

Regulation of *Saccharomyces cerevisiae* genetic engineering on the production of acetate esters and higher alcohols during Chinese Baijiu fermentation

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Abstract Acetate esters and higher alcohols greatly influence the quality and flavor profiles of Chinese Baijiu (Chinese liquor). Various mutants have been constructed to investigate the interactions of *ATF1* overexpression, *IAH1* deletion, and *BAT2* deletion on the production of acetate esters and higher alcohols. The results showed that the overexpression of *ATF1* under the control of the *PGK1* promoter with *BAT2* and *IAH1* double-gene deletion led to a higher production of acetate esters and a lower production of higher alcohols than the overexpression of *ATF1* with *IAH1* deletion or overexpression of *ATF1* with *BAT2* deletion. Moreover, deletion of *IAH1* in *ATF1* overexpression strains effectively increased the production of isobutyl acetate and isoamyl acetate by reducing the hydrolysis of acetate esters. The decline in the production of higher alcohol by the *ATF1* overexpression strains with *BAT2* deletion is due to the interaction of *ATF1* overexpression and *BAT2* deletion. Mutants with varying abilities of producing acetate esters and higher alcohols were developed by genetic engineering. These strains have great potential for industrial application.

Keywords *Saccharomyces cerevisiae* · Chinese Baijiu · Acetate ester · Higher alcohol

Introduction

Chinese Baijiu (Chinese liquor), one of the six most famous distillates worldwide, has been a traditional alcoholic beverage in China for thousands of years. The annual output of Chinese Baijiu has increased steadily in recent years and exceeds 10 million metric tons in China. Chinese Baijiu is typically made from grains, mainly sorghum, by soaking, steaming, fermenting, distilling, aging, and blending [36]. During fermentation, flavor compounds result from the metabolic activity of the microbial community [20, 27]. Higher alcohols and aromatic esters, two of the most abundant and most important groups of volatile flavor compounds in Chinese Baijiu, are significant parameters in determining the quality and flavor profiles of the beverage.

Higher alcohols markedly influence the smell and taste of alcoholic beverages [25]. However, high concentration of higher alcohols in Chinese Baijiu leads to fusel oil taste and is potentially harmful to human health. This characteristic may cause cerebral paralysis [31]. Flavor-active esters, which are responsible for the highly desired fruity aroma, are the most important compounds for the flavor of Chinese Baijiu [6]. Appropriate control of the higher alcohol content and increasing the production of beneficial esters are always considered during fermentation of Chinese Baijiu.

Higher alcohols are mostly produced by *Saccharomyces cerevisiae* in traditional Chinese Baijiu fermentation [29]. The flavor substance originates from the biosynthetic pathway [2] or the Ehrlich pathway in yeast cells [1]. In the Ehrlich pathway, branched-chain alcohols such as isobutanol, isoamylol, active amyl alcohol, and β -phenylethanol are

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products from the degradation of corresponding branched-chain amino acids (BCAAs) [14]. BCAAs are converted to the corresponding α -keto acids through the initial transamination step, which is catalyzed by the corresponding mitochondrial and cytosolic amino acid aminotransferases encoded by *BAT1* and *BAT2* [2–4, 13]. Lilly et al. [16] showed that the transaminases encoded by *BAT1* and *BAT2* significantly influenced the production of branched-chain alcohols. We previously showed that *BAT2* deletion remarkably affected the formation of higher alcohols in Chinese rice wine; by contrast, *BAT1* deletion had little impact [33].

Flavor-active esters are produced by the microbial community and synthesized by esterifying enzyme in natural mixed culture starters (Daqu, Xiaoqu, etc.) at later stages of the traditional fermentation process [5]. Esters are mainly biosynthesized using aroma-producing yeast, bacteria, and molds with high esterification abilities instead of ethanol-fermenting yeast *S. cerevisiae* [28, 35]. Implementing effective measures to increase the content of beneficial esters produced by *S. cerevisiae* is motivated. *S. cerevisiae* strains that can produce high amounts of beneficial esters can reduce grain expense, increase efficiency, and lower costs by shortening the fermentation period. In *S. cerevisiae*, alcohol acetyltransferase encoded by *ATF1* (known as AATase I or Atf1p) is a key enzyme in acetate ester synthesis [37]. Acetate ester, such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana-like aroma) and isobutyl acetate (fruity-like aroma), is one of the major beneficial esters [30]. Alcohols and acetyl-coenzyme A are catalyzed to acetate esters by Atf1p [21–23]. Previous studies have shown that the deletion or overexpression of *ATF1* significantly affects the production of acetate esters in beer, wine, and sake [12, 15, 17, 26]. In addition, esterase encoded by *IAH1* is one of the hydrolyzing enzymes that catalyzes the cleavage of ester bonds in *S. cerevisiae* [19]. Fukuda et al. [7] reported that, instead of enhancing the activity of AATases, the concentration of isoamyl acetate was significantly improved by deleting the acetate-hydrolyzing esterase gene *IAH1* to avoid isoamyl acetate cleavage. The important balance activity between AATases and esterases for the ester accumulation by *S. cerevisiae* was further proved in [8].

