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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 *Δku80*. 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.

Importance

Ethyl acetate is a common bulk chemical that is currently produced from petrochemical sources. Several Eat1-containing yeast strains naturally produce high amounts of ethyl acetate and are potential cell factories for the production of biobased ethyl acetate. Rational design of the underlying metabolic pathways may result in improved production strains, but it requires fundamental knowledge on the function of Eat1. A key feature is the location of Eat1 in the yeast cell. The precursors for ethyl acetate synthesis can be produced in multiple cellular compartments through different metabolic pathways. The location of Eat1 determines the relevance of each pathway, which will provide future targets for the metabolic engineering of bulk ethyl acetate production in yeast.

Introduction

Ethyl acetate is a valuable bulk chemical and an important aroma compound in fermented foods (1). 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 (2). Several non-*Saccharomyces* yeast species produce ethyl acetate from carbohydrates at a much higher yield than *S. cerevisiae* (3). Ethyl acetate yields up to 51.4 % of the theoretical pathway maximum have been reported in *Kluyveromyces marxianus* (4). Other bulk ethyl acetate producing yeasts include *Wickerhamomyces anomalus* (5, 6), *Cyberlindnera fabianii* (7) and *Kluyveromyces lactis* (8).

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* (9, 10). A *S. cerevisiae* strain lacking *atf1* and *atf2* produced 50 % less ethyl acetate compared to the parental strain (11). Homologs of Atf1 and Atf2 are present in bulk ethyl acetate producing yeasts (12, 13).

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 (3, 14). 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 (19). Ethyl acetate is formed under conditions where the efficiency of the TCA cycle is impaired by e.g. iron or oxygen limitation (15, 16). As a consequence, acetyl-CoA cannot enter the TCA cycle and accumulates in the mitochondria. It is assumed that yeasts use an AAT-catalysed

67 reaction to relieve the acetyl-CoA accumulation and regenerate free CoA (3, 18). Ethyl
68 acetate is formed in the process. This hypothesis would imply that mitochondrial acetyl-CoA
69 accumulation causes ethyl acetate production (17).

70 Ethanol is the second substrate needed for ethyl acetate synthesis by AATs. Crabtree-
71 negative yeasts typically do not form ethanol under aerobic conditions. However,
72 unfavourable conditions, such as iron limitation, lead to ethanol formation in *K. marxianus*
73 even in the presence of oxygen (16, 20). Ethanol is produced from pyruvate via acetaldehyde
74 in the cytosol (Figure 1). The acetaldehyde may also be converted to acetate and further to
75 cytosolic acetyl-CoA via acetyl-CoA synthetase (Figure 1, Reaction II). This reaction is
76 essential in most yeasts as it supplies acetyl-CoA for fatty acid synthesis (21). However,
77 during aerobic growth on sugars, the acetyl-CoA flux in the cytosol is much lower compared
78 to the mitochondria (22). It is therefore unlikely that it contributes significantly to bulk ethyl
79 acetate synthesis. Moreover, bulk ethyl acetate synthesis in yeast does not occur in the
80 absence of oxygen (17). Under anaerobic conditions, carbohydrate catabolism occurs in the
81 cytosol, and mitochondrial acetyl-CoA cannot accumulate. These observations strongly
82 suggest that acetyl-CoA used to synthesise ethyl acetate is derived from the mitochondria.

83 Atf1, Atf2 and their homologs appear to be cytosolic, or located in the endoplasmic reticulum
84 (23–25). A translocation step would therefore be required to transfer acetyl-CoA from the
85 mitochondria to the cytosol. Some yeasts are able to translocate acetyl-CoA to the cytosol in
86 the form of citrate. This shunt relies on the presence of ATP-citrate lyase, which converts
87 citrate to acetyl-CoA and oxaloacetate at the expense of one ATP (Figure 1, Reaction III).
88 The reaction is typically present in oleaginous yeasts, such as *Yarrowia lipolytica* or
89 *Rhodosporidium toruloides* (26, 27). It is not known if ATP-citrate lyase is present in any of
90 the yeasts that produce high amounts of ethyl acetate. Without this enzyme, transport of
91 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 (28). 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* (29). This included the hypothetical protein YGR015C, which was later identified as the *S. cerevisiae* homolog of Eat1 (28). The *S. cerevisiae* Eat1 was tracked to the mitochondria (29), 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 (28).

