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Enhanced Production of Isobutyl and Isoamyl Acetate Using *Yarrowia lipolytica*

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Abstract

Short-chain esters, particularly isobutyl acetate and isoamyl acetate, hold significant industrial value due to their wide-ranging applications in flavors, fragrances, solvents, and biofuels. In this study, we demonstrated the biosynthesis of acetate esters using *Yarrowia lipolytica* as a host by feeding alcohols to the yeast culture. Initially, we screened for optimal alcohol acyltransferases for ester biosynthesis in *Y. lipolytica*. Strains of *Y. lipolytica* expressing *atf1* from *Saccharomyces cerevisiae*, produced 251 mg/L or 613 mg/L of isobutyl acetate or of isoamyl acetate, respectively. We found that introducing additional copies of *ATF1* enhanced ester production. Furthermore, by increasing the supply of acetyl-CoA and refining the culture conditions, we achieved high production of isoamyl acetate, reaching titers of 3404 mg/L. We expanded our study to include the synthesis of a range of acetate esters, facilitated by enriching the culture medium with various alcohols. This study underscores the versatility and potential of *Y. lipolytica* in the industrial production of acetate esters.

Keywords: acetate ester, *Yarrowia lipolytica*, alcohol acyltransferase, metabolic engineering

1. Introduction

Short-chain esters, which are volatile organic compounds, are commonly found in a variety of botanical sources, such as flowers, ripened fruits, and yeast cultures undergoing fermentation.[1,2] They have numerous industrial applications, serving as essential components in the production of flavors, fragrances, solvents, and potential biofuels.[3] Isobutyl acetate is a promising candidate for biofuel applications due to its superior volatility, reduced polarity, and diminished hygroscopicity

compared to its alcohol-based counterparts, like isobutanol.[4] Isoamyl acetate, also known as 3-methyl-1-butyl acetate or banana oil due to its characteristic scent, had a substantial market value, estimated at $5 billion in 2019.[5] Ethyl valerate, an isomeric counterpart of isoamyl acetate, demonstrates excellent compatibility with traditional fuel sources such as gasoline or diesel, highlighting the potential of isoamyl acetate as a viable biofuel substitute.[6]

Certain microorganisms have the ability to synthesize esters through the enzymatic condensation of alcohols with acyl-CoA substrates, a process mediated by alcohol acyltransferases (AATs).[7-9] Acetate esters, among the most common esters in nature, originate from the ubiquitous and vital metabolic intermediary, acetyl-CoA. The yeast *Saccharomyces cerevisiae* has shown the ability to metabolize sugars into ethyl acetate, which significantly contributes to the organoleptic profile of wines and beers.[10,11] Ethyl acetate biosynthesis has also been observed in other yeasts such as *Wickerhamomyces anomalu*s and *Kluyveromyces marxianus*, achieving ethyl acetate production with yields of 0.18 g/g-sugar and 0.29 g/g-sugar, respectively.[12,13] The physiological parameters governing the production of ethyl acetate by these yeasts have been characterized; *W. anomalus* produces ethyl acetate under conditions of oxygen limitation,[14] whereas *K. marxianus* does so in response to iron limitation.[15] Despite numerous research findings, significant challenges remain in the strategic development of strains optimized for enhanced production.[16]

The transition toward microbial synthesis offers a sustainable alternative to traditional petrochemical-based processes and extraction from natural sources. Microorganisms have been successfully engineered to produce a wide range of bioactive compounds and industrially relevant chemicals from renewable substrates, such as sugars and CO_2. This field has witnessed significant advancements.[15-21] For instance, *Escherichia coli,* when engineered with the isobutanol biosynthetic pathway and alcohol acyltransferase (Atf1p), has demonstrated a notable capacity for isobutyl acetate production, reaching up to 36 g/L.[4] Furthermore, the biosynthesis of isoamyl acetate has been achieved through the condensation of isoamyl alcohol with acetyl-CoA, with reported titers reaching 8.8 g/L.[16]

Yarrowia lipolytica is a nonconventional yeast species that is phylogenetically distinct from the extensively studied *S. cerevisiae*. It is characterized by its easy genetic tractability, robust growth kinetics, and GRAS status by FDA. These characteristics make it an ideal host for the biosynthesis of pharmacologically active compounds such as resveratrol,[22-24] itaconic acid,[25,26] and triacetic acid lactone.[27,28] Furthermore, *Y. lipolytica* is adept at lipid and fatty acid biosynthesis owing to its inherent mechanisms for acetyl-CoA and malonyl-CoA syntheses.[29-31] Despite these promising applications, refining microbial ester production represents a complex challenge, necessitating extensive research to identify and manipulate the metabolic pathways involved, thereby enabling the sustainable exploitation of ester synthesis.

This study investigated the production of esters using *Y. lipolytica* as a production platform. We first screened for AATs that are optimally suited for ester biosynthesis in *Y. lipolytica*. Subsequently, we attempted to increase the intracellular pool of acetyl-CoA and the expression levels of AAT, affirming the potential of *Y. lipolytica* as a viable microbial factory for ester production. This approach underscores the strategic manipulation of metabolic pathways to enhance the yield and productivity of target biochemicals.

2. Materials and methods

2.1 Strains and media

The study utilized various strains, all of which are detailed in Table 1. The parental strain

employed was *Y. lipolytica* PO1fΔku70Δku80.[28] The yeast strains were cultivated in YPD medium (BD Diagnostic Systems, Sparks, MD, USA) or YNB medium (Formedium, Norfolk, UK).

2.2 Construction of plasmids and transformation

The plasmids and primers used in this study are listed in Tables S1 and S2, respectively. The plasmid pCRISPRyl[32] was procured from Addgene (Watertown, MA, USA). A 20-base seed sequence, along with the NGG PAM sequence (N20NGG) in the *Y. lipolytica* genome, was selected using CHOPCHOP (https://chopchop.cbu.uib.no/). The homologous recombination (HR) donor sequences included donor arms approximately 500 bp in length, both upstream and downstream of the Cas9 cutting site and the intended insertion sites. A Cas9 protein expression plasmid, targeting *A08*, was assembled using the KOD-Plus-Mutagenesis Kit (TOYOBO, Co. Ltd., Osaka, Japan), following the manufacturer's instructions. pCRISPRyl was used as a template with the primer pair fw-Cas9-A08-inv/rev-Cas9-A08-inv. The resultant plasmid was named pCRISPRyl_A08. Other plasmids for Cas9 targeting were similarly constructed and are summarized in Table S1.