Therefore, *S. cerevisiae* that can produce high amounts of acetate esters and low concentration of higher alcohols is very important in the Chinese Baijiu industry. Most studies that examined the effects of *ATF1*, *IAH1*, and *BAT2* on the production of flavor compounds have investigated one or two genes and focused especially on beer and wine fermentation. Moreover, *ATF1* overexpression, *IAH1* deletion, and *BAT2* deletion have different effects on the flavor compound production in various yeasts [7, 8, 15, 17, 24, 26, 32–34]. In *S. cerevisiae* used for Chinese Baijiu fermentation, the effects of *ATF1* overexpression with *BAT2* deletion and/or

IAH1 deletion on the production of acetate esters and higher alcohols remain unclear. Furthermore, comparison of combined mutations of *ATF1* overexpression, *BAT2* deletion, and *IAH1* deletion remains unclear.

In this study, we constructed the *ATF1* overexpression Chinese Baijiu yeast strains with *BAT2* and/or *IAH1* deletion. In addition, the *ATF1* overexpression strain, deletion mutant strains of *IAH1* and/or *BAT2*, and *ATF1* double-overexpression strain with *BAT2* and *IAH1* double-gene deletion were successfully constructed. The effects of *ATF1* overexpression, *BAT2* deletion, and *IAH1* deletion on the flavor compounds were investigated by corn hydrolysate fermentation and Chinese Baijiu fermentation. The interactions of *ATF1* overexpression, *BAT2* deletion, and *IAH1* deletion were further investigated. The results suggest that, compared with other mutant strains, overexpression of *ATF1* with double-gene deletion of *BAT2* and *IAH1* in yeast cells could further increase the ratio of ester to higher alcohol. Combined mutations of *ATF1* overexpression, *BAT2* deletion, and *IAH1* deletion showed diverse effects on the flavor compounds. This study introduced a method for the future optimization of yeast strains for Chinese Baijiu production. In addition, this study has great reference value for future optimization of yeast strains for other alcoholic beverages.

Materials and methods

Strains and plasmids

All strains and plasmids as well as their relevant genotypes used in this study are listed in Table 1.

Media and culture conditions

Escherichia coli cells were grown at 37 °C in Luria–Bertani medium (0.5% yeast extract, 1% tryptone, and 1% NaCl), supplemented with 100 μ g/mL ampicillin to select positive *E. coli* transformants.

Saccharomyces cerevisiae cells were cultured at 30 °C in a yeast extract peptone dextrose (YEPD) medium (1% yeast extract, 2% peptone, and 2% glucose). To select the positive yeast transformants, 1000 μ g/mL G418 (Promega, Madison, USA) was added to the YEPD plates for the yeast culture. To select Zeocin-resistant yeast strains, 500 mg/L Zeocin (Promega, Madison, USA) was added to the YEPD plates. Then, the YEPD medium was used for the *Cre* expression in the yeast transformants.

All solid media contained 2% agar powder (Solarbio, Beijing, China).