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 dye 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

128 supplementation. The strain producing the Eat1-yEGFP fusion was still able to synthesise
129 ethyl acetate (Figure 3). Surprisingly, the ethyl acetate titre achieved by *K. lactis* CBS 2359
130 Δku80 *eat1-yEGFP* was 3.75-fold higher compared to *K. lactis* CBS 2359 Δ*ku80*. The reason
131 for this increase is not clear, but it demonstrated that the Eat1-yEGFP fusion is still
132 functional. These results show that ethyl acetate itself is primarily a mitochondrial product of
133 *K. lactis* CBS 2359 Δ*ku80*.

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

135 As Eat1 is located in the mitochondria of *S. cerevisiae* and *K. lactis* CBS 2359 Δ*ku80,* we
136 were wondering whether it is located in the mitochondria of other bulk ethyl acetate
137 producing yeasts as well. The expression of GFP-fused proteins in these yeasts is
138 cumbersome because of their poor genetic accessibility. We attempted to transform *W.*
139 *anomalus* DSM 6766 and *K. marxianus* DSM 5422, but were not successful. To gain further
140 insight on the location of ethyl acetate synthesis in other yeast strains, we compared the
141 natural producers *in vivo* instead. We reasoned that if the conditions that trigger bulk ethyl
142 acetate formation are similar, the underlying pathways are likely shared as well, including the
143 cellular location of Eat1. However, there are no studies that accurately compare ethyl acetate
144 production by multiple yeasts under the same conditions. Moreover, many studies on bulk
145 ethyl acetate synthesis in yeast often did not control or measure parameters such as oxygen
146 levels or ethyl acetate evaporation (3). This makes metabolic comparisons between different
147 yeast species impossible. To resolve the issue, we examined bulk ethyl acetate production in
148 five yeast species under the same controlled conditions. We used aerobic, iron-limited
149 chemostats to induce ethyl acetate production in *W. anomalus* DSM 6766, *C. fabianii* CBS
150 5640, *C. jadinii CECT* 1946, *K. marxianus* DSM 5422, and *K. lactis* CBS 2359. When 1 mM
151 $FeSO_4$ was added to the medium, all five yeasts fully consumed the glucose, and virtually no
152 ethyl acetate or other fermentation products were formed (Figure 4). To induce ethyl acetate
153 production, iron was omitted from the medium. Sufficient iron impurities were present to
154 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 $g_{EA}/g_{glucose}$.

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 (16, 20). *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

182 produced ethyl acetate under the same iron-limited conditions as *K. lactis* CBS 2359, which
183 suggests that the ethyl acetate produced by these yeasts is of mitochondrial origin as well.

184 *In silico* indications for Eat1 localisation in yeast

185 Acetyl-CoA used for bulk ethyl acetate synthesis is produced in the mitochondria. We
186 investigated whether the five yeast strains are able to transport acetyl-CoA from the
187 mitochondria to the cytosol via citrate. The enzyme needed for the realisation of this pathway
188 is the cytosolic ATP-citrate lyase (Figure 1). We used BlastP to search for homologs of three
189 fungal ATP-citrate lyase proteins in *W. anomalus*, *C. jadinii*, *C. fabianii*, *K. marxianus* and *K.*
190 *lactis.* The ATP-citrate lyase homologs originated from *Yarrowia lipolytica* CLIB 122,
191 *Aspergillus nidulans* FGSC A4 and the *Rhodosporidium toruloides* IFO 0880. None of the
192 bulk ethyl acetate producing yeasts contained apparent ATP-citrate lyase homologs. The
193 absence of the ATP-citrate lyase suggests that ethyl acetate-producing yeasts cannot
194 transport acetyl-CoA to the cytosol (22, 26). Bulk ethyl acetate synthesis by Eat1 is therefore
195 more likely to be located in the mitochondria.

