan - *Wickerhamomyces anomalus*, Wci - *Wickerhamomyces ciferrii*, Kma - *Kluyveromyces marxianus*, Kla - *Kluyveromyces lactis*, Cja - *Cyberlindnera jadinii*, Cfa - *Cyberlindnera fabianii*, Huv - *Hanseniaspora uvarum*, Ecy - *Eremothecium cymbalarie,* Sce - *Saccharomyces cerevisiae*, ND – not determined

Next, we assessed whether acetate and propanoate ester production were increased by other *eat* genes. We overexpressed 15 members of the *eat* family in *S. cerevisiae* CEN.PK2-1D, originating from nine yeast species (Figure 2B). It was found that 10 out of 15 overexpression strains showed increased production of acetate and propanoate esters, while medium chain fatty acid (MCFA) ester production was not affected. This was comparable to the overexpression of the *S. cerevisiae eat1*. Ordering the ester production profiles of the overexpression strains according to a phylogenetic tree of the Eat protein sequences revealed that three groups seem to exist within the Eat family (Figure 2B). Two groups consist of *eat1* genes that are able to increase ester production. The third group consists of genes which had only a negligible effect on the ester profile compared to the other two *eat* groups. It is likely that these enzymes are not involved in ester synthesis in yeast. Curiously, *W. anomalus* and *W. ciferrii eat1* are more closely

related to the non-producing *eat* genes, but still increased ester production in *S. cerevisiae* CEN.PK2-1D. Most yeasts contain only one ester producing *eat* gene. The only exception thus far was *H. uvarum,* which possesses two ester-producing *eat* genes. *S. cerevisiae* CEN.PK2-1D (pCUP1:Huv2) also showed a different ester production profile compared to the other *eat1* expressing strains. The strain showed a 1291- and 590-fold increase in isoamyl acetate and phenylethyl acetate production, respectively (Supplementary Figure 2). These were some of the highest increases in ester production observed in this experiment. Huv *eat2* was also the only gene that significantly (>5-fold) increased the production of isoamyl propanoate. The remaining *eat1* homologs evoked comparable ester production profiles to *S. cerevisiae* CEN.PK2-1D (pCUP1: Sce Eat1).

Effect of eat disruption on in vivo ester production in S. cerevisiae

S. cerevisiae eat1 clearly has the potential to produce acetate and propanoate esters *in vivo*, while the effect of *imo32* is unconfirmed. To determine the impact of *eat1* and *imo32* on the total ester production, we disrupted the two genes in combination with the other four known *S. cerevisiae* AAT genes (*atf1, atf2, eht1, eeb1)* in *S. cerevisiae* CEN.PK2-1D by CRISPR-Cas9 (DiCarlo et al., 2013; Mans et al., 2015). A total of 14 disruption strains were generated; six single knockouts (Δ*atf1,* Δ*atf2,* Δ*eht1,* Δ*eeb1,* Δ*eat1,* Δ*imo32*), two double knockouts (Δ*atf1*Δ*atf2,* Δ*eht1*Δ*eeb1*), two triple knockouts (Δ*atf1*Δ*atf2*Δ*eat1,* Δ*eht1*Δ*eeb1*Δ*eat1*), one quadruple knockout (Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*), two quintuple knockouts (Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*, Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*imo32*), and finally a sextuple disruption strain in which all six genes were disrupted (Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*Δ*imo32*). We determined their ester production profiles under three cultivation conditions. Two were based on routine laboratory cultivations in minimal YSg medium and rich YPD-80 medium while shaking. The latter medium was used previously to assess the effects of AAT disruptions on ester production (Verstrepen et al., 2003b). The third cultivation condition simulated industrial white wine fermentations and was performed statically. We first assessed the fermentation performance of the strains and found that there were only small differences in growth, glucose consumption or ethanol production in either YSg medium or YPD-80 medium (Supplementary Figure 3). However, the

strains showed substantial differences in their ester production profiles. Three acetate esters (ethyl-, isoamyl- and phenylethyl acetate) and three ethyl MCFA esters (ethyl-octanoate, 9-decenoate, and decanoate) were measured under these cultivation conditions.

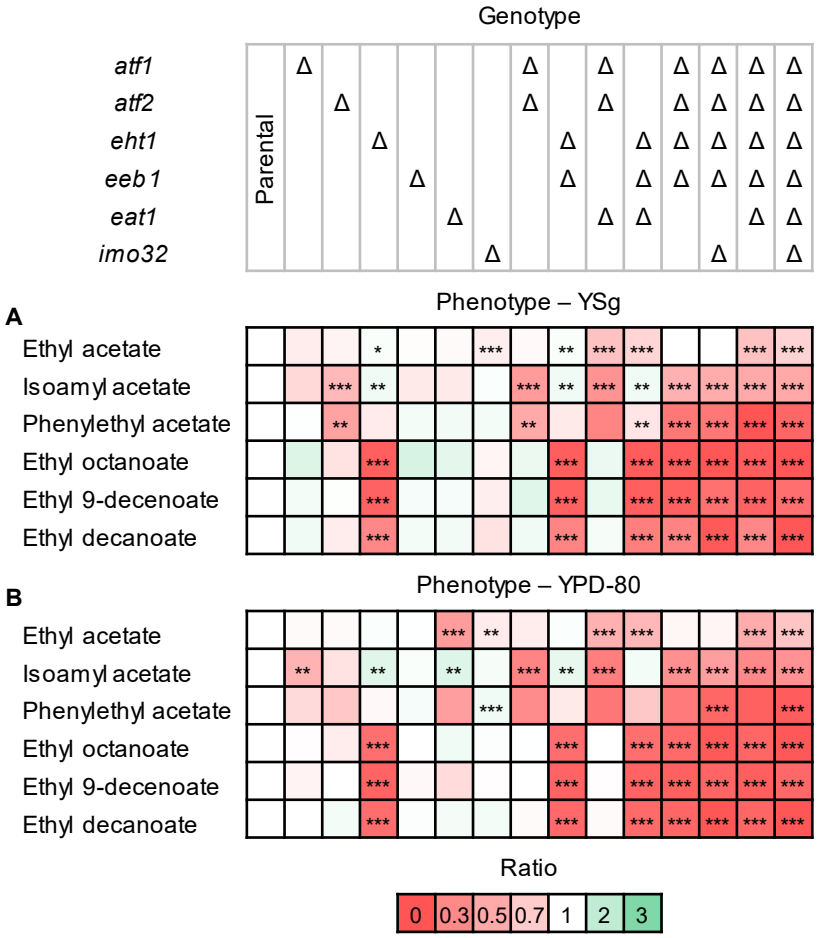


Figure 3: Ester production profiles of *S. cerevisiae* CEN.PK2-1D AAT disruption strains. A – strains grown on YSg (minimal) medium. B – strains grown on YPD-80 (rich medium). Strains were cultivated in 10 mL medium while shaking. Ester production was measured by HS-SPME GC-MS. Values represent the ratio between the area of the ester peak relative to the peak measured in the parental strain. The values are averages of two technical and two biological replicates. Values represent the average of two biological replicates. Stars indicate statistical significance: * α=0.1, ** α=0.05, *** α=0.01. The p(critical) values after the Bonferroni adjustment were 0.0067, 0.0033 and 0.0007, respectively.

Single deletions of *atf1* and *atf2* resulted in a slightly lower acetate ester production in both YSg (Figure 3A) and YPD-80 medium (Figure 3B). In contrast, CEN.PK2-1D Δ*eht1* produced less MFCA esters, but showed no differences in acetate ester levels in either media (Figure 3). The effects of these single disruptions were observed previously

(Saerens et al., 2006; Verstrepen et al., 2003b), although the effect of the *atf1* and *atf2* deletions on acetate ester production in these studies was stronger. This may be due to the repression of *atf1* under aerobic conditions (Mason and Dufour, 2000). Disruption of *eeb1* did not show any detectable effect on ester production in YSg or YPD-80 medium (Figure 3). CEN.PK2-1D Δ*eat1* showed significantly reduced levels of ethyl acetate in YPD-80 medium (Figure 3B). In some cases, the roles of the AAT genes in ester production became more apparent when multiple genes were disrupted simultaneously. For example, the ethyl acetate production of CEN.PK2-1D Δ*eat1* grown on YSg medium was similar to the CEN.PK2-1D parental strain. However, ethyl acetate production was reduced in CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eat1*, CEN.PK2-1D Δ*eht1*Δ*eeb1*Δ*eat1* and CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1* compared to their respective strains without the *eat1* deletion ($p<0.0007$). The gene disruptions mostly showed a cumulative effect. The quintuple disruption strain CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1* produced the least esters. However, ester production was not completely abolished, indicating that other ester forming reactions exist in *S. cerevisiae* CEN.PK2-1D. This was the most apparent in the case of ethyl acetate and isoamyl acetate, where more than 50% ethyl acetate production remained in some cases (Supplementary Figure 4). Imo32 was never associated with ester production but is a putative homolog of *eat1* that may contribute to ester synthesis. The single deletion strain CEN.PK2-1D Δ*imo32* produced 10% less ethyl acetate in both YSg and YPD-80 media. This effect disappeared when *imo32* was disrupted in CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1* and CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1* (Figure 3). The strain where all six genes were disrupted still produced similar amounts of esters compared to CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*. This indicates that *imo32* does not play a direct role in ester production in *S. cerevisiae* CEN.PK2-1D grown on YSg and YPD-80 media. The production of other esters, such as phenylethyl acetate and all MCFA esters was almost completely eliminated in CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1* and CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*Δ*imo32* grown on YSg or YPD-80 medium (Supplementary Figure 4). The effect of the AAT disruptions was then investigated under more industrially relevant conditions. The strains were cultivated statically in white grape juice, which contained 112.1 g/L sugars

(58.8 g/L glucose and 53.3 g/L fructose). The gene disruptions affected the fermentation performance of the strains. This was most apparent in the strain where all AA genes were disrupted. The growth, sugar consumption, ethanol formation and CO_2 formation were approximately 10 % lower (Supplementary Figure 5). However, the differences in ester production were more dramatic. Five additional esters (isobutyl acetate, ethyl hex-4-enoate, ethyl hexanoate, isoamyl octanoate, and ethyl dodecanoate) were detected in white grape juice compared to YSg and YPD-80 medium (Figure 3).

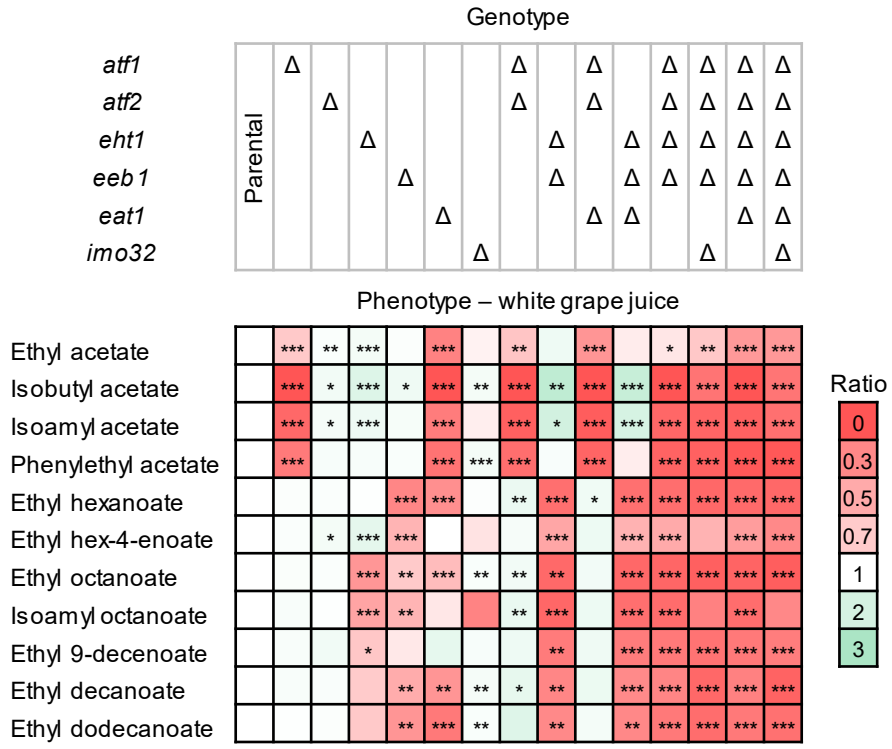


Figure 4: Ester production profiles of *S. cerevisiae* CEN.PK2-1D AAT disruption strains in white grape juice. Strains were cultivated statically in 50 mL medium for 7 days. Ester production was measured by HS-SPME GC-MS. Values represent the ratio between the area of the ester peak relative to the peak measured in the parental strain. The values are averages of two technical and two biological replicates. Stars indicate statistical significance: * α=0.1, ** α=0.05, *** α=0.01. The p(critical) values after the Bonferroni adjustment were 0.0067, 0.0033 and 0.0007, respectively.

The CEN.PK2-1D AAT disruption strains behaved differently in white grape juice (Figure 4) compared to the cultivation in YSg and YPD-80 media. The most notable difference was observed in CEN.PK2-1D Δ*eat1*, which showed a decreased production of both acetate and MCFA esters. This observation is not in line with the behaviour

of CEN.PK2-1D Δ*eat1* in laboratory media (Figure 3), or the overexpression experiment (Figure 3), where only an effect on acetate and propanoate ester production was observed. The deletion of *eat1* did not show the same effect on MCFA ester production in CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eat1,* compared to CEN.PK2-1D Δ*atf1*Δ*atf2*. Rigorous genotyping and three independent repeats of the fermentation confirmed that the observed results are reproducible and not caused by technical issues. Moreover, CEN.PK2-1D Δ*eat1* served as the precursor strain for generating other disruption strains that did not show this behaviour. Disruption of a*tf2* and *eeb1* also resulted in different production profiles in white grape juice, compared to YSg and YPD-80. CEN.PK2-1D Δ*atf2* did not show a difference in the ester production profile compared to the parental strain, whereas it reduced acetate ester production in YSg and YPD-80 cultures. In contrast, the disruption of *eeb1*, which had no significant effect in laboratory media, resulted in a broad decrease of MCFA esters in white grape juice, which was consistent with previously published results (Saerens et al., 2006). Deletion of *atf1* and *eht1* showed reduced production of acetate and MCFA esters, respectively, which is comparable to the profiles observed in laboratory media (Figure 3). The quintuple disruption strain in CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1* again produced the least esters. The disruption of *imo32* either did not have an impact on ester production in wine juice, or these differences were not statistically significant (Figure 4). Residual levels of esters were still detected (Supplementary Figure 6), matching the observations in YSg and YPD-80 media (Figure 3).

Discussion

Ester production by *S. cerevisiae* is critical for the flavour and aroma of fermented foods. Industrial yeasts strains can vary dramatically in both the amounts of esters produced, as well as the ratios between the different esters (Gallone et al., 2016). We attempted to correlate the differences in ester production to variations of the amino acid sequences and CNV of known *S. cerevisiae* AATs. We observed no clear correlation, indicating that other factors determine the differences in ester production. These may include factors such as different expression levels of the AAT genes or varying levels of esterase activity (Lilly et al., 2006; Verstrepen et al., 2003b). The

role of Eat1 on ester production was instead determined *in vivo* in *S. cerevisiae* CEN.PK2-1D.

Strains in which *eat1* was deleted exhibited significantly lower acetate ester levels, while overexpression of the gene increased the production of acetate as well as propanoate esters. This shows that Eat1 clearly contributes to the *in vivo* synthesis of these esters. The Eat1 family seems to prefer acetyl-CoA and propionyl-CoA as the acyl-donor. However, the *S. cerevisiae* Eat1 is located in the mitochondria (Huh et al., 2003) where mostly acetyl-CoA is produced. On the other hand, MCFA acyl-CoA synthesis predominantly takes place in the cytosol (Marchesini and Poirier, 2003; Tehlivets et al., 2007). It is therefore possible that Eat1 might be able to use other acyl-CoAs, but they are inaccessible under *in vivo* conditions. Contrary to acyl-CoA, alcohols can freely diffuse throughout the cell, and may react with the acyl-donor anywhere in the cell. This may explain why Eat1 seems to be unspecific towards alcohols but prefers short chain acyl-CoAs. The substrate preferences of the enzymes should be determined *in vitro*. Under *in vivo* conditions, the Eat family produces a similar spectrum of esters as Atf1, although ethyl propanoate (pineapple aroma) was increased significantly more0 by Eat1. There is variation in the individual ester levels produced by the Eat1 family as well. For example, expression of the *H. uvarum* Eat2 in *S. cerevisiae* had a bigger impact on isoamyl acetate and phenylethyl acetate production compared to ethyl acetate. In many cases, a high ratio of isoamyl acetate over ethyl acetate results in more pleasant aromas of fermented products (Verstrepen et al., 2003a).

Atf1, Atf2, Eht1, Eeb1 and Eat1 are not the only enzymes contributing to ester synthesis in *S. cerevisiae*. The production of some esters was almost completely abolished in the quintuple disruption strain CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*, while some were still produced at reduced levels compared to the parental strain. Esters that were almost completely abolished include isobutyl acetate, phenylethyl acetate and ethyl propanoate and most MCFA esters. On the other hand, there were still considerable levels of ethyl acetate and isoamyl acetate produced even when all five AAT genes were disrupted. This is somewhat surprising since previous reports showed

that ethyl acetate production was reduced by 50% when either *atf1* or *eat1* were disrupted (Kruis et al., 2017; Verstrepen et al., 2003b). The CEN.PK2-1D Δ*atf1*Δ*atf2*Δ*eat1* strain was therefore not expected to produce ethyl acetate anymore. The additional disruption of *imo32* did not explain this residual ester production. It is clear that there are other mechanisms producing ethyl acetate and isoamyl acetate in *S. cerevisiae*. It is possible that the esters are synthesised by other unknown AATs or through entirely different enzymatic reactions. In *S. cerevisiae,* ester synthesis has also been linked to hemiacetal dehydrogenation and reverse esterase activity (Masayuki Kusano et al., 1998; Park et al., 2009).

The three cultivation conditions strongly influenced the effects of the AAT disruptions on strain ester profiles. In CEN.PK2-1D Δ*eat1* grown in YPD-80 or white grape juice produced less esters even as a single disruption. In white grape juice the single deletion of *eat1* even reduced MCFA ester production. This effect was unexpected since it was not observed in any other strain where *eat1* was disrupted. The three independent repetitions of the experiment consistently showed the same result. Atf2 and Eeb1 also behaved differently in the three media. The deletion of *atf2* showed decreased acetate ester production when grown in YSg and YPD-80 while shaking, but not in the static white grape juice fermentations. Strains where *eeb1* was disrupted showed the opposite trend. It is possible that the effect of the *atf2*, *eeb1* and other AAT genes is controlled by the presence of oxygen. In several cases, strain genotype also influenced the effect individual AAT disruptions had on the total ester profile. For example, in YSg medium, the effect of *eat1* disruption was not apparent in CEN.PK2-1D Δ*eat1*. However, when the gene was disrupted in various combinations with the remaining four AATs, it caused a decrease in acetate ester production. This indicates that in some cases, the effect of a single AAT disruption may be masked by the remaining AATs, which is plausible as they compete for the same substrates; although not all AATs have the same cellular location (Huh et al., 2003; Lin and Wheeldon, 2014). There is a significant knowledge gap on the expression, function and interaction of yeast AATs, especially under industrially relevant conditions. Further research into these aspects might reveal the reasons behind the unexpected behaviour of some of the disruption strains. Nevertheless, the general trend observed both in the overexpression, as well as

the deletion strains is that Eat1 is a major source of acetate and propanoate esters and is able to utilise a wide range of alcohols.

The esters produced by Eat1 and the other AATs present in yeast are key flavour compounds in fermented products. We have shown that Eat1 contributes to the synthesis of acetate and propanoate esters in *S. cerevisiae*. However, we have also demonstrated that even when all known AATs were disrupted, ester synthesis was not completely abolished. This study provided a better understanding of ester production in yeast but has also focused our attention to the still existing knowledge gap on ester formation by *S. cerevisiae*.

Materials and methods

Strain and plasmid construction

Strains and plasmids used and created in this study are listed in Table 1 and Table 2, respectively. Plasmids p414-TEF1p-Cas9-CYC1t and p426-SNR52p-gRNA.CAN1.Y-SUP4t were gifts from George Church (Addgene plasmids #43802 and #43803, respectively). The plasmid pROS13 was a gift from A.J.A. van Maris (Euroscarf plasmid #P30790). Plasmid p426-*atf1* and p426-*eat1* were constructed by introducing the appropriate gRNA sequence into the plasmid through PCR. A 5' phosphoryl group was introduced on the 5' ends of the PCR product through 5' phosphorylated primers. The PCR product was then purified using the DNA Clean & Concentrator™-5 (Zymo research) and ligated with T4 ligase (NEB) according to the manufacturer's protocol. Plasmid p426-*atf2* was constructed from a PCR amplified p426 backbone (Q5, NEB) and a synthetic gBlock (IDT), containing the Atf2 gRNA sequence and 50 bp homologous regions overlapping the linear plasmid backbone. The two fragments were assembled using Gibson assembly® (NEB), following the manufacturer's instructions. pROS13-*eht1* and pROS13-*eeb1* were constructed by introducing the appropriate gRNAs as described previously (Mans et al., 2015). gRNAs were designed with the help of ChopChop (Labun et al., 2016). Plasmids, ligations and Gibson assemblies were routinely transformed to NEB® 5-alpha chemically competent cells (NEB) according to the supplier protocol. Correct plasmid construction was confirmed by sequencing (GATC, Macrogen). *S. cerevisiae* plasmid transformations were performed as described

previously (Gietz and Woods, 2002). Gene disruptions in *S. cerevisiae* CEN.PK2-1D (p414-TEF1p-Cas9-CYC1t) were performed by co-transformation with the appropriate p426 or pROS13 plasmid, together with a synthetic dsDNA repair fragment (IDT), as described previously (DiCarlo et al., 2013). The 140 bp repair fragment consisted of 70 bp homology directly upstream and downstream of the start and stop codon. Successful disruptions were confirmed by PCR, using genomic DNA as template (Lõoke et al., 2011). Plasmids derived from p426-SNR52p-gRNA.CAN1.Y-SUP4t were cured by growing the strains in the presence of 1 mg/mL 5-fluoroorotic acid (Boeke et al., 1984). Plasmids derived from pROS13 were cured by overnight growth without antibiotics and subsequent streaking on selective and non-selective plates. Colonies unable to grow on selective plates were deemed cured and used for the next round of gene disruption. Plasmid p414-TEF1p-Cas9-CYC1t was cured by counter-selection with 0.5 mg/ml 5-fluoroanthranilic acid (Toyn et al., 2000). Cured strains were used for physiological characterisation.