Table 1 Microbial strains and plasmids used in the current study

Strains or plasmids	Relevant characteristic	References or sources
Strains		
<i>Escherichia coli</i>		
DH5α	Φ80 <i>lacZΔM15 ΔlacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA relA1</i>	Stratagene
<i>Saccharomyces cerevisiae</i>		
AY15	Commercial liquor yeast strain	Angel yeast, China
α5 ^a	<i>MATα</i> , haploid yeast strain from AY15	This study
a8 ^a	<i>MATa</i> , haploid yeast strain from AY15	This study
α5-IAH1	<i>MATα</i> , Δ <i>IAH1::loxP-KanMX-loxP</i>	This study
α5-BAT2	<i>MATα</i> , Δ <i>BAT2::loxP-KanMX-loxP</i>	This study
α5-IAH1-BAT2	<i>MATα</i> , Δ <i>IAH1::loxP</i> , Δ <i>BAT2::loxP-KanMX-loxP</i>	This study
α5+ATF1	Yep352-A	This study
α5-IAH1+ATF1	<i>MATα</i> , Δ <i>IAH1::PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study
α5-BAT2+ATF1	<i>MATα</i> , Δ <i>BAT2::PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study
α5-IAH1-BAT2+ATF1	<i>MATα</i> , Δ <i>BAT2::PGK1_p-ATF1-PGK1_T-loxP</i> , Δ <i>IAH1::loxP-KanMX-loxP</i>	This study
α5-IAH1+ATF1-BAT2+ATF1	<i>MATα</i> , Δ <i>BAT2::PGK1_p-ATF1-PGK1_T-loxP</i> , Δ <i>IAH1::PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study
a8-BAT2	<i>MAT a</i> , Δ <i>BAT2::loxP-KanMX-loxP</i>	This study
a8+ATF1	Yep352-A	This study
a8-IAH1+ATF1	<i>MATa</i> , Δ <i>IAH1::PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study
a8-BAT2+ATF1	<i>MATa</i> , Δ <i>BAT2::PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study
a8-IAH1-BAT2+ATF1	<i>MATa</i> , Δ <i>BAT2::PGK1_p-ATF1-PGK1_T-loxP</i> , Δ <i>IAH1::loxP-KanMX-loxP</i>	This study
AY15-BAT2	Diploid yeast strain, Δ <i>BAT2::loxP-KanMX-loxP</i>	This study
AY15+ATF1	Yep352-A	This study
AY15-IAH1+ATF1	Diploid yeast strain, Δ <i>IAH1::PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study
AY15-BAT2+ATF1	Diploid yeast strain, Δ <i>BAT2::PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study
AY15-IAH1-BAT2+ATF1	Diploid yeast strain, Δ <i>BAT2::PGK1_p-ATF1-PGK1_T-loxP</i> , Δ <i>IAH1::loxP-KanMX-loxP</i>	This study
Plasmids		
pPGK1	Ap ^f , containing <i>PGK1_p-PGK1_T</i> expression cassette	[15]
pUG6	<i>E. coli/S. cerevisiae</i> shuttle vector, containing Amp ⁺ and <i>loxP-KanMX-loxP</i> cassette	[10]
pUC19	Ap ^f , cloning vector	Invitrogen (Carlsbad, CA, USA)
Yep352	URA3 ⁺ , Amp ^f ori control vector	Invitrogen (Carlsbad, CA, USA)
Yep352-A	<i>KanMX PGK1_p-ATF1-PGK1_T</i>	This study
pSH-Zeocin	Zeo ^f , Cre expression vector	[18]
pUC-PAK	Ap ^f , Kan ^f , containing <i>PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP</i>	This study

^a α5 and a8 are, respectively, high ethanol-producing α mating-type haploid and a mating-type haploid derived from the AY15 strain

Plasmid constructions

The plasmid pUC19 was used as the backbone to construct recombinant plasmid pUC-PAK. The plasmid pUG6 was used as the template to amplify the *KanMX* gene for G418 resistance and transformation fragments. Plasmid pPGK1 which contained the promoter (*PGK1_p*) and terminator (*PGK1_T*) was utilized to prepare the overexpression cassette. The plasmid Yep352 was used as the backbone to construct recombinant plasmid Yep352-A. The plasmid was

prepared from DH5α using a Plasmid Mini Kit II (D6945, Omega, Norcross, USA). Genomic yeast DNA was prepared from the industrial yeast strain α5 using a yeast DNA kit (D3370-01, Omega, Norcross, USA). The polymerase chain reaction (PCR) primers used in this study are listed in Online Resource 1.

The plasmid pUC-PAK was constructed as follows. The yeast phosphoglycerate kinase I gene *PGK1* fragment (1771 bp) was amplified via PCR from the plasmid pPGK1 with PGK-F and PGK-R primers. The *PGK1* fragment was

inserted into the *Xba*I site of pUC19 to generate plasmid pUC-P. The *ATF1* fragment (1578 bp), which was amplified via PCR from genomic yeast DNA of α 5 with ATF1-F and ATF1-R primers, was inserted into the *Xho*I site of plasmid pUC-P, between *PGK1_P* and *PGK1_T*. The pUC-PA plasmid was then constructed. The PCR-generated *loxP-KanMX-loxP* fragment (1613 bp) from plasmid pUG6 with Kan-F and Kan-R primers was inserted into the *Kpn*I site of plasmid pUC-PA to construct the final recombinant plasmid pUC-PAK.

The plasmid Yep352-A, which is an episomal plasmid with *ATF1* under the control of *PGK1_P* and *PGK1_T*, was constructed as follows. The fragment *PGK1_P-ATF1-PGK1_T-loxP-KanMX-loxP* was amplified via PCR from the plasmid pUC-PAK with PGK-U and KAN-D primers. The fragment was inserted into the *Eco*RI and *Sph*I sites of Yep352 to generate plasmid Yep352-A.

