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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.

1. 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 flavor 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, 1953; Urit et al., 2013). 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., 2004a) 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) (Fig. 1). The thermodynamic equilibrium of the esterase reaction strongly favors 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

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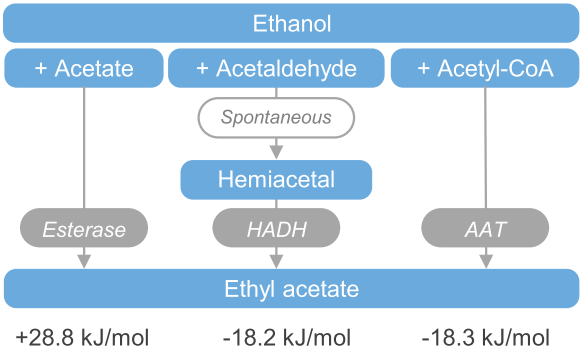

Fig. 1. Possible enzymatic reactions associated with ethyl acetate formation in yeast. Numbers represent $\Delta G_r'^m$ values of the reactions and were calculated with the Equilibrator tool (Flamholz et al., 2014).

catalyze the condensation of acetyl-CoA and an alcohol. The $\Delta G_r'$ of the latter two reactions are strongly negative and therefore more likely to be responsible for biological ethyl acetate production than esterases.

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 *Δatf1Δatf2* strain of *S. cerevisiae* still retained 50% of its ethyl acetate production, suggesting the presence of an additional ester producing enzyme (Verstrepen et al., 2003). 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 flavor 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., 2004a; 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 flavor compound.

2. Materials and methods

2.1. Strains and plasmids construction

The strains and plasmids that were used in this study are given in Tables 1 and 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 *Δku80* (Kooistra et al., 2004) homolog of *wanomala_5543* (KLLA0_E24421g) was disrupted by homologous recombination with a disruption cassette

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 *Δku80*	*Δku80*	Kooistra et al. (2004)
Kluyveromyces lactis CBS 2359 *Δku80ΔklaEat1*	*Δku80Δeat1*	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 *Δygr015C*	MATa can1D::cas9-natNT2 URA3 TRP1 LEU2 HIS3 *Δygr015C*	This study
Escherichia coli BL21 (DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5	NEB
Escherichia coli NEB® 5-alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB