



Modulating acetate ester and higher alcohol production in *Saccharomyces cerevisiae* through the cofactor engineering

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Abstract

Flavor production by esters or by higher alcohols play a key role in the sensorial quality of fermented alcoholic beverages. In *Saccharomyces cerevisiae* cells, the syntheses of esters and higher alcohols are considerably influenced by intracellular CoA levels catalyzed by pantothenate kinase. In this work, we examined the effects of cofactor CoA and acetyl-CoA synthesis on the metabolism of esters and higher alcohols. Strains 12 α -BAP2 and 12 α +ATF1 were generated by deleting and overexpressing *BAP2* (encoded branched-chain amino acid permease) and *ATF1* (encoded alcohol acetyl transferases), respectively, in the parent 12 α strains. Then, 12 α -BAP2+CAB1 and 12 α -BAP2+CAB3 strains were obtained by overexpressing *CAB1* (encoded pantothenate kinase Cab1) and *CAB3* (encoded pantothenate kinase Cab3) in the 12 α -BAP2 strain, and 12 α -BAP2+CAB1+ATF1 and 12 α -BAP2+CAB3+ATF1 were generated by overexpressing *ATF1* in the pantothenate kinase overexpression strains. The acetate ester level in 12 α -BAP2 was slightly changed relative to that in the control strain 12 α , whereas the acetate ester levels in 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 were distinctly increased (44–118% for ethyl acetate and 18–57% for isoamyl acetate). The levels of *n*-propanol, methyl-1-butanol, isopentanol, isobutanol, and phenethylol levels were changed and varied among the six engineered strains. The levels of acetate esters and higher alcohols can be modulated by changing the CoA and acetyl-CoA levels. The method proposed in this work supplies a practical means of breeding yeast strains by modulating acetate ester and higher alcohol production.

Keywords CoA · Acetyl-CoA · *Saccharomyces cerevisiae* · Cofactor engineering · Acetate ester · Higher alcohols

Kun-Qiang Hong and Xiao-Meng Fu have contributed equally to the article.

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Introduction

Many researchers have mainly performed pathway manipulation (pathway deletion, addition, and modification) through traditional metabolic engineering methods to achieve their desired metabolic engineering goals. However, some control parameters involved in cellular metabolism limit the

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potential and efficiency of pathway manipulation strategies. Cofactor level is the parameter used for regulating flux through various metabolic pathways. Cofactor engineering is evidently an important tool for preparing metabolic engineering products [22, 27, 28].

Several works reported that the cofactor nicotinamide adenine dinucleotide (NAD⁺) involves over 300 redox reactions [2]. Berrios-Rivera et al. confirmed that NAD⁺ cofactor manipulation system can be used to improve the production of industrially useful compounds [2, 3]. Meanwhile, other important cofactors include coenzyme A (CoA) and its derivatives, such as acetyl-CoA, malonyl-CoA, and acetoacetyl-CoA.

CoA is an essential cofactor for a large number of enzymes involved in the transfer of acyl groups in living organisms. CoA-dependent enzymes play critical roles for the catabolism of sugars and fatty acids as well as in anabolic reactions, such as biosynthesis of sterols, fatty acids, and polyketides. Acetyl-CoA is the most important derivative of CoA and is also needed for histone acetylation in eukaryotic chromatin and for gluconeogenesis from C₂ substrates in many microorganisms [26]. The biosynthesis of CoA contains a consecutive pathway, and pantothenate (vitamin B₅), as a precursor, is generated from the carbon backbone of amino acids in *Saccharomyces cerevisiae* [17]. Pantothenate is converted into 4'-phosphopantothenate by pantothenate kinase Cab1 (encoded by *YDR531W* (*CAB1*) gene). Together with cysteine, 4'-phosphopantothenate is then transformed into 4'-phosphopantothenoylecysteine, which is subsequently converted into 4'-phosphopantetheine by pantothenate kinase Cab3 (encoded by the *YKL088W* (*CAB3*) gene). Finally, 4'-phosphopantetheine is converted to dephospho-CoA and CoA [27]. In the consecutive pathway of CoA synthesis in yeast cells, Cab3 is the sole protein, serves as a subunit of phosphopantothenoylecysteine decarboxylase, and can interact with itself and other Cab proteins [24, 26]. Pantothenate kinase Cab1 (encoded by *CAB1*), the rate-limiting enzyme of CoA biosynthesis, does not interact with other Cab proteins [24, 26]. *CAB1* overexpression in yeast and *Escherichia coli* increases pantothenate kinase (PanK) activity approximately 50- and 25-fold, respectively [27]. Therefore, we overexpressed *CAB1* or *CAB3* to increase CoA level in yeast cells. Additionally, yeast cell can synthesize CoA via an endogenous pathway starting from amino acids methionine and valine, which is transaminated into 2-ketoisovalerate, a precursor for pantoate production [28, 29]. Moreover, Grauslund et al. found that branched-chain amino acid permease (encoded by *BAP2* gene) has an immediate effect on the uptake of valine and methionine in yeast cells [15]. Therefore, we hypothesized that CoA level can be increased by deleting *BAP2*.