Yeast transformation and screening

The transformation fragments *IA-PGK1_P-ATF1-PGK1_T-loxP-KanMX-loxP-IB* and *BA-PGK1_P-ATF1-PGK1_T-loxP-KanMX-loxP-BB* were amplified by PCR from the recombinant plasmid pUC-PAK with II-F/IK-R and BB-F/BK-R primers, respectively. The transformation fragments *IF-loxP-KanMX-loxP-IB* and *BF-loxP-KanMX-loxP-BB* were amplified by PCR from the plasmid pUG6 with IK-F/IK-R and BK-F/BK-R primers, respectively. The fragments and plasmid Yep352-A were then transferred to yeast cells using the lithium acetate/PEG procedure [9]. The recombinant strains were selected using the YEPD medium supplemented with G418 (α -type haploid, 1000 μ g/mL; a-type haploid, 1400 μ g/mL; and diploid, 1200 μ g/mL) and verified through PCR with the primers listed in Online Resource 1. The marker gene *KanMX* in the engineered strain was removed using the *Cre/loxP* recombination system [11].

Construction of the recombinant diploid yeast strains

Recombinant diploid yeast strain was constructed by hybridizing a-type and α -type haploid recombinants. Haploid a- and α -type cells were cultured in 5 mL of YEPD medium at 30 °C for 24 h, respectively. Then, they (0.5 mL each) were added to a tube containing 5 mL of YEPD. Yeast cells were cultivated and hybridized at 30 °C for 24 h. The recombinant diploid strain was selected using the MacConkey medium (0.1% glucose, 0.18% NaCl, 0.82% sodium acetate, 0.25% yeast extract, and 2% agar) and verified through PCR with the primers MAT-F/MAT- α /MAT-a. The resulting diploid yeast strains with spore formation were verified under a microscope (Olympus, Tokyo, Japan).

Growth curve determination

Yeast cells were precultured in 5 mL of YEPD medium at 30 °C for 12 h and then transferred into the YEPD medium and cultured at 30 °C for 16 h. The optical density (OD 600) was determined every 1 h using a Bioscreen Automated Growth Curves analysis system (OY Growth Curves Ab Ltd., Helsinki, Finland).

Real-time quantitative PCR (RT-qPCR)

The total RNA of yeast was extracted using a Yeast RNAiso Kit (Takara Biotechnol, Dalian, China) and then reverse-transcribed using a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Biotechnol, Dalian, China). Changes in gene expression level were assessed by RT-qPCR using an SYBR *Premix Ex Taq* II (Tli RNaseH Plus) (Takara Biotechnol, Dalian, China). The PCR primers used are listed in Online Resource 1. The PCR program was composed of pre-denaturation for 30 s at 95 °C, amplification using 40 cycles of denaturation for 5 s at 95 °C and annealing and polymerization for 30 s at 60 °C, and melt curve stage for 15 s at 95 °C and 1 min at 60 °C. The result was quantitatively analyzed using the $2^{-\Delta\Delta C_t}$ method. The actin gene (*ACT1*) was used as a housekeeping gene. The PCR primers used are listed in Online Resource 1.

Fermentation experiments

The corn hydrolysate medium was prepared by gelatinizing a mixture of 60 g of corn flour and 130 mL of water at 65 °C for 20 min in a 250-mL conical flask. Then the mixture was liquefied at 90 °C for 90 min with thermostable α -amylase (10 U/g corn weight, 2×10^5 U/mL; Novozymes, Copenhagen, Denmark) and subsequently saccharified at 60 °C for 30 min with a saccharifying enzyme (150 U/g corn weight, 10×10^5 U/mL; Novozymes, Copenhagen, Denmark). The resulting medium was cooled to 30 °C at room temperature. Yeast cells were precultured in 4 mL of 8 Brix degree (°Bx) corn hydrolysate medium at 30 °C for 24 h. Then, the yeasts were transferred into 36 mL of 12°Bx corn hydrolysate in a 100-mL conical flask and cultured at 30 °C for 16 h. The second precultured yeast (15 mL) was transferred to the prepared corn hydrolysate medium. Moreover, the recombinant strains which harbored Yep352-A were cultured using medium supplemented with G418 (α -type haploid, 1000 μ g/mL; a-type haploid, 1400 μ g/mL; diploid, 1200 μ g/mL). The mixture was fermented at 30 °C until the weight loss of CO₂ after interval 12 h was less than 1 g.