An HR donor for *ATF1* expression was constructed as follows: A DNA fragment was amplified using the primer pair fw-pHR-atf1-insert/rev-pHR-atf1-insert, with a codon-optimized *atf1* fragment (Invitrogen, Waltham, MA, USA; Table S3) as a template. The fragment was ligated into the *Asc*I and *Nhe*I sites of the plasmid pHR_A08_hrGFP. Using this resultant plasmid as a template, the region encoding UAS1B8-TEF(136) promoter-PEX10-CYCterminator was amplified using the primer pair fw-Avr2-xyr6/rev-Spe1-xyr6. The upstream and downstream regions of *xyr6* were amplified with the primer pairs fw-xyr6-1/rev-xyr6-2 and fw-xyr-3/rev-xyr6-4, respectively. These three amplified fragments were conjugated using overlap extension PCR. Other fragments were similarly constructed. To construct HR donor DNA to disrupt *DGA1*, the upstream and downstream regions were amplified using the primer pairs fw-dga1-knock_out-1/rev-dga1-knock_out-2 and fw-dga1-knock_out-3/rev-dga1-knock_out-4, respectively. The two amplified fragments were conjugated using overlap extension PCR. Other HR donor DNAs were similarly constructed.

All strains were constructed using the lithium acetate method[28] by co-transformation of 100 ng of the pCRISPRyl vector series with 500 ng of the respective HR donor DNA. Before the transformation, the cells were incubated overnight at 30°C with shaking at 220 rpm in test tubes containing 5 mL of YPD medium. The transformants were then spread onto YNB plates supplemented with 125 mg/L uracil when necessary.

2.3 Culture conditions and analytical conditions

For the production of esters, the strains were initially precultured in 5 mL of YPD medium for 48 h at 30°C. This starter culture was used to inoculate 5 mL of YNB medium supplemented with 20g/L glucose and 5 g/L of ethanol or appropriate alcohol at an initial $O.D._{600}$ of 0.3 in a 50-mL conical tube. After overlaying with 2 mL of oleyl alcohol, the tubes were wrapped in PTFE tape to seal the threading and then capped to ensure complete anaerobic conditions. The resulting culture was agitated for 72 h at 30°C and 220 rpm.

After cultivation, oleyl alcohol overlay was collected and stored at room temperature for ester analysis. The amount of ester was evaluated using a gas chromatograph (GC-2025,

Shimadzu, Kyoto, Japan) equipped with an SH-stabilwax column (30 m, 0.25-mm internal diameter; Shimadzu). Helium was used as the carrier gas at a flow rate of 2 mL/min. The injection volume was set at 1 μL with a split ratio of 1:50. The oven temperature was initially maintained at 40°C for 1 min, after which it was gradually increased to 120°C at a rate of 20°C/min and further to 250°C at a rate of 120°C/min.

3. Results and discussion

3.1. Construction of acetate ester-producing *Y. lipolytica* strains

We investigated the ability of the PO1f strain to produce acetate esters. A concentration of 5 g/L each of ethanol, isobutanol, and isoamyl alcohol was introduced into the culture medium, followed by an overlay of oleyl alcohol. The cells were subsequently incubated at 30°C for 72 h. As shown in Figure 1, the PO1f strain synthesized only 35 mg/L of ethyl acetate and failed to produce isobutyl acetate or isoamyl acetate. Consequently, we attempted to identify potential acetate ester-producing genes and integrated into *Y. lipolytica*. Our selection focused on four genes—*ATF1*, *ATF2*, *EAT1*, and *EHT1*—originating from *S. cerevisiae*. Atf1p and Atf2p are responsible for ethyl acetate production in yeast,[9] whereas Eat1p and Eht1p contribute to ester production.[33] Furthermore, ester production has been reported in budding yeast expressing *ATF1*, demonstrating that *ATF1* and *ATF2* are suitable for ester production when *E. coli* is used as a host.[4,34] Additionally, *EAT1*, derived from *K. marxianus*, or *W. anomalus*, or *Wickerhamomyces ciferrii*, has been reported to be effective in enhancing ethyl acetate production in *S. cerevisiae* as a host.[12] In our study, these genes were introduced into the *XYR6* or *XYR11* locus of POf1 (Table 1). The constructed strains were cultivated in the presence of 5 g/L of ethanol, isobutanol, and isoamyl alcohol, respectively, with an oleyl alcohol overlay. The results (Figure 1) indicated a slight enhancement in ethyl acetate production across all genes, peaking at 61 mg/L. Conversely, *Y. lipolytica* strains expressing *ATF1* exhibited a significant increase, producing 251 mg/L of isobutyl acetate or 613 mg/L of isoamyl acetate. These findings indicate the ability of Atf1p to synthesize esters in *Y. lipolytica*, thereby enabling the high-yield production of isobutyl and isoamyl acetates.

The heterologous expression of *ATF1* in *E. coli* has been observed to exhibit a higher activity in ester production compared to *ATF2*.[12] Our findings suggest that *ATF1* is well-suited for the production of isobutyl acetate or isoamyl acetate in *Y. lipolytica*. Interestingly, in *E. coli* expressing *ATF1*, a larger quantity of isobutyl acetate is produced compared to isoamyl acetate.[34] However, when *Y. lipolytica* was used as the host, the titer of isoamyl acetate exceeded that of isobutyl acetate (Figure 1). The reported kinetic parameters corroborate this observation, demonstrating that the kcat/km value for isoamyl alcohol is higher than that for isobutanol.[34] This disparity may be attributed to the membrane-dependent enzymatic activity of *S. cerevisiae* Atf1p and Atf2p, which localize to lipid droplets via amphipathic helices present at both their N- and C-termini.[35-37] The observed variance in activity in *Y. lipolytica* could potentially be attributed to a difference in enzyme localization, differing from that observed in its native host. The strain expressing *ATF1* was designated as EST-1 and was used in subsequent experiments.

Table 1. Strains used in this study.

Strain	Genotype	Source

Escherichia coli Nova Blue	endA1, hsdR17, (rK-mK+), supE44, thi-1, gyrA96, relA1, lac, recA1/F' [proAB+, lac lq Z ΔM15, Tn10(tetr)]	Novagen
PO1f	*MATA ura3-302 leu2-270 xpr2-322 axp2-deltaNU49 XPR2::SUC2*	ATCC (MYA-2613)
EST-1	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1*	This study
atf2	PO1f Δ*ku70Δku80,* Δ*XYR6*::ATF*2*	This study
eat1	PO1f Δ*ku70Δku80*, Δ*XYR6::EAT1*	This study
eht1	PO1f Δ*ku70Δku80*, Δ*XYR11::EHT1*	This study
Kma	PO1f Δ*ku70Δku80*, Δ*XYR6::EAT1*	This study
Wan	PO1f Δ*ku70Δku80*, Δ*XYR11::EAT1*	This study
Wci	PO1f Δ*ku70Δku80*, Δ*XYR6::EAT1*	This study
EST-2	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1,* Δ*A08::ATF1*	This study
EST-3	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1,* Δ*A08::ATF1,* Δ*XYR11::ATF1*	This study
EST-4	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1,* Δ*D17::PEX10*	This study
EST-5	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1,* Δ*C18::POR1*	This study
EST-6	PO1f Δ*ku70Δku80*, Δ*XYR6*::*ATF1*, Δ*D17*::*PEX10*, Δ*C18*::*POR1*	This study
EST-7	PO1f Δ*ku70Δku80*, Δ*XYR6*::*ATF1*, Δ*D17*::*PEX10*, Δ*C18*::*POR1,*Δ*inte3::ACS1*	This study
EST-8	PO1f Δ*ku70Δku80*, Δ*XYR6*:*ATF1*, Δ*D17*::*PEX10*, Δ*C18*::*POR1,* Δ*inte3::PDC2*	This study
EST-9	PO1f Δ*ku70Δku80*, Δ*XYR6*::*ATF1*, Δ*D17*::*PEX10*, Δ*C18*::*POR1, Δinte3::PDC2, ΔXDH::ALD5*	This study
EST-13	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1,* Δ*A08::ATF1,* Δ*XYR11::ATF1,* Δ*DGA1*	This study
EST-14	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1,* Δ*A08::ATF1,* Δ*XYR11::ATF1,* Δ*DGA2*	This study
EST-15	PO1f Δ*ku70Δku80*, Δ*XYR6::ATF1,* Δ*A08::ATF1,* Δ*XYR11::ATF1,* Δ*IRO1*	This study

EST-16	PO1f Δ*ku70Δku80*,Δ*XYR6::ATF1*, Δ*A08::ATF1*, Δ*XYR11::ATF1*, Δ*OIL1*	This study
EST-17	PO1f Δ*ku70Δku80*,Δ*XYR6::ATF1*, Δ*A08::ATF1*, Δ*XYR11::ATF1*, Δ*TGL4*	This study

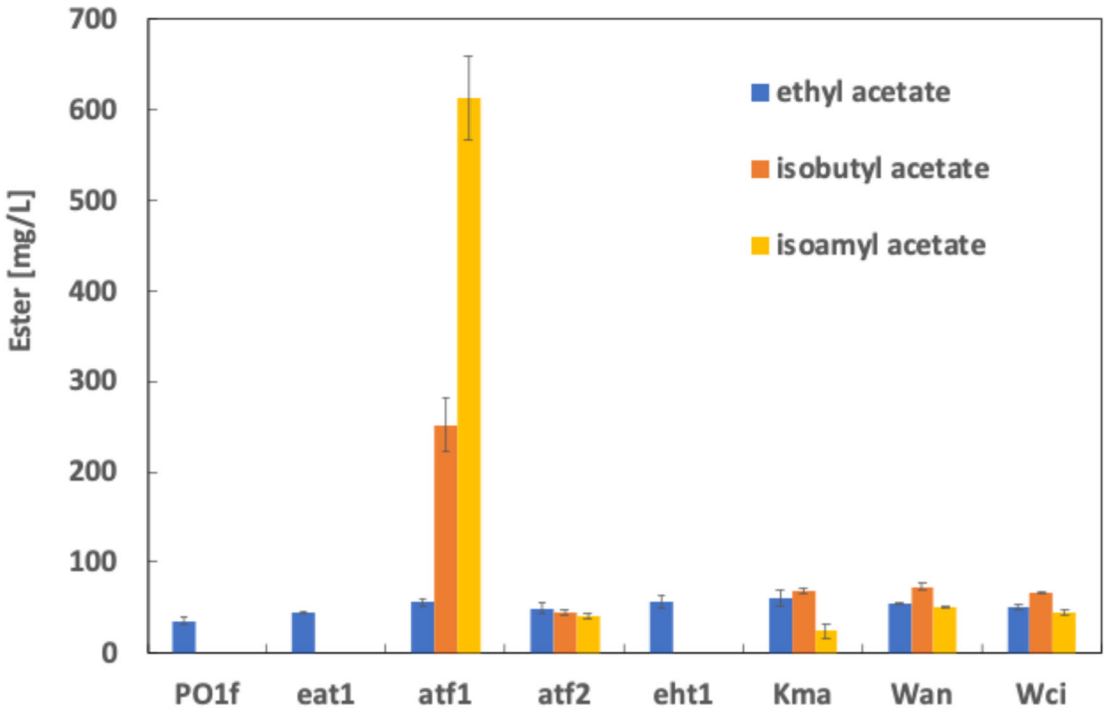


Figure 1. Ester production using AAT-expressing *Y. lipolytica* after 72 h of anaerobically cultivation. Ethanol, isobutanol, or isoamyl alcohol (5 g/L) were supplemented in YNB medium containing 20g/L glucose. Abbreviations: Wan; *eat1* from *Wickerhamomyces anomalus*, Wci; *eat1* from *Wickerhamomyces ciferrii*, Kma; *eat1* from *Kluyveromyces marxianus*. Data are expressed as the means and standard deviations of three independent experiments.

3.2. Effects of improved acetyl-CoA synthesis by enhanced β-oxidation or pyruvate bypass pathway

To optimize ester production, we deliberately enhanced genes involved in the β-oxidation pathway, a crucial metabolic route in *Y. lipolytica*, as depicted in Figure 2. In this organism, fatty acid degradation proceeds through β-oxidation within peroxisomes, yielding acetyl-CoA and acyl-CoA, which are essential intermediates utilized in the tricarboxylic acid cycle and glyoxylate shunt.[38] We focused on two key genes: *PEX10* (YALI0C01023) and *POR1* (YALI0D12628).

Overexpression of *PEX10*, responsible for peroxisome biogenesis, has been linked to enhanced peroxisomal proliferation.[39] Meanwhile, upregulation of *POR1*, a vital regulator in fatty acid metabolism has been shown to enhance polyketide synthesis.[40,41] In *Y. lipolytica*, increased expression of both genes has been associated with an increase in the production of TAL (triacetic acid lactone).[28,42] Given the central role of acetyl-CoA in these pathways, we hypothesized that enhancing *PEX10* and *POR1* expression would increase ester biosynthesis.

To evaluate this hypothesis, we engineered three strains: EST-4 with elevated *PEX10* expression, EST-5 with increased *POR1* expression, and EST-6 with upregulated expression of both genes. Contrary to expectations, all modified strains exhibited reduced ester synthesis compared to the control EST-1 (Figure 3). This decrease was particularly pronounced in the production of isobutyl acetate or isoamyl acetate, suggesting limited availability of the acetyl-CoA substrate. Given that ester synthesis is predominantly active under microaerobic conditions, it is conceivable that elevated expression of *PEX10* and *POR1* may hinder peroxisomal activity.

In *Y. lipolytica*, the conversion of pyruvate to acetaldehyde is facilitated by pyruvate decarboxylase (*PDC*) (YALI0D06930), followed by the transformation of acetaldehyde to acetate via acetaldehyde dehydrogenase (*ALD*) (YALI0D07942), ultimately leading to acetyl-CoA synthesis through acetyl-CoA synthetase (*ACS*)(YALI0F05962).[43] A previous study demonstrated that upregulation of enzymes in this pathway enhances free fatty acids[31], triacylglycerols[31] or TAL production in *Y. lipolytica*.[27] Building upon this, our study aimed to further augment ester production by enhancing the expression of *PDC2*, *ALD5*, and *ACS1*, thereby increasing acetyl-CoA biosynthesis. We engineered the strains EST-7 (EST-6/*Δinte3::ACS1*), EST-8 (EST-6/*Δinte3::PDC2*), and EST-9 (EST-6/*Δinte3::PDC2,Δxdh::ALD5*). However, all strains exhibited decreased ester production (Figure 3). Notably, ester production in the EST-7 strain was comparable to that in the EST-5 and EST-6 strains. Minimal acetate accumulation was detected in the culture media, suggesting an insufficient acetate supply. Additionally, expression levels of *PDC2* and *ALD5* were inversely associated with ester production. These findings indicate that although acetyl-CoA supply was adequate, alternative strategies are necessary for efficient ester synthesis.