196 The sub-cellular location of proteins can also be predicted *in silico* based on their primary
197 sequence. We predicted the location of nine Eat1 homologs originating from nine bulk ethyl
198 acetate producing yeast species (28). The mitochondrial citrate synthase (Cit1) and the
199 cytosolic Atf1 and Atf2 from *S. cerevisiae* were included in the analysis as controls. Seven
200 tools were used in the analysis: MitoProt II (30), MultiLoc 2 (31), Yloc (32), WoLF PSORT
201 (33), Protein Prowler (34) and BacCelLo (35) and MitoFates (36). The Eat1 homologs were
202 generally predicted as mitochondrial (Figure 5). The Eat1 homologs of *K. lactis, K. marxianus*
203 and *Wickerhamomyces ciferri* were predicted as mitochondrial by all seven tools. The least
204 mitochondrial localisation predictions were given to the *W. anomalus* and *C. fabianii* Eat1
205 homologs. Most of the non-mitochondrial predictions were assigned by Protein Prowler, Mito
206 Prot II and MitoFates. These tools also did not identify the *S. cerevisiae* Eat1 protein as
207 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 (31, 37).

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 (28). 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 (17). 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 (2). 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 (19, 38). It is possible that the low ethyl acetate production in *S. cerevisiae* is caused by the low mitochondrial acetyl-CoA flux.

234 *W. anomalus* DSM 6766, *C. fabianii* CBS 5640, *C. jadinii CECT* 1946, *K. marxianus* DSM
235 5422 and *K. lactis* CBS 2359 are all Crabtree-negative yeasts. Under aerobic conditions,
236 such yeasts convert glucose to cytosolic pyruvate and further to mitochondrial acetyl-CoA.
237 Conditions such as iron limitation repress the synthesis of enzymes in the TCA cycle and
238 respiratory chain (39, 40). As a consequence, ethanol is produced in the cytosol even under
239 aerobic conditions (20, 41). In the mitochondria, iron limitation leads to the accumulation of
240 acetyl-CoA. The accumulation may be resolved by Eat1 in the mitochondria by forming ethyl
241 acetate (14, 18, 41). The mitochondrial localisation of Eat1 thus agrees with its proposed
242 physiological function of preventing the accumulation of acetyl-CoA in yeast. However, more
243 research is needed to confirm this hypothesis. It has been shown that Eat1 can function as a
244 thioesterase *in vitro* (28). This activity could relieve acetyl-CoA accumulation by releasing
245 acetate instead of ethyl acetate. However, it is possible that ethyl acetate formation provides
246 ancillary benefits to the yeasts. It has been shown that ethyl acetate inhibits the growth of
247 competitive microorganisms (42) and helps yeast disperse in the environment by attracting
248 fruit flies (43).

249 The demonstration that Eat1 is a mitochondrial enzyme is critical for improving ethyl acetate
250 production by microorganisms. Yeasts like *K. marxianus* and *W. anomalus* are naturally able
251 to produce ethyl acetate at high yields. However, they also produce considerable amounts of
252 ethanol and acetate as by-products. The acetyl-CoA and ethanol used for ethyl acetate
253 synthesis are produced in the mitochondria and cytosol, respectively. Cytosolic pyruvate is
254 the precursor of both substrates. Efficient ethyl acetate production would therefore require
255 precise control over the pyruvate flux so that acetyl-CoA and ethanol production are
256 stoichiometrically balanced. Alternatively, Eat1 can be used to produce ethyl acetate in
257 heterologous hosts. In such cases, consideration should be given to identifying the
258 localisation pre-sequence. In eukaryotes, N-terminal pre-sequences are cleaved from the
259 nascent protein during translocation to the mitochondria, giving rise to the mature protein
260 (44). Unrelated hosts may not be able to perform this cleavage. The presence of the N-

261 terminal localisation sequence has resulted in lower protein activity and stability in some
262 cases (45, 46). Proper N-terminal processing of the Eat1 proteins may therefore improve the
263 activity of the protein in heterologous hosts.