For the solid fermentation of sorghum, 80 g of sorghum was immersed in 200 mL of water at 60 °C in a 500-mL conical flask. The mixture was liquefied at 90 °C for 60 min with thermostable α -amylase (5 U/g sorghum weight), and

subsequently sterilized at 0.1 MPa for 30 min. Then the mixture was saccharified at 60 °C for 30 min with a saccharifying enzyme (50 U/g sorghum weight), and acid proteinase (15 U/g sorghum weight; 2×10^5 U/g, Novozymes, Copenhagen, Denmark) was added at 40 °C for 30 min. The resulting medium was cooled to 30 °C at room temperature. Yeast cells were precultured in 4 mL of YEPD medium at 30 °C for 24 h and then transferred into 36 mL of YEPD medium at 30 °C for 16 h. The natural mixed culture starter Daqu (20 g) and 20 mL of the second precultured yeast were transferred to the prepared sorghum medium. Moreover, the strain AY15+ATF1 was cultured using the medium supplemented with G418 (1200 µg/mL). The mixture was fermented at 30 °C for 1 day and then at 35 °C until the weight loss of CO₂ after an interval of 12 h was less than 1 g.

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

All fermentations were performed in triplicate.

Gas chromatography analysis

GC has been widely used in the analysis of volatile compounds in Chinese Baijiu [5]. Samples from the corn hydrolysate and sorghum media were distilled after fermentation and then used for GC.

The volatile compounds were analyzed on an Agilent 7890C GC with an HP-INNOWax polyethylene glycol column (30 m × 320 µm internal diameter and 0.5 µm coating thickness; Lab Alliance). GC was equipped with an Agilent G4513A autosampler, injector, and a flame ionization detector (FID). Nitrogen was used as carrier gas at a constant flow rate of 2 mL/min. The injector temperature was 200 °C, split ratio was 10:1, and injection volume was 1 µL. The FID was operated at 200 °C. The oven temperature program was as follows: 50 °C for 8 min and then the temperature was increased to 120 °C at 5 °C/min. The final temperature was maintained for 5 min. *N*-butyl acetate was used as the internal standard. An internal calibration curve was constructed for each compound measured using a specific amount of authentic standards. These chemicals were purchased from Merck.

Results

Overexpression of *ATF1* and deletion of *IAH1* increased acetate ester production and reduced higher alcohol production

IAH1 genes of α5 strain were, respectively, replaced with the constructed cassette *IA-PGK1_P-ATF1-PGK1_T-loxP-KanMX-loxP-IB*

and the fragment *IF-loxP-KanMX-loxP-IB*. We obtained engineered strains α5-IAH1+ATF1 and α5-IAH1. In addition, the engineered strain α5+ATF1, which harbored the plasmid Yep352-A, was constructed. The effects of *ATF1* overexpression and *IAH1* deletion on ester and higher alcohol productions were investigated in the liquid fermentation of corn hydrolysate. The data of fermented samples analyzed by GC are presented in Fig. 1.