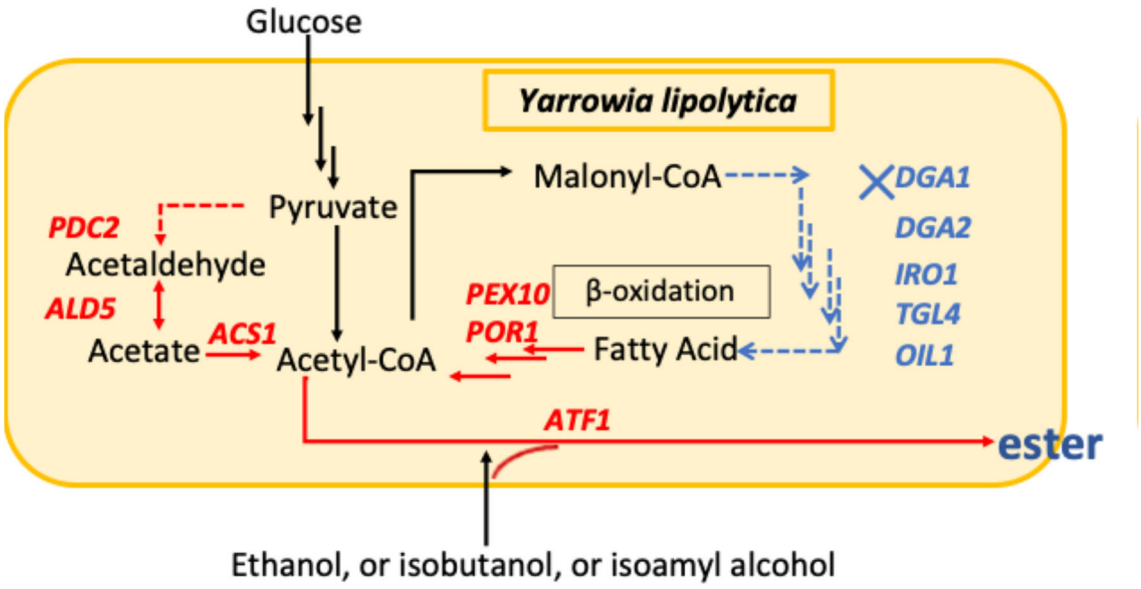
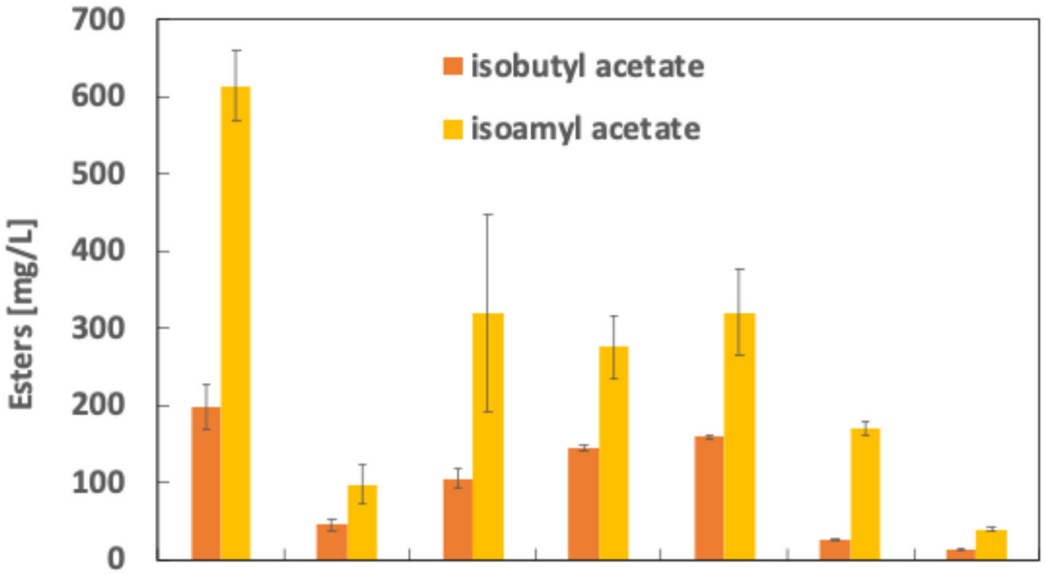


Figure 2. Metabolic engineering of ester-producing *Yarrowia lipolytica*. Red indicates the expression of genes involved in ester synthesis, including *PEX10*, *POR1, PDC2, ALD5*, and *ACS1*. Blue X indicates the disruption of *OIL1*, *DGA1, DGA2*, *IRO1*, and *TGL4*. Abbreviations: *ATF1*, alcohol acetyltransferase from *Sacchromyces cerevisiae* (YOR377W); *pex10*, peroxin-10; *por1*, primary oleate regulator I; *PDC2*, pyruvate carboxylase; *ALD5*, acetaldehyde dehydrogenase; *ACS1*, acetyl-CoA synthetase; *OIL1*, oleaginicity inducing LD protein; *DGA1*, diacylglycerol O-acyltransferaseI; *IRO1*, phospholipid:diacylglycerol acyltransferase; *TGL4*, triacylglycerol lipase.



Expression	EST-1	EST-4	EST-5	EST-6	EST-7	EST-8	EST-9
PEX10	-	+	-	+	+	+	+
POR1	-	-	+	+	+	+	+
ACS	-	-	-	-	+	-	-
PDC2	-	-	-	-	-	+	+
ALD5	-	-	-	-	-	-	+

Figure 3. Modifying ester production through the β-oxidation and pyruvate bypass pathways. Isobutanol or isoamyl alcohol (5 g/L) were supplemented in YNB medium containing 20g/L glucose. and anaerobically cultivated for 72 h. Data are expressed as the means and standard deviations of three independent experiments.

3.3. Pull strategy by introducing additional copies *atf1*

The pull strategy, which involves the overexpression of genes downstream in the desired pathway, improves the efficiency of converting precursors into the target compound.[43,44] This strategy has

been shown to effectively increase the metabolic flux toward lipid biosynthesis in *Y. lipolytica*,[45] through enhanced precursor production and the activation of subsequent precursor-consuming reactions. In this study, we engineered two new strains, EST-2 and EST-3, by introducing an additional *ATF1* into the *A08* and *XYR11* loci, respectively. The ester biosynthesis results of these strains are presented in Figure 4. Strain EST-2 produced 1414 mg/L of isoamyl acetate, whereas strain EST-3 yielded 2918 mg/L of the same ester. These quantities represent a 2.3-fold and 4.7-fold increase in production compared to the control strain EST-1, respectively. Furthermore, the EST-3 strain synthesized 1075 mg/L of butyl acetate, a quantity that is 4.3-fold higher than that produced by the EST-1 strain. These results highlight the efficacy of amplifying *ATF1* expression in enhancing ester synthesis. Cell growth are almost same levels among the strains. It is suggested that a pull strategy, which enhances ester production, is more suitable for *Y. lipolytica* than a push strategy, which enhances the supply of acetyl-CoA.