Table 1: Strains used and created during this study

Strain	Origin
Escherichia coli	
NEB5-α	NEB
Saccharomyces cerevisiae	
CEN.PK2 1-D	(Entian and Kötter, 2007)
CEN.PK2 1-D Δ*atf1*	This study
CEN.PK2 1-D Δ*atf2*	This study
CEN.PK2 1-D Δe*ht1*	This study
CEN.PK2 1-D Δe*eb1*	This study
CEN.PK2 1-D Δ*eat1*	This study
CEN.PK2 1-D Δ*imo32*	This study
CEN.PK2 1-D Δ*atf1*Δ*atf2*	This study
CEN.PK2 1-D Δ*eeb1*Δ*eht1*	This study
CEN.PK2 1-D Δ*atf1*Δ*atf2*Δ*eat1*	This study
CEN.PK2 1-D Δ*eat1*Δ*eeb1*Δ*eht1*	This study
CEN.PK2 1-D Δ*atf1*Δ*atf2*Δ*eeb1*Δ*eht1*	This study
CEN.PK2 1-D Δ*atf1*Δ*atf2*Δ*eeb1*Δ*eht1*Δ*imo32*	This study
CEN.PK2 1-D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*	This study
CEN.PK2 1-D Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*Δ*imo32*	This study

Table 2: Plasmids used and created in this study

Construct	Characteristics	Origin
p414-TEF1p-Cas9-CYC1t	Plasmid harbouring SpyCas9	(DiCarlo et al., 2013)
p426-SNR52p-gRNA.CAN1.Y-SUP4t	Plasmid expressing the sgRNA	(DiCarlo et al., 2013)
p426-*atf1*	p426-SNR52p-gRNA.CAN1.Y-SUP4t expressing the *atf1* targeting gRNA	This study
p426-*atf2*	p426-SNR52p-gRNA.CAN1.Y-SUP4t expressing the *atf2* targeting gRNA	This study
p426-*eat1*	p426-SNR52p-gRNA.CAN1.Y-SUP4t expressing the *eat1* targeting gRNA	This study
p426-*imo32*	p426-SNR52p-gRNA.CAN1.Y-SUP4t expressing the *imo32* targeting gRNA	This study
pROS13	Dual gRNA expression plasmid	(Mans et al., 2015)
pRos13-*eht1*	pROS12 expressing *eht1* targeting gRNA	This study
pRos13-*eeb1*	pROS12 expressing *eeb1* targeting gRNA	This study
pCUP1	pYES2 (Invitrogen) where the GAL1 promoter was replaced with the CUP1 promoter	(Kruis et al., 2017)
pCUP1: Wan Eat1	Expression of *Wickerhamomyces anomalus* DSM 6766 *eat1*	(Kruis et al., 2017)
pCUP1: Wan Eat2	Expression of *Wickerhamomyces anomalus* DSM 6766 *eat2*	(Kruis et al., 2017)
pCUP1: Wci Eat1	Expression of *Wickerhamomyces ciferrii* CBS 111 *eat1*	(Kruis et al., 2017)
pCUP1: Wci Eat2	Expression of *Wickerhamomyces ciferrii* CBS 111 *eat2*	(Kruis et al., 2017)
pCUP1: Kma Eat1	Expression of *Kluyveromyces marxianus* DSM 5422 *eat1*	(Kruis et al., 2017)
pCUP1: Kla Eat1	Expression of *Kluyveromyces lactis* CBS 2359 *eat1*	(Kruis et al., 2017)
pCUP1: Cja Eat1	Expression of *Cyberlindnera jadinii* DSM 2361 *eat1*	(Kruis et al., 2017)
pCUP1: Cja Eat2	Expression of *Cyberlindnera jadinii* DSM 2361 *eat2*	(Kruis et al., 2017)
pCUP1: Cfa Eat1	Expression of *Cyberlindnera fabianii* CBS 5640 *eat1*	(Kruis et al., 2017)
pCUP1: Cfa Eat2	Expression of *Cyberlindnera fabianii* CBS 5640 *eat2*	(Kruis et al., 2017)
pCUP1: Huv Eat1	Expression of *Hanseniaspora uvarum* CECT 11105 *eat1*	(Kruis et al., 2017)
pCUP1: Huv Eat2	Expression of *Hanseniaspora uvarum* CECT 11105 *eat2*	(Kruis et al., 2017)
pCUP1: Ecy Eat1	Expression of *Eremothecium cymbalarie* CBS 270.75 *eat1*	(Kruis et al., 2017)
pCUP1: Sce Eat1	Expression of *S. cerevisiae* NCYC 2629 *eat1*	(Kruis et al., 2017)
pCUP1: Sce Imo32	Expression of *S. cerevisiae* NCYC 2629 IMO32 (*eat2* homolog)	(Kruis et al., 2017)

Cultivation conditions

Escherichia coli and *S. cerevisiae* cultures were routinely cultivated in LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) and YPD medium (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone), respectively. 15 g/L bacteriological agar was added to make plates. Media were supplemented with 50 µg/mL ampicillin and 200 µg/mL geneticin when appropriate. *E. coli* and *S. cerevisiae* were cultivated at 37°C and 30°C, respectively, unless stated otherwise. *S. cerevisiae* gene disruptions were performed in YNB-HL medium (5 g/L glucose, 6.7 g/L Yeast Nitrogen Base, vitamins and trace elements (Verduyn et al., 1992), 125 mg/L histidine-HCl and 500 mg/L leucine). 75 mg/L tryptophan and 150 mg/L uracil were added to YNB-HL as needed.

S. cerevisiae strains harbouring pCUP1 derived plasmids were characterised in YSg medium (20 g/L glucose, 6.7 g/L Yeast Nitrogen Base without amino acids, 1.92 g/L medium supplements without uracil). Single colonies were picked into 10 mL YS-glc and incubated overnight while shaking at 150 rpm. The following day, 100 µL of the preculture was transferred to 10 mL fresh YS-glc in a 50 mL Greiner tube while shaking at 150 rpm. 1 mM $CuSO_4$ was added for gene induction. Cultures were sampled after 24 hours. Samples were frozen at -20 °C until analysis. Experiments were performed as biological duplicates.

S. cerevisiae gene disruption strains were assayed in YPD-80 (YPD medium with 80 g/L glucose) and YSg-ura (YSg medium supplemented with 150 mg/L uracil). Single colonies were picked into 10 mL medium and incubated overnight while shaking at 150 rpm. The following day, 100 µL of the preculture was transferred to 10 mL fresh medium in a 50 mL Greiner tube while shaking at 150 rpm for 24 hours. 2 mL sample was transferred to a 10 mL glass vial, sealed, and frozen at -20 °C until analysis. Experiments were performed as biological duplicates. Samples were analysed as technical duplicates.

White grape juice fermentation

White grape juice was prepared by diluting the Arsegan® white grape juice concentrate five times. 1 g/L yeast extract was added to the medium which was filter sterilised. Subsequent HPLC analysis revealed that 58.8 g/L glucose and 53.3 g/L fructose were

initially present in the medium. Precultures were grown in 10 mL medium in a 50 mL Greiner tube while shaking overnight at 150 rpm. The next day, 2 mL of the preculture was transferred to 50 mL fresh medium in a 100 mL Schott bottle. The bottles were closed with a rubber stopper and a water lock. Cultures were cultivated statically at 20°C for 7 days. 2 mL sample was transferred to a 10 mL glass vial, sealed, and frozen at -20 °C until analysis. Experiments were performed as biological duplicates. Samples were analysed as technical duplicates.

Analytical

OD_{600} was measured in flat-bottom 96-well plates (Greiner) in 200 µL sample using a Synergy MX microplate reader (BioTek). The light path was calculated as 0.632 cm and used to adjust the OD_{600} values to the standard 1 cm light path. CO_2 production in white grape juice fermentations was estimated by measuring the weight of the bottles with the closed water lock before and after the fermentation. The difference was assumed to be due to loss of CO_2 (van Rijswijck et al., 2017).

Sugars were measured by High Pressure Liquid Chromatography (HPLC) on an ICS5000 HPLC system (Thermo Scientific) equipped with a DP pump, AS-AP autosampler and a VWD UV detector (Dionex), at 210 nm and an RI detector (Shodex) at 35°C. An HPX-87H cation-exchange column (Aminex) was used with a mobile phase of 0.016 N H_2SO_4. The HPLC was operated at 0.8 mL/min and 60°C. A final concentration of 2 mM DMSO in 0.04 N H_2SO_4 was used as internal standard.

Ethanol was measured by GC-FID as described before (Kruis et al., 2017) on a Shimadzu 2010 gas chromatograph equipped with a temperature controlled 20i-s autosampler. 0.5 µL of liquid sample was injected on a Stabilwax column (30 m x 0.25 mm, 0.5 µm coating, Restek). The column temperature was held at 60°C for 1 min and increased to 120°C at a rate of 20°C/min. The split ratio was 20. 2 mM 1-butanol was used as internal standard.

The ester production profile of the yeast strains was assessed using Headspace-Solid Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME GC-MS) as described previously, with minor adaptations (van Rijswijck et al., 2017). A Trace

1300 Gas Chromatograph (Thermo Fisher) with a TriPlus RSH autosampler (Thermo Fisher) and an ISQ QD mass spectrometer (Thermo Fisher) was used for analysis of volatiles. Frozen samples were incubated at 60°C for 10 min. Volatile compounds were extracted for 20 min at 60°C using an SPME fibre (Car/DVB/PDMS, Supelco). The compounds were desorbed from the fibre for 2 min onto a Stabilwax®-DA column (30 m length, 0.25 mm ID, 0.5 µm d_f, Restek). The PTV was heated to 250 °C and operated in split mode at a ratio of 1:25. The GC oven temperature was kept at 40 °C for 2 min, raised to 240°C with a slope of 10°C/min and kept at 240°C for 5 min. Helium was used as carrier gas at a constant flow rate of 1.2 ml/min. Mass spectral data were collected over a range of m/z 33–250 in full-scan mode with 3.0030 scans/s. Data was analysed using Chromeleon® 7.2. The ICIS algorithm was used for peak integration and the NIST main library to match the mass spectral profiles with the profiles of NIST. Peak areas were calculated using the MS quantification peak (highest m/z peak per compound).

Statistical analysis

Statistical analysis was performed using R (R Foundation for Statistical Computing, 2016). A one tailed Student's t-test was performed. The p(critical) values were adjusted according to the Bonferroni method (Dunn, 1961) to account for multiple comparisons.

Bioinformatics

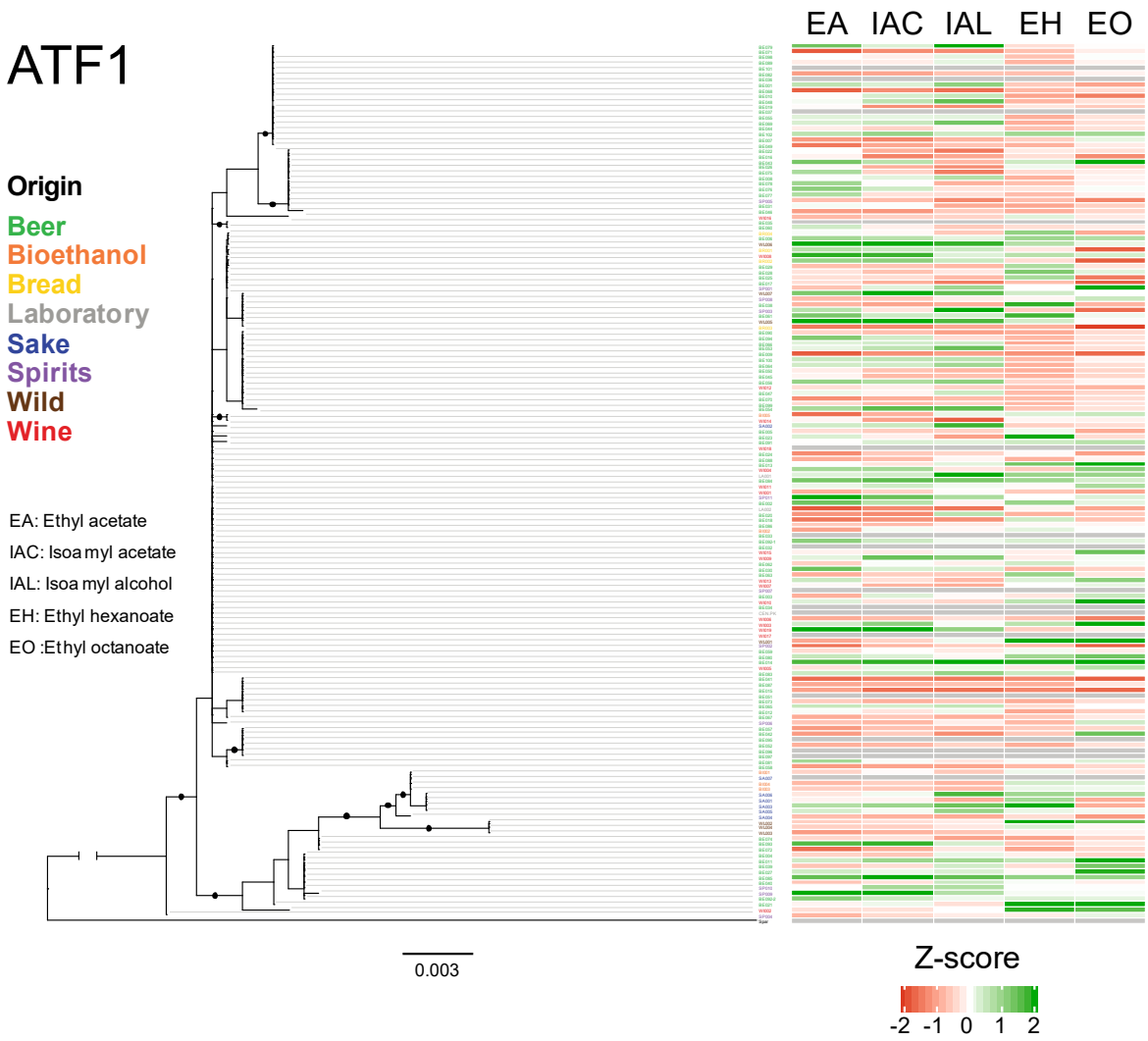
The protein-coding nucleotide sequences of *atf1*, *atf2*, *eat1*, *eeb1*, *eht1* and *imo32* were retrieved from the 157 *S. cerevisiae* strains sequenced in Gallone et al. (2016) (*de novo* assemblies downloaded from NCBI BioProject accession: PRJNA323691). Local BLAST databases were set up for all the genomes and BLASTN (vBLAST+ 2.5.0) searches were performed (1E-04 E-value cut-off). The protein coding sequences of each gene from *S. cerevisiae* CEN.PK2-1D were used as queries. Multiple sequence alignments (MSAs) were obtained for each gene using MAFFT (v7.187), with default settings and 1,000 refinement iterations (Katoh and Standley, 2013). Maximum likelihood (ML) trees were constructed for each MSA using RAxML (v8.2.8), under the GTRGAMMA model (Stamatakis, 2014). Rapid bootstrapping (2000 bootstrap replicates) and search for the best-scoring ML tree were conducted in one single run

(Pattengale et al., 2009). Trees were visualised and rooted in FigTree (v1.4.2) using *Saccharomyces paradoxus* (Scannell et al., 2011) as outgroup (http://tree.bio.ed.ac.uk/software/figtree/). Shannon entropy (H) was calculated for every position within the protein translated alignment of each gene (Shannon, 1948). Gaps were excluded from the calculation. Estimated ploidy and copy number variation data were obtained from Gallone et al. (2016).

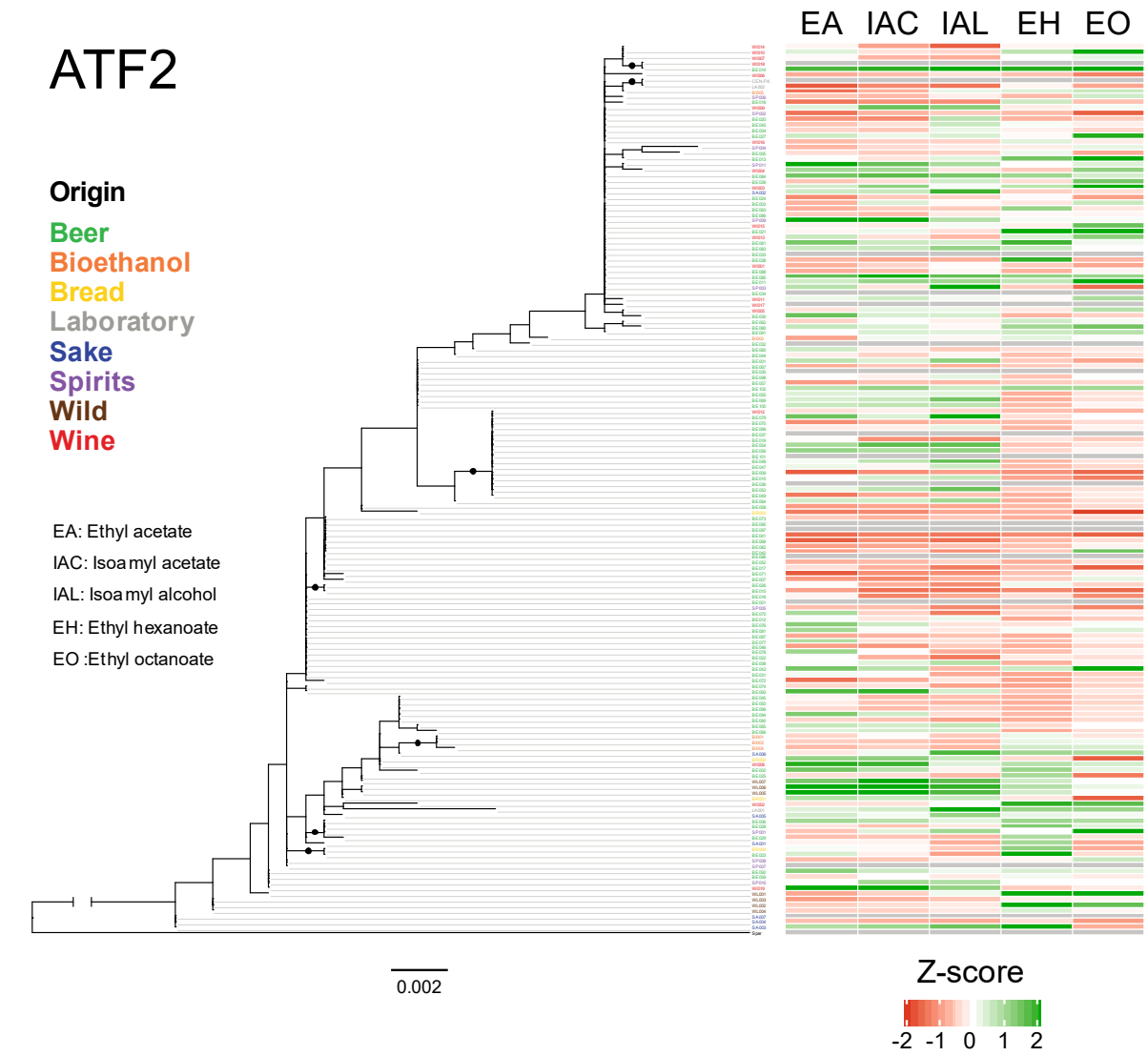
Acknowledgements

We would like to acknowledge the BE-Basic foundation and AkzoNobel Speciality Chemicals for funding the research. We thank dr. George Church for providing the plasmids p414-TEF1p-Cas9-CYC1t and p426-SNR52p-gRNA.CAN1.Y-SUP4t. We additionally thank dr. Jean-Marc Daran and dr. Jack Pronk for the stimulating discussions. Research in the laboratory of K.J.V. is supported by VIB, AB-InBev-Baillet Latour Fund, FWO, VLAIO, and European Research Council (ERC) Consolidator Grant CoG682009. K.J.V. acknowledges funding from the Human Frontier Science Program (HFSP) grant 246 RGP0050/2013. J.S. acknowledges funding from FWO grant 12W3918N.

Supplemental material



Supplementary Figure 1: Ester production profiles in a collection of industrial *Saccharomyces cerevisiae* strains (Gallone et al., 2016) sorted according to the best phylogenetic hypothesis of Atf1, Atf2, Eht1, Eeb1, Eat1 and Imo32 protein coding sequences (panels a, b, c, d, e ,f, respectively). Colour codes indicate origin of the strains. Branches supported by more than 60% of 2000 bootstrap replicates are highlighted by black dots.



Supplementary Figure 1, continued: Ester production profiles in a collection of industrial *Saccharomyces cerevisiae* strains (Gallone et al., 2016) sorted according to the best phylogenetic hypothesis of Atf1, Atf2, Eht1, Eeb1, Eat1 and Imo32 protein coding sequences (panels a, b, c, d, e ,f, respectively). Colour codes indicate origin of the strains. Branches supported by more than 60% of 2000 bootstrap replicates are highlighted by black dots.

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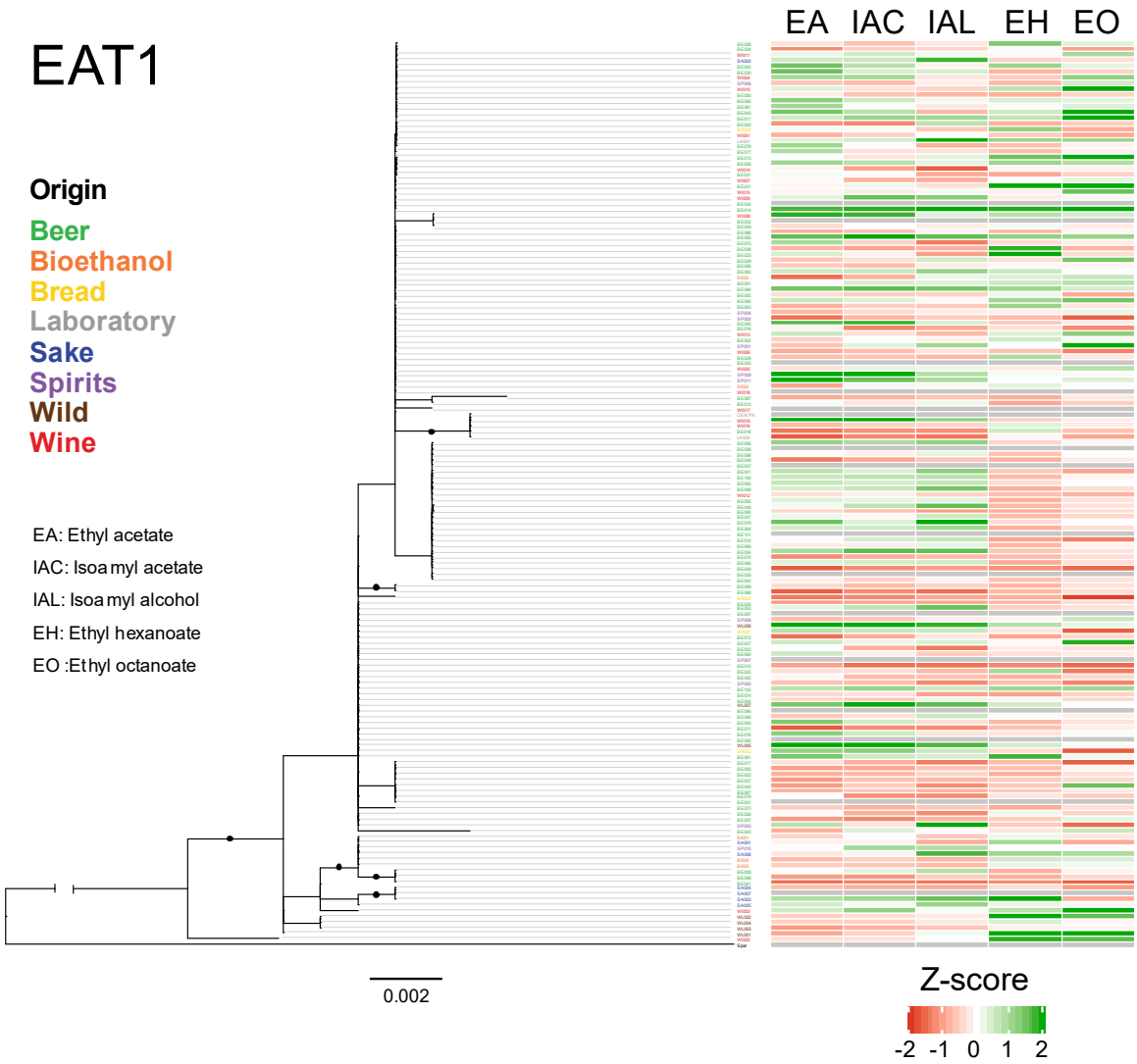
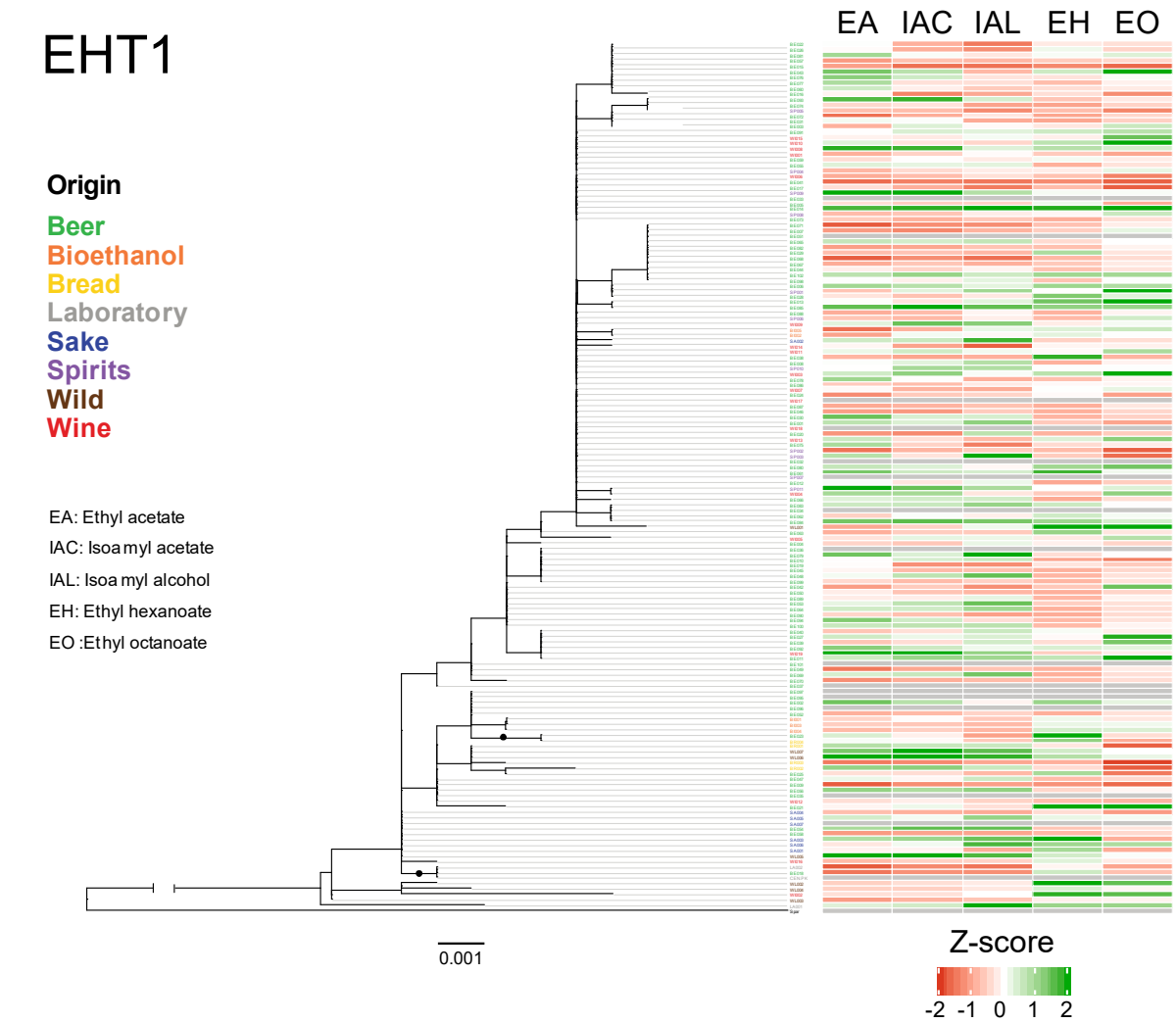