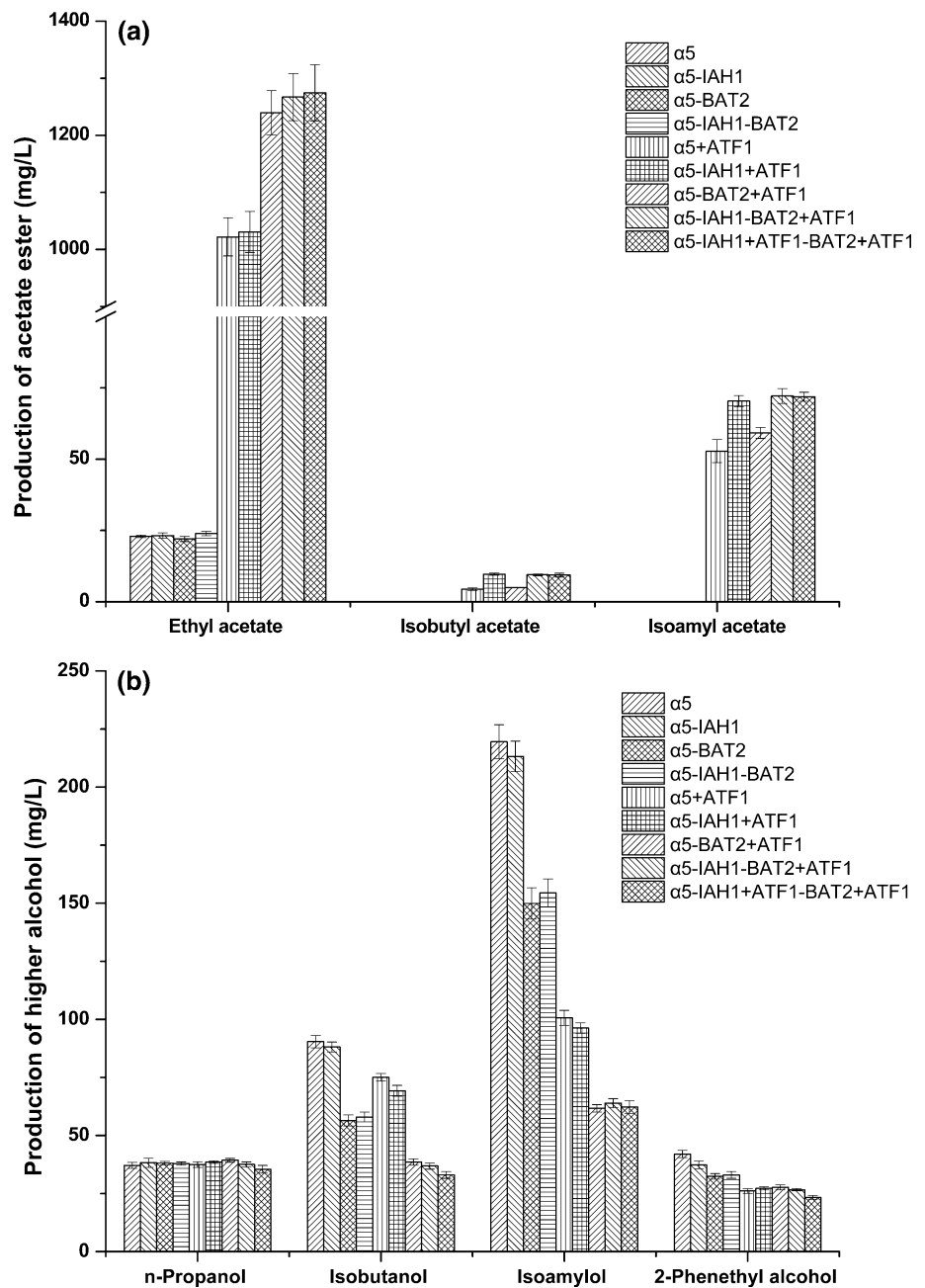
No significant distinction was observed between the engineered strain α5-IAH1 and parental strain α5. The production of ethyl acetate by α5-IAH1+ATF1 was ~44.93-fold higher than that by α5. The concentration of higher alcohols produced by α5-IAH1+ATF1 was 40.58% less than that produced by α5. The productions of isobutanol, isoamylol (isoamyl alcohol and active amyl alcohol), and β-phenylethanol by α5-IAH1+ATF1 were significantly lower than those produced by the parental strain. Moreover, the productions of ethyl acetate and higher alcohols by α5+ATF1 were similar to that of α5-IAH1+ATF1. The productions of isobutyl acetate and isoamyl acetate by α5-IAH1+ATF1 increased to 9.66 and 70.37 mg/L, respectively. The productions of isobutyl acetate and isoamyl acetate by α5+ATF1 were 54.04 and 24.93% less than those of α5-IAH1+ATF1. By contrast, no isobutyl acetate or isoamyl acetate was detected in the fermentation sample of α5.

Overexpression of *ATF1* and deletion of *BAT2* increased acetate ester production and further reduced higher alcohol production

Thus, *BAT2* genes of α5 strain were replaced with the constructed cassette *BA-PGK1_P-ATF1-PGK1_T-loxP-KanMX-loxP-BB*, and the fragment *BF-loxP-KanMX-loxP-BB*. The engineered strains α5-BAT2+ATF1 and α5-BAT2 were obtained. Fermentation data presented in Fig. 1a suggest that the production of ethyl acetate by the engineered strain α5-BAT2+ATF1 was ~54.03-fold higher than that by α5. The production of isobutyl acetate and isoamyl acetate also increased to 4.98 and 59.17 mg/L, respectively. Meanwhile, the concentration of higher alcohols produced by α5-BAT2+ATF1 was 57.02, 39.59, 30.09, and 27.67% less than those produced by α5, α5-BAT2, α5+ATF1, and α5-IAH1+ATF1 (Fig. 1b).