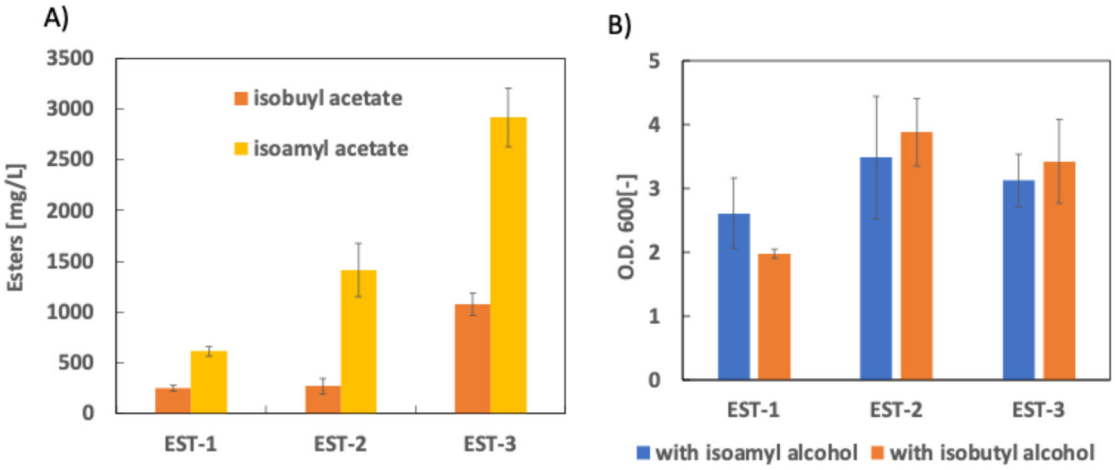


Figure 4. Enhancing ester production by introducing additional copies of *ATF1*. A) Isobutyl acetate or isoamyl acetate production. B) Cell growth. Isobutanol or isoamyl alcohol (5 g/L) were supplemented in YNB medium containing 20g/L glucose and anaerobically cultivated for 72 h. Data are expressed as the means and standard deviations of three independent experiments.

3.4. Improving acetyl-CoA synthesis by modifying fatty acid biosynthesis pathways

To increase the accumulation of acetyl-CoA, we assumed that reducing the flux toward the competing fatty acid biosynthesis pathway would be crucial. Prior research has shown that the inhibition of fatty acid synthesis by cerulenin, a fatty acid synthesis inhibitor, results in a substantial accumulation of malonyl-CoA.[28] Given that acetyl-CoA is a direct precursor to malonyl-CoA, we inferred that a high accumulation of malonyl-CoA would lead to increased levels of acetyl-CoA. Consequently, we aimed to increase the production of acetic esters by disrupting genes associated with the fatty acid biosynthetic pathway.

We focused on four types of genes: *DGA1*, *DGA2*, *IRO1*, and *TGL4*. The genes *DGA1* and *DGA2* encode diacylglycerol O-acyltransferase, which is involved in triacylglycerol (TAG) synthesis, a primary storage lipid in oleaginous yeast. Similarly, *IRO1* gene encodes phospholipid:diacylglycerol acyltransferase, whereas *TGL4* encodes triacylglycerol lipase, responsible for converting TAG into glycerol.[46-48] Studies in *Y. lipolytica* with disrupted *DGA1*

and *DGA2* have shown a reduction in total lipid content by over 50%.[46] In *Rhodotorula toruloides,* disruption of *DGA1* led to an 11% increase in TAL production using acetyl-CoA as a precursor, whereas disruption of *IRO1* resulted in a 19% increase in TAL production.[49] Furthermore, we focused on *oil1*, implicated in the production of the Oil1p protein (oleaginicity inducing LD protein). The *Y. lipolytica,* in which oil1 was deleted, the amount of TAG accumulated decreased, whereas in strains overexpressing oil1, the amount of TAG accumulated increased.[50]

We engineered strains based on EST-3, disrupting *DGA1*, *DGA2*, *IRO1*, *TGL4*, and Oil1 (resulting in strains EST-13 to EST-17), aiming to inhibit fatty acid synthesis and increase the accumulation of acetyl-CoA. The aim was to enhance the production of acetate ester. After a 72 h cultivation period, we confirmed that the Δ*DGA1*(EST-13) and Δ*DGA2* (EST-14) strains increased the production of isoamyl acetate (Figure 5). The targeted disruption of *DGA1* or *DGA2* is hypothesized to reduce fatty acid biosynthesis, thereby increasing the pool of acetyl-CoA available for acetate ester synthesis. This genetic manipulation shifts metabolic fluxes to favor ester production by reallocating precursor substrates. However, double deletion of *DGA1* and *DGA2* (*ΔDGA1ΔDGA2)* decreased ester production (data not shown). In contrast, strains EST-16 and EST-17 exhibited a decrease in ester production levels. This can be attributed to the role of *tgl4*, which is involved in triacylglycerol degradation. The disruption of tgl4 did not inhibit TAG synthesis, suggesting that the expected increase in acetyl-CoA supply did not occur, possibly due to the continued synthesis of TAG. Furthermore, Oil1 is presumed to exert a negative regulatory effect on lipase activity. The deletion of *oil1* may have increased lipase activity, leading to ester hydrolysis. These gene disruptions did not seem to have a significant impact on cell growth. These findings imply a complex regulatory mechanism governing lipid metabolism and ester production, warranting further investigation to elucidate the precise molecular pathways involved.

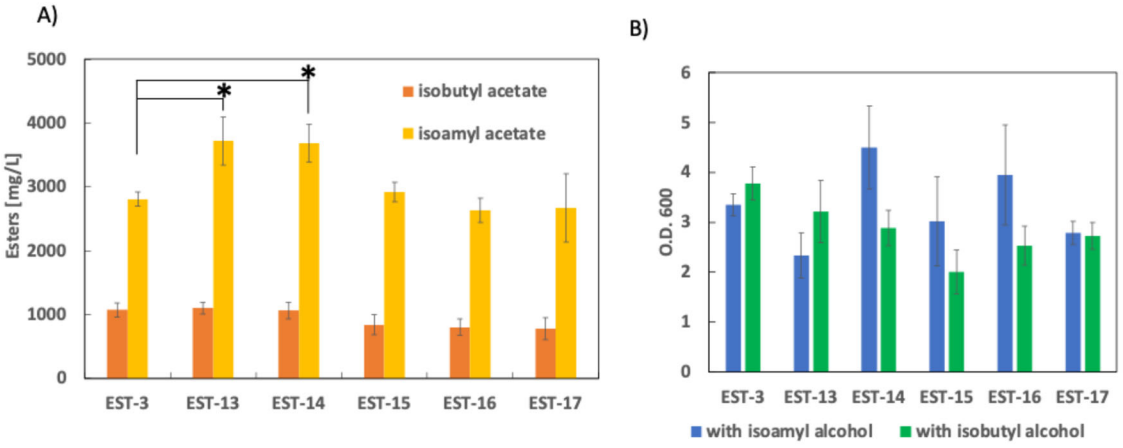


Figure 5. Improving ester production by gene deletions related the fatty acid synthesis pathway. A) Isobutyl acetate or isoamyl acetate production. B) Cell growth. Isobutanol or isoamyl alcohol (5 g/L) were supplemented in YNB medium containing 20g/L glucose and anaerobically cultivated for 72 h. Data are expressed as the means and standard deviations of three independent experiments. * $p < 0.01$, compared with EST-3.