Supplementary Figure 1, continued: Ester production profiles in a collection of industrial *Saccharomyces cerevisiae* strains (Gallone et al., 2016) sorted according to the best phylogenetic hypothesis of Atf1, Atf2, Eht1, Eeb1, Eat1 and Imo32 protein coding sequences (panels a, b, c, d, e ,f, respectively). Colour codes indicate origin of the strains. Branches supported by more than 60% of 2000 bootstrap replicates are highlighted by black dots.



Supplementary Figure 1, continued: Ester production profiles in a collection of industrial *Saccharomyces cerevisiae* strains (Gallone et al., 2016) sorted according to the best phylogenetic hypothesis of Atf1, Atf2, Eht1, Eeb1, Eat1 and Imo32 protein coding sequences (panels a, b, c, d, e ,f, respectively). Colour codes indicate origin of the strains. Branches supported by more than 60% of 2000 bootstrap replicates are highlighted by black dots.

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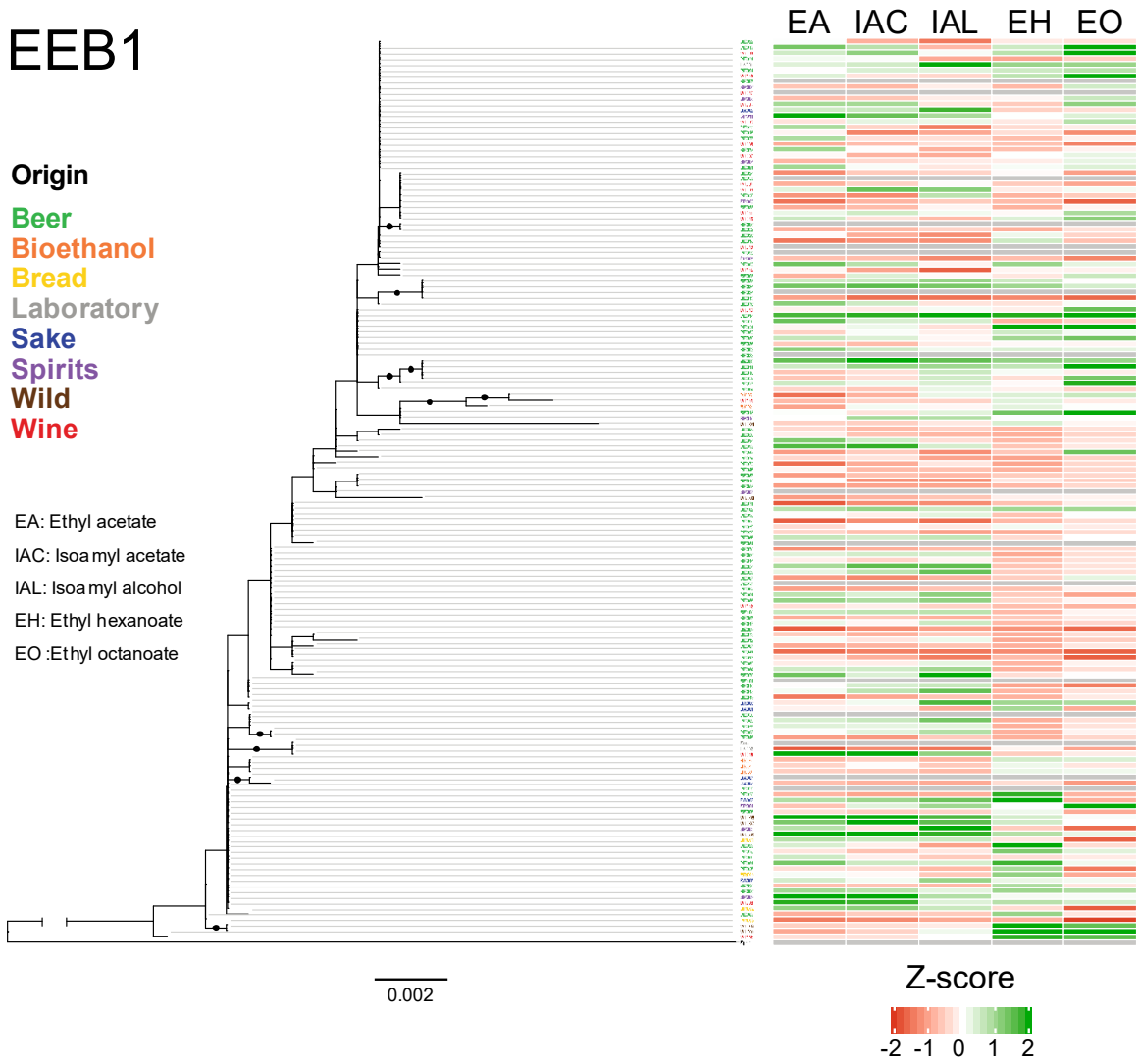

Supplementary Figure 1, continued: Ester production profiles in a collection of industrial *Saccharomyces cerevisiae* strains (Gallone et al., 2016) sorted according to the best phylogenetic hypothesis of Atf1, Atf2, Eht1, Eeb1, Eat1 and Imo32 protein coding sequences (panels a, b, c, d, e ,f, respectively). Colour codes indicate origin of the strains. Branches supported by more than 60% of 2000 bootstrap replicates are highlighted by black dots.

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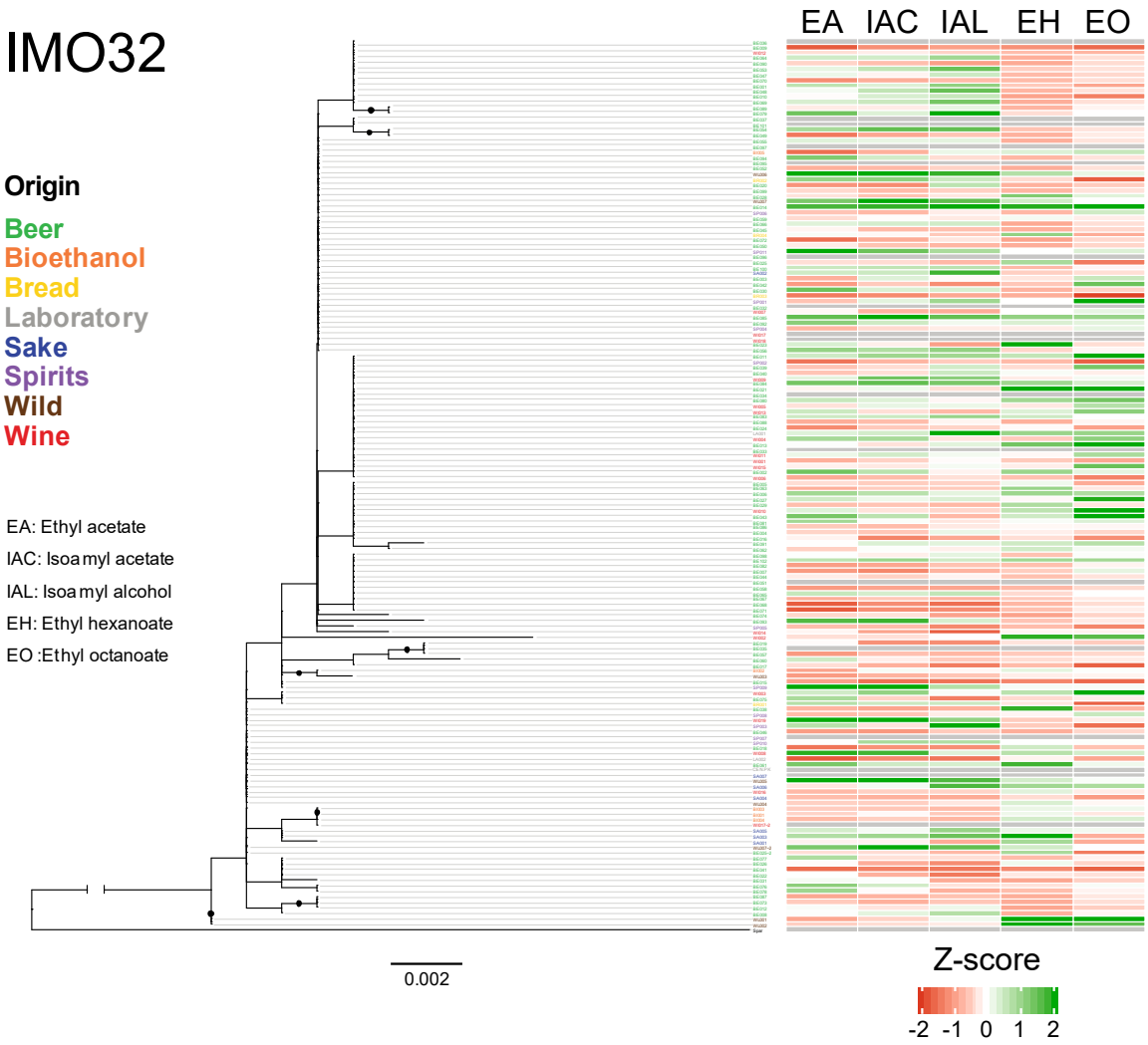
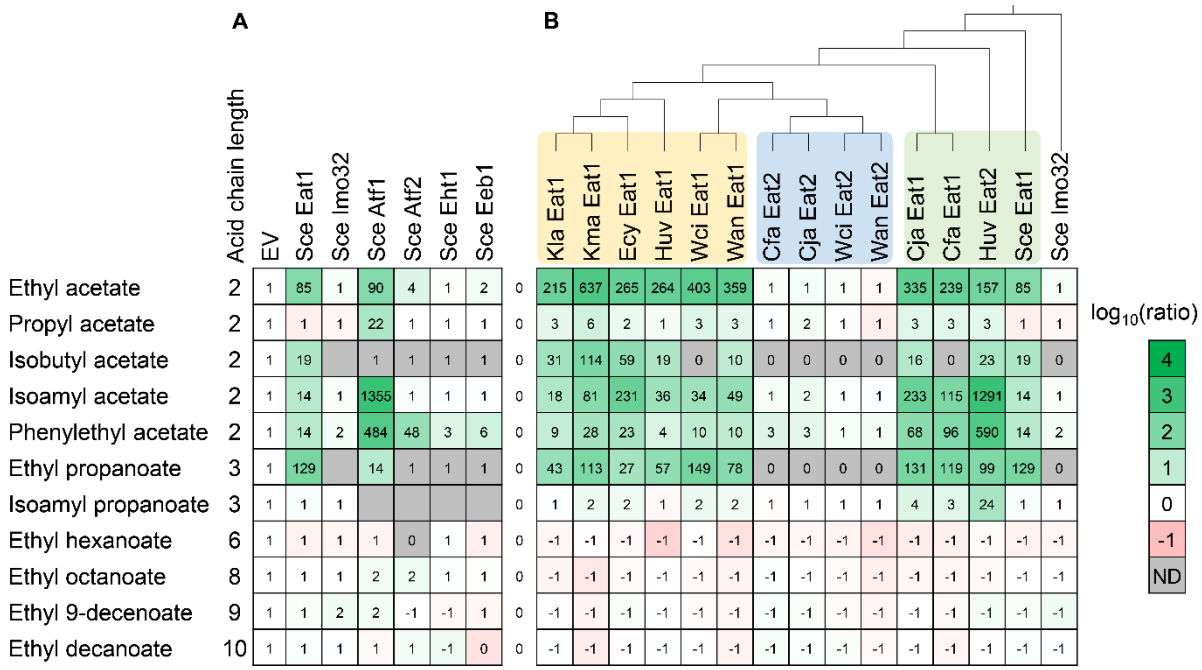
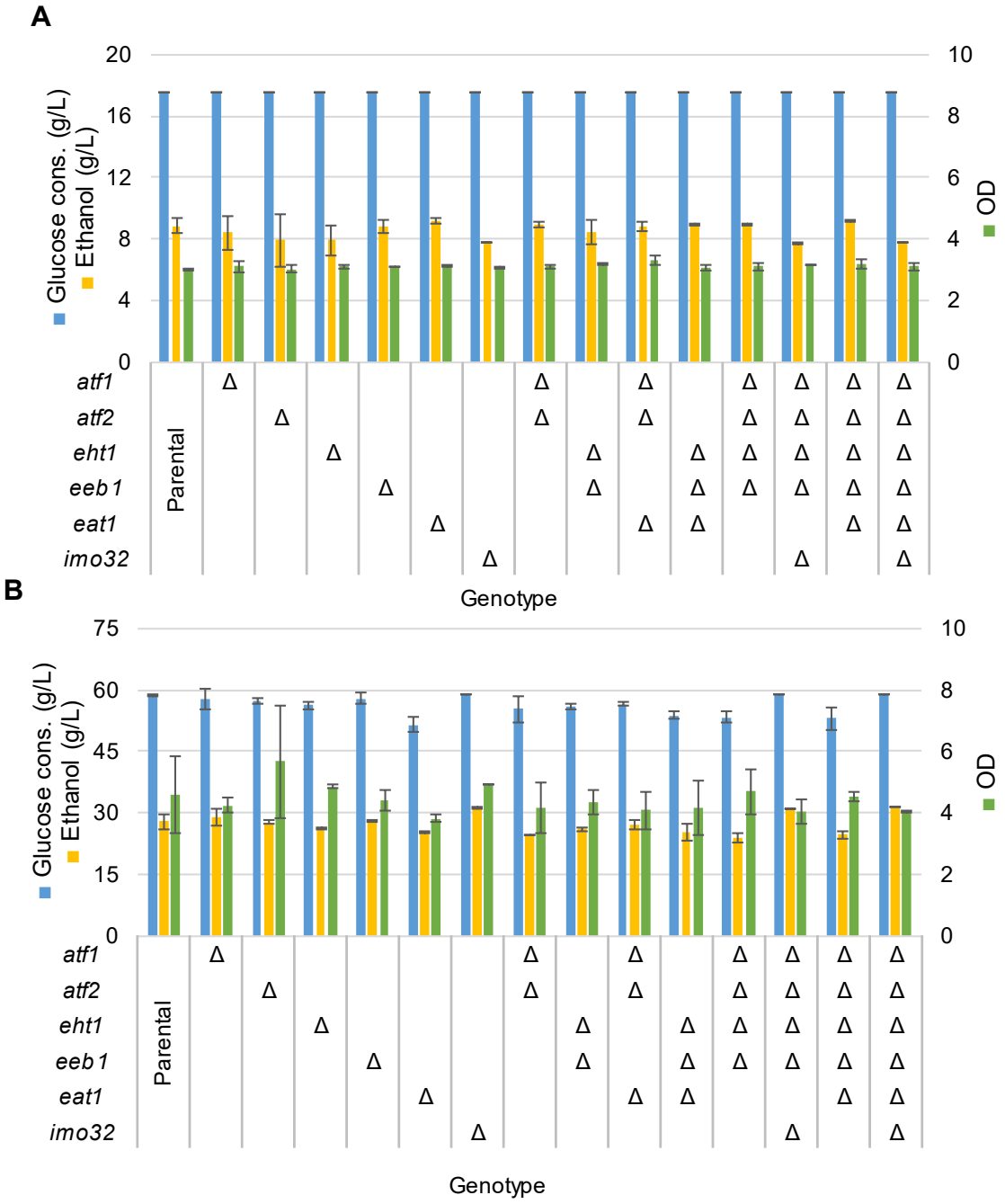


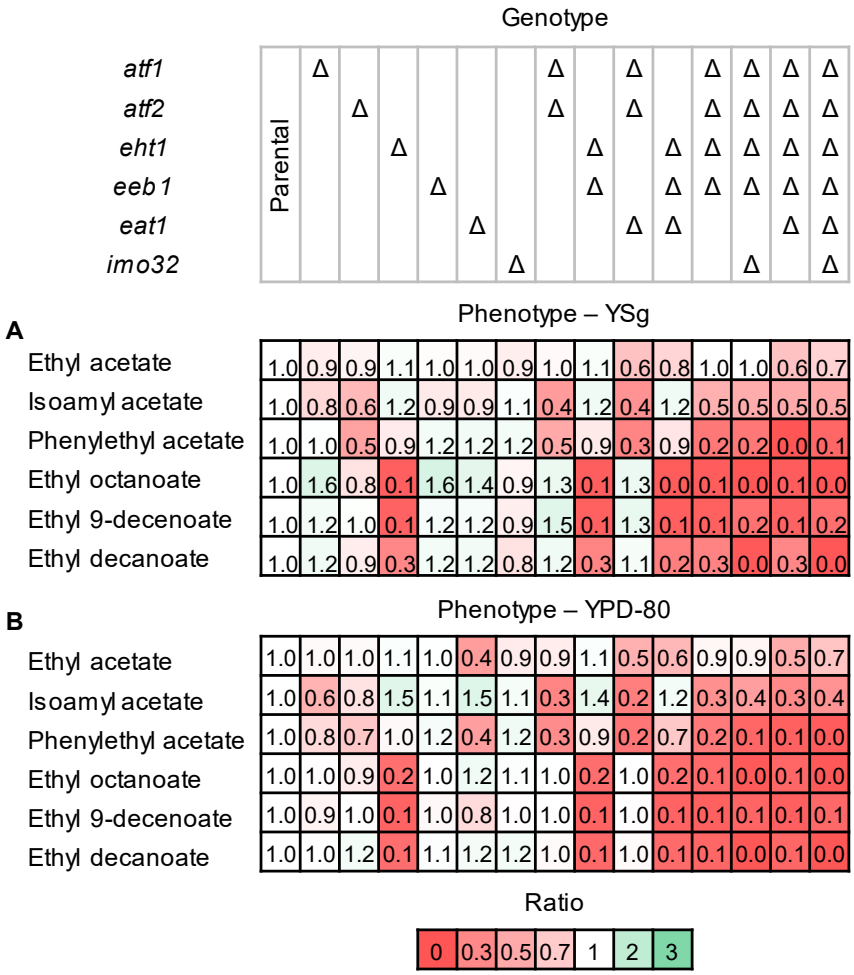
Supplementary Figure 1, continued: Ester production profiles in a collection of industrial *Saccharomyces cerevisiae* strains (Gallone et al., 2016) sorted according to the best phylogenetic hypothesis of Atf1, Atf2, Eht1, Eeb1, Eat1 and Imo32 protein coding sequences (panels a, b, c, d, e ,f, respectively). Colour codes indicate origin of the strains. Branches supported by more than 60% of 2000 bootstrap replicates are highlighted by black dots.