These results indicate that the production of higher alcohols (isobutanol, isoamylol, and β-phenylethanol) remarkably declined because of the overexpression of *ATF1* or the deletion of *BAT2*. Moreover, compared with the overexpression of *ATF1* without *BAT2* deletion, overexpression of *ATF1* with *BAT2* deletion could further reduce higher alcohol production and increase ethyl acetate production in yeast cells. In addition, overexpression of *ATF1* with *IAH1* deletion produced higher concentrations of isobutyl acetate and isoamyl acetate than overexpression of *ATF1* without *IAH1*

Fig. 1 Flavor compound productions of α mating-type strains in the liquid fermentation of corn hydrolysate. **a** Production of acetate ester; **b** production of higher alcohol. Data are average values and standard deviations from at least three independent tests



deletion. To construct the strain with *ATF1* overexpression and both *IAH1* and *BAT2* deletion, the *kan^r* marker gene was removed from the engineered strain $\alpha 5$ -BAT2+ATF1 using the Cre/*loxP* recombination system to produce the mutant strain $\alpha 5$ -BAT2+ATF1-1.

Overexpression of *ATF1* and double-gene deletion of *BAT2* and *IAH1* further increased the ratio of ester to higher alcohol

The *IAH1* gene of $\alpha 5$ -BAT2+ATF1-1 was replaced with the fragment *IF-loxP-KanMX-loxP-IB* to further increase

ester production and reduce higher alcohol production. The engineered strain $\alpha 5$ -IAH1-BAT2+ATF1 was obtained. In addition, the engineered strain $\alpha 5$ -IAH1-BAT2 was constructed, using the fragment *BF-loxP-KanMX-loxP-BB* amplified via PCR from the plasmid pUG6. Figure 1a shows that the concentration of ethyl acetate produced by $\alpha 5$ -IAH1-BAT2+ATF1 was 1267.04 mg/L, which was nearly the same as that produced by $\alpha 5$ -BAT2+ATF1. The concentrations of isobutyl acetate and isoamyl acetate were 9.46 and 72.11 mg/L, which were nearly the same as those produced by $\alpha 5$ -IAH1+ATF1. Meanwhile, negligible differences in higher alcohol contents were found between

the fermentation samples of $\alpha 5$ -IAH1-BAT2+ATF1 and $\alpha 5$ -BAT2+ATF1 (Fig. 1b).

The results indicate that compared with other engineered and parental strains, the strain with overexpression of *ATF1* and double-gene deletion of *BAT2* and *IAH1* could produce higher ratio of ester to higher alcohol. This strain combines the advantages of $\alpha 5$ -IAH1+ATF1 and $\alpha 5$ -BAT2+ATF1.

We confirmed that overexpression of the *ATF1* gene could enhance the synthesis of acetate ester. Thus, *IAH1* gene of $\alpha 5$ -BAT2+ATF1-1 was replaced with the fragment *BA-PGK1_p-ATF1-PGK1_T-loxP-KanMX-loxP-BB*. The engineered strain $\alpha 5$ -IAH1+ATF1-BAT2+ATF1 was obtained. However, no obvious differences in the productions of ester and higher alcohol were observed between $\alpha 5$ -IAH1-BAT2+ATF1 and $\alpha 5$ -IAH1+ATF1-BAT2+ATF1.

mRNA levels

It is noteworthy that there were significant differences in acetate ester and higher alcohol productions among the engineered strains. We quantified the mRNA expression levels of *ATF1*, *BAT2*, and *IAH1* to clarify the relationship among these genes (Fig. 2a, b, c). The results of RT-qPCR suggest that the *ATF1* expression levels of $\alpha 5$ -IAH1+ATF1, $\alpha 5$ -BAT2+ATF1, $\alpha 5$ -IAH1-BAT2+ATF1, and $\alpha 5$ -IAH1+ATF1-BAT2+ATF1 were 23.62-, 26.29-, 27.99-, and 29.13-fold higher than that of $\alpha 5$, respectively (Fig. 2a). The overexpression of *ATF1* gene under the control of the *PGK1* promoter in *S. cerevisiae* significantly increased the gene expression level. In addition to $\alpha 5$ -IAH1+ATF1, which showed the lowest mRNA level, other *ATF1* overexpression strains with different *ATF1* expression levels showed similar capacities of producing ethyl acetate. The synthesis pathways of acetate esters catalyzed by *ATF1*-coded alcohol O-acetyltransferase may reach the highest efficiency by overexpressing *ATF1* with the *PGK1* promoter. Compared with $\alpha 5$ -BAT2+ATF1, the lower mRNA level of *ATF1* in $\alpha 5$ -IAH1+ATF1 may be due to the gene locus where the fragment was inserted. Moreover, the mRNA level of *ATF1* in $\alpha 5$ -IAH1+ATF1-BAT2+ATF1 was similar to that in $\alpha 5$ -BAT2+ATF1, which may be restricted by the concentration of substrate acetyl-CoA.