3.5. Production of other acetate esters with a high carbon/nitrogen ratio in culture medium

Increasing the C/N ratio in the growth medium has a profound impact on cellular metabolism,

favoring lipid biosynthesis while inhibiting cell growth.[28,51] By adjusting this ratio, we can intensify metabolic flux toward fatty acid and lipid synthesis, thereby enhancing the production of acetyl-CoA, a crucial precursor for acetate esters. To assess ester production, we cultivated the EST-14 strain in YNB+Ura+Leu medium supplemented with 50 g/L glucose, 5 g/L yeast extract, and 10 g/L peptone, maintaining a (C:N ratio of 11:1)[51]. After 72 h of cultivation, the EST-14 strain achieved a high production of isoamyl acetate, reaching 3404 mg/L (Figure 6). This outcome suggests that under anaerobic respiration conditions, a higher carbon source consumption is necessary for energy generation, promoting both microbial growth and metabolite production.

We further explored the potential for producing other acetate esters using the EST-14 strain. Cultivating this strain in a medium with a C:N ratio of 11:1, supplemented with 5 g/L of propanol, butanol, and amyl alcohol each, yielded promising results. As depicted in Figure 6, we successfully produced 144 mg/L of ethyl acetate, 332 mg/L of propyl acetate, 896 mg/L of butyl acetate, and 2542 mg/L of amyl acetate. Cell growth exhibited a slight decrease with the increasing carbon number of the added alcohol. By introducing the corresponding alcohols as supplements, our engineered strain demonstrated the capability to produce a total of six different acetate esters. This achievement underscores the potential applicability of our developed strains in industrial production settings, offering the flexibility to tailor production to specific esters required for various applications.

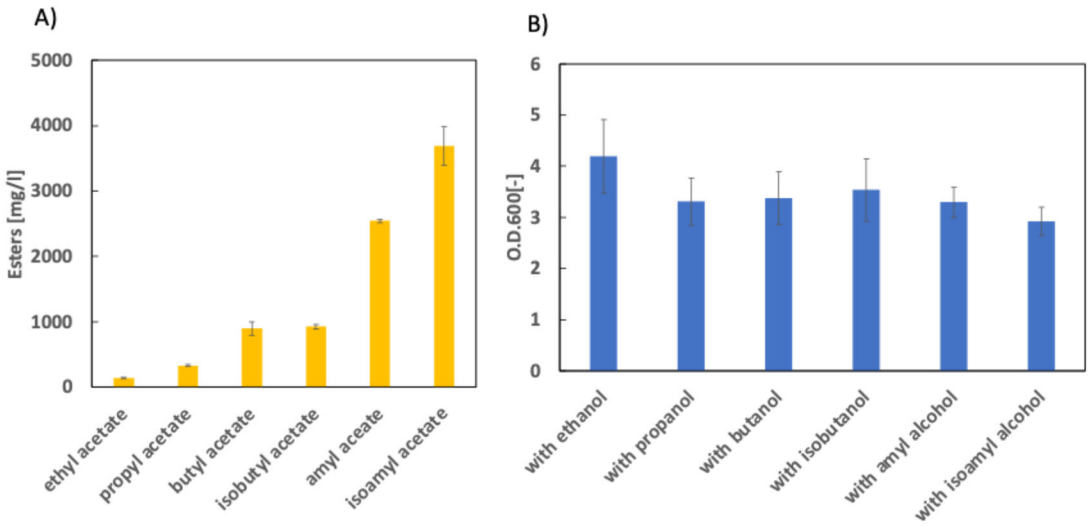


Figure 6. Ester production using EST-14 strain after 72 h of anaerobically cultivation. A) Ester production. B) Cell growth. Ethanol, propanol, butanol, isobutanol, amyl alcohol or isoamyl alcohol (5 g/L) were supplemented in YNB medium containing 50 g/L glucose, 5 g/L yeast extract, and 10 g/L peptone. Data are expressed as the means and standard deviations of three independent experiments.

4. Conclusion

Our study demonstrated the ability of *Y. lipolytica* to biosynthesize significant short-chain acetate esters, specifically isobutyl acetate or isoamyl acetate. This was achieved by genetically engineering the yeast to overexpress *atf1* from *S. cerevisiae.* Efforts to enhance acetyl-CoA synthesis and implement a pull strategy by adding extra *atf1* copies proved particularly effective in increasing ester yields. We achieved a notably high production of isoamyl acetate, reaching 3404 mg/L. Furthermore, by modifying the culture medium with different alcohols, we expanded the range of esters produced. Metabolic engineering efforts have enabled alcohol synthesis from glucose, facilitating the direct production of esters from glucose. Although *Y. lipolytica* has been reported to produce isoamyl alcohol at a level of 1.8 mg/L,[52] there remains a tangible need to enhance the titer. Employing a co-culture system may offer a promising solution for ester production without the need for exogenous alcohol supplementation. Our research not only underscores the potential of *Y. lipolytica* in sustainable microbial ester production but also paves the way for future advancements in microbial synthesis for industrial applications.

Authorship contribution statement

Ayumi Koshiba: Conceptualization, Investigation, Writing − original draft. Mariko Nakano: Investigation. Yuuki Hirata: Investigation. Rie Konishi: Investigation. Yuta Matsuoka: Investigation. Yuta Miwa: Investigation. Ayana Mori: Investigation. Akihiko Kondo: Resources. Tsutomu Tanaka: Conceptualization, Investigation, Writing − review & editing, Funding acquisition, Project administration.