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

270

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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* (47) was a gift from Paul Hooykaas (Leiden University). Plasmid pCY 3040-01 was a gift from Anne Robinson (Addgene plasmid # 36217). Plasmid pUG75 (48) was obtained from Euroscarf (Plasmid #P30671). *K. lactis* CBS 2359 *Δku80 eat1-yEGFP* was constructed by integrating the *yegfp* gene (49) 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 (50). 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 *Δku80* and *K. lactis* CBS 2359 *Δku80 eat1-yEGFP* was assessed as reported previously (28). The cells were grown in 250-mL Erlenmeyer flasks containing 50 mL YM (yeast minimal) medium adapted from Thomas and Dawson, 1978 (18). 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), Ca, $ZnSO_4$ *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 (51). 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 (52) 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 (51). 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 (53).

Bioinformatics

The subcellular locations of proteins was predicted with six tools: MitoProt II (30), MultiLoc 2 (31), Yloc (32), WoLF PSORT (33), Protein Prowler (34) and BacCelLo (35). Where applicable, the prediction settings were set to fungal. MitoFates (36) 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 (54–56).

Analytical

Glucose and organic acids were analyzed 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 aluminum tray before weighing.

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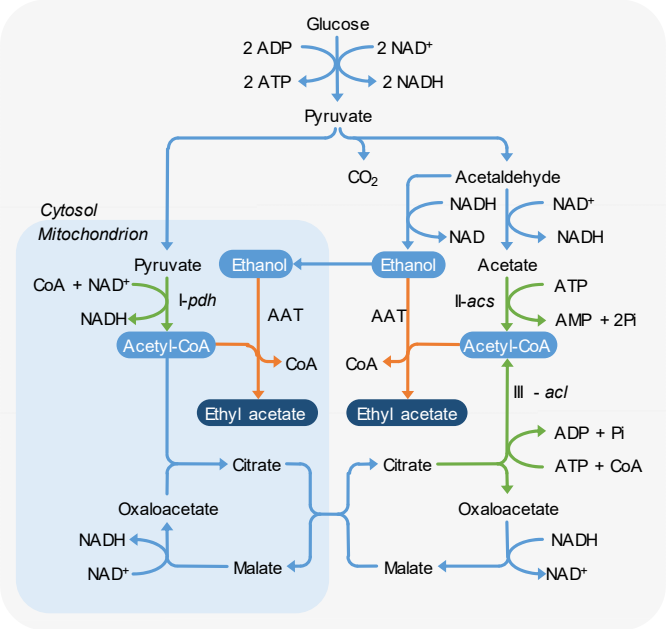
562 **Acknowledegements**

563 We would like to acknowledge the BE-Basic foundation and AkzoNobel Specialty Chemicals
564 for funding the research.

565



566

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

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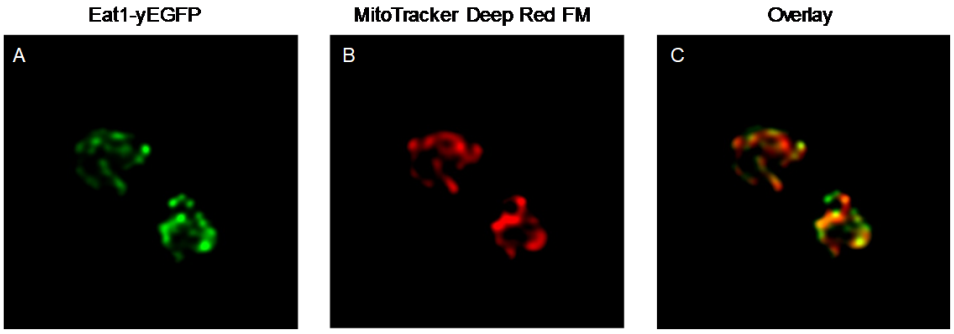


Figure 2. Visualisation of Eat1 in the mitochondria of *K. lactis* CBS 2359 Δ*ku80 eat1-yegfp*. (A) Visualisation of Eat1-yEGFP. (B) Mitochondria visualised by MitoTracker Deep Red FM. (C) Overlay of the two signals. The images shown are representative of the entire cell population.