During fermentation, *S. cerevisiae* produces a broad range of aroma-active substances, which considerably affect the

complex flavors of fermented alcoholic beverages [34]. Acetate ester plays a key role in these aroma-active substances, the synthesis of which is catalyzed by alcohol acetyl transferase I (encoded by *ATF1*) [4, 11, 12]. Although the physiological role of acetate ester biosynthesis has not been demonstrated yet, one factor has been suggested to be the result of the metabolic process that balances the acetyl-CoA/CoA ratio [19]. Some studies demonstrated that isoamyl acetate production can be boosted by increasing intracellular CoA and acetyl-CoA concentrations. Vadali et al. found that the engineered strain expressing yeast *ATF2* and *panK* genes produced six-fold more isoamyl acetate than the control strain expressing only the *ATF2* gene and increased intracellular CoA and acetyl-CoA levels in *E. coli* [30]. Additionally, Cordente et al. demonstrated that the CoA/acetyl-CoA ratio is crucial for determining acetate ester synthesis [6]. Meanwhile, CoA and acetyl-CoA limit higher alcohol production with yeast. Recently, the biosynthetic pathway for 1-butanol production was reconstructed in *S. cerevisiae* by increasing flux toward cytosolic acetyl-CoA for butanol production. The increase resulted in a 6.5-fold improvement in butanol titers relative to the previously reported results for yeast [23]. Furthermore, the selection or enrichment of C6 and C8 acyl-CoA increases the production of *n*-hexanol by tenfold [23], and a CoA-dependent pathway can be used to synthesize isobutanol and *n*-butanol [18]. However, studies on the catabolism of the branched-chain amino acids in *S. cerevisiae* showed that different enzymes are involved in the decarboxylation of keto acids to corresponding higher alcohols [4, 8, 10]. The deletion of *BAP2* has been demonstrated to reduce leucine, isoleucine, and valine uptake by 25–50% but reduces or increases the uptake of the eight other L-a-amino acids [15], which might subsequently affect the synthesis of CoA and higher alcohol yield. Therefore, the synthesis of acetate ester and higher alcohols may be related to the CoA and acetyl-CoA levels.

Considering that *BAP2*, *CAB1*, and *CAB3* influence CoA synthesis, we deleted *BAP2* in yeast strain 12 α work and investigated the effect of the deletion on CoA, acetate ester, and higher alcohol formation during fermentation. Then, we overexpressed *CAB1* and *CAB3* in yeast strain 12 α -*BAP2* to increase CoA level and overexpressed *ATF1* in yeast strains 12 α -*BAP2*+*CAB1* and 12 α -*BAP2*+*CAB3* to enhance acetate ester synthesis in these strains and compare with the acetate ester synthetic ability in 12 α +*ATF1*. Herein, we report the modulation of acetate ester and higher alcohols production by influencing CoA and acetyl-CoA synthesis.

Materials and methods

Strains and plasmids

The genetic properties of all the strains and plasmids used in this work are listed in Table 1.

Medium

Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl, pH 7.0) was used to incubate *E. coli* at 37 °C, and ampicillin (100 mg/L) was used for plasmid selection. Yeast strains were cultured in a YEPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) or SC-ura3 medium (6.7 g/L yeast nitrogen base without amino acids, supplemented with all the auxotrophic requirements except uracil, and 20 g/L glucose) at 30 °C. Meanwhile, 5-fluoroorotic acid (5-FOA) medium (6.7 g/L yeast nitrogen base without amino acids supplemented with all the auxotrophic requirements, 20 g/L glucose, and 2 g/L 5-fluoroorotic acid) was used only for the selection of the uracil auxotrophic transformants of the yeast cells. A KAC medium (20 g/L potassium acetate, 20 g/L agar) was used in the induction of hybridization in yeast for spore production, generation of haploid yeast, and recovery of the mutant *URA3* gene. A liquid YEPD medium was used for cell growth determination, *ATF1* transcription level analysis, NAD(H) level analysis, and CoA and acetyl-CoA quantification. All the solid media used in this study contained 2% agar.

Plasmid and yeast strains construction

Restriction enzymes, DNA polymerase, and DNA ligase used in cloning were purchased from Beijing Solarbio

Science & Technology Co., Ltd. (Beijing, China). Yeast transformation was conducted with the lithium acetate procedure as previously reported [14]. Standard molecular genetic techniques were used for nucleic acid manipulation [7, 35]. The primers used are listed in Table S1.