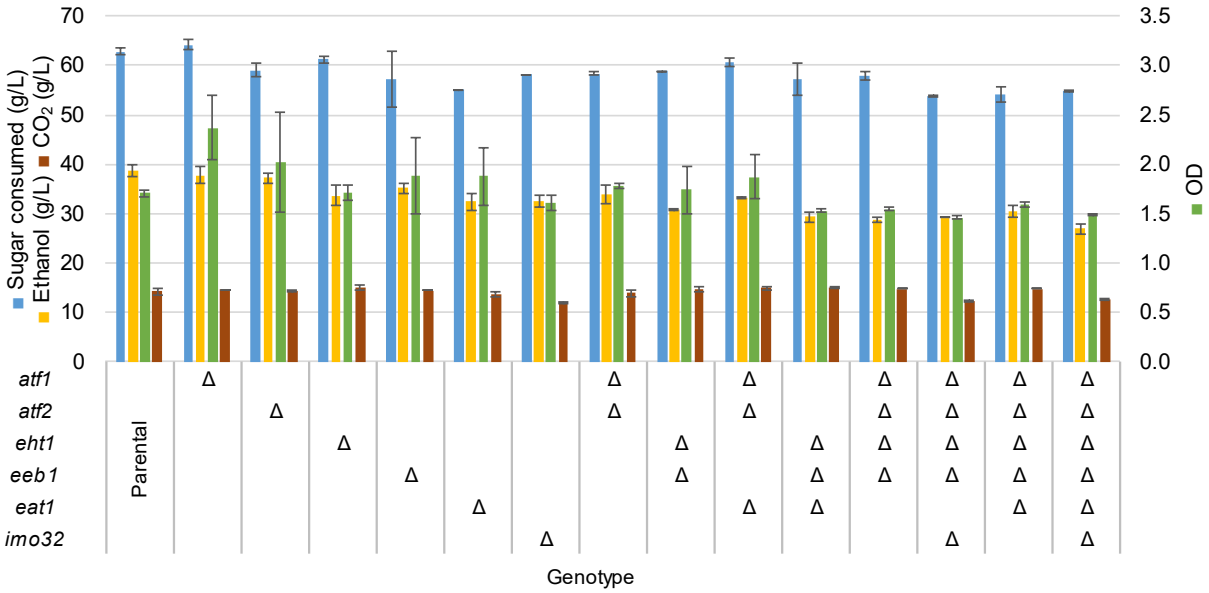
Supplementary Figure 2: Overexpression of 15 *eat1* homologs from nine yeast species in *S. cerevisiae* CEN.PK2-1D. The colours of the squares represent the log ratio between the ester peak area of an overexpression strain relative to the area detected in the empty vector (EV) strain. The numbers in the squares represents the ratio between the ester peak area of an overexpression strain relative to the area detected in the empty vector (EV) strain. The values are averages of two biological replicates. The Eat1 homologs form three phylogenetic groups. Two groups (yellow and green) increased ester production while one (blue) had no effect on ester production. Abbreviations: Wan - *Wickerhamomyces anomalus*, Wci - *Wickerhamomyces ciferrii*, Kma - *Kluyveromyces marxianus*, Kla - *Kluyveromyces lactis*, Cja - *Cyberlindnera jadinii*, Cfa - *Cyberlindnera fabianii*, Huv - *Hanseniaspora uvarum*, Ecy - *Eremothecium cymbalarie,* Sce - *Saccharomyces cerevisiae*



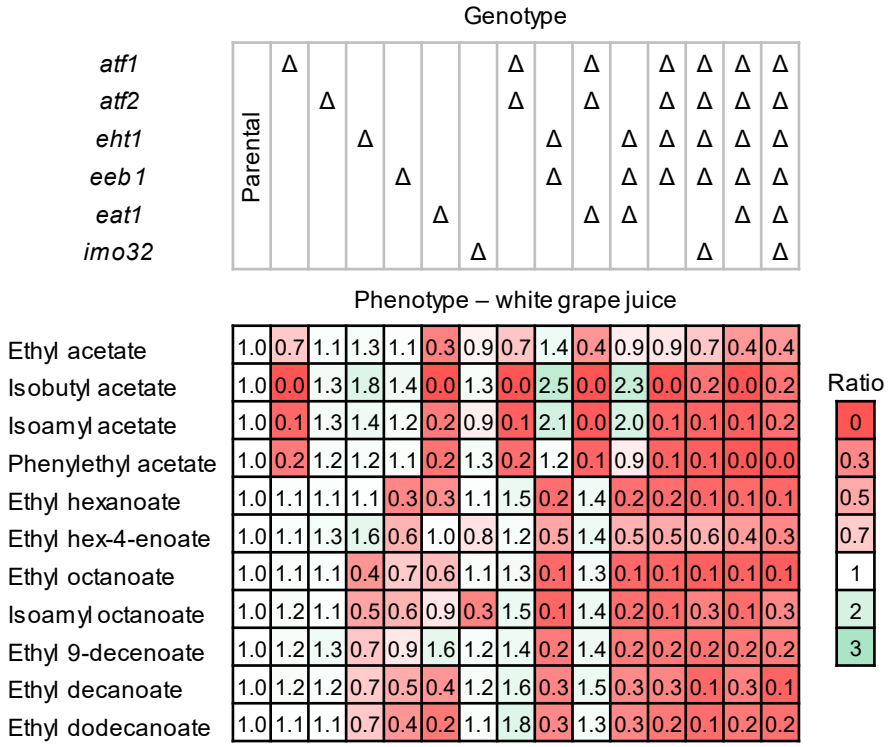
Supplementary Figure 3: Fermentation profile of *S. cerevisiae* CEN.PK2-1D AAT disruption strains. A – strains grown on YSg (minimal) medium. B – strains grown on YPD-80 (rich medium). Strains were cultivated in 10 mL medium while shaking. Sugars and ethanol were measured by HPLC and GC, respectively after 20 hours of cultivation. The values shown are averages of two biological duplicates. Error bars represent the standard deviation.



Supplementary Figure 4: Ester production profiles of *S. cerevisiae* CEN.PK2-1D AAT disruption strains. A – strains grown on YSg (minimal) medium. B – strains grown on YPD-80 (rich medium). Strains were cultivated in 10 mL medium while shaking. Ester production was measured by HS-SPME GC-MS. The numbers in the squares represent the ratio between the area of the ester peak relative to the peak measured in the parental strain. The values are averages of two technical and two biological replicates.



Supplementary Figure 5: Fermentation profile of *S. cerevisiae* CEN.PK2-1D AAT disruption strains in white grape juice. Strains were cultivated statically in 50 mL medium for 7 days. The medium initially contained 112.1 g/L fermentable sugars. Sugars and ethanol were measured by HPLC and GC, respectively. CO_2 production was measured as the loss of weight before and after the fermentation. The values shown are averages of two biological duplicates. Error bars represent the standard deviation.



Supplementary Figure 6. Ester production profiles of *S. cerevisiae* CEN.PK2-1D AAT disruption strains in white grape juice. Strains were cultivated statically in 50 mL medium for 7 days. Ester production was measured by HS-SPME GC-MS. The numbers in the squares represent the ratio between the area of the ester peak relative to the peak measured in the parental strain. The values are averages of two technical and two biological replicates.

CHAPTER 6

Microbial ester production: From enzymes and pathways to applications

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† - these authors contributed equally to the work

Submitted to Biotechnology Advances

Abstract

Sustainable production of bulk chemicals is one of the major contemporary challenges. This includes esters, which are currently produced mainly through unsustainable, energy intensive processes. Esters are versatile compounds that are currently used in a wide range of applications, such as fragrance compounds, industrial solvents, lubricants and biodiesels. Microorganisms are naturally able to produce many of the esters that are used in industry today. This trait could potentially be exploited to develop novel biobased processes aimed at sustainable ester production. This can only be achieved if the biological ester production is well understood. This review provides an overview of the current state of biobased ester production, focusing on microorganisms as production hosts. The natural ester production in microorganisms is discussed, with focus on the enzymes behind the synthesis. Alcohol acyltransferases (AATs) are the most established class of ester producing-enzymes, and have been applied in metabolic engineering strategies aimed at improving biobased ester production. An overview of these strategies is provided and notable examples are discussed more detail. The value of esters for the biobased production of other compounds, such as alcohols and carboxylic acids is addressed as well. Finally, the major challenges that lie ahead are summarised.

Introduction

Carboxylate esters are versatile bulk (or commodity) 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 odour of fermented beverages like wine and beer (Saerens et al., 2010). Natural food additives rely on strains that produce large quantities of esters (Berger, 2009). Ethyl acetate, isoamyl acetate or propyl acetate are common fragrance and aroma compounds (Table 1) (Carroll et al., 2016; Rodriguez et al., 2014). They are also used as industrial solvents due to their low toxicity and biodegradability. They are able to dissolve a variety of compounds, or can be used as plasticizers and polymer additives. (Białecka-Florjańczyk and Florjańczyk, 2007; Durrans and Davies, 1971; Löser et al., 2014). Further, they find applications as lubricants, coatings and are explored for their potential as drop-in fuels or biodiesels (Table 1) (Chuck and Donnelly, 2014; Kalscheuer et al., 2006; Lange et al., 2010).

Traditional ester production processes make use of the Fischer-Speier esterification. 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 et al., 2006). To allow the reaction to proceed, the water is continuously removed from the system in energy intense distillation or adsorption steps.

Table 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. Abbreviations: SCFA – short chain fatty acid, MCFA - medium chain fatty acid, LCFA – long chain fatty acid, FAME – fatty acid methyl ester, FAEE – fatty acid ethyl ester.

	Short alcohols			Long alcohols
	SCFA (C2-C4)	MCFA (C6-C12)	LCFA (C14+)	
Applications	**Solvents, plasticizers, lubricants**			**Waxes, coatings, adhesives, cosmetics**
	Ethyl acetate, Butyl propanoate, Pentyl acetate	Hexanedioates (adipates), Decanedioates (sebacates), Ethyl heptanoate	Ethyl stearate	Dodecyl hexadecanoate, Undecyl hexadecanoate
	Drop-in fuels		**Biodiesel**	
	Butyl butyrate	Ethyl pentanoate, Isobutyl hexanoate	FAME, FAEE	
	Aroma compounds, fragrances			
	Ethyl acetate, Isoamyl acetate	Pentanoates		

The conversion of biomass, particularly sugars, into useful products could provide a sustainable route to bulk chemical synthesis and has received much attention in recent years (Donate, 2014; van Haveren et al., 2008; Vennestrøm et al., 2011; Werpy and Petersen, 2004). Bulk chemicals, such as esters 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 titres, 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)

Microbial conversion systems will be the key in developing efficient ester-producing bioprocesses. Their vast array of enzymes is able to perform hundreds of chemical conversions at ambient conditions. To catalyse multiple metabolic pathways simultaneously, enzymes have evolved high specificity for their substrates. The

formation of by-product in industrial microbial bioprocesses is thereby limited (Quax, 2014).

This review article focusses 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 how the physical and chemical properties of esters 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 summarised.

Microbial ester production

Esters are produced by a number of microorganisms for various reasons. Ethyl acetate is a major fermentation product of certain yeast species which contributes to balancing their central carbon metabolism under sub-optimal growth conditions (Fredlund et al., 2004b; Kruis et al., 2018; Löser et al., 2014). High concentrations of ethyl acetate also repress the growth of competitive organisms (Fredlund et al., 2002, 2004c). In some bacterial species volatile esters serve as metabolic intermediates during growth on alkanes or cyclic alcohols (Iwaki et al., 2002; Kotani et al., 2007). Wax esters are produced as intracellular storage compounds in *Acinetobacter baylyi* and *Marinobacter hydrocarbonoclasticus* (Ishige et al., 2003; Wältermann and Steinbüchel, 2005). Wax esters are also produced as an anaerobic fermentation product in *Euglena gracilis* (Muller et al., 2012). Volatile esters, such as isoamyl acetate are produced to help yeast disperse in the environment by attracting insects (Christiaens et al., 2014). Some specialised esters can even act as bacterial virulence factors (Onwueme et al., 2005). Because of the structural and chemical diversity of esters, no singular physiological role can be defined for ester synthesis. Some of the proposed roles are even still debated. Nonetheless, natural ester formation in microorganisms has been applied in industry, most notably 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-flavours when present in high amounts (Liu et al., 2004). Ethyl acetate is the most abundant volatile ester in food. Its concentration ranges from 52 to 99 mg/L in dairy products and from 8 to 63.5 mg/L in beer and wine (Liu et al., 2004; Saerens et al., 2010). Other volatile esters, such as isoamyl acetate, phenylethyl acetate, ethyl hexanoate and many others do no exceed concentrations above 1 mg/L. These concentrations lie around or just above the human detection threshold (Dzialo et al., 2017; Saerens et al., 2010) and therefore greatly affect the aroma of food products.

The amounts of esters naturally produced by microorganisms from sugars are typically low, although some exceptions exist, such as bulk ethyl acetate-producing yeast and wax ester accumulation in *Euglena gracilis*. This microalgae 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 synthesise ethyl acetate from sugars or ethanol (Armstrong et al., 1984a; Löser et al., 2014; Meersman et al., 2016; Tabachnick and Joslyn, 1953a, 1953b; 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; Kruis et al., 2018; Urit et al., 2012). 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 described in most detail. Several strains have been identified that form ethyl acetate from whey sugars (Kallelmhiri et al., 1993; Löser et al., 2011), glucose (Willetts, 1989), and cassava bagasse supplemented with glucose (Medeiros et al., 2001). *K. marxianus* is able to catabolise lactose and can utilise whey, a side stream of the cheese industry to produce ethyl acetate. Moreover, the 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., 2014, 2011; Urit et al., 2013b).

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., 2013a).

Ester forming reactions

Four main enzymatic ways of ester biosynthesis have been described; esterases (Figure 2a), hemiacetal dehydrogenation (HADH, Figure 2b), Baeyer-Villiger monooxygenases (BVMOs, Figure 2c) and alcohol acyltransferases (AATs, Figure 2d). The reactions of AATs and esterases are redox neutral. On the other hand, BVMOs and HADH require NAD(P)H or NAD(P) to catalyse ester production, respectively (Figure 2). Other ester-forming enzyme classes exist, but are not discussed in detail in this review. These include S-adenosyl methionine (SAM) dependent O-methyltransferases and polyketide synthase associated proteins (Menendez-Bravo et al., 2017, 2014; Sherkhanov et al., 2016).

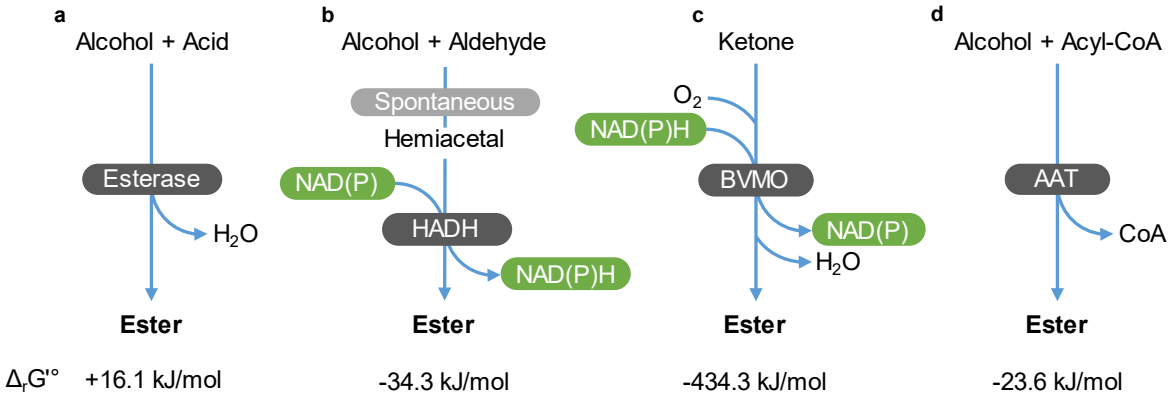

Figure 2. Enzymatic pathways that result in ester production. The $\Delta_rG'°$ of the reactions were estimated using Equilibrator (Flamholz et al., 2014) under standard conditions and pH 7. Abbreviations: AAT – alcohol acyltransferase, HADH – hemiacetal dehydrogenation, BVMO – Baeyer-Villiger monooxygenase

Esterases

Esterases, including lipases, are ubiquitous enzymes that have been identified in all domains of life. In aqueous environments, they catalyse the hydrolysis of ester bonds, resulting in the formation of an alcohol and a carboxylic acid. Water-content, 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 unfavourable (Figure 2a). Hence, industrial production of esters via esterases is typically performed in non-aqueous systems using organic solvents or high substrate concentrations (Bornscheuer, 2018; Khan and Rathod, 2015). Nonetheless, several studies have reported *in vivo* reverse esterase activity as the cause of ester formation by microorganisms (Abeijón Mukdsi et al., 2018, 2009; Costello et al., 2013; Hong et al., 2018; Rojas et al., 2002). 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 characterised and showed significant reverse esterase activity *in vitro* (Kashima et al., 1998). However, when the experimental conditions of the reverse esterase assay (pH 3.0, 0.5 M ethanol, 25 mM acetate) are included in a thermodynamics calculation (Flamholz et al., 2014), the $\Delta_r G'$ of the reaction becomes negative, making it thermodynamically favourable, but only when ester concentrations are low.

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 catalyse the reaction are in some cases referred to as hemiacetal dehydrogenases (M Kusano et al., 1998; Kusano et al., 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 et al., 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. Aldehydes are toxic compounds for most organisms (Kunjapur and Prather, 2015). Hemiacetal dehydrogenation may act as mechanism to detoxify aldehydes by converting them to esters via hemiacetals (Yurimoto et al., 2005). This activity has been observed for methyl formate synthesis in *Pichia methanolica*, *Candida boidinii* and *S. cerevisiae* (M Kusano et al., 1998; Murdanoto et al., 1997b; Yurimoto et al., 2004). Hemiacetal dehydrogenation may also contribute to ethyl acetate formation in *Neurospora crassa, S. cerevisiae, Cyberlindnera jadinii,* and *Kluyveromyces marxianus*, but this has not

been confirmed *in vivo* (Kusano et al., 1999; M Kusano et al., 1998; Löbs et al., 2017; Park et al., 2007).

Baeyer-Villiger monooxygenases

BVMOs are flavin-containing enzymes that require NAD(P)H to perform their reaction. They catalyse 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 et al., 2015). In nature, BVMOs participate in the synthesis of secondary metabolites (Frank et al., 2007; Wen et al., 2005) or enable utilisation of unconventional carbon sources, such as alkanes, ketones or cyclic alcohols (Britton and Markovetz, 1977; Iwaki et al., 2002; van der Werf and Boot, 2000). They catalyse the conversion of ketones to esters, which are further hydrolysed to readily metabolisable alcohols and acids. Such pathways enable a *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 (de Gonzalo et al., 2010; Fraaije et al., 2002; Kamerbeek et al., 2003; Leisch et al., 2011; Torres Pazmiño et al., 2010).

Alcohol acyltransferases

AATs are a large and diverse group of enzymes. They are the main source of ester production in plants, yeast, filamentous fungi and some bacteria (Aharoni et al., 2000; Beekwilder et al., 2004; Holland et al., 2005; Nagasawa et al., 1998; Saerens et al., 2006; Shalit et al., 2001; Stribny et al., 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 2). 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 (Kruis et al., 2017; Shi et al., 2012; Stribny et al., 2016).

Table 2: Characteristics of the most studied and applied microbial AATs.

	AtfA (WS)	Atf1	Atf2	Eht1	Eeb1	Eat1
Source, *species examples*	Bacteria, *A. baylyi*	Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*	Yeast, *S. cerevisiae*	Yeast, *W. anomalus* *K. marxianus*
Typical ester product	Wax esters and TAG	Acetate esters of various alcohols	Acetate esters of various alcohols	MCFA ethyl esters	MCFA ethyl esters	Ethyl acetate, other acetate esters
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)	MCFA detoxification (Saerens et al., 2006)	MCFA detoxification (Saerens et al., 2006)	Relieving acetyl-CoA accumulation (Kruis et al., 2018; Löser et al., 2014)
Cellular location	Lipophilic inclusion in the bacterial cytosol (Stöveken et al., 2005)	ER, Cyt. lipid droplets (Lin and Wheeldon, 2014; Verstrepen et al., 2004)	ER, Cyt. lipid droplets (Lin and Wheeldon, 2014)	ER, Mit. outer membrane (Huh et al., 2003; Zahedi et al., 2006)	Unknown	Mitochondria (Huh et al., 2003; Kruis et al., 2018)
[2]Protein family	WES acyl-transferase-like domain (PF03007)	AATase domain (PF07247)	AATase domain (PF07247)	α/β-hydrolase family 4	α/β-hydrolase family 4	α/β-hydrolase family 6
Catalytic or conserved regions	HXXXXD, DFGWG (Rottig and Steinbuchel, 2013)	HXXXXD (Nancolas et al., 2017)	HXXXXD (Nancolas et al., 2017)	Ser-Asp-His triad (Saerens et al., 2006)	Ser-Asp-His triad (Saerens et al., 2006)	Ser-Asp-His triad (Kruis et al., 2017)
Side activities	Unknown	Thioesterase (*in vitro*) (Nancolas et al., 2017)	Unknown	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, Kruis et al. man. in prep.)
[1]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)	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. in submission)
[1]Acyl-CoA specificity	Broad: Long acyl-CoA (C14-C18), also shorter and longer acyl-CoA (*in vitro*) (Stöveken et al., 2005)	Acetyl-CoA only (*in vitro*) (Nancolas et al., 2017)	Acetyl-CoA *(in vivo)* (Tiwari et al., 2007; Verstrepen et al., 2003b)	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*), Propionyl-CoA (*in vivo*) (Kruis et al. in submission)

1 – Acyl-CoA and alcohol specificities were taken from *in vivo* studies when *in vitro* information was not available. 2 – The PF number represent the classification according to PFAM (Bateman et al., 2000), the α/β-hydrolase family number are taken according to the classification in the ESTHER database (Lenfant et al., 2013). Abbreviations: TAG – triacylglycerides MCFA – medium chain fatty acid, AATase – alcohol acyltransferase ER – endoplasmic reticulum WES – wax ester synthase

Much research on ester producing AATs has focused on yeast (Table 2). One of the few other well studied microbial AATs is the TAG and wax ester-producing AtfA (sometimes referred to a wax synthase – WS) from *Acinetobacter baylyi*. (Rottig and Steinbuchel, 2013; Stöveken et al., 2005). Five yeast AATs have been described thus far. The *S. cerevisiae* Eht1 and Eeb1 produce MCFA ethyl esters as a way to detoxify MCFAs that accumulate during fatty acid synthesis (Saerens et al., 2006; Tehlivets et al., 2007). The *S. cerevisiae* Atf1 and Atf2 are responsible for a large part of acetate ester production in this yeast (Verstrepen et al., 2003b). Acetate ester formation by *S. cerevisiae* Atf1 was associated to attracting insects as a means of dispersing 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 recently discovered Eat1 family contributes to the synthesis of acetate esters as well, particularly the high amounts of ethyl acetate produced by yeasts such as *K. marxianus* and *W. anomalus* (Kruis et al., 2017; Löser et al., 2014). Bulk ethyl acetate production was associated to suboptimal growth conditions under which excess acetyl-CoA accumulates in yeast mitochondria (Löser et al., 2015; Thomas and Dawson, 1978). Ethyl acetate synthesis by Eat1 may relieve this accumulation by converting acetyl-CoA to ethyl acetate. The localisation of Eat1 in yeast mitochondria supports this hypothesis (Huh et al., 2003; Kruis et al., 2018). The cellular location of the remaining AATs has not been clearly linked to their proposed biological functions. The *S. cerevisiae* Atf1 and Atf2 localise to the endoplasmic reticulum (ER), but are also associated to lipid droplets in the cytosol (Huh et al., 2003; Lin and Wheeldon, 2014; Verstrepen et al., 2004). Curiously, Atf1 and Atf2 homologs from *W. anomalus* and *K. lactis* did not localise to lipid droplets when expressed in *S. cerevisiae* (Lin and Wheeldon, 2014). Eht1 was traced to the ER and to the outer mitochondrial membrane of *S. cerevisiae* (Huh et al., 2003; Zahedi et al., 2006), while the location of its paralog Eeb1 has not been determined (Table 2).

The catalytic mechanisms of AATs 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 (Galaz et al., 2013; Morales-Quintana et al., 2011; Navarro-Retamal et al., 2016). Microbial AATs belong to two structurally

unrelated protein families (Table 2). 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 characterised in plants (D'Auria, 2006). Its members share the conserved HXXXD and DFGWG motifs (D'Auria, 2006; Galaz et al., 2013) Curiously, Atf1 and Atf2 lack the DFGWG motif (Nancolas et al., 2017), which is most likely not directly involved in the catalytic mechanism (Morales-Quintana et al., 2015, 2013). It should be noted that Atf1 and Atf2 are not closely related to AtfA (Rottig and Steinbuchel, 2013) and they belong to different AAT subfamilies (Table 2). 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 (Knight et al., 2014; Kruis et al., 2017; Saerens et al., 2006). This fold is typical for esterases, a large group of hydrolytic enzymes that includes proteases, lipases and peroxidases (Rauwerdink and Kazlauskas, 2015). Eht1 and Eeb1 are only distantly related to Eat1 (Kruis et al., 2017).

Despite their structural differences, AATs show remarkably similar catalytic (mis)behaviour. Thioesterase and/or esterase activities have been observed in Eht1, Eeb1 and Eat1 *in vitro* (Table 2). For *W. anomalus* and *K. marxianus* Eat1, ethyl acetate hydrolysis was also observed *in vivo* (Kruis et al., manuscript in preparation). 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). However, thioesterase activity has also been demonstrated in the *S. cerevisiae* Atf1 (Nancolas et al., 2017), even though it does not resemble α/β-hydrolases. It was able to act as a thioesterase on longer acyl-CoA substrates, while it could only utilise acetyl-CoA to produce esters in the AAT reaction. While the 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* and *in vivo* (Kruis et al., 2017, Kruis et al. manuscript in preparation). 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 and the unwanted behaviour of AATs should be investigated further to prevent unwanted product or substrate degradation.