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Conflict of interest statement

The authors have no conflict of interest to declare.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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References

1. Sugimoto N, Engelgau P, Jones AD, Song J, Beaudry R. Citramalate synthase yields a biosynthetic pathway for isoleucine and straight- and branched-chain ester formation in ripening apple fruit. *Proc Natl Acad Sci U S A*. 2021;118:e2009988118. doi:10.1073/pnas.2009988118

2. Sumby KM, Grbin PR, Jiranek V. Microbial modulation of aromatic esters in wine: current

362 knowledge and future prospects. Food Chem. 2010;121:1-16.
363 3. Sun L, Xin F, Alper HS. Bio-synthesis of food additives and colorants-a growing trend in
364 future food. *Biotechnol Adv*. 2021;47:107694. doi:10.1016/j.biotechadv.2020.107694
365 4. Rodriguez GM, Tashiro Y, Atsumi S. Expanding ester biosynthesis in Escherichia coli. *Nat*
366 *Chem Biol*. 2014;10:259-265. doi:10.1038/nchembio.1476
367 5. Lee JW, Trinh CT. Towards renewable flavors, fragrances, and beyond. *Curr Opin*
368 *Biotechnol*. 2020;61:168-180. doi:10.1016/j.copbio.2019.12.017
369 6. Lange JP, Price R, Ayoub PM, Louis J, Petrus L, Clarke L, Gosselink H. Valeric biofuels: a
370 platform of cellulosic transportation fuels. *Angew Chem Int Ed Engl*. 2010;49:4479-4483.
371 doi:10.1002/anie.201000655
372 7. Layton DS, Trinh CT. Engineering modular ester fermentative pathways in Escherichia coli.
373 *Metab Eng*. 2014;26:77-88. doi:10.1016/j.ymben.2014.09.006
374 8. Mason AB, Dufour JP. Alcohol acetyltransferases and the significance of ester synthesis in
375 yeast. *Yeast*. 2000;16:1287-1298. doi:10.1002/1097-0061(200010)16:14<1287::AID-
376 YEA613>3.0.CO;2-I
377 9. Yoshimoto H, Bogaki T. Mechanisms of production and control of acetate esters in yeasts.
378 *J Biosci Bioeng*. 2023;136:261-269. doi:10.1016/j.jbiosc.2023.06.009
379 10. Malcorps P, Dufour JP. Short-chain and medium-chain aliphatic-ester synthesis in
380 Saccharomyces cerevisiae. *Eur J Biochem*. 1992;210:1015-1022. doi:10.1111/j.1432-
381 1033.1992.tb17507.x
382 11. Rojas V, Gil JV, Piñaga F, Manzanares P. Studies on acetate ester production by non-
383 saccharomyces wine yeasts. *Int J Food Microbiol*. 2001;70:283-289. doi:10.1016/s0168-
384 1605(01)00552-9
385 12. Kruis AJ, Levisson M, Mars AE, van der Ploeg M, Garcés Daza F, Ellena V, Kengen SWM,
386 van der Oost J, Weusthuis RA. Ethyl acetate production by the elusive alcohol acetyltransferase from
387 yeast. *Metab Eng*. 2017;41:92-101. doi:10.1016/j.ymben.2017.03.004
388 13. Urit T, Li M, Bley T, Löser C. Growth of Kluyveromyces marxianus and formation of ethyl
389 acetate depending on temperature. *Appl Microbiol Biotechnol*. 2013;97:10359-10371.
390 doi:10.1007/s00253-013-5278-y
391 14. Fredlund E, Blank LM, Schnürer J, Sauer U, Passoth V. Oxygen- and glucose-dependent
392 regulation of central carbon metabolism in Pichia anomala. *Appl Environ Microbiol*. 2004;70:5905-
393 5911. doi:10.1128/AEM.70.10.5905-5911.2004
394 15. Urit T, Stukert A, Bley T, Löser C. Formation of ethyl acetate by Kluyveromyces marxianus
395 on whey during aerobic batch cultivation at specific trace element limitation. *Appl Microbiol*
396 *Biotechnol*. 2012;96:1313-1323. doi:10.1007/s00253-012-4107-z
397 16. Seo H, Giannone RJ, Yang YH, Trinh CT. Proteome reallocation enables the selective de

398 novo biosynthesis of non-linear, branched-chain acetate esters. *Metab Eng*. 2022;73:38-49.
399 doi:10.1016/j.ymben.2022.05.003
400 17. Keasling J, Garcia Martin H, Lee TS, Mukhopadhyay A, Singer SW, Sundstrom E.
401 Microbial production of advanced biofuels. *Nat Rev Microbiol*. 2021;19:701-715.
402 doi:10.1038/s41579-021-00577-w
403 18. Pontrelli S, Chiu TY, Lan EI, Chen FY, Chang P, Liao JC. Escherichia coli as a host for
404 metabolic engineering. *Metab Eng*. 2018;50:16-46. doi:10.1016/j.ymben.2018.04.008
405 19. Han T, Nazarbekov A, Zou X, Lee SY. Recent advances in systems metabolic engineering.
406 *Curr Opin Biotechnol*. 2023;84:103004. doi:10.1016/j.copbio.2023.103004
407 20. Brooks SM, Alper HS. Applications, challenges, and needs for employing synthetic biology
408 beyond the lab. *Nat Commun*. 2021;12:1390. doi:10.1038/s41467-021-21740-0
409 21. Nielsen J, Tillegreen CB, Petranovic D. Innovation trends in industrial biotechnology.
410 *Trends Biotechnol*. 2022;40:1160-1172. doi:10.1016/j.tibtech.2022.03.007
411 22. Liu M, Wang C, Ren X, Gao S, Yu S, Zhou J. Remodelling metabolism for high-level
412 resveratrol production in Yarrowia lipolytica. *Bioresour Technol*. 2022;365:128178.
413 doi:10.1016/j.biortech.2022.128178
414 23. He Q, Szczepańska P, Yuzbashev T, Lazar Z, Ledesma-Amaro R. De novo production of
415 resveratrol from glycerol by engineering different metabolic pathways in Yarrowia lipolytica. *Metab*
416 *Eng Commun*. 2020;11:e00146. doi:10.1016/j.mec.2020.e00146
417 24. Sáez-Sáez J, Wang G, Marella ER, Sudarsan S, Cernuda Pastor M, Borodina I. Engineering
418 the oleaginous yeast Yarrowia lipolytica for high-level resveratrol production. *Metab Eng*. 2020;62:51-
419 61. doi:10.1016/j.ymben.2020.08.009
420 25. Diankristanti PA, Ng IS. Microbial itaconic acid bioproduction towards sustainable
421 development: insights, challenges, and prospects. *Bioresour Technol*. 2023;384:129280.
422 doi:10.1016/j.biortech.2023.129280
423 26. Rong L, Miao L, Wang S, Wang Y, Liu S, Lu Z, Zhao B, Zhang C, Xiao D, Pushpanathan
424 K, Wong A, Yu A. Engineering Yarrowia lipolytica to produce itaconic acid from waste cooking oil.
425 *Front Bioeng Biotechnol*. 2022;10:888869. doi:10.3389/fbioe.2022.888869
426 27. Markham KA, Palmer CM, Chwatko M, Wagner JM, Murray C, Vazquez S, Swaminathan
427 A, Chakravarty I, Lynd NA, Alper HS. Rewiring Yarrowia lipolytica toward triacetic acid lactone for
428 materials generation. *Proc Natl Acad Sci U S A*. 2018;115:2096-2101. doi:10.1073/pnas.1721203115
429 28. Matsuoka Y, Fujie N, Nakano M, Koshiba A, Kondo A, Tanaka T. Triacetic acid lactone
430 production using 2-pyrone synthase expressing Yarrowia lipolytica via targeted gene deletion. *J Biosci*
431 *Bioeng*. 2023;136:320-326. doi:10.1016/j.jbiosc.2023.07.006
432 29. Blazeck J, Hill A, Liu L, Knight R, Miller J, Pan A, Otoupal P, Alper HS. Harnessing
433 Yarrowia lipolytica lipogenesis to create a platform for lipid and biofuel production. *Nat Commun*.