The plasmid YIplac211 was used as the backbone for the construction of recombinant plasmid YIplac211-UPD. Detailed information about plasmid construction is presented in Fig. S1A [9]. *URA3* gene, the upstream and downstream fragment of *BAP2*, was amplified from the yeast strain 12 α and transformed into 12 α -u to delete *BAP2* gene, generating 12 α -BAP2 (Fig. S2). Then, 12 α -BAP2+CAB1 and 12 α -BAP2+CAB3 with overexpressed pantothenate kinase were generated by overexpressing *CAB1* and *CAB3*, respectively, in the 12 α -u strain (Fig. S3). Additionally, the plasmid YIplac211-UPD was linearized and transformed into 12 α -u and 12a-u for the generation of 12 α +ATF1 and 12a+ATF1 (Fig. S1B). The process of inducing yeast for spore production and haploid generation is critical to yeast genetics and breeding [25]. Finally, we hybridized 12 α -BAP2+CAB1 and 12 α -BAP2+CAB3 with 12a+ATF1 on a KAC plate to enhance the synthesis of acetate ester and to generate 12 α -BAP2+CAB1+ATF1 and 12 α -BAP2+CAB3+ATF1 (Fig. S4), respectively. Haploid was verified by colony PCR using three primers of MAT-F, MAT-a, and MAT-a. Transformants with *URA3* gene were plated on SC-ura3 medium (with *ura3* gene plated on 5-FOA medium), and the genotypes were verified by PCR using specific primers (Fig. S2, Table. S1).

Quantification of CoA and acetyl-CoA levels

Preparation of cell extract

Cell extract preparation was conducted through the modified protocol of Boynton et al. [3]. Cell culture quantity of

Table 1 *Saccharomyces cerevisiae* strains used in this study

Strains or plasmid	Genotype	Source or reference
<i>Saccharomyces cerevisiae</i> , 12 α	Commercial liquor yeast strain	Angel Yeast Co., Ltd
12 α -u	<i>MATα ura3</i>	This work
12 α +ATF1	<i>MATα URA3, pATF1-pPGK1-ATF1</i>	This work
12 α -BAP2	<i>MATα ura3, ΔBAP2::URA3</i>	This work
12 α -BAP2+CAB1	<i>MATα ura3, ΔBAP2::URA3-PGK1p-CAB1-PGKt</i>	This work
12 α -BAP2+CAB3	<i>MATα ura3, ΔBAP2::URA3-PGK1p-CAB3-PGKt</i>	This work
12 α -BAP2+CAB1+ATF1	<i>MATα URA3, ΔBAP2::PGK1p-CAB1-PGKt, pATF1-pPGK1-ATF1</i>	This work
12 α -BAP2+CAB3+ATF1	<i>MATα URA3, ΔBAP2::PGK1p-CAB3-PGKt, pATF1-pPGK1-ATF1</i>	This work
<i>Escherichia coli</i> DH5 α	Φ 80 <i>lacZ</i> Δ M15 Δ lacU169 <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA relA1</i>	Stratagene
YIplac211	<i>Amp^r URA3</i>	[13]
YIplac211-UPD	<i>Amp^r URA3</i>	This work

40 OD units ($\text{OD}_{600 \text{ nm}} \times \text{Vol (mL)} = 40$; logarithmically growing cells in YEPD liquid medium at 30 °C) was collected, chilled immediately on ice, and centrifuged at 8000 RPM at 4 °C for 10 min. The cell pellet was resuspended in 1 mL of 6% perchloric acid for cell lysis. Potassium carbonate (0.3 mL of 3 M) was added while vortexing for the neutralization of the acid. The solution was centrifuged for the pelleting of the cell debris. The supernatant was collected and filtered through a 0.2 µm PVDF membrane syringe filter and stored chilled for the analysis of CoA and acetyl-CoA compounds through HPLC.

Analysis of CoA and acetyl-CoA compounds

CoA and acetyl-CoA quantification was conducted through the modified protocol of Boynton [3]. The separation of the CoA compounds was performed with a thermostable HPLC system (Agilent Technologies, Beijing, China) equipped with a Spectrasystem P4000 pump, UV2000 detector, and a 5 mm octyldecyl silane column (Cell Technologies, Inc., Houston, TX). The two mobile phase solvents used were buffer A (0.2 M sodium phosphate, pH 5.0) and buffer B (acetonitrile) at a flowrate of 1 mL/min ($V_{\text{bufferA}}:V_{\text{bufferB}} = 1:9$). The method of quantifying intracellular CoA and acetyl-CoA was modified as previously described [3, 31].