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 such cases, *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 localisation of the AAT may mask the true substrate specificity of the enzyme, which can only be determined *in vitro*. Several enzyme assays have been developed that couple the release of CoA during the AAT reaction (Figure 2d) to NADH formation, or a coloured reaction (Knight et al., 2014; Kruis et al., 2017; Lin et al., 2016). Given the current knowledge that AATs can often display thioesterase activity, care should be taken in interpreting the outcomes 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 hydrolyse longer acyl-CoA substrates as a thioesterase (Nancolas et al., 2017). Direct measurements of the ester products may provide a reliable alternative (Kruis et al., 2017; Nancolas et al., 2017; Stöveken et al., 2005).

AATs seem to be relatively unspecific towards the alcohol substrate (Table 2). For instance, the *S. cerevisiae* Atf1 and Atf2 have a broad specificity towards primary alcohols *in vitro* (Nancolas et al., 2017; Stribny et al., 2016). This was reflected *in vivo* when the genes were overexpressed in *S. cerevisiae* and led to a broad increase in acetate ester production (Rodriguez et al., 2014; Verstrepen et al., 2003b). The overexpression of several *eat1* genes from different yeasts in *S. cerevisiae* also resulted in increased levels of various acetate esters (Kruis et al., submitted). AtfA shows a particularly broad substrate specificity towards alcohols and can accept 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

synthesise ethyl esters *in vivo* (Knight et al., 2014; Saerens et al., 2006; van Nuland et al., 2017a). Recently, Eeb1 was shown to utilise 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 et al., 2016). Even the antibiotic resistance marker chloramphenicol acetyltransferase (Cat), which normally acetylates the antibiotic chloramphenicol, was able to form low amounts of esters in *E. coli*. (Rodriguez et al., 2014).

The specificity of AATs towards their acyl-CoA is higher compared to alcohols in some cases. *S. cerevisiae* Atf1 utilised acetyl-CoA exclusively 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 much lower compared to the variety of accepted alcohols (Table 2) (Stöveken et al., 2005). The *S. cerevisiae* Eht1 and Eeb1 show a broader range of MCFA-CoA substrates they can accept *in vitro* and *in vivo* (Table 2). 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). Since then it has been determined that the *S. cerevisiae* Eht1 is more active towards octanoyl-CoA (Knight et al., 2014; Lin et al., 2016). Nevertheless, all studies agree that the *S. cerevisiae* Eht1 and Eeb1 are show activity against a broad range of MCFA-CoA substrates. In one study, the *S. cerevisiae* Eht1 was even able to utilise caffeoyl-CoA, which contains an aromatic ring in its structure (Wang et al., 2017).

Most studies on the AAT activity and specificity have been limited to a small set of enzymes (Table 2). The increasing number of genome sequencing projects has enabled the identification of a number of AAT homologs from other organisms. For example, since the initial identification of the Eat1 family (Kruis et al., 2017), at least five new yeast species have been sequenced that contain putative homologs of *eat1.* However, most newly identified AAT enzymes remain to be experimentally characterised. The homologs of the *S. cerevisiae* Atf1 in *Saccharomyces pastorianus, Kluyveromyces lactis* and *W. anomalus* have been studies to some extent. They evoked an increase in acetate

ester production similar to the *S. cerevisiae* Atf1 or Atf2 (Kruis et al., 2017; Van Laere et al., 2008; Verstrepen et al., 2003b). However, homologs of the same enzyme in different yeasts can exhibit altered specificities for their substrates. For example, the Atf1 and Atf2 homologs of *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* differed in their alcohol preferences compared to the *S. cerevisiae* Atf1 and Atf2 (Stribny et al., 2016). Similar differences in fatty acid ethyl ester (FAEE) formation-capacities were observed when various homologs of the *A. baylyi* AtfA were overexpressed in *E. coli* (Röttig et al., 2015). Enzymes with altered substrate specificities may enable the development of novel metabolic engineering strategies.

Metabolic engineering of *de novo* ester synthesis

Competing with the petrochemical industry is challenging due to the low 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 competitive biobased production processes. Considerable efforts have therefore been invested into improving this production through metabolic engineering. The crucial factors in any metabolic engineering strategy are the selection of a suitable catalyst and a sufficient supply of metabolic precursors.

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; Kumar et al., 2016; Levisson et al., 2009; Stergiou et al., 2013). However, the reverse esterase reaction is thermodynamically unfavourable 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 conditions (pH 7).

BVMOs have been used extensively for the biotransformation of cyclic ketones into lactones (cyclic esters) (Leisch et al., 2011). Several BVMOs exist that are also active towards aliphatic ketones, and are interesting catalysts for the production of volatile esters (Ceccoli et al., 2017; Pereira et al., 2018; Rehdorf et al., 2009). However, most bioconversions via BVMOs were performed in cell free systems. There are only a few reports where BVMOs were used *in vivo* for the formation of aliphatic esters (Pereira et al., 2018). This may be because BVMOs are often difficult to functionally express *in vivo* (Seo et al., 2016). Moreover, BVMO-catalysed ester production depends on the supply of ketones, which are not common microbial metabolites. For these reasons, esterase and HADH and BVMO reactions are not discussed further in this review as options for *in vivo* metabolic engineering of ester synthesis.

AATs have dominated the field of metabolic engineering of *in vivo* ester production. This reaction is thermodynamically favourable 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 to 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. Several studies have added precursors for ester synthesis, such as acids or alcohols to the cultivation media (Gao et al., 2018; Horton and Bennett, 2006; Kruis et al., 2017; Rodriguez et al., 2014; Singh et al., 2007; Vadali et al., 2004). Such studies have been useful for studying ester formation capacities of e.g. engineered strains. However, *de novo* ester synthesis from renewable substrates, such as glucose is often preferred. To ensure efficient alcohol, acyl-CoA and ester synthesis from glucose, the microbial metabolism needs to be modified (Figure 3).

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 and NADPH), and metabolic energy (ATP) are released (Figure 3a). In the presence of oxygen, ATP is also produced in the Electron transport chain (ETC). The carbon compounds, reducing equivalents, and ATP are subsequently used to produce acyl-CoAs (Figure 3b) and alcohols (Figure 3c). Finally, the ester is formed by an AAT (Figure 3d).

Several variants of glycolysis exist (Figure 3a). The main differences between them are the number and types of reducing equivalents produced, and their ATP yield (X. Chen et al., 2016; Stincone et al., 2015). The Embden-Meyerhof-Parnas (EMP) pathway is the conventional glycolytic pathway, which produces NADH and yields 2 ATP per glucose (Figure 3a). A parallel glycolytic route is the pentose phosphate pathway (PPP), which has a lower ATP yield, but produces exclusively NADPH (Figure 3a). A third, less established glycolytic pathway is the Entner-Duodoroff (ED) pathway, present in e.g. *Pseudomonas putida* and *Zymomonas mobilis* which produces both NADH and NADPH (Rutkis et al., 2013; Sudarsan et al., 2014). The final product of these glycolytic pathways is pyruvate, which can be used for e.g. alcohol synthesis (Figure 3c), or converted further to acetyl-CoA (Figure 3a).

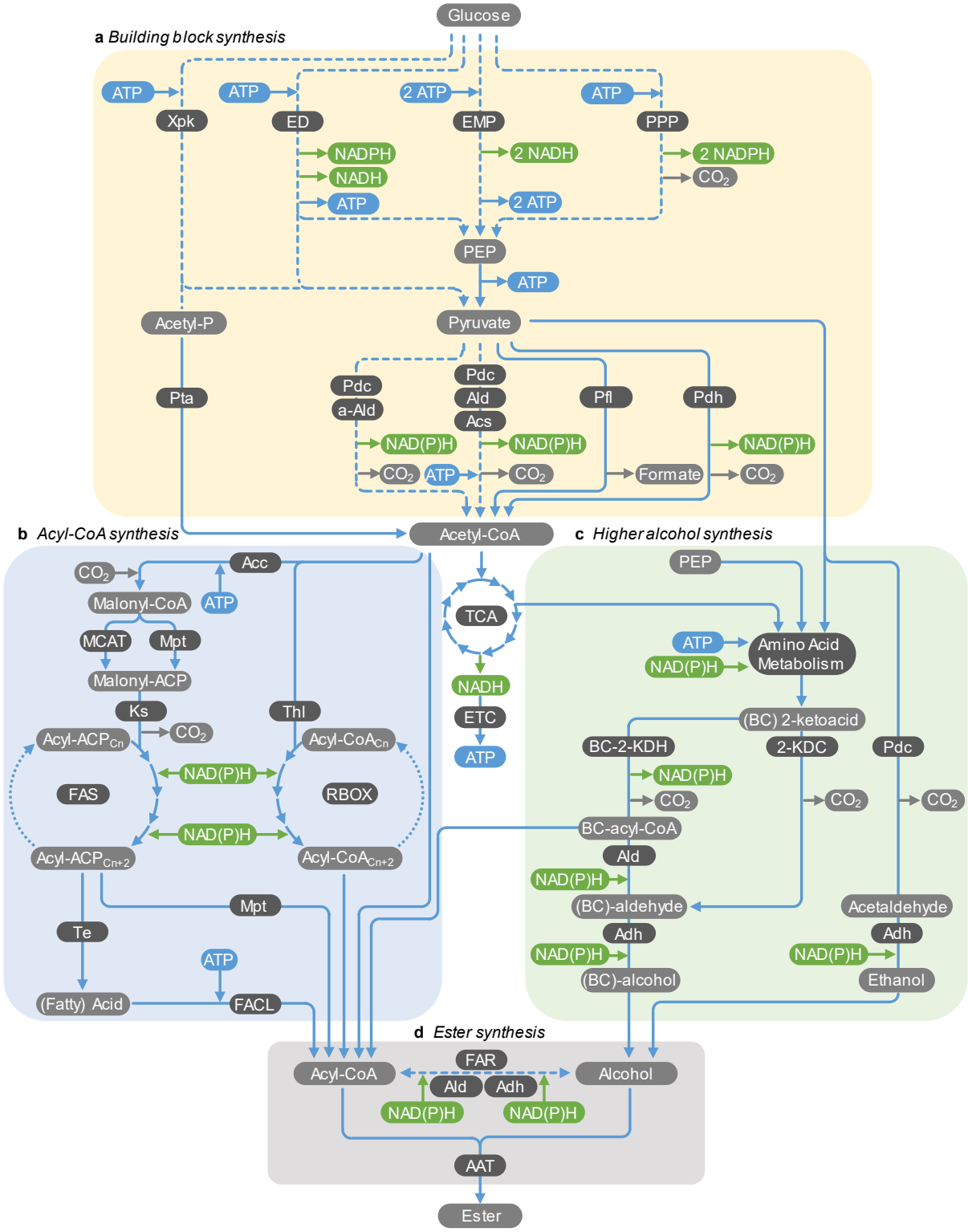

Figure 3: Overview of the general metabolic pathways converting glucose to esters. (a) – Formation of basic carbon building blocks, redox equivalents and ATP from glucose. (b) – Generalised formation of longer chain acyl-CoAs from acetyl-CoA. (c) – Generalised formation of branched alcohols from 2-ketoacids. (d) The final ester formation reaction catalysed by an AAT. Also shown is the interconversion of acyl-CoAs and alcohols. Abbreviations: NOG – non-oxidative glycolysis. ED-Entner-Duodoroff pathway, EMP – Embden-Meyerhof-Parnas pathway, PPP – Pentose phosphate pathway, Xpk – phosphoketolase, MCAT – Malonyl CoA-

acyl carrier protein transacylase, PEP - Phophoenolpyruvate, Pta – phopshotransacetylase, Pdc – Pyruvate decarboxylase, a-Ald – acetylating acetaldehyde dehydrogenase, Ald – acetaldehyde dehydrogenase, Acs – acetyl-CoA synthetase, Pfl – pyruvate formate lyase, Pdh – pyruvate dehydrogenase, ACP – Activated carrier protein, Acc – acetyl-CoA carboxylase, Ks – ketoacyl synthase, Thl - thiolase. FAS – fatty acid synthesis, RBOX – reverse β-oxidation, Te - thioesterase, Mpt – malonyl/palmitoyl transferase, Facl – fatty acid CoA ligase, TCA – tricarboxylic acid cycle, ETC – electron transport chain, BC-– branched chain, BC-2-KDH – branched chain 2-ketoacid dehydrogenase, 2-KDC – 2-ketoacid decarboxylase, Adh – alcohol dehydrogenase, FAR – Fatty acid reductase, AAT – alcohol acetyl transferase. Dashes indicate multi-enzyme conversion pathways.

Acetyl-CoA can be used directly to produce a variety of acetate esters, or it can be condensed into longer acyl-CoA moieties (Figure 3b) (Barney, 2014; Park et al., 2009). The oxidative decarboxylation of pyruvate to acetyl-CoA has been engineered and reviewed extensively (Krivoruchko et al., 2015; van Rossum et al., 2016b). Pyruvate formate lyase (Pfl) and Pyruvate dehydrogenase (Pdh) catalyse the conversion in a single enzymatic step. Other pathways convert pyruvate to acetyl-CoA in a series of reactions (Figure 3a). 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-aAld) to produce acetyl-CoA directly (Figure 3). 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 (Knappe and Sawers, 1990). Since formate is not fully oxidised, no extra reducing equivalents are released in the reaction (Figure 3a). 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 3a) (Bocanegra et al., 1993; Inui et al., 1989; Miyagi et al., 2009).

Most acetyl-CoA forming reactions do not consume ATP, with the exception of acetyl-CoA formation via Pdc-Ald-Acs (Figure 3a). This pathway is the main acetyl-CoA forming pathway the *S. cerevisiae* cytosol (Zaldivar et al., 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 (aAld) or Pdh (Figure 3a) (Kozak et al., 2014a; van Rossum et al., 2016a). The availability of cytosolic acetyl-CoA in yeast has also been increased by introducing the ATP-citrate lyase, which converts mitochondrial-derived citrate to acetyl-CoA and oxaloacetate at the cost of ATP. Expression of a phosphoketolase (Xpk) also led to 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 3a).

The requirements for acetyl-CoA, reducing equivalents, and ATP supply are determined by the biosynthetic pathways leading to the acyl-CoA and alcohol (Figure 3cb). These compounds are biotechnologically relevant for a number of applications, such as production of sustainable chemical building blocks, fragrances or biodiesels. Their biobased production has been the focus of numerous studies which have been reviewed extensively (Abbott et al., 2009; Beld et al., 2015; L. Chen et al., 2016; Choi et al., 2014; Marella et al., 2018; Nozzi et al., 2014; Sheng and Feng, 2015). The metabolic engineering approaches that were used for engineering acyl-CoA and alcohol supply can also be extended to ester synthesis.

Synthesis of long and branched acyl-CoA substrates

The microbial metabolism is able to produce a variety of structurally diverse acyl-CoA substrates. Linear acyl-CoAs are produced from acetyl-CoA and are based on fatty acid metabolism (Figure 3b). Two main pathways have been used to produce (fatty) acyl-CoAs *de novo*; fatty acid synthesis (FAS) and the reversed β-oxidation (RBOX) (Figure 3b). 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 3b). 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 two carbon atoms per cycle (Figure 3b). 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 (Johansson et al., 2009; Schweizer and Hofmann, 2004). The free fatty acid can be activated to an acyl-CoA by a fatty acid CoA-ligase (FACL) at the cost of ATP (Figure 3b) (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 disrupting the catabolic β-oxidation pathway that degrades fatty acyl-CoAs, increasing the activity of rate limiting steps, such as acetyl-CoA carboxylase (Acc) (Figure 3b), or removing the regulatory mechanisms that repress the FAS cycle (Beld et al., 2015; Duan et al., 2011; Pan et al., 2017; Runguphan and Keasling, 2014; Valle-Rodríguez et al., 2014).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 3a) in the cytosol of *Y. lipolytica* increased the lipid titre 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 3a). To circumvent the carbon loss, the metabolism of the yeast was rewired for more efficient NADPH supply. This allowed reaching the highest lipid titre to date of 99 g/L (Qiao et al., 2017).

The reversed β-oxidation (RBOX) provides an energetically efficient 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 oxidises fatty acyl-CoAs (Houten and Wanders, 2010). There are three key differences between the RBOX and FAS reactions (Kallscheuer et al., 2017). The RBOX is energetically more efficient because the Thiolase (Thl)-catalysed entry of acetyl-CoA into the cycle avoids the ATP-consuming malonyl-CoA formation (Figure 3b). In the RBOX, NADH instead of NADPH is used to reduce the growing acyl-CoA. Lastly, the RBOX operates via CoA,

and not ACP-intermediates (Figure 3b). 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 NADH while the reaction is ATP neutral (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 RBOX 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 (Clomburg et al., 2015; Dellomonaco et al., 2011; Kallscheuer et al., 2017; Kim et al., 2016).

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 3c). They are derived from BC-2-ketoacids, which are in turn derived from the amino acid metabolism (Hazelwood et al., 2008). BC-amino acids can be converted to BC-2-ketoacids and further to BC-acyl-CoAs. The reaction is catalysed by the BC-2-ketoacid dehydrogenase (BC-2-KDH) and is analogous to acetyl-CoA formation by Pdh (Figure 3ac) (Mooney et al., 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 et al., 2014). BC-acyl-CoAs can also be generated by converting BC-alcohols via an appropriate aldehyde dehydrogenase (Ald) and alcohol dehydrogenase (Adh) (Rodriguez et al., 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 3abc). 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) catalyse the reactions, respectively (Figure 3ac). In *E. coli*,

ethanol is produced via oxidative decarboxylation of pyruvate to acetyl-CoA by pyruvate dehydrogenase (Pdh) (Figure 3a). In the next step, acetyl-CoA is reduced with 2 NAD(P)H, first to acetaldehyde, and then to ethanol (Figure 3d). The reactions are catalysed 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 convert pyruvate to ethanol via acetyl-CoA are analogous to the steps that form higher and fatty alcohols. 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 3cd) (Becerra et al., 2015). Fatty acyl-CoAs that are produced in the FAS and RBOX cycles (Figure 3b) 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 et al., 2013) (Figure 3d). By introducing FAR genes into *S. cerevisiae*, fatty alcohols, such as 1-hexadecanol could be produced from fatty acyl-CoAs (Wang et al., 2016).

Alcohols, such as 1-propanol, isobutanol, isoamyl alcohol, 1-butanol and 2-phenylethanol are derived from 2-ketoacids (also referred to as the 2-keto pathway). This pathway is analogous to the conversion of pyruvate to ethanol via Pdc and Adh (Peralta-Yahya et al., 2012). As discussed above, 2-ketoacids originate from the amino acid metabolism (Figure 3c). Amino acids or their precursors can be converted to specific 2-ketoacids, which are decarboxylated by an 2-ketoacid decarboxylase (2-KDC) to an aldehyde and reduced to an alcohol by an Adh (Figure 3c) (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 et al., 2014; Tai et al., 2015; Yuan et al., 2016). This approach enabled the production of 22 g/L isobutanol from glucose at 86 % of the maximum yield (Atsumi et al., 2008). The synthesis of amino acid-derived alcohols relies heavily on the supply of NADPH (Figure 3c). 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

Engineered ester production invariably requires the supply of alcohols, acyl-CoAs and the selection of a suitable AAT. Microbial AATs (Table 2) have been used extensively in this context. Plant AAT genes have also been expressed in microbial hosts to evoke ester production (Horton and Bennett, 2006; Layton and Trinh, 2016a; Noh et al., 2018). These AATs are not included in this review. The selection of the AAT with the appropriate specificity depends on the alcohol and the acyl-CoA substrates. For example, the *S. cerevisiae* Atf1 and Atf2 are specific for acetyl-CoA, but not alcohols and have been used to produce a variety of acetate esters (Horton and Bennett, 2006; Layton and Trinh, 2016b; Rodriguez et al., 2014; Vadali et al., 2004; van Nuland et al., 2017b). On the other hand, *A. baylyi* AtfA and its homologs have been used to engineer the production of wax esters and FAEE (Table 3). The selection of an efficient AAT catalyst is critical. Many studies therefore perform a screening of several AATs before selecting the optimal one (Röttig et al., 2015; Tai et al., 2015). Other engineering approaches targeting the AAT reaction are not commonplace, but they can be effective. For example, co-localising the *S. cerevisiae* Atf1 with the acetyl-CoA generating Ald-Acs pathway (Figure 3a) resulted in a 2-fold increase in ethyl acetate formation (Table 3) (Lin et al., 2017). In another study, removing the destabilising N-terminal sequence of two Eat1 homologs improved the *in vivo* performance of the AATs by 11-fold (Kruis et al. manuscript in preparation).

Most engineering efforts have focused on designing optimal metabolic pathway towards ester production (Table 3). A common first step is the removal of competing pathways (Shen et al., 2011; Wu et al., 2017). As a consequence, the carbon flux is channelled towards the desired products. Established modifications that lead away from common by-products, such as lactate and acetate in *E. coli* are not discussed in detail here and are also not indicated in Table 3. Another commonly utilised strategy to improve ester production is to increase the availability of basic building blocks, such as acetyl-CoA or reducing equivalents. By expressing the Xpk-Pta pathway (Figure 3a) in *S. cerevisiae*, the production of FAEE could be improved 1.6-fold (Table 3). This improvement was attributed to the combined effect of additional acetyl-CoA and NADPH supply, which fuel the FAS cycle (de Jong et al., 2015). Increased precursor

supply could also be achieved by moving the *A. baylyi* AtfA to the endoplasmic reticulum or peroxisome where they are more abundant (Table 3). This strategy resulted in a 15- to 19-fold improved FAEE synthesis.

The redox equivalent-forming reactions (Figure 3a) can also influence the efficiency of ester production. For example, the conversion of 1 mol glucose to 1 mol ethyl acetate generates an excess of reducing equivalents. In yeast, these equivalents are produced in the form of NADH. To maintain the redox balance of the cell, they must be oxidised in the ETC (Figure 3), which requires oxygen to function. Ethyl acetate production in yeast can therefore only proceed under aerobic conditions, which may be economically disadvantageous (Garcia-Ochoa and Gomez, 2009). In contrast, bacteria such as *E. coli* can produce formate instead of NADH during acetyl-CoA formation by Pfl (Figure 3a). In this pathway, no NADH accumulates *E. coli* can therefore synthesise ethyl acetate efficiently under anaerobic conditions (Table 3) (Kruis et al., manuscript in preparation). By using the *W. anomalus* Eat1, 63.4 % of the maximum theoretical yield was achieved under anaerobic conditions (Table 3).

Efficient ester synthesis often requires more than just increased availability of basic building blocks or redox equivalents. This became clear when 2-KDC (Figure 3c) was introduced in *E. coli*. This enzyme catalyses the step in the 2-keto pathway which converts 2-keto acids to higher aldehydes that can then be converted to higher alcohols (Figure 3c). When a 2-KDC enzyme was introduced in *E. coli* along with the *S. cerevisiae* Atf1, the acetate ester amounts detected were below 30 mg/L (Table 3) (Rodriguez et al., 2014). Efficient ester synthesis only commenced after the entire 2-keto pathway towards isobutanol production was introduced (Atsumi et al., 2008; Rodriguez et al., 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 et al., 2015).

Table 3: Overview of notable successes in metabolic engineering of microbial ester production.