2014;5:3131. doi:10.1038/ncomms4131

30. Soong YV, Coleman SM, Liu N, Qin J, Lawton C, Alper HS, Xie D. Using oils and fats to replace sugars as feedstocks for biomanufacturing: challenges and opportunities for the yeast Yarrowia lipolytica. *Biotechnol Adv*. 2023;65:108128. doi:10.1016/j.biotechadv.2023.108128

31. Xu P, Qiao K, Ahn WS, Stephanopoulos G. Engineering Yarrowia lipolytica as a platform for synthesis of drop-in transportation fuels and oleochemicals. *Proc Natl Acad Sci U S A*. 2016;113:10848-10853. doi:10.1073/pnas.1607295113

32. Schwartz CM, Hussain MS, Blenner M, Wheeldon I. Synthetic RNA polymerase III promoters facilitate high-efficiency CRISPR-Cas9-Mediated genome editing in Yarrowia lipolytica. *ACS Synth Biol*. 2016;5:356-359. doi:10.1021/acssynbio.5b00162

33. Malcorps P, Dufour JP. Short-chain and medium-chain aliphatic-ester synthesis in Saccharomyces cerevisiae. *Eur J Biochem*. 1992;210:1015-1022. doi:10.1111/j.1432-1033.1992.tb17507.x

34. Tai YS, Xiong M, Zhang K. Engineered biosynthesis of medium-chain esters in Escherichia coli. *Metab Eng*. 2015;27:20-28. doi:10.1016/j.ymben.2014.10.004

35. Verstrepen KJ, Van Laere SD, Vercammen J, Derdelinckx G, Dufour JP, Pretorius IS, Winderickx J, Thevelein JM, Delvaux FR. The Saccharomyces cerevisiae alcohol acetyl transferase Atf1p is localized in lipid particles. *Yeast*. 2004;21:367-377. doi:10.1002/yea.1100

36. Lin JL, Wheeldon I. Dual N- and C-terminal helices are required for endoplasmic reticulum and lipid droplet association of alcohol acetyltransferases in Saccharomyces cerevisiae. *PLoS One*. 2014;9:e104141. doi:10.1371/journal.pone.0104141

37. Zhu J, Lin JL, Palomec L, Wheeldon I. Microbial host selection affects intracellular localization and activity of alcohol-O-acetyltransferase. *Microb Cell Fact*. 2015;14:35. doi:10.1186/s12934-015-0221-9

38. Beopoulos A, Mrozova Z, Thevenieau F, Le Dall MT, Hapala I, Papanikolaou S, Chardot T, Nicaud JM. Control of lipid accumulation in the yeast Yarrowia lipolytica. *Appl Environ Microbiol*. 2008;74:7779-7789. doi:10.1128/AEM.01412-08

39. Tan X, Waterham HR, Veenhuis M, Cregg JM. The Hansenula polymorpha PER8 gene encodes a novel peroxisomal integral membrane protein involved in proliferation. *J Cell Biol*. 1995;128:307-319. doi:10.1083/jcb.128.3.307

40. Luo X, Affeldt KJ, Keller NP. Characterization of the Far transcription factor family in Aspergillus flavus. *G3 (Bethesda)*. 2016;6:3269-3281. doi:10.1534/g3.116.032466

41. Poopanitpan N, Kobayashi S, Fukuda R, Horiuchi H, Ohta A. An ortholog of farA of Aspergillus nidulans is implicated in the transcriptional activation of genes involved in fatty acid utilization in the yeast Yarrowia lipolytica. *Biochem Biophys Res Commun*. 2010;402:731-735. doi:10.1016/j.bbrc.2010.10.096

42. Flores CL, Rodríguez C, Petit T, Gancedo C. Carbohydrate and energy-yielding metabolism in non-conventional yeasts. *FEMS Microbiol Rev*. 2000;24:507-529. doi:10.1111/j.1574-6976.2000.tb00553.x

43. Sun T, Yu Y, Wang L, Qi Y, Xu T, Wang Z, Lin L, Ledesma-Amaro R, Ji XJ. Combination of a push-pull-block strategy with a heterologous xylose assimilation pathway toward lipid overproduction from lignocellulose in Yarrowia lipolytica. *ACS Synth Biol*. 2023;12:761-767. doi:10.1021/acssynbio.2c00550

44. Zahrl RJ, Prielhofer R, Ata Ö, Baumann K, Mattanovich D, Gasser B. Pushing and pulling proteins into the yeast secretory pathway enhances recombinant protein secretion. *Metab Eng*. 2022;74:36-48. doi:10.1016/j.ymben.2022.08.010

45. Tai M, Stephanopoulos G. Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production. *Metab Eng*. 2013;15:1-9. doi:10.1016/j.ymben.2012.08.007

46. Shi T, Li Y, Zhu L, Tong Y, Yang J, Fang Y, Wang M, Zhang J, Jiang Y, Yang S. Engineering the oleaginous yeast Yarrowia lipolytica for β-farnesene overproduction. *Biotechnol J*. 2021;16:e2100097. doi:10.1002/biot.202100097

47. Athenstaedt K. YALI0E32769g (DGA1) and YALI0E16797g (LRO1) encode major triacylglycerol synthases of the oleaginous yeast Yarrowia lipolytica. Biochim Biophys Acta. 2011;1811:587-596. doi:10.1016/j.bbalip.2011.07.004

48. Fakas S. Lipid biosynthesis in yeasts: a comparison of the lipid biosynthetic pathway between the model nonoleaginous yeast Saccharomyces cerevisiae and the model oleaginous yeast Yarrowia lipolytica. *Eng Life Sci.* 2017;17:292-302. doi:10.1002/elsc.201600040

49. Cao M, Tran VG, Qin J, Olson A, Mishra S, Schultz JC, Huang C, Xie D, Zhao H. Metabolic engineering of oleaginous yeast Rhodotorula toruloides for overproduction of triacetic acid lactone. *Biotechnol Bioeng*. 2022;119:2529-2540. doi:10.1002/bit.28159

50. Bhutada G, Kavšček M, Hofer F, Gogg-Fassolter G, Schweiger M, Darnhofer B, Kordiš D, Birner-Gruenberger R, Natter K. Characterization of a lipid droplet protein from Yarrowia lipolytica that is required for its oleaginous phenotype. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2018;1863:1193-1205. doi:10.1016/j.bbalip.2018.07.010

51. Ma Y, Liu N, Greisen P, Li J, Qiao K, Huang S, Stephanopoulos G. Removal of lycopene substrate inhibition enables high carotenoid productivity in Yarrowia lipolytica. *Nat Commun*. 2022;13:572. doi:10.1038/s41467-022-28277-w

52. Zhao Y, Liu S, Lu Z, Zhao B, Wang S, Zhang C, Xiao D, Foo JL, Yu A. Hybrid promoter engineering strategies in Yarrowia lipolytica: isoamyl alcohol production as a test study. *Biotechnol Biofuels*. 2021;14:149. doi:10.1186/s13068-021-02002-z