Mensuration of NAD(H) levels

NAD(H) level detection kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Extraction of NAD⁺ and NADH

A total of five million yeast cells were collected in microcentrifuge tube, and 0.5 mL of acid extraction solution was added (acid extraction solution for NAD⁺ and alkaline extraction solution for NADH). The mixture was broken with ultrasonication for 1 min (strength by 20% or 200 W, ultrasonic 2 s, stop 1 s), boiled for 5 min (cover tightly to prevent moisture loss), and cooled on ice. Then, the mixture was centrifuged at 10,000g at 4 °C for 10 min. Exactly 200 µL of supernatant was removed and placed into another microcentrifuge tube, and the same volume of alkaline extract was added for neutralization (alkaline extraction solution for NAD⁺ and acid extraction solution for NADH). The mixture was centrifuged at 10,000g at 4 °C for 10 min. The supernatant was placed on ice for the measurement of NAD(H) level.

Analysis of NAD⁺ and NADH levels

The spectrophotometer (Shimadzu Co. Ltd., China) was preheated for over 30 min, and the wavelength was regulated to 570 nm. According to the sequence presented in Table S2, various solutions were added to 1.5 mL brown microcentrifuge tubes. Finally, the solutions were mixed, and the absorbance at 570 nm was measured. The following formulas were used to calculate the NAD(H) level according to the instructions of the kit.

$$\Delta A_M = A_m - A_c, \quad (1)$$

$$\Delta A_{S1} = A_{s1} - A_b, \quad (2)$$

$$\Delta A_{S2} = A_{s2} - A_b, \quad (3)$$

$$\text{NAD}^+ \text{ (nmol/g, FW)} = \frac{\Delta A_M \times V_E}{\Delta A_{S1} \div C_S \times W} = \frac{1.25 \times \Delta A_M}{\Delta A_{S1} \times W}, \quad (4)$$

$$\text{NADH (nmol/g, FW)} = \frac{\Delta A_M \times V_E}{\Delta A_{S2} \div C_S \times W} = \frac{1.25 \times \Delta A_M}{\Delta A_{S2} \times W}. \quad (5)$$

A_m —the absorbance of measuring tube; A_{s1} —the absorbance of standard tube 1 (NAD⁺); A_{s2} —the absorbance of standard tube 2 (NADH); A_c —the absorbance of control tube; A_b —the absorbance of blank tube; C_S —the standard solution concentration of NAD⁺ or NADH, 1.25 nmol/mL; V_E —total volume of extracting solution, 1 mL; W and FW —yeast cell fresh weight, g.

Real-time quantitative PCR

The mRNA of the yeast precultured in the YEPD medium was extracted using the Yeast RNAiso Kit (Takara Biotechnology, Dalian, China), and the relative expression level of key genes was assessed by real-time quantitative PCR (RT-qPCR) [9]. The primers used to amplify the key genes and the reference gene *ACT1* are listed in Table S1. RT-qPCR was conducted using a Roche LightCycler 480 Real-Time PCR machine, and the final data were calculated through the threshold cycle ($2^{-\Delta\Delta C_t}$) method [21].

Fermentation experiments

The fermentation medium was prepared by mixing 60 g of corn powder (with 88% dry matter) and 135 mL of water, which had been prewarmed to 65 °C for 20 min in a 250 mL conical flask. Then, the mixture was incubated at 90 °C for 90 min with thermostable α -amylase (2×10^5 U/mL; Novozymes Biotechnology Co. Ltd., Tianjin, China). The temperature of the mixture was lowered to 65 °C, and 5 M

H₂SO₄ was used to adjust the pH to 4.2–4.4; subsequently, saccharifying enzyme (200 U/mL; Novozymes Biotechnology Co. Ltd., Tianjin, China) was added. The medium was incubated at 65 °C for 20 min, and then acid proteinase (2×10^4 U/mL; Novozymes Biotechnology Co. Ltd., Tianjin, China) and nutrient salt (150 g/L MgSO₄, 75 g/L KH₂PO₄, and 81 g/L Urea) were added. The mixture was cooled down to 40 °C for 20 min and then lowered to 30 °C and was immediately used for fermentation. Yeast cells were precultured in 4 mL of 8 °Bx corn hydrolysate medium at 30 °C for 24 h and then transferred into 36 mL of 12 °Bx corn hydrolysate in a 50 mL conical flask and stored at 30 °C for 16 h. Exactly 15 mL of the second precultured yeast solution was transferred to the prepared liquid fermentation medium. The flask was tightly sealed with parafilm for oxygen-limited fermentation, and a carbon dioxide-releasing channel was created on parafilm with a 0.5 mm injection needle. The fermentation was processed till the weight loss of CO₂ over the interval of 12 h was < 1 g. All the fermentations were performed in triplicate.

The fermentation performance of CO₂ weight loss, residual sugar, and ethanol production was determined using an analytical balance, Brix hydrometer, and oenometer, respectively. Production of volatile flavor compounds, including higher alcohols and esters, was determined using gas chromatography (GC) analysis.