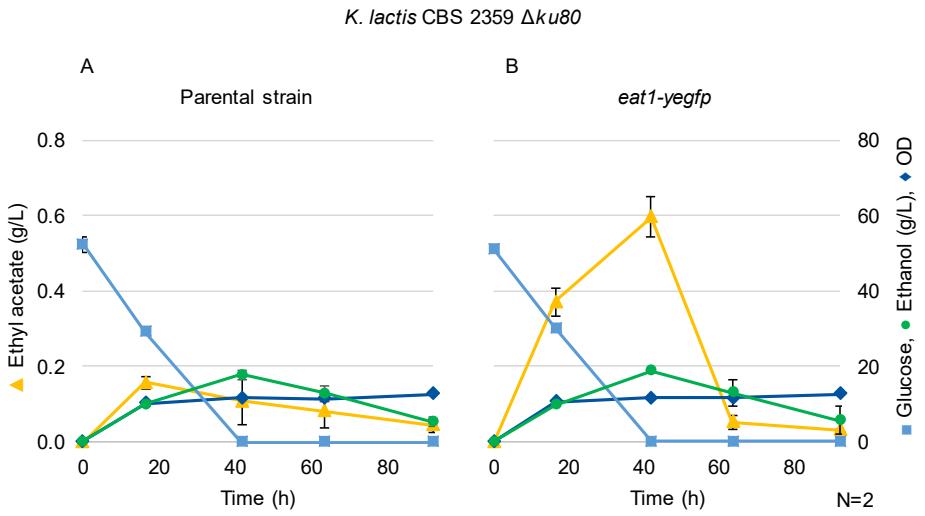
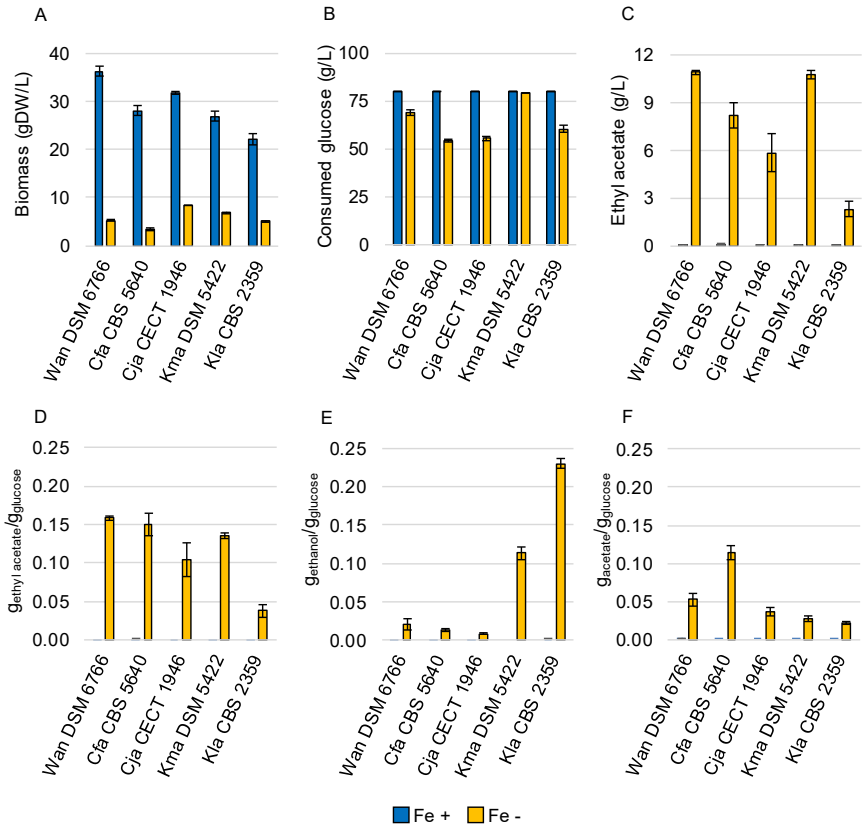