Target ester	Organism	Goal/Strategy	AAT catalyst	Acyl-CoA supply	Alcohol supply	Effect/Titre	Notes	References
2-Methyl-1-butyl acetate, Phenylethyl acetate	*E. coli*	Acetate ester prod.	Sce Atf1	Native Acetyl-CoA	Introduction of only 2-KDC	app. 20 mg/L		(Rodriguez et al., 2014)
Isobutyl acetate	*E. coli*	Isobutyl acet. prod.	Sce Atf1	Native Acetyl-CoA	2-keto pathway to isobutanol	17.2 g/L	80% of th. max. yield, Hexadecane layer	(Rodriguez et al., 2014)
Isobutyl acetate	*E. coli*	Isobutyl acet. prod.	Sce Atf1	Native Acetyl-CoA	2-keto pathway for isobutanol	36 g/L	42 % max. yield	(Tai et al., 2015)
Isoamyl acetate	*E. coli*	Isoamyl acet. prod.	Sce Atf1	Native Acetyl-CoA	2-keto pathway to isoamyl al.	386 mg/L	10.8% th. max.	(Tai et al., 2015)
Ethyl acetate	*E. coli*	Anaerobic ester production	Wan Eat1	Native Acetyl-CoA (via Pfl)	Native ethanol	3.3 g from 11 g glucose	63% of max, Gas stripping	Kruis et al. man. in prep.
Ethyl acetate	*S cerevisiae*	Pathway colocalization	Sce Atf1	Ald-Acs targeted to Atf1 (lipid droplet)	Native ethanol	2-fold increase		(Lin et al., 2017)
Isobutyl Isobutyrate	*E. coli*	Branched acid esters prod.	Sce Eht1 Cat	Branched acyl-CoA synthesis via BC-2-KDH,	Native conversion of isobutyryl-CoA to isobutanol	27.1 mg/L		(Rodriguez et al., 2014)
MCFA ethyl ester	*S cerevisiae*	Implement. RBOX	Native AATs	Introduced RBOX	Native ethanol	Increased MCFA ethyl esters	Quantity not reported	(Lian and Zhao, 2015)
FASBE	*S cerevisiae* *E. coli*	Combination of FAS and 2-keto pathway	WS	Δ repressor of fatty acyl-CoA synthesis OR Δ β-oxidation	2-keto pathway to isobutanol, isoamyl alcohol, amyl alcohol	230mg/L FASBE 1000 mg/L FASBE	Combination of higher alcohol synthesis and fatty acyl-CoA production	(Guo et al., 2014; Teo et al., 2015)
FAEE	*E. coli*	Increased FAS activity	Aba AtfA	FAS – overexpressed thioesterase and MCAT – initiating step to FAS	Introduced Pdc-Adh	674mg/L FAEE (C12-C18)	9.4% of th. max yield	(Steen et al., 2010)
FAEE	*S cerevisiae*	Additional acetyl-CoA and NADPH	Mhy WS2	FAS Xpk+Pta bypass	Native ethanol	4.6 mg/g CDW	1.6-increase	(de Jong et al., 2014)
FAEE	*Y. lipolytica*	Targeting to Per or ER	Per-AbAtfA ER-AbAtfA	Native acyl-CoA metab. in compartments	Native ethanol	110.9 mg/L 136.5 mg/L	15-fold increase 19-fold increase	(Xu et al., 2016)
WE	*A baylyi*	Novel FAR	Native WS	Native fatty acyl-CoA	Overexpression of novel FAR	0.45 g/l	Highest WE titre to date on glucose only	(Lehtinen et al., 2018)
WE	*A baylyi*	Product diversification	Native WS	Native fatty acyl-CoA	Changed by Pflu LuxCDE	Shift from C18 to C16 WE		(Santala et al., 2014)

The metabolic modifications are described. Routine gene disruptions aimed at common by-products are not listed and can be found in the original references. Abbreviations: FASBE – fatty acid short- and branched-chain esters, Wan – *W. anomalus,* Sce – *S. cerevisiae*, Cat – chloramphenicol acetyltransferase, Fve – *Fragarria vesca*, WS – wax synthase, Aba – *Acinetobacter baylyi*, Mhy – *Marinobacter hydrocarbonoclasticus*, Per - peroxisome, WE – Wax esters, ER – Endoplasmic reticulum, Pflu – *Pseudomonas fluorescens*

The production of FAEE in *E.* coli and yeast can be limited by the supply of fatty acids in a similar way. The activity of the 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 committing steps in the pathway, Malonyl CoA-acyl carrier protein transacylase (MCAT) as well as the final Te that terminated the FAS cycle (Figure 3b). 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).

The production of esters via the AAT reaction is flexible as any alcohol can hypothetically be reacted with any acyl-CoA. This gives much space for product diversification and the design of novel esters that may not exist in nature (Table 2). For example, 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), which can be applied as biodiesels. In *A baylyi*, the spectrum of wax esters could be adapted by expressing alternative FAR enzymes. These enzymes convert 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).

Beyond esters as final products

The various engineering strategies and increased understanding of microbial ester synthesis should boost developments towards biobased production of esters as high value compounds and bulk chemicals. Due to their close connection to organic acids and alcohols, the biobased production of these compounds could profit from enhanced ester production as well. Alcohols and acids are valuable bulk chemicals in their own right (Chen and Nielsen, 2016). Furthermore, esterification intermediates improve

microbial production of α,ω-diols from n-alkanes as they act as a protective group during conversions.

The esters platform

By adding a hydrolysis step, organic acid and alcohol derivatives of 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, purely based on their physical properties.

The absence of hydrogen-bond donating or accepting groups in esters generally lowers their solubility in water compared to their alcohol and acid derivatives. The result is decreased solubility in water. As a rule of thumb, a minimum titre of 50g/L is considered acceptable when implementing a bio-based process or the DSP steps would become too cost intense (Van Dien, 2013). Especially regarding alcohols and acids, product toxicity often prevents these titres to be reached. The presence of 4 g/L (50 mM) 2-Butanol already negatively affected the growth rates for *S. cerevisiae, E. coli* and *B. subtilis* with increasing strength, while concentrations of 16 g/L butanol inhibit growth and continuation of the fermentation even in solventogenic *Clostridia* (Ezeji et al., 2005; Pereira et al., 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 6g/L, respectively (Vázquez et al., 2011). Only for ethanol, fairly tolerant hosts were found in e.g. *S. cerevisiae*, readily growing in the presence of more than 100 g/L ethanol (Casey and Ingledew, 1986; Ghareib et al., 1988).

Based on their physical characteristics, a relation was found between compound polarity and microbial toxicity. As molecular toxicity is primarily related to cell membrane disruption and disturbances of membrane potentials, polar and apolar groups are key contributors (Sikkema et al., 1995). Information on a compounds polarity is provided by the logP value, which describes how well a compound distributes over an octanol phase in comparison to an aqueous phase (Harnisch et al., 1983). Several studies could link the logP value to the molecular toxicity of a compound for microorganisms during fermentation (Heipieper et al., 1994; Inoue and Horikoshi,

1991; Laane et al., 1987; Straathof, 2003). Straathof and colleagues correlated both the logP and the aqueous solubility of a compound to the critical concentration C^{aq}_{crit} 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 logP correlation of some industrially important esters, acids and alcohols highlight the above-mentioned bottleneck of reaching insufficient titres and dealing with rather severe toxic effects of the products (Table 4).

As a rule of thumb, compounds with a logP between 0.7 and 4 are considered toxic to an organism. At higher logP 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 2nd phase imposes on the microorganism (Bar and Gainer, 1987). At lower logP values the toxic effects are circumvented as the compound is so hydrophilic that it is no longer interfering with the cell membrane.

Coincidentally, most of the interesting ester examples mentioned in Table 1 end up in the toxic range based on their corresponding logP values (Table 4). Nevertheless, microbial production of these esters may be more promising than microbial production of their alcohol or acid precursors. Comparing the C-mol-based Heat of Vaporization (H_{vap}), the energy needed to evaporate a compound of interest, esters consistently require less energy input than their acid or alcohol counterpart (Table 4). Moreover, in-situ product removal by, for instance gas stripping or phase extraction has proven an efficient way to increase final yields and titres. Introducing a biphasic system using hexadecane, enabled a yield of 80%, reaching a final titre of 17g/L isobutyl acetate (Rodriguez et al., 2014). With gas stripping final titres of 38 g/L ethyl acetate or 36 g/L isobutyl acetate were obtained (Tai et al., 2015; Urit et al., 2011). For ethyl acetate this corresponds to titres twice as high has 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.

Table 4: Characteristic parameters of several esters and their alcohol and acid derivatives. logP (oct/w) values and the heat of vaporization (H_{vap}) were retrieved from PubChem database, the critical concentration (C_{rit}) was calculated based on the Straathof correlation (Straathof, 2003).

Compound	#C	logP (oct/w)	C_{crit} (M)	H_{vap} (kJ C-mol^{-1})
Ethanol	2	-0.31	2.0700	21.20
Acetic Acid	2	-0.17	1.6787	25.80
Ethyl acetate	4	0.73	0.1686	8.83
Butyric acid	4	0.79	0.1343	14.50
Butanol	4	0.88	0.1604	13.10
Butyl acetate	6	1.78	0.0228	7.17
Ethyl butyrate	6	1.85	0.0149	7.00
Hexanoic acid	6	1.92	0.0861	11.88
Hexanol	6	2.03	0.0577	10.17
Ethyl hexanoate	8	2.40	0.0044	6.08
Butyl butyrate	8	2.83	0.0021	5.32
Butyl hexanoate	10	3.30	0.0005	5.88

Acetic acid, ethanol and ethyl acetate are abundant industrial compounds, with moderate toxicity (logP <0.7). While a lot of research has been performed on improving ethanol production itself, the latest advances focus on *in-situ* product removal via gas stripping (Qureshi et al., 2012; Taylor et al., 1998). A similar approach has been pursued for ethyl acetate production (Urit et al., 2011). In direct comparison to ethanol, a stripping process would require less energy as the ester is 30% more volatile at 25°C. While both compounds have fairly comparable boiling points around 77°C, higher volatilities for ethyl acetate relate to a more favourable Henry coefficient, resulting in an about 25-fold more beneficial liquid/gas distribution at 25°C (derived from (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 titres to encourage further research (Huang and Yang, 1998). Latest technologies and challenges for the bulk production of acetic acid have been summarized recently and predict yields and DSP efficiency and costs as major challenges (Vidra and Németh, 2018). 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 favours the ester derivative once again (Figure 4). Despite the fact that all compounds are fairly toxic to any production host (logP below 4), in-situ product removal 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 in-situ product removal strategies were compared (Ezeji et al., 2005; W.J. Groot et al., 1992; 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 cost with still significant losses of 25 and 22%, respectively (Oudshoorn et al., 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.

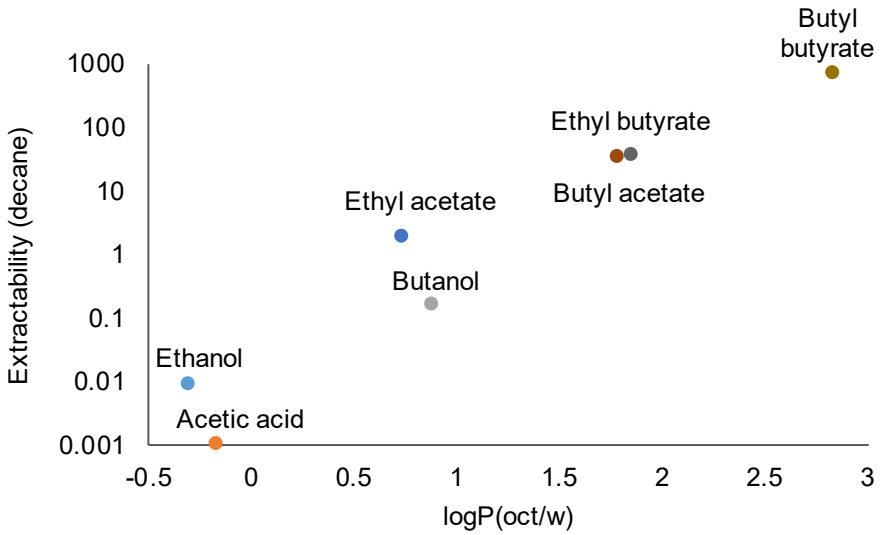

Figure 4: Correlation between logP (oct/w) values and extractability using decane as organic phase. Extractability was estimated using the LSER approach (Vitha and Carr, 2006). Solute parameters were calculated by ACDLabs (Labs, 2018) while solvent parameters were exported from (Stovall et al., 2005).

One benefit of the proposed approaches however, is undebatable in all cases. It enables tuneable 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.

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 of short to medium-chain alcohols or carboxylic acids. Alkane monooxygenases can ω-oxidize primary alcohols and fatty acids, but the efficiencies are low with medium-chain molecules (Clomburg et al., 2015; Fujii et al., 2006; Gudiminchi et al., 2012; Honda Malca et al., 2012; Scheps et al., 2011; van

Nuland et al., 2017b) 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 (Julsing et al., 2012; Schrewe et al., 2014, 2011; van Nuland et al., 2017b). 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, our group equipped *E. coli* 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 et al., 2017b). 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 et al., 2017b).

For the production of α,ω-diols another challenge has to be tackled. Alkane monooxygenases tend to overoxidized 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 et al., 2004; Hashiguchi et al., 2014; Konnick et al., 2014).

Challenges and perspectives

This review has provided an overview of microbial ester production both on a fundamental level, as well as within the potential applications in the bulk chemical arena. As always, there are some major challenges still ahead before biobased ester production can move to the industrial level. AATs are currently at the core of metabolic engineering strategies aimed at improved ester production. However, these enzymes are still not understood well. Studies that focus solely on understanding the

fundamental aspects of AAT-functioning are limited. For example, it is still not clear why many AATs display thioesterase and esterase activities. More importantly, it is unclear how to control these activities. In case of Eat1, ethanol is the determining factor that shifts the enzyme from a hydrolase to an AAT. The controlling factors in other AATs have not been established yet, which may hinder the development of efficient bioprocesses. It is therefore critical that such fundamental questions are answered.

Another major challenge is how to fully employ the vast array of AATs that are available in nature. Metabolic engineering studies have hitherto utilised a relatively limited subset of AATs. However, it has been shown that even homologs of the same AATs in different organisms can show remarkable differences in their substrate specificities. Mining the unknown AATs may provide us with enzymes that are able to perform conversion that are currently either not possible or inefficient. It could also help identify AATs that are more specific than the ones employed currently. 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. A mix of various final products could increase the costs of DSP.

The advances in the metabolic engineering of ester production are closely linked to the developments in the engineering of acyl-CoA (acids) and alcohols. The AAT reaction itself is relatively simple. As long as an acyl-CoA and alcohol are provided, an ester can be formed. The metabolic diversity of the alcohol and acyl-CoA pathways indicate that a practically limitless number of esters can be designed, assuming the appropriate AAT specificity. The real challenge of engineering ester production will be to balance the supply of the alcohol and acyl-CoA substrates. Ideally a 1:1 ratio of the two would be produced to ensure efficient conversion of sugars into esters. Both substrates are produced in complex and intertwined metabolic pathways. Efficient and balanced

production of the alcohol and the acyl-CoA in terms of carbon, energy and redox balance should be investigated further.

The commercialisation of ester production will also require efficient DSP development. In many cases, ester production can be a benefit as they can be removed by gas stripping or extracted into an organic phase. In some cases, they may even form their own phase. Based on their physical properties, it should be feasible to evaluate which esters are suitable candidates for biobased production processes with facilitated DSP. The challenge of extracting esters from the cell will also have to be tackled. Small, volatile esters can freely diffuse from the cell. This may not be the case for large, insoluble esters, such as wax esters of FAEE. These compounds 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 favourable 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 7

Thesis summary and General discussion

Thesis summary

Ester production by microorganisms is of great importance to biotechnological processes. These volatile compounds impart pleasant, fruity aromas to beer, wine, and other fermented products. Yeast in particular are well known for their ability to produce volatile esters. The genetic and enzymatic intricacies of ester production have been the focus of many studies, mainly in *S. cerevisiae*. Despite this, ester synthesis in yeast is not fully understood. This holds particularly true for yeasts that produce high amounts of ethyl acetate from sugars, such as *Kluyveromyces marxianus* and *Wickerhamomyces anomalus*. As was introduced in **Chapter 1**, this ability has been described more than a century ago, but the enzymatic mechanisms behind the synthesis were unclear. Circumstantial evidence suggested that an alcohol acetyltransferase (AAT) was responsible for the ethyl acetate formation. Ethyl acetate is a versatile commodity chemical that is currently produced in unsustainable processes. Understanding the enzymes responsible for bulk ethyl acetate synthesis in yeast could enable rational design of novel production strains. This, in turn could facilitate the development of biobased ethyl acetate production processes. This thesis has made several important breakthroughs in the field of ester production in yeast, particularly ethyl acetate. The most significant of these was the discovery of the elusive enzyme responsible for bulk ethyl acetate synthesis in yeast.

The identification of the ethyl acetate-producing enzyme in *W. anomalus* is described in **Chapter 2**. The purified enzyme showed AAT activity with ethanol and acetyl-CoA and was therefore named Ethanol acetyltransferase 1 (Eat1). Production of Eat1 in *Escherichia coli* enabled efficient ethyl acetate production in *E. coli*. The enzyme could thus potentially be used to develop new biobased ethyl acetate production processes. However, Eat1 was also able to function as a thioesterase and esterase active against acetyl-CoA and ethyl acetate, respectively. It was observed that the presence of ethanol was able to repress the hydrolytic activities, at which point the AAT activity was dominant. How ethanol can control the activities of Eat1 is unclear. It highlights that sufficient ethanol concentrations must be present to produce ethyl acetate with Eat1. It is also shown that Eat1 homologs are present in other bulk ethyl acetate-producing yeasts. These homologs are only distantly related to known AATs. Eat1 is therefore

proposed to compose a novel alcohol acetyltransferase family. The discovery of this novel enzyme family was the cornerstone of the research presented in this thesis.

The identification enabled further studies on the physiology of Eat1 and bulk ethyl acetate production in the native yeasts in **Chapter 3**. The cellular location of Eat1 in *Kluyveromyces lactis* was determined. The enzyme localised to the mitochondria of the yeast. This observation opposed the literature consensus which assumed that bulk ethyl acetate synthesis occurred in the yeast cytosol. Cytosolic acetyl-CoA flux in yeast is low and would presumably not support the synthesis of high amounts of ethyl acetate. The localisation of Eat1 in the mitochondria could better explain how bulk ethyl acetate synthesis occurs. The current hypothesis suggests that bulk ethyl acetate is an overflow product of yeast under iron limited conditions. Under these conditions, the entry of acetyl-CoA into the TCA cycle is impaired and acetyl-CoA accumulates. Eat1 is then proposed to relieve the accumulation by forming ethyl acetate. The TCA cycle and the major flux of acetyl-CoA in yeast are located in the mitochondria, where Eat1 is also located. It is thereby established that bulk ethyl acetate is a mitochondrial product of certain yeasts.

Chapter 4 describes the engineering of efficient ethyl acetate production in *E. coli* using Eat1 as the catalyst. Unlike yeast, the metabolism of *E. coli* can support the synthesis of ethyl acetate under anaerobic conditions. This removes the need for aeration, which is costly on a large scale. Establishing the anaerobic ethyl acetate pathway was faced with several bottlenecks, and removing them was the main theme of this chapter. The pathway towards ethyl acetate could be improved by disrupting by-product formation and optimising the expression levels of *eat1*. Further improvements could be made by removing the N-terminal mitochondrial localisation sequence of Eat1. These sequences typically destabilise proteins unless they are removed. These approaches did improve ethyl acetate formation by Eat1 to the point where ethanol levels were no longer sufficient to repress the hydrolytic activities of Eat1. To prevent ester hydrolysis the volatility of ethyl acetate was used to strip it from the fermenter. The result was ethyl acetate production at 63.4 % of the pathway maximum.

We then looked beyond ethyl acetate as a bulk chemical in Chapter 5 and investigated the role Eat1 has in general ester formation by yeasts, particularly *S. cerevisiae*. The formation of esters is industrially relevant also in *S. cerevisiae* as these compounds contribute to the aroma of fermented foods. *S. cerevisiae* naturally produces a variety of alcohols and thus provided a convenient platform to compare Eat1 homologs from different yeast. Expression of various *eat1* genes resulted in an increase of various in acetate and propionate ester levels. By disrupting the *S. cerevisiae eat1* gene the inverse effect was observed. Eat1 therefore seems to contribute to acetate ester synthesis in *S. cerevisiae* as well. In this chapter, a *S. cerevisiae* strain was generated where all known AAT genes were disrupted. Opposite to the expectations, ester production persisted in this strain, showing that even more ester-producing mechanisms exist.

Chapter 6 focuses on the complex field of ester production as bulk chemicals, from the fundamentals of microbial ester production. Much research has been devoted to understanding the ester-producing processes in microorganisms. This is a daunting task as the structures and functions of esters in microorganisms are in many cases unrelated. Most esters are produced via the AAT reaction. With the exception of Eat1, which was identified in this thesis, other AATs have been studied for a long time. Still, relatively little is known about their structure and catalytic mechanisms, likely because there are no crystal structures available yet. Nevertheless, they have been applied extensively in metabolic engineering of ester production. The AAT reaction is simple. As long as a suitable alcohol and acyl-CoA are provided, the AAT will catalyse the ester formation. The real challenge of engineering ester formation is in the efficient supply of alcohols and acyl-CoA substrates. Some remarkable success has been made in recent years which is outlined in the review. In many cases, esters are less toxic and more readily extractable than the alcohols and acids they are composed of. They could therefore serve as a platform compound.

In summary, this thesis contributed significantly to the knowledge of ester synthesis in yeast. The progress made on the production of ethyl acetate specifically can be used to develop new biotechnological, more sustainable production processes for this versatile compound. At the same time, much remains to be discovered.

General discussion

The research presented in this thesis focused on the elusive enzyme that is responsible for bulk ethyl acetate production in several yeast species, such as *Kluyveromyces lactis, Kluyveromyces marxianus*, *Wickerhamomyces anomalus* and *Cyberlindnera jadinii*. *W. anomalus* was the first yeast where bulk ethyl acetate formation was described in 1892 (Beijerinck, 1892). The identity of the ethyl acetate-forming enzyme remained a mystery until we related it to the hypothetical protein Wanomala_5543 in *W. anomalus* and showed that it possesses alcohol acetyltransferase activity (AAT). We named the enzyme Eat1 (Ethanol acetyltransferase) based on the substrates (ethanol and acetyl-CoA). The discovery of Eat1 in *W. anomalus* led to the identification of a new family of yeast AATs that is predominantly present in bulk ethyl acetate-producing yeasts (Chapter 2). We showed that Eat1 is located in the mitochondria of bulk ethyl acetate producing yeasts, such as *Kluyveromyces lactis* and *Wickerhamomyces anomalus* (Chapter 3). In the mitochondria, Eat1 presumably contributes to balancing the central carbon metabolism when yeast grow under unfavourable conditions. Ethyl acetate is produced as an overflow product in the process. We then applied Eat1 for ethyl acetate production in *Escherichia coli* under anaerobic conditions. We chose the optimal AAT catalyst, removed its destabilising localisation pre-sequence, optimised its expression levels. We then observed that Eat1 was able to hydrolyse ethyl acetate *in vivo*. To prevent this, we used gas stripping to remove the ester. The combined effect of the optimisation approaches was the production of ethyl acetate at 63.4 % of the theoretical pathway maximum. In Chapter 4 we looked beyond ethyl acetate and investigated the role Eat1 plays in general ester production in *S. cerevisiae*. We found that it contributes mostly to the synthesis of a variety of acetate esters. We also observed that more ester-forming mechanism exist in *S. cerevisiae* (Chapter 4). Finally, we performed an extensive literature review of ester-forming mechanisms. We outlined the metabolic engineering approaches aimed at improving the production of ester as bulk chemicals in microbial systems. AATs have dominated the field of metabolic engineering for ester production, although other enzymatic reactions, such as Baeyer-Villiger monooxygenases have also led to efficient

ester production. We highlighted that ester production may benefit production of other biobased compounds as well, such as alcohols and organic acids (Chapter 6).

In the course of this research, the status of the *W. anomalus* Eat1 (and its homologs in other yeasts) changed from hypothetical protein to the experimentally annotated Eat1 AAT. We determined some of the fundamental aspects of Eat1, such as the presence of the α/β-hydrolase fold, its Ser-Asp-His catalytic triad, and the remarkable feature that it catalyses three distinct enzyme conversions: Eat1 has AAT, esterase and thioesterase activity. However, these were only the first steps in understanding the function of Eat1, and many unanswered questions remain.