Gas chromatography analysis

Gas chromatography (GC) has been extensively used to determine the abundance of volatile compounds [33]. The corn broth after fermentation was distilled and used for GC analysis, which was performed on an Agilent 7890C GC with AT.LZP-930 column (50 m × 320 μm internal diameter and 1 μm coating thickness). Nitrogen was used as the carrier gas, and the GC conditions were as follows: injector temperature, 200 °C; the split ratio, 5:1; and injection volume, 1.0 μL with the constant rate of 1 mL/min. The oven temperature program used was 50 °C (8 min), followed by an increase to 120 °C at 5 °C/min. The final temperature was maintained for 8 min. Ethyl acetate, isoamyl acetate, isobutyl acetate, lactic acetate, and alcohol were purchased as standards from Merck Serono Co. Ltd.

Results

Characterization of the engineered strains

Previous studies have shown that pantothenate kinase overexpression increases intracellular CoA and acetyl-CoA levels and influences ester or higher alcohol synthesis [6, 16]. In this study, we confirmed that the modulation of CoA and

acetyl-CoA levels is an effective way of regulating acetate ester and higher alcohol production. First, to research the relationship among uptake of branched-chain amino acid, CoA, and acetyl-CoA levels and the synthesis of higher alcohols, we replaced *BAP2* with *URA3* to generate the strain 12α–BAP2 (Fig. S2). The genes of *CAB1* and *CAB3* were overexpressed in the strain of 12α–BAP2 to further increase the levels of CoA and acetyl-CoA. The 12α–BAP2+CAB1 and 12α–BAP2+CAB3 strains were generated, respectively (Fig. S3). Our previous work showed that the overexpression cassette of *pATF1-pPGK1-ATF1* can increase AATase I activity and acetate ester titer [9]. Therefore, to enhance the synthesis of acetate ester and verify the effect of CoA and acetyl-CoA levels on acetate ester titer, we used the cassette of *pATF1-pPGK1-ATF1* to overexpress the *ATF1* gene in 12α, 12α–BAP2+CAB1, and 12α–BAP2+CAB3 and generate 12α+ATF1, 12α–BAP2+CAB1+ATF1, and 12α–BAP2+CAB3+ATF1, respectively (Figs. S1B and S4).

The growth performance of each engineered strain and that of the parent strain 12α were tested separately at 30 °C in YEPD liquid media. We did not obtain any remarkable distinctions among the growth rates of all the engineered strains and the parent strain at this temperature (data not shown). These data revealed that all the engineered strains exhibited similar growth performance relative to that of the parent strain 12α, revealing that genetic manipulation does not impair the growth property of the yeast strain.

Quantification of CoA and acetyl-CoA levels

The intracellular CoA and acetyl-CoA levels were studied in the control strain 12α and six engineered strains in YEPD liquid media at 30 °C. Table 2 shows the intracellular CoA and acetyl-CoA levels of the strains during the logarithmic growth phase. In the control strain 12α, CoA level was approximately 0.246 μmol/L of the dry cell weight initially, and the ratio of acetyl-CoA/CoA was 2.024. The ratio of acetyl-CoA/CoA in 12α–BAP2, 12α+ATF1, 12α–BAP2+CAB1, 12α–BAP2+CAB3, 12α–BAP2+CAB1+ATF1, and 12α–BAP2+CAB3+ATF1 were 1.742, 2.261, 2.085, 2.794, 2.559, and 2.239, respectively. The ratio in 12α–BAP2 was decreased by 13.93%, whereas the ratios in 12α+ATF1, 12α–BAP2+CAB1, 12α–BAP2+CAB3, 12α–BAP2+CAB1+ATF1, and 12α–BAP2+CAB3+ATF1 had variable increases relative to the ratio of the parent strain 12α (Fig. 1).

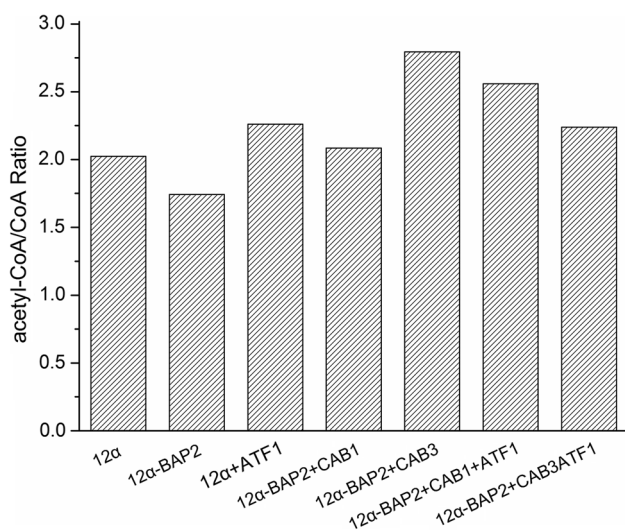
Measurement of NAD(H) levels

The cofactor NAD⁺ involves numerous redox reactions, including the synthesis of acetate ester and higher alcohol production [1, 2]. We inferred that the total levels of NAD(H) also changed in the engineered strains; therefore,