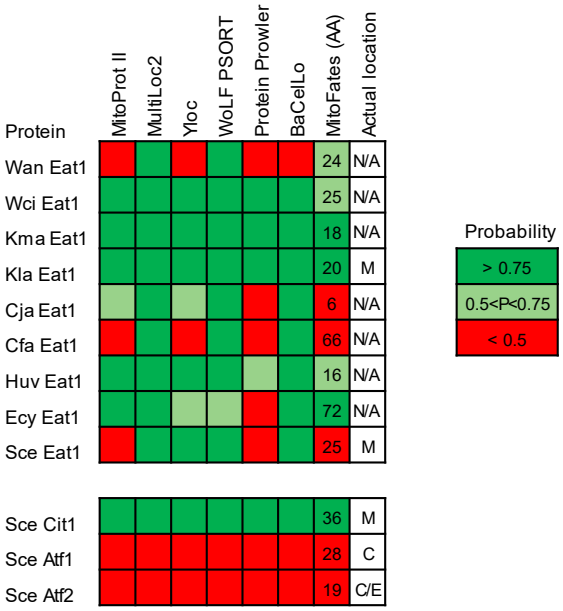
Figure 3. Fermentation profiles of (A) *K. lactis* CBS 2359 Δku80 and (B) *K. lactis* CBS 2359 Δku80 *eat1-yEGFP* growing in shake flasks in 50 ml YM medium without iron supplementation. The numbers shown are the averages of biological duplicates. Error bars represent the standard deviation. Ethyl acetate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



586
587 Figure 4. Cultivation parameters of five different yeast species that were grown in aerobic,
588 pH-controlled chemostats in the presence or absence of 1 mM of $FeSO_4$. The numbers
589 shown are averages of three steady states. Error bars indicate standard deviation. The
590 amount of ethyl acetate that was removed by gas stripping was determined by headspace
591 measurements and was added to the amount that was measured in the liquid phase. (A)
592 Biomass concentration. (B) Glucose consumption. (C) Ethyl acetate titre. (D, E, F) The yields
593 of ethyl acetate, ethanol and acetate on glucose consumed, respectively.

594

595



596

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

605

606 Table 1: Strains and plasmids used and created during this study.

Strain	**Characteristics**	**Source**
Cyberlindnera fabianii CBS 5640	Wild type strain	CBS
Cyberlindnera jadinii CECT 1946	Wild type strain	CECT
Kluyveromyces marxianus DSM 5422	Wild type strain	DSMZ
Wickerhamomyces anomalus DSM 6766	Wild type strain	DSMZ
Kluyveromyces lactis CBS 2359	Wild type strain	CBS
Kluyveromyces lactis CBS 2359 *Δku80*	Disruption of KU80	(47)
Kluyveromyces lactis CBS 2359 *Δku80 eat1-yegfp*	Disruption of KU80, Eat1 labelled with a C-terminal yEGFP	This study
E. coli NEB5α	Cloning strain	New England Biolabs
Plasmids		
pUG75	HygR marker template	(48)
pCY-3040-01	yEGFP template	(49)
pYES2		Invitrogen
pYES2-KlaEat1-yEGFP-hphMX-1000	Plasmid carrying the *yegfp* integration cassette targeting the 3'end of the *K. lactis eat1* locus with 1000 bp homologous regions	This study

607