The three faces of Eat1

Eat1 is able to catalyse the formation of esters (AAT), but also the degradation of esters (esterase) and acetyl-CoA (thioesterase). The ratio of the activities is controlled by ethanol. The *in vitro* assays performed with the *W. anomalus* Eat1 (Chapter 2) suggest that when 15-20 mM ethanol is present, the AAT reaction is preferred over esterase and thioesterase. This observation appeared to agree very well with *in vivo* analyses (Chapter 4). The maximum measured AAT activity was only 0.018 $\mu Mol_{ethyl\ acetate}/min/mg_{purified\ protein}$ when 45 mM ethanol and 1 mM acetyl-CoA were present. At this ethanol concentration, the innate esterase activity of Eat1 was almost entirely repressed (Chapter 2). This would suggest that Eat1 is an esterase-turned-AAT once the concentration of ethanol reached a certain level. However, when ethanol was absent, the ethyl acetate-hydrolysing activity of Eat1 was 0.8 $\mu Mol/min/mg_{purified\ protein}$, which is almost 45-fold higher compared to the conditions where the AAT activity was dominant. The low *in vitro* AAT activity of Eat1 was surprising and it is not clear how this enzyme is able to produce ethyl acetate *in vivo* efficiently. It does, however, highlight the fact that sufficient ethanol levels must be present during ethyl acetate formation *in vivo* to prevent ester degradation (Chapters 1 and 5).

The esterase activity of Eat1 was somewhat expected since the enzyme belongs to the α/β-hydrolase superfamily. Enzymes with this fold mostly perform hydrolytic reactions in aqueous environments (Rauwerdink and Kazlauskas, 2015). Eat1 seems to be an exception to this rule under certain conditions, but it is not the only one. The paralogs

Eht1 and Eeb1 are yeast AATs that form medium chain fatty acid (MCFA) ethyl esters. Like Eat1, they contain an α/β-hydrolase fold and showed AAT, esterase and thioesterase activities (Knight et al., 2014; Saerens et al., 2006). Despite the shared characteristics, Eat1 is only distantly related to Eht1 and Eeb1 (Chapter 2). Ethanol is likely the factor that also controls the activity of Eht1 and Eeb1, but this remains to be confirmed experimentally.

The hypothetical catalytic mechanism of Eat1 and other acyltransferases resembles the canonical mechanism observed in other enzymes containing the α/β-hydrolase fold and a Ser-Asp-His triad (Rauwerdink and Kazlauskas, 2015). The reaction is initiated by the nucleophilic attack of Ser on the acetyl moiety of acetyl-CoA and the formation of the acetyl-protein intermediate. In the next step, the acetyl group is transferred to ethanol and ethyl acetate is released in the AAT reaction. When water is the acceptor of the acetyl group, Eat1 performs the thioesterase reaction. Alternatively, ethyl acetate can be the donor of the acetyl group and is hydrolysed in the reaction. The exclusion of water over ethanol determines whether Eat1 acts as an AAT or a hydrolase. Similar behaviour has been observed in other esterase-like acyl transferases. The preference for the acyltransferase reaction 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, favour binding of alcohols over water, or decrease the reactivity of water compared to alcohols (Jiang et al., 2011; Lejon et al., 2008; Rauwerdink and Kazlauskas, 2015). It is tempting to speculate which mechanism promotes AAT activity in Eat1, but such information on the catalytic mechanism can only be obtained by studying the crystal structure of the enzyme and its acetyl-protein intermediate (Mirza et al., 2005). Attempts to obtain the crystal structure of Eat1 are ongoing.

The low AAT activity of Eat1

One of the most puzzling questions throughout this thesis was the apparent loss of Eat1 AAT activity *ex vivo*. This was very surprising given the high rate of ethyl acetate formation in live yeast. In continuous iron-limited cultivations, *W. anomalus* was able to generate 224.7 $mg_{ethyl\ acetate}/h/g_{CDW}$ (Chapter 3). This corresponds to 0.042

$\mu Mol_{ethyl\ acetate}/min/mg_{CDW}$. Dry yeast biomass is composed of approximately 40 % protein (Winkler et al., 2011). Assuming 100 % efficiency during protein extraction, the expected specific ethyl acetate-forming AAT activity in cell free extracts (CFE) would therefore be approximately 0.1 $\mu Mol_{ethyl\ acetate}/min/mg_{protein}$. This is high, considering that Eat1 represents only a fraction of the total protein content.

Despite numerous attempts, we were never able to detect any AAT activity for ethyl acetate synthesis in CFE of *W. anomalus*. The concentrated CFE (containing at least 5 $mg_{protein}/mL$) was prepared by French press, incubated with ethanol and acetyl-CoA and analysed by gas chromatography to detect ethyl acetate. We cultivated *W. anomalus* on either glucose or ethanol since both substrates support bulk ethyl acetate formation (Fredlund et al., 2004b; Gray, 1949). We also varied a number of assay parameters in an attempt to induce ethyl acetate formation *in vitro*. These included varying the amount of CFE, ethanol concentration (20-100 mM), acetyl-CoA concentration (1-5 mM), buffer types (Tris-HCl or phosphate buffer), addition of protease inhibitors (with and without Phenylmethylsulphonyl fluoride – PMSF), reducing agents (dithiothreitol - DTT) and various metals (e.g. Mg, Ca, Fe, Zn, Cu, Mn). We even went as far as adding ATP, ADP and NADH to the mixture, even though there is no clear role for them in the AAT reaction. None of these approaches resulted in *in vitro* ethyl acetate formation in CFE of *W. anomalus*. We also performed similar, although less extensive *in vitro* assays for other ethyl acetate-forming reactions (Chapter 2), also without success.

The reason for the activity loss is difficult to determine, but the most apparent explanation would be that the optimal conditions of the assay have not been met. Low enzyme stability *in vitro* may also explain why no AAT activity was observed in CFE of *W. anomalus*. However, purified Eat1 was able to produce ethyl acetate *in vitro* for several hours, albeit at a slow rate (Chapter 2). We were also able to use the same *W. anomalus* Eat1 protein preparations two years after purification without any apparent loss in activity *in vitro*. The proteins were stored at -20 °C in phosphate buffer (pH 7.5) containing 200 mM NaCl. This would indicate that while the AAT activity of Eat1 is low, the proteins do remain stable over long periods. An explanation may be

that the CFE contains unknown compounds that inhibit the activity of Eat1. Alternatively, esterase activity present in yeast CFE may degrade any ethyl acetate before it can be detected (Fukuda et al., 1998; Lilly et al., 2006; Rojas et al., 2002; Stribny et al., 2016). Eat1 possesses an innate ability to hydrolyse ethyl acetate, which may have interfered with AAT activity measurements as well. However, the ethanol concentrations in the *in vitro* assays were higher than 20 mM, which was sufficient to repress ester hydrolysis by Eat1 (Chapter 2). Considering the low *in vitro* AAT activity of purified Eat1, where no esterase activity was present, it is more likely that we could not detect Eat1-derived AAT activity in yeast CFE because the enzyme does not perform well in the here used *in vitro* AAT assay conditions.

Ethyl acetate-forming AAT activity was reported in CFE of *C. jadinii* grown in iron-limited continuous cultures, but the experimental data presented in the study did not allow accurate estimations of ethyl acetate production rates, either *in vivo* or *in vitro*. The methods also do not exclude the presence of whole cells in the CFE. Moreover, the AAT assay was carried out at pH 4.6, which is low considering Eat1 is located in mitochondria (Chapter 3), where pH values are approximately 7.5 or higher (Orij et al., 2009). Other authors have measured AAT activity in CFE of *C. jadinii* and found it was considerably lower compared to hemiacetal dehydrogenation (Kusano et al., 1999).

Such contradictory results are common within studies on the molecular mechanisms behind bulk ethyl acetate synthesis. Much of the confusion may be attributed to the fact that Eat1-catalysed AAT activity has not been detected (accurately) in CFE. Instead, the ethyl acetate formation has been related to other enzymatic mechanisms, such as HADH and reversed esterases (Löser et al., 2014; Park et al., 2009). Optimising the AAT assay in CFE is a key issue that would help clarify the confusion in the field and enable accurate measurements of ethyl acetate production by yeast. Alternatively, the *eat1* genes could be disrupted in the various yeasts to determine their contribution to ethyl acetate synthesis. This may be challenging to accomplish due to limited genetic tools available for many bulk ethyl acetate-producing yeasts, with the exception of *K. lactis* and *K. marxianus* (Juergens et al., 2018; Kooistra et al., 2004; Löbs et al., 2017).

We identified Eat1, but are there more undiscovered ester producing enzymes?

Eat1 seems to be the key enzyme in bulk ethyl acetate synthesis in yeasts such as *K. lactis*, *K. marxianus* and *W. anomalus*. However, it is clear that it was not the only undiscovered ethyl acetate-producing enzyme. For example, when *eat1* was disrupted in *K. lactis*, roughly 20 % of the ethyl acetate synthesis remained (Kruis et al., 2017). We additionally disrupted the *atf1* homolog of *K. lactis*, but this did not affect the residual ethyl acetate formation in the *K. lactis* Δ*atf*Δ*eat1* double deletion strain (unpublished results). The disruption of *atf1* also did not reduce ethyl acetate production in *K. marxianus* (Löbs et al., 2017). The role of Atf1 and Atf2 homologs in bulk ethyl acetate production is likely minor (Chapter 2). The origin of ester production is also not entirely understood in *S. cerevisiae* (Chapter 5). Atf1, Atf2 and Eat1 all contribute to ethyl acetate synthesis in this yeast. Approximately 50% of ethyl acetate and 80% of isoamyl acetate were previously attributed to Atf1 and Atf2 activity (Verstrepen et al., 2003b). The deletion of *eat1* in *S. cerevisiae* also decreased ethyl acetate production by 50% (Chapter 2). We were therefore surprised that the *S. cerevisiae* Δ*atf1*Δ*atf2*Δ*eat1* triple deletion strain was still able to produce both ethyl acetate and isoamyl acetate. This production was present even when all known *S. cerevisiae* AAT genes were disrupted (Chapter 5). Other esters such as phenylethyl acetate and ethyl octanoate were almost entirely absent in *S. cerevisiae* Δ*atf1*Δ*atf2*Δ*eht1*Δ*eeb1*Δ*eat1*. The source of the remaining ethyl acetate and isoamyl acetate is not clear. It is possible that other AATs exist in both *S. cerevisiae* and *K. lactis*, or that other activities, such as reverse esterase or HADH are responsible (Masayuki Kusano et al., 1998; Park et al., 2009). The reversed esterase reaction is thermodynamically unfavourable in aqueous environments and may result only in low level ester production (Chapter 2, Chapter 6). Recently, ethyl acetate forming-HADH activity of the *K. marxianus* alcohol dehydrogenase Adh7 has been demonstrated *in vitro*, but disrupting the gene did not affect ethyl acetate production (Löbs et al., 2017). High HADH activity was also reported in CFE extracts of *C. jadinii* (Kusano et al., 1999), but has not been shown *in vivo*. Hence, the role of HADH in ethyl acetate formation is still unclear. It does not seem to play a role in *K. marxianus*, but this has not been confirmed in *S. cerevisiae* or *C. jadinii*. The latter yeast is able to produce

high concentrations of acetaldehyde from ethanol (Armstrong et al., 1984b). Both compounds are needed to synthesise ethyl acetate via HADH activity.

A fourth biological reaction that could contribute to ethyl acetate synthesis is Baeyer-Villiger monooxygenation (BVMO). These enzymes convert ketones into esters. Yeast are generally not known for their BVMO activity, but several *Candida* species use them to catabolise alkanes via alcohols and ketones (Beier et al., 2014). It has not been shown if these BVMOs can form ethyl acetate. A second requirement for ethyl acetate synthesis via BVMO is the availability of 2-butanone. This ketone is not known as a major product of yeast. In beer fermentations with *S. cerevisiae,* 2-butanone was measured at only 12.35 µg/L (Saison et al., 2009). Moreover, 2-butanone oxidation via BVMO would likely result in the formation of both ethyl acetate and methyl propionate (Pereira et al., 2018). The latter has not been reported as a product of any bulk ethyl acetate producing yeast. It is therefore considered unlikely that BVMOs play a role in bulk ethyl acetate synthesis in yeast.

The remaining ethyl acetate and isoamyl acetate production in yeast remains unexplained at this point and should be the target of future research. Our own experience with identifying the elusive Eat1 has shown that a rational approach can be effective. On the other hand, random and semi-rational approaches can also facilitate identifying novel enzymes. Recently, genomic saturation mutagenesis combined with polygenic trait analysis was successful in identifying three loci in *S. cerevisiae* that are related to ethyl acetate synthesis (Abt et al., 2016). The authors did not mention whether known ethyl acetate-producing AATs, such as Atf1 were also identified in the study. Such methods may identify other novel ethyl acetate producing genes in the future.

The physiological role of ester production by Eat1 in yeast

Several roles of ester production in *S. cerevisiae* have been proposed and linked to the activity of specific AATs. The activity of Atf1, which forms acetate esters, attracts insects and helps yeast disperse in the environment (Christiaens et al., 2014). It was also proposed that Atf1 and Atf2 play a role in lipid and sterol metabolism (Mason and Dufour, 2000). Eht1 and Eeb1 produce MCFA ethyl esters, presumably to

counteract the toxic effects of MCFA (Saerens et al., 2006), which are released during fatty acid synthesis (Tehlivets et al., 2007). The main established role of Eat1 in yeast is (bulk) ethyl acetate formation, but its true physiological role is unknown. Despite the seemingly important function of the various AAT activities, ester formation does not seem to be essential for yeast growth. A *S. cerevisiae* strain lacking all five known AATs was still able to grow aerobically and anaerobically, although fermentation performance such as sugar consumption and ethanol yield were reduced by approximately 10% when all AAT genes were disrupted (Chapter 5). This indicates that ester production in *S. cerevisiae* improved the fermentation performance of the yeast but was not critical under the tested conditions. This is consistent with previous reports (Saerens et al., 2006; Verstrepen et al., 2003b).

The biological function of Eat1 in yeasts such as *K. marxianus, W. anomalus* and *K. lactis* may differ from *S. cerevisiae*, considering that these yeasts produce much more ethyl acetate. The bulk production of ethyl acetate has been linked to acetyl-CoA accumulation under iron limited conditions. This hypothesis was first proposed by Thomas and Dawson, 1978, who suggested that an AAT relieves acetyl-CoA accumulation by forming ethyl acetate. At the time, the enzymes involved were not identified and the hypothesis could not be tested. We were able to show that Eat1 is the main source of ethyl acetate production in bulk ethyl acetate producing yeast (Chapter 2). It functions as an AAT and is located in mitochondria, where acetyl-CoA accumulation is most likely to occur (Chapter 3). These findings support the hypothesis that ethyl acetate is indeed an overflow metabolite derived from acetyl-CoA accumulation. Excessive amounts of acetyl-CoA may reduce the availability of free CoA, which is needed for the formation of other mitochondrial metabolites, such as succinyl-CoA.

Bulk ethyl acetate production in yeast only occurs under specific environmental conditions. These include iron and oxygen limitation in the presence high substrate concentrations (Fredlund et al., 2004b; Löser et al., 2014; Thomas and Dawson, 1978). Such conditions may occur in nature, but it is questionable whether they can be maintained over longer time periods. Ethyl acetate synthesis through Eat1 may only

be a transient reaction that helps maintain the homeostasis of acetyl-CoA in the mitochondria. The low affinity for acetyl-CoA (apparent K_m=2.43± 0.24 mM) may ensure that Eat1 is only active when the acetyl-CoA levels are high (Chapter 2). However, cultivation under controlled laboratory conditions may create a stable environment where mitochondrial acetyl-CoA is continuously in excess, resulting in Eat1-catalysed ethyl acetate production. It is tempting to speculate that bulk ethyl acetate formation by yeast is in fact an artefact of laboratory cultivation. When *eat1* was disrupted in *K. lactis*, there was no apparent effect on the phenotype of the yeast (under the tested conditions), other than reduced ethyl acetate production (Chapter 2). Considering the proposed hypothesis that Eat1 plays a role in maintaining the carbon metabolism in mitochondria (Löser et al., 2014), a more dramatic effect was expected. The effect of *eat1* disruption was similar in *S. cerevisiae*. However, *K. lactis* and *S. cerevisiae* produced only low amounts of ethyl acetate under the tested conditions, suggesting that the carbon flux through Eat1 was not high. Yeasts such as *K. marxianus* or *W. anomalus* form significantly more ethyl acetate. These strains may therefore give better insights into the effect the disruption of *eat1* has on yeast growth and physiology.

The reason why some yeasts have acquired the ability to produce high titres of ethyl acetate is not clear. If the true physiological function of Eat1 is to prevent accumulation of excess acetyl-CoA in the mitochondria, ethyl acetate synthesis is not strictly needed. The same effect could hypothetically be achieved by a thioesterase reaction, where acetate is released as the product. It is possible that the preference of Eat1 to produce ethyl acetate over acetate (in the presence of ethanol) is an evolutionary coincidence. However, it is also possible that ethyl acetate production provides yeast with ancillary benefits, such as inhibiting the growth of competitive organisms (Fredlund et al., 2004c). Studies on the expression levels of *eat1* under various growth conditions may reveal if the primary activity of Eat1 is the production of ethyl acetate, or if it fulfils other physiological roles. The only information on expression levels of *eat1* in bulk ethyl acetate producing yeasts was obtained in *W. anomalus* under two growth conditions (Chapter 2). In *W. anomalus* grown in continuous cultures, *eat1* was almost fully repressed under aerobic conditions whereas

it was highly expressed under oxygen-limited conditions under which ethyl acetate was produced. Studying the dynamics of *eat1* expression may answer important questions about the physiological role of Eat1. For example, the regulation of *eat1* expression is not understood. It is possible that *eat1* is upregulated as part of a general response when *W. anomalus* switched from an aerobic to a respiro-fermentative metabolism. Alternatively, the expression of *eat1* may be controlled by other factors. At the moment, it is not possible to answer these relevant questions because gene expression data in bulk ethyl acetate producing yeasts is lacking. In contrast, gene expression data is available for *S. cerevisiae*. A compendium of microarray data in *S. cerevisiae* continuous cultivations allows the comparison of *eat1* expression under 55 growth conditions. These include various combinations of aeration (aerobic or anaerobic), growth limiting nutrient (e.g. glucose, ethanol, iron), and culture pH (Knijnenburg et al., 2009). Examining the expression data of *eat1* (courtesy of Dr. Jean-Marc Daran) showed that the gene was expressed under all tested conditions. The expression levels also did not vary significantly between the conditions. This includes fully aerobic, glucose limited conditions, when the expression of *eat1* in *W. anomalus* was fully repressed (Chapter 2). Again, this suggests that Eat1 may have different roles in *S. cerevisiae* and *W. anomalus*. However, more experimental data is needed to determine this.

The most significant contribution of this thesis on a fundamental level was the identification of the elusive Eat1. This AAT has explained how *W. anomalus*, *K. marxianus* and *K. lactis* are able to produce high levels of ethyl acetate from sugars (Tabachnick and Joslyn, 1953a; Urit et al., 2013b). We have determined some of the basic properties of Eat1, but there is much still to be discovered. This includes confirming whether the main physiological role of Eat1 in yeast is counteracting excessive concentrations of mitochondrial acetyl-CoA, or if it has other functions. Eat1 is also able to catalyse the AAT, the thioesterase and the esterase activity. We have determined that the presence of ethanol (and presumably other alcohols) promotes the AAT activity. However, the precise catalytic mechanism is not known. A crystal structure, which is not available at the moment, would provide additional information on how Eat1, that so closely resembles an esterase, is able to function efficiently as an

AAT in aqueous environments. This knowledge may provide indications on how to decrease esterase and thioesterase activities and improve the AAT activity of Eat1.

Application of Eat1 for improved ester production

The discovery of Eat1 may have implications on the food-fermentation industry. By overexpressing and disrupting *eat1* in *S. cerevisiae* CEN.PK2-1D we showed that the gene contributes to acetate and propionate ester production (Chapter 5). This phenotype was demonstrated in *S. cerevisiae* CEN.PK2-1D, which is a laboratory strain with several auxotrophies that facilitate genetic modifications (Entian and Kötter, 2007; van Dijken et al., 2000). It is possible that the fermentation performance of *S. cerevisiae* CEN.PK2-1D does not fully reflect that of the industrial *S. cerevisiae* strains. The impact of Eat1 on ester production should therefore be determined in strains that are used in industrial fermentations. The genetic accessibility of these strains may be problematic (Steensels et al., 2014). We therefore attempted to correlate the primary amino acid sequence and copy number variation (CNV) of Eat1 with ester production in industrial *S. cerevisiae* strains (Chapter 5). We did not observe any correlation, indicating that differences in ester production profiles are not caused by AAT variation on a genomic level. Comparative transcriptomics of *eat1* and other AAT genes may offer additional insights into the dynamics of ester production in industrial *S. cerevisiae* strains (Mendes et al., 2017). It should be noted that ester production in *S. cerevisiae* and other yeast strongly depends on the production of the alcohol and acyl-CoA precursors (Chapter 6), as well as esterase activity (Dzialo et al., 2017; Lilly et al., 2006).

Eat1 has been added to the repertoire of AATs that are useful for the metabolic engineering of ester production. We have demonstrated that ethyl acetate can be produced efficiently in *E. coli* expressing either the *K. marxianus* or *W. anomalus eat1* (Chapter 4). Such pathways have previously been engineered in *E. coli* and *S. cerevisiae*, but they mostly relied on Atf1 as the AAT catalyst. Although Atf1 and Eat1 are both able to produce acetate esters, the ethyl acetate titres achieved by *E. coli* expressing *atf1* were low compared to e.g. isoamyl acetate (Rodriguez et al., 2014). This may indicate that the affinity of Atf1 for ethanol is lower compared to isoamyl

alcohol. When we compared the ethyl acetate production capacity of *atf1* and *eat1* under the same conditions, the strains expressing *eat1* consistently produced more ethyl acetate (Chapter 4). Eat1 therefore seems more suitable for ethyl acetate production than Atf1. Whether this is true for other esters remains to be determined.

The capacity of Eat1 to produce esters other than ethyl acetate has not been studied in detail yet. Overexpression of several *eat1* genes in *S. cerevisiae* resulted in the production of a range of acetate and propionate esters (Chapter 5). This suggests that Eat1 members may catalyse the production of esters such as isoamyl acetate, phenylethyl acetate and ethyl propionate in *E. coli* as well. There is a selection of ester-producing Eat1 homologs from various yeast available at the moment. New Eat1 candidates are also being identified as more yeast genomes become available. Since our initial identification of the Eat1 family in *W. anomalus*, putative Eat1 homologs have been found in *Lachancea fermentati, Hanseniaspora osmophila, Hanseniaspora guillermondii, Hanseniaspora opuntiae, Hanseniaspora valbyensis, Pachysolen tannophilus* and *Babjeviella inositovora*. Bulk ethyl acetate production in these yeasts has not been described. The presence of putative Eat1 homologs indicates that they may have this capacity and could be useful for industrial ethyl acetate production. These yeasts are also a source of novel Eat1 homologs that may differ in their kinetic parameters and substrate specificities.

Optimising the metabolic pathway towards ethyl acetate production

Ethyl acetate is currently produced from petrochemical resources in energy intensive and unsustainable processes (Löser et al., 2014). Production of ethyl acetate from biobased sources, such as sugars are an interesting alternative that was explored in this thesis. The key factors that determine whether such a process is economically viable are yield, productivity and operational costs. *K. marxianus* and other bulk ethyl acetate producing yeasts are able to efficiently convert sugars into ethyl acetate. The ethyl acetate yields in *K. marxianus* reached up to 56 % of the pathway maximum (Urit et al., 2013a). The maximum recorded ethyl acetate productivity was 5.33 g/L/h (Urit et al., 2011). With the discovery of Eat1, these yields and productivities may be improved in natural ethyl acetate producing yeast, assuming that genetic modifications

are possible. A major challenge of engineering ethyl acetate production in yeast may be caused by the spatial separation of acetyl-CoA and ethanol formation. Ethanol and acetyl-CoA are needed to form ethyl acetate. Eat1 and most of the acetyl-CoA produced by yeast are located in the mitochondria, while ethanol is formed in the cytosol (Chapter 3). Precise fine tuning will be needed to ensure that the carbon flux is distributed equally between the cytosol and the mitochondria to ensure optimal ethyl acetate synthesis. Alternatively, cytosolic acetyl-CoA synthesis could be increased by introducing e.g. the pyruvate dehydrogenase complex or a phosphoketolase (de Jong et al., 2014; Kozak et al., 2014b, 2014a).

A drawback of using yeast as cell factories for ethyl acetate production is their dependence on aeration. This constraint is imposed by the maximum yield of the ethyl acetate pathway that can be achieved in organisms that metabolise glucose via the Embden-Meyerhof-Parnas (EMP) pathway (Chapter 4). Such organisms, which include yeast and *E. coli*, can only form 1 $mol_{ethyl\ acetate}/mol_{glucose}$ and cannot achieve the maximum yield of 1.2 $mol_{ethyl\ acetate}/mol_{glucose}$. As a result, excess reducing equivalents (NADH) are formed. In yeast the excess NADH can only be oxidised in the respiratory electron transport chain (ETC), which requires oxygen to function. Production of ethyl acetate in yeast is therefore limited to aerobic cultivations, which increases the operational costs of the bioprocess (Garcia-Ochoa and Gomez, 2009). This may present a problem for ethyl acetate, a bulk chemical product with a relatively low price of \$800-\$1200/ton (OrbiChem, 2013). Anaerobic fermentations can avoid the costs of oxygen supply, while maintaining high yield and productivity (Weusthuis et al., 2011).

E. coli is able to produce 1 $mol_{ethyl\ acetate}/mol_{glucose}$ under anaerobic conditions because it can excrete the excess reducing equivalents as formate (Chapter 4). The maximum ethyl acetate yield achieved by *E. coli* under anaerobic conditions was 63.4 ± 3.4 % of the pathway maximum. This was an improvement compared to the yields achieved by *K. marxianus*. However, the pathway maximum of 1 $mol_{ethyl\ acetate}/mol_{glucose}$ imposed by the EMP pathway was still not achieved. It is critical that these process parameters are optimised to make the biobased ethyl acetate production economically viable. This could be achieved by further optimisation of the metabolic pathway in *E. coli*. In

Chapter 4, *eat1* was expressed from plasmids, which are maintained by the presence of antibiotics. This is both expensive and negatively impacts culture growth (Diaz Ricci and Hernández, 2000). The genomic integration of *eat1* would eliminate the need for antibiotics and would likely improve the growth and performance of the strain. Placing *eat1* under promoters that do not need expensive inducers such as IPTG or m-toluate would also help decrease the costs of the process. However, it is likely that the expression levels of *eat1* would have to be optimised again. A way to achieve this may be through varying promotor strengths or by optimising the ribosome binding site of *eat1* (Bonde et al., 2016; Mutalik et al., 2013; Salis et al., 2009). In case of Eat1, the yields and productivities in *E. coli* were also improved by removing the mitochondrial localisation tag (Chapter 4). The position of the N-terminal truncation greatly affected the efficiency of ethyl acetate production by Eat1. Several Eat1 variants should therefore be screened to identify the best performing one. However, the entire sequence space of the *K. marxianus* and *W. anomalus* N-terminus was not explored. It is possible that more active variants of trEat1 exist. Moreover, Eat1 homologs from different yeast had varying capacities for ethyl acetate synthesis. For instance, the *W. anomalus* Eat1 outperformed its *K. marxianus* counterpart in *E. coli* (Chapter 4). The AAT reaction may additionally be improved through metabolic channelling by e.g. fusing Eat1 with Pfl or AdhE, which produce acetyl-CoA and ethanol, respectively. This principle improved ethyl acetate production in *S. cerevisiae* by almost 2-fold (Lin et al., 2017).

Optimising the process parameters of ethyl acetate production

A substantial benefit of ethyl acetate is its high volatility relative to other compounds produced by microorganisms. The volatility facilitates *in situ* product removal (ISPR), where ethyl acetate is continuously removed from the liquid by gas stripping (Chapter5, Chapter 6). The ethyl acetate can then be collected by condensation. The optimal ethyl acetate stripping rate will need to be determined, which should prevent product inhibition and ester hydrolysis in the culture. However, it should not be too high or the ethyl acetate concentration in the gas outflow may become too low to facilitate energy-efficient product recovery. The temperature of the fermentation plays a key role in determining the stripping rate (Urit et al., 2013a). At increased

temperatures, ethyl acetate evaporates more readily. As a consequence, the gas stripping flow can be lower, while still maintaining efficient ethyl acetate removal from the fermentation broth (Chapter 5, Chapter 6). The ethyl acetate production in *E. coli* under anaerobic conditions described in Chapter 4 was performed at 30 °C. Increasing the temperature to 37 °C, which is the optimal growth temperature of *E. coli* may result in increased growth rates, higher productivities and improved removal of ethyl acetate from the broth. The beneficial effect of ethyl acetate production at elevated temperatures (42 °C) was already demonstrated in *K. marxianus*. In the latter study, the biomass-specific productivity of ethyl acetate increased from 0.4 $g_{ethyl\ acetate}/g_{cell\ dry\ weight}/h$ to 0.67 $g_{ethyl\ acetate}/g_{cell\ dry\ weight}/h$ (Urit et al., 2013a). The volumetric productivity increased accordingly. High temperatures may become a problem for mesophilic organisms, such as *E. coli*. To facilitate ethyl acetate evaporation at lower temperatures, a vacuum could be used instead of low temperatures. However, this would increase the operational costs.

Selection of an optimal production host is also key for an efficient bioprocess. Despite active ethyl acetate stripping in an industrial process, a certain residual concentration would always be present in the liquid. If this exceeds the tolerance levels of the host organism, the efficiency of the bioprocess would be affected. Ethyl acetate became toxic for *E. coli* under anaerobic conditions at approximately 10 g/L (113 mM). However, *E. coli* was still able to grow (at reduced rates) in the presence of 15 g/L (170 mM) ethyl acetate (Wilbanks and Trinh, 2017). In *K. marxianus* and *W. anomalus,* ethyl acetate fully inhibited yeast growth at a concentration between 12 g/L and 17 g/L (136 mM and 190 mM) (Urit et al., 2013b). Such concentrations of ethyl acetate in the liquid have not been reached yet, but this will likely change on large scale. Other organisms with higher tolerances could be used to produce ethyl acetate, such as *Deinococcus geothermalis*, which could withstand saturating concentrations of ethyl acetate (Kongpol et al., 2008). Alternatively, the solvent tolerance of yeast or *E. coli* could be improved by rational or random evolution (Caforio et al., 2018; Dragosits and Mattanovich, 2013).

The ethyl acetate yields achieved by *E. coli* under anaerobic conditions were high (63.4 ± 3.4 %). On the other hand, the maximum volumetric productivities were low (1.3 $mmol_{ethyl\ acetate}/L/h$ or 0.11 $g_{ethyl\ acetate}/L/h$). For comparison, the highest ethyl acetate yield achieved by *K. marxianus* was 56 % of the pathway maximum, with a typical productivity of approximately 3 g/L/h (Löser et al., 2014). This corresponded to approximately 0.4 $g_{ethyl\ acetate}/g_{dry\ cell\ weight}/h$ (Urit et al., 2013b, 2013a). Our biomass measurements did not allow accurate determination of the biomass-specific productivities of ethyl acetate (Chapter 4). However, a rough estimation can be made by converting OD_{600} measurements to $g_{dry\ cell\ weight}$. In *E. coli*, one OD_{600} unit corresponds to approximately 0.3 $g_{dry\ cell\ weight}/L$ (Soini et al., 2008). Based on this conversion, the estimated maximum biomass-specific production rate of ethyl acetate was between 0.7 and 0.8 $g_{ethyl\ acetate}/g_{dry\ cell\ weight}/h$. This was higher than the typical biomass-specific productivity of *K. marxianus* (Urit et al., 2013b, 2013a). However, the biomass growth under anaerobic conditions is significantly slower compared to aerobic conditions, indicating that the volumetric productivities may be low until enough biomass is formed. Alternatively, high biomass concentrations of *E. coli* can be achieved by an initial aerobic phase. The subsequent production of ethyl acetate can then be performed under anaerobic conditions, which should result in increased productivities.

The most established anaerobic bioprocesses that produce bulk chemicals on an industrial scale are ethanol and lactate production. The yield of lactate achieved 89 % of the theoretical maximum with a productivity of 22.5 g/L/h (Tejayadi and Cheryan, 1995), while ethanol was produced at 87 % of the theoretical maximum and a productivity of 10.6 g/L/h (W. J. Groot et al., 1992). These numbers can serve as a reference for the yields and productivities needed for the scale-up of biobased ethyl acetate production. At the current stage, the ethyl acetate yields are approaching 70 % of the theoretical pathway maximum, while the productivities still need to be optimised. Optimisation of these process parameters has been a major challenge in the biobased industry not just for ethyl acetate production, but for biobased bulk chemicals in general (Chapter 6).

Beyond ethyl acetate production

In *E. coli* grown under anaerobic conditions, 1 mol of glucose can be converted to 1 mol ethyl acetate and 2 mol formate (Figure 1A). Formate, like other weak organic acids, can become toxic at high concentrations (Vázquez et al., 2011). Moreover, constant excretion of acids into the medium may increase the costs of pH control during large scale cultivation. Both problems can be omitted by converting the formate into H_2 and CO_2. In *E. coli,* this reaction is performed by the formate hydrogen lyase complex - Fhl (Rossmann et al., 1991). The only by-products of the reaction are CO_2 and biomass. This pathway produces two added-value products, ethyl acetate and H_2, assuming that the gases can be separated (Figure 1B).

Ethyl acetate may also serve as an intermediate for the production of other bulk chemicals. Biobased production of acetic acid as a bulk chemical is particularly challenging due to its low market price $500/ton (Orbichem, 2013) and the negative impact it has on microbial growth (Roe et al., 2002). The acetate titres in microbial fermentations typically do not exceed 100 g/L, while the extraction processes are expensive and energy intensive (Vidra and Németh, 2018). Such a process is only economically viable for the production of food-grade acetic acid, which reaches a higher market price of $1000-$1200/ton (Vidra and Németh, 2018). As an alternative, ethyl acetate can be chemically hydrolysed into ethanol and pure acetic acid (Figure 1C, Chapter 6). Ethanol and H_2 are produced as by-products in the process. The co-production of ethanol, a biobased chemical with a well-established market and acetic acid may in fact result in an economically feasible process for the production of biobased acetic acid.

Ethyl acetate and H_2 can also be converted to ethanol as the sole product through ethyl acetate hydrogenolysis (Lu et al., 2016). Zeachem, a US based company has used a similar reaction to form ethanol from lignocellulosic sugars, mainly glucose. In the first phase, sugars are converted to acetic acid by homoacetogenic bacteria. The acetate is then recovered and chemically esterified with ethanol to form ethyl acetate. In the following hydrogenolysis, ethyl acetate reacts with H_2 to form ethanol. Part of the ethanol is recycled for the next cycle of acetate esterification, and a part is extracted

as the final product (Verser and Eggeman, 2013). A similar process could be envisioned for converting 1 mol ethyl acetate and 2 mol H_2 to 2 mol ethanol (Figure 1D). This reaction reaches the maximum theoretical yield of 2 $mol_{ethanol}/mol_{glucose}$. The same yield can be achieved by the current bio-ethanol process using by *S. cerevisiae* as well. However, the extraction and purification of ethanol is energy intensive (Hill et al., 2006). The glucose to ethanol via ethyl acetate may provide a new alternative to bio-based ethanol production.

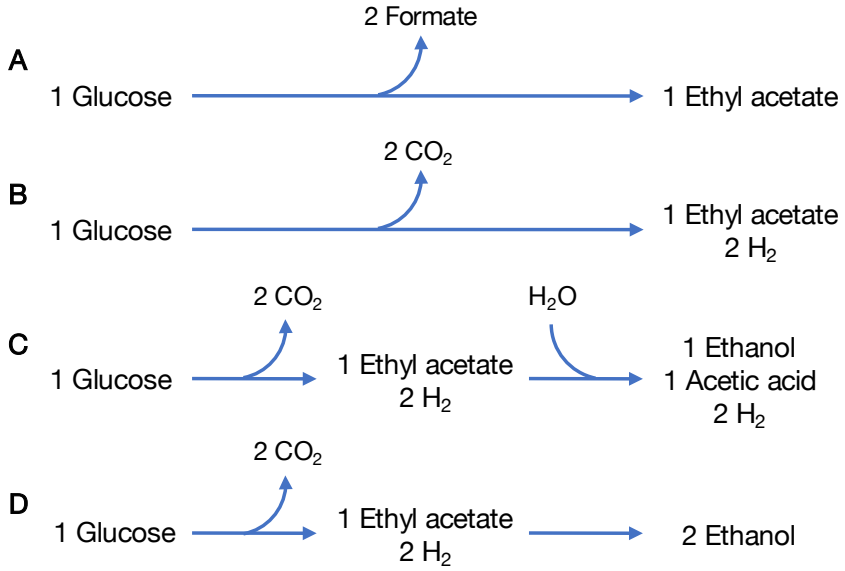

Figure 1: Versatility of anaerobic conversion of glucose into ethyl acetate.

Future outlook

This thesis has identified a novel AAT family in yeast that can efficiently catalyse ethyl acetate formation. This has opened new possibilities for the biobased production of this interesting bulk chemical. Future research should focus on optimising this production and implementing the biobased ethyl acetate-production process on an industrial level. Efforts should focus on expanding the metabolic pathways of ethyl acetate production, so that the maximum theoretical yield of 1.2 $mol_{ethyl\ acetate}/mol_{glucose}$ can be achieved. The process parameters should also be optimised to facilitate efficient *in situ* ethyl acetate recovery (Chapter 6). Moreover, it should be investigated if Eat1 can be applied for the synthesis of other industrially interesting esters and unlock the full potential of this new AAT.

BACK MATTER

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About the author

List of Publications

List of completed training activities

Acknowledgements

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About the author

Aleksander (Alex) Johannes Kruis was born on March 6th 1989 in Kranj, Yugoslavia (in a small territory now known as Slovenia). In 2011, he obtained a BSc degree in Biotechnology (with honours) from the University of Ljubljana. He then travelled to the Netherlands and started the MSc study of Biotechnology at Wageningen University, specialising in Cellular and Molecular Biotechnology. Alex obtained his MSc degree (*cum laude*) in 2013.

During his MSc studies, Alex worked on two research projects. In the first, he worked on sustainable production of succinic acid in thermophilic bacteria at the Laboratory of Microbiology at Wageningen University. He was supervised by Prof. Dr. John van der Oost, Dr. Richard Kranenburg, Dr. Elleke Bosma and Tom van de Weijer. In the second research project he worked at the Biobased Chemicals department of Wageningen University under the supervision of Dr. Ruud Weusthuis and Dr. Astrid Mars. He worked on elucidating the mechanisms behind bulk ethyl acetate synthesis in the yeast *Wickerhamomyces anomalus*.

In September 2013, Alex started his PhD research at Wageningen University under the supervision of Prof. Dr. John van der Oost, Dr. Ruud Weusthuis and Dr. Servé Kengen. He worked at the Laboratory of Microbiology and collaborated closely with the Bioprocess Engineering department, as well as the Food and Biobased Research institute. He continued his work on bulk ethyl acetate production in yeast in a project co-funded by AkzoNobel Speciality Chemicals and the BE-Basic consortium. The goal of the project was to develop ways to produce ethyl acetate as a bulk chemical from sustainable sources. Alex focused on identifying and studying the enzymes responsible for bulk ethyl acetate synthesis in yeast. He then applied the enzymes in the bacterium *Escherichia coli* to engineer and optimise ethyl acetate production under anaerobic conditions. During his PhD studies Alex helped supervise the Wageningen University IGEM team, which achieved 2nd place in this synthetic biology competition.

Currently, Alex is employed as a PostDoc researcher at the Bioprocess Engineering department of Wageningen University, where he is continuing his work on biobased ethyl acetate production using microorganisms.

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van Rijswijck, I.M.H., Kruis, A.J., Wolkers-Rooijackers, J. C.M., Abee, T., Smid E.J. Linking esterase activities to aroma profiles of *Cyberlindnera fabianii*, *Pichia kudriavzevii* and *Saccharomyces cerevisiae* - *submitted*

Koopal, B.*, Kruis, A.J.*, Claassens, N.J., Nobrega, F.L., van der Oost, J., Incorporation of a synthetic amino acid into dCas9 improves control of gene silencing - *under review at ACS Synthetic Biology*

* - contributed equally

List of patent applications

Kruis, A.J., Levisson, M., Mars, A.E., V., Kengen, S.W.M., van der Oost, J., Sanders, J.P.M., Weusthuis, R.A., 2016. Alcohol acetyl transferases for ethyl acetate production. 2017. Patent Application No. 16201644.8

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Overview of completed training activities

Discipline specific meetings and courses

Meetings and Conferences:

- BE Basic project symposia (BE-Basic), The Netherlands, annual *, ***
- NBV Biotechnology Conference (NBV), The Netherlands, 2014*
- NBV Biotechnology Conference (NBV), The Netherlands 2016**
- NKVM meetings (NKVM), The Netherlands, annual*
- ECO-BIO (Elsevier), The Netherlands, 2016*
- Metabolic engineering XI (SBE), Japan, 2016*
- ISSY 33 (Microbiological Society), Ireland, 2017** best oral presentation prize

*poster presentation **oral presentation ***pitch

Courses:

- Metabolomics (BSDL), Brazil, 2015
- Microbial Physiology and Ferm. Technology (BSDL), The Netherlands, 2015
- Masterclass Biobased Innovation (SENSE), The Netherlands, 2013
- Metabolic Engineering (VLAG), The Netherlands, 2015

General courses

- VLAG PhD week (VLAG), The Netherlands, 2014
- Competence assessment (WGS), The Netherlands, 2013
- Scientific Writing (WGS), The Netherlands, 2014
- BE-BIC workshop (BE-Basic), The Netherlands, 2013
- Scientific Artwork with Photoshop and Illustrator (WGS), The Netherlands, 2016
- Effective behaviour in your prof. surroundings (WGS), The Netherlands, 2016
- PhD Carousel (WGS), The Netherlands, 2014
- Engaging Projects Workshop (AKZO Nobel), The Netherlands, 2014
- Brain Training (WGS), The Netherlands, 2015
- Career perspectives (WGS), The Netherlands, 2016

Optional activities:

- Preparation of research proposal (The Netherlands), 2013
- Weekly group meetings (MIB-BacGen), The Netherlands, 2013-2018
- Monthly departmental meetings (MIB), The Netherlands, 2013-2018
- PhD study trip to California with organisation (MIB), 2015
- PhD study trip to Portugal (BPE), 2013

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First of all, I would like to thank all my supervisors for giving me the opportunity to work on this PhD project. As you all probably realised quite soon, I can be somewhat stubborn at times, so thank you all for accepting, or at least tolerating that. It was great to help start the project from the ground up and see it evolve to the point where it is now.

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During my PhD project we also established some collaborations with people within WUR, as well as outside. Thanks to **Irma, Judith** and **Eddy** we were able to look at the role that Eat1 has in ester production in yeast. It is a pity that we only realised we worked on somewhat related projects when we were both almost done. Nevertheless, I enjoyed working together. Also, thanks to **Brigida, Jan** and **Kevin** from Belgium for performing a genomic analysis on ester producing genes including *eat1* in yeast. This added an interesting dimension to our shared paper. We technically have not met in person, but hopefully this will change in the future. I would also like to thank **Jan-Willem** at the Laboratory of Biochemistry for all you help with the confocal microscope. Thanks also to **Simone, Michail** and **Jennifer** from Exeter for trying to determine the crystal structure of Eat1. Unfortunately, Eat1 is not cooperating yet but let us hope it will soon.

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Luckily my other paranymph, **Anna** already has this skill. You started your PhD on anaerobic ethyl acetate production when I was finishing mine (the contract part at least), and we immediately got along. Once I moved to BPE we started to collaborate closely and made a good team. The collaboration has worked well in the lab, with me focusing on molecular biology and you on fermentations and calculations. We also get along well out of the lab. This is in no small part due to our mutual affinity for beers, gin tonics or any other alcoholic beverage. You were instrumental in helping me finish our shared chapters in time for my deadline and I really appreciate your effort. I also really value our daily coffee-trip to Albert Heijn after lunch (and other times as well). I am also happy that I was able to gently re-educate your tendency to whistle while walking and working. Thanks for being my friend and my paranymph.

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Carrie, I consider myself lucky that MolEco did not want to make space for you in one of their offices when you first arrived. Instead you ended up in our BacGen office and contributed greatly to the frequency and duration of our general office conversations. You enjoyed so much that you did not want to leave in the end. We also had many fun walks to the Albert Heijn to get some coffee and many random conversations that improved my mood a lot during the last phases of finishing my thesis. I think we were also the only people to genuinely annoy Nico by bombarding him with the little aqua-balls for half an hour. That in itself was quite an achievement, considering his high tolerance to office antics. Thank you, a lot, for organising together with Teunke the surprise dinner after I handed in my thesis. We were supposed to do sports that evening but went for food and drinks instead, which I believe summarises our friendship quite well.

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coffee, even though sometimes me and Anna had to force you to join. Thanks also for all the fun nights having drinks at the department and outside. As always, I am happy to supply the alcohol. Thank you also for your critical examination of the concepts for my thesis cover and pointing out the phallic symbolism, although it only seems obvious to you.

During my PhD and PostDoc, I moved three times, which resulted in a collection of office mates. You all contributed to a fun and pleasant environment during my PhD studies. **Elleke**, you were first my supervisor during my MSc thesis (together with Tom) and then became my office mate. We had a lot of fun running around and training our condition for the Veluweloop and other competitions. You were always very optimistic and enthusiastic about work and life in general and it created a good atmosphere in the office. I can say the same for **Tom**, although he was generally more mischievous than Elleke. Thank you for all the help with running the fermenters and for running the BacGen lab. Thanks also for always organising the MIB Veluweloop team and for hiding my shoes around the Dreijen whenever you could. **Tijn**, thanks for organising the office beers and providing a good distraction from work from time to time. Hope California is treating you well! **Wen**, you were also our officemate for a while. However, we moved to the Helix soon after, so our time together was short (but sweet). I am proud of you that you are finally starting to master sarcasm. And I really feel bad about flying that drone into your face. Also, *pat pat*. Thanks also to **Yannis**, **Amos** and **Christa** for contributing to the office environment on the Dreijen.

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About the cover

The line on the cover represents in simple terms the progress through my PhD and some of the major events or themes that occurred during the process. The first critical part was the identification of the Eat1 family, which is represented by the outline of its phylogenetic relationship to other yeast AATs (taken from Chapter 2, Fig 5c). The identification resulted in low ethyl acetate production, as depicted by the two peaks of the chromatogram. The first, low peak is ethyl acetate and the second, high peak is ethanol. A major goal of the project was improving ethyl acetate production in *E. coli*. One successful way to achieve that was by truncating the N-terminus of the protein, which is represented by the increasingly shorter white lines at the point where the line bends (Based on Chapter 4, Fig. 4a). The line then continues and eventually forms the shape of a wine bottle. This connects to the role Eat1 has in ester production in fermented products (Related to the topic of Chapter 5). Finally, at the end of the line (end of the PhD), another chromatogram is shown. In this one, the first peak (ethyl acetate) is bigger than the second peak (ethanol), representing the improved ethyl acetate production that was achieved in this thesis.

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