Table 2 CoA/acetyl-CoA levels of engineered strains and control strain^a

Yeast strains	CoA level ($\mu\text{mol/L}$ of DCW)	Acetyl-CoA level ($\mu\text{mol/L}$ of DCW)	Acetyl-CoA/CoA
12 α	0.246 \pm 0.012	0.498 \pm 0.024	2.024
12 α -BAP2	0.248 \pm 0.014	0.432 \pm 0.023	1.742
12 α +ATF1	0.276 \pm 0.015	0.624 \pm 0.012	2.261
12 α -BAP2+CAB1	0.257 \pm 0.010	0.536 \pm 0.046	2.085
12 α -BAP2+CAB3	0.408 \pm 0.025	1.140 \pm 0.065	2.794
12 α -BAP2+CAB1+ATF1	0.506 \pm 0.021	1.295 \pm 0.095	2.559
12 α -BAP2+CAB3+ATF1	0.296 \pm 0.009	0.663 \pm 0.012	2.239

^aData are presented as the means and standard deviations of three independent experiments

**Fig. 1** Acetyl-CoA/CoA ratio of engineered and control strains

the total levels of NAD⁺ and NADH were measured as described in the “**Materials and methods**” section. Table S3 shows the levels of NAD⁺ and NADH in control and engineered strains. The NAD⁺ and NADH levels (nmol/mg of fresh cell weight) were 0.142 and 0.050 for the control strain 12 α , 0.101 and 0.020 for 12 α -BAP2, 0.247 and 0.084 for 12 α +ATF1, 0.218 and 0.117 for 12 α -BAP2+CAB1, 0.449 and 0.085 for 12 α -BAP2+CAB3, 0.605 and 0.012 for 12 α -BAP2+CAB1+ATF1, and 0.864 and 0.051 for 12 α -BAP2+CAB3+ATF1. The total NAD(H) levels of 12 α -BAP2 slightly decreased, whereas those of the other engineering strains increased by 72%, 74%, 178%, 221%, or 377%. These results show that the NAD(H) levels were influenced by the change in CoA and acetyl-CoA levels.

Analysis of mRNA levels

We performed RT-qPCR assay to quantify the mRNA levels of the target genes and analyze the transcription levels of *ATF1*, *BAP2*, *CAB1*, and *CAB3* of the engineered strains. As shown in the data in Fig. S5, the RT-qPCR results

exhibit that the *ATF1* expression levels of 12 α -BAP2, 12 α +ATF1, 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 were 0.91-, 1.69-, 0.65-, 0.58-, 4.31-, and 3.17-fold higher than that of 12 α , respectively (Fig. S5). Compared with the parental strain, the *BAP2* expression levels of *BAP2* deletion strains were nearly zero. The *CAB1* expression levels of *CAB1* overexpression strains 12 α -BAP2+CAB1 and 12 α -BAP2+CAB1+ATF1 were 3.32- and 5.67-fold higher than those of 12 α , respectively. The *CAB3* overexpression strains 12 α -BAP2+CAB3 and 12 α -BAP2+CAB3+ATF1 were 20.10- and 11.10-fold higher than those of 12 α , respectively.

Fermentation properties of engineered strains

The fermentation properties of engineered strains were further monitored and compared with 12 α in the liquid fermentation of corn hydrolysate under the conditions described in the “**Materials and methods**” section. The free glucose concentration in the medium was approximately 264 g/L at the initiation of fermentation. We did not obtain any remarkable distinction in glucose consumption in the engineered strains. The fermentation performance of each strain, including time of fermentation (114 h), carbon dioxide loss (approximately 23 g), and ethanol, organic acid, and ester production, were all tested for the assessment of the fermentation ability of the strains in liquid fermentation medium. Data collected from three independent biological replicates for each strain are shown in Table S5. The preceding data all proved that the fermentation properties of the resulting yeast strains were not influenced by the cofactor engineering.

Furthermore, the effect of CoA and acetyl-CoA level variation on acetate ester regulation and higher alcohol production was investigated. As shown in Table S4 and Fig. 2a, b, the final concentration of ethyl acetate (mg/L) was 29.28 for 12 α -BAP2, 45.47 for 12 α -BAP2+CAB1, and 45.11 for 12 α -BAP2+CAB3. Compared with that of the control strain, the level of ethyl acetate was slightly changed in 12 α -BAP2, but its levels in 12 α -BAP2+CAB1 and 12 α -BAP2+CAB3

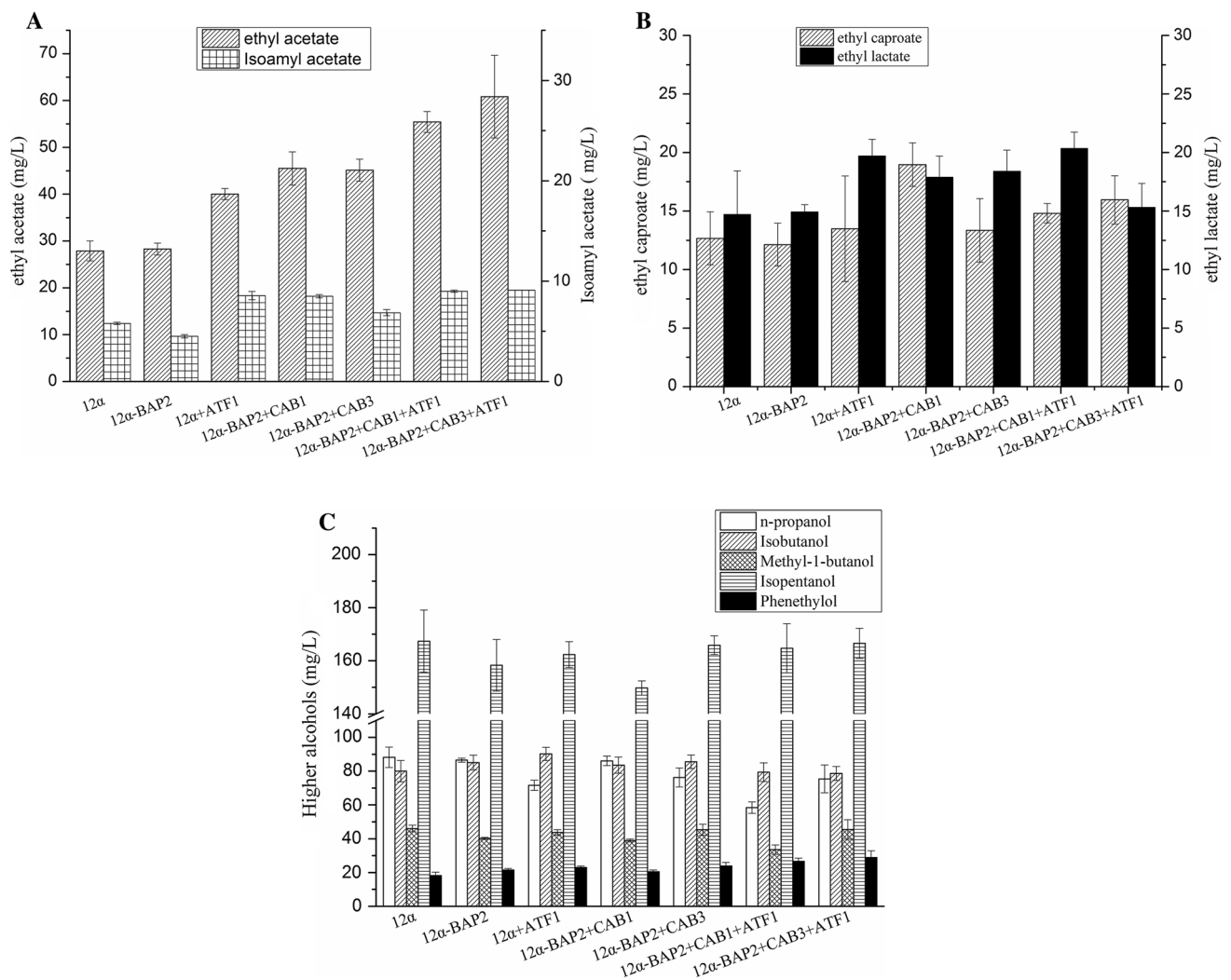


Fig. 2 Concentration of acetate ester and higher alcohols in the engineered and control strains. **a** Ethyl acetate and isoamyl acetate levels; **b** ethyl caproate and ethyl lactate levels; **c** higher alcohols levels. The

experiments were repeated three times. Data are the average of three independent experiments. Error bars represent \pm SD

were increased by 63% and 62%, respectively. The *ATF1* was overexpressed in strains 12 α -BAP2+CAB1 and 12 α -BAP2+CAB3, resulting in the ethyl acetate levels (mg/L) of 55.44 for 12 α -BAP2+CAB1+ATF1 and 60.84 for 12 α -BAP2+CAB3+ATF1, which are higher than those of parent strain 12 α and only *ATF1* overexpression strain 12 α +ATF1. Moreover, the isoamyl acetate level (mg/L) in 12 α -BAP2 was decreased to 4.51 mg/L, and its levels in 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α +ATF1, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 were increased to 8.50, 6.86, 8.56, 9.00, and 9.11, respectively. Additionally, the levels of ethyl caproate and ethyl lactate in 12 α -BAP2 were considerably changed and those in 12 α +ATF1, 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 were slightly increased. The GC analysis revealed that the

modulation of CoA and acetyl-CoA levels was beneficial for regulating the acetate ester production in yeast fermentation.

The levels of *n*-propanol, isobutanol, methyl-1-butanol, isopentanol, and phenethylol were also detected, as shown in Table S4 and Fig. 2c. The *n*-propanol concentrations (mg/L) in the strains 12 α -BAP2, 12 α +ATF1, 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 were 86.52, 71.65, 86.10, 76.27, 58.37, and 75.41, respectively, which were lower than 88.20 mg/L of the parent strain. The isobutanol concentrations (mg/L) for the parent and engineered strains were found to be 80.02, 85.08, 90.20, 83.54, 85.58, 79.49, and 78.69. The methyl-1-butanol concentrations (mg/L) for 12 α -BAP2, 12 α +ATF1, 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 were 40.17, 43.70, 38.94, 45.30,

33.52, and 45.48, respectively, which were all lower than 46.03 of 12 α . The isopentanol concentrations (mg/L) in engineered strains were 158.32, 162.33, 149.76, 165.87, 164.76, and 166.59, respectively, which were all lower than those of parent strain. The phenethylol concentrations (mg/L) of 12 α -BAP2, 12 α +ATF1, 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 were 21.44, 22.96, 20.44, 23.91, 26.63, and 28.91, respectively. These results revealed that the synthesis of higher alcohols was influenced by the change of CoA and acetyl-CoA levels in yeast cell.

Discussion

This work focused on regulating the synthesis of acetate ester and higher alcohols through cofactor engineering. Although numerous works have been performed to analyze the CoA and acetyl-CoA levels in different microorganisms and their roles in the production of different bio-products [5, 25, 30], this work is the first attempt to examine the relationship among the uptake of branched-chain amino acid, the intracellular CoA and acetyl-CoA levels, and the synthesis of acetate esters and higher alcohols. Owing to the decrease in valine uptake caused by *BAP2* gene deletion in yeast cell [15], the acetyl-CoA/CoA levels might be affected by the endogenous pathway starting from amino acids [28, 29, 32]. Meanwhile, the essential genes *CAB1* and *CAB3* in the consecutive pathway are crucial for catalyzing CoA biosynthesis [8, 26], which can be explained by the higher levels of engineered strains 12 α -BAP2+CAB1, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1+ATF1, and 12 α -BAP2+CAB3+ATF1 with *CAB1* or *CAB3* gene overexpression than those of 12 α -BAP2 strains. Additionally, we found that the NAD(H) levels were raised corresponding to the increase in CoA and acetyl-CoA in the strains with overexpression *ATF1*, *CAB1*, or *CAB3* gene. The increase in NAD(H) levels may be due to the change in relevant reaction and corresponding enzyme levels during the yeast fermentation, which may lead to the increase in NAD(H) levels in these strains. Conclusively, the metabolic mechanism of CoA and acetyl-CoA should be further studied.

In this work, the decrease in the *n*-propanol, methyl-1-butanol, and isopentanol levels and the increase in isobutanol and phenethylol levels may be due to the reduced uptake of leucine, isoleucine, and valine and the increased uptake of phenylalanine and threonine in the strains with *BAP2* deletion. The decreased uptake of valine decreased acetyl-CoA/CoA ratio and isoamyl acetate level. The increase in acetyl-CoA/CoA ratio caused the upregulation of acetate ester in the strains 12 α -BAP2+CAB1 and 12 α -BAP2+CAB3. The increase in acetate ester levels in the strain 12 α +ATF1 is consistent with previous studies [9, 20]. Owing to the

increase in acetyl-CoA/CoA ratio, which is beneficial for the synthesis of acetate ester, 12 α -BAP2+CAB1 and 12 α -BAP2+CAB3 exhibited the highest levels than those of other engineered strains. The change of higher alcohol levels in 12 α +ATF1, 12 α -BAP2+CAB, 12 α -BAP2+CAB3, 12 α -BAP2+CAB1, and 12 α -BAP2+CAB3 may be caused by the uptake of branched-chain amino acids, acetyl-CoA and CoA levels, or others factors [1, 13, 33]. Further research is needed to improve the understanding of gene-regulating mechanisms, cofactor levels, and biosynthetic pathways of volatile flavor during yeast fermentation.

Overall, this is the first successful comprehensive manipulation of CoA and acetyl-CoA levels as a novel metabolic engineering tool to modulate the production of acetate esters and higher alcohols only in yeast cell. This method is effective to better control volatile flavor levels and ensure a satisfactory test in the end product. The success of the CoA and acetyl-CoA manipulation shows that cofactor manipulation has considerable potential, which can be explored with pathway manipulation. Cofactor manipulation might alleviate some of the limitations of pathway manipulations and provide a new strategy for realizing metabolic engineering goals. Additionally, our protocols would be securely applied because the resulting self-cloning strains do not contain any heterologous sequences in its genome.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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