Compared with the parental strain, the *BAT2* expression levels of *BAT2* deletion strains and *IAH1* expression levels of *IAH1* deletion strains were nearly zero. Furthermore, no obvious differences in the *BAT2* expression levels were observed among *IAH1* one-gene deletion and parental strains (Fig. 2b). It is noteworthy that the *IAH1* expression level of $\alpha 5$ -BAT2+ATF1 was ~1.56-fold higher than that of other *BAT2* one-gene deletion strains and the parental strain (Fig. 2c). In addition, the *BAT2* expression level was not affected by *ATF1* overexpression or *IAH1*

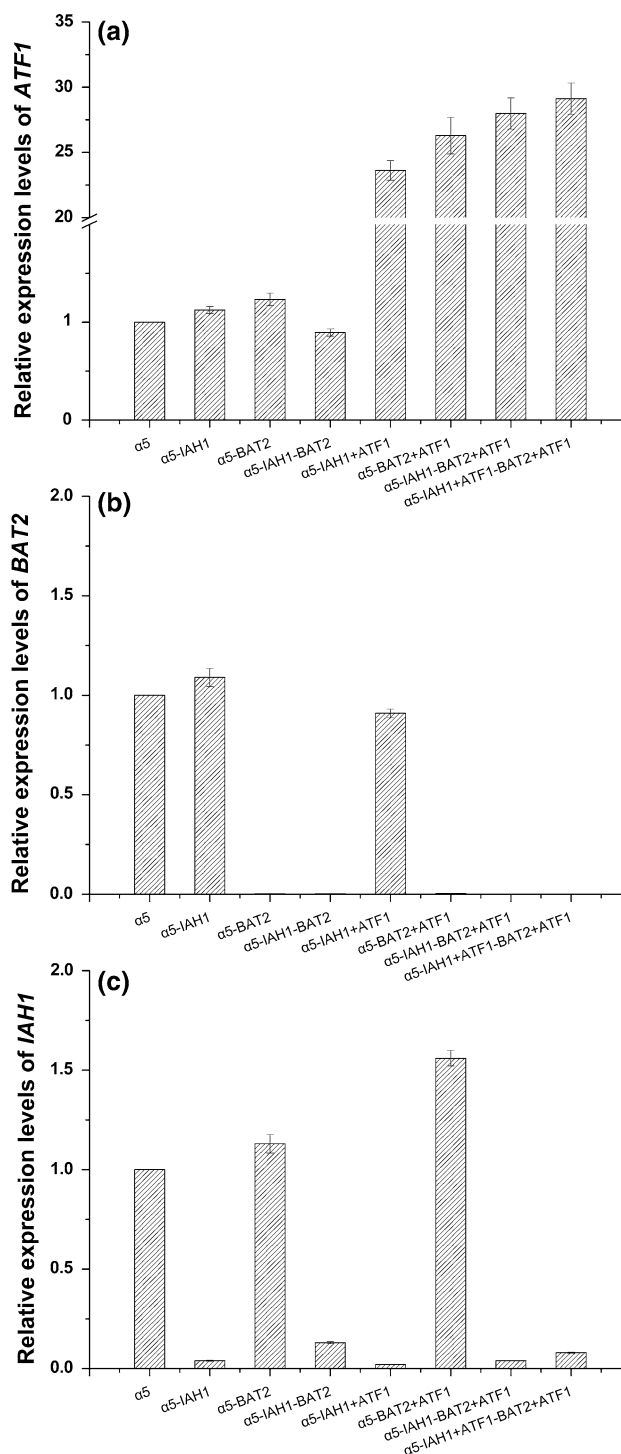


Fig. 2 Determination of expression levels in the α mating-type mutant strains and the parental strain $\alpha 5$. **a** The expression levels of *ATF1*; **b** the expression levels of *IAH1*; **c** the expression levels of *BAT2*. Data are average values and standard deviations from at least three independent tests

deletion. The results indicate that the expression level of *IAH1* gene was relatively upregulated because of the *ATF1* overexpression.

Growth curve and fermentation properties

The growth abilities of engineered strains showed a slight difference compared with their parental strains (Fig. 3a). Fermentation properties, including residual sugar and weight loss of CO₂, were further monitored (Table 2). The *ATF1* overexpression strains produced 5.03–7.79% less ethanol contents than the parental strain in the liquid fermentation. This result may be due to more synthesis of ethyl acetate from ethanol and acetyl-CoA in the *ATF1* overexpression strains.

Construction of recombinant diploid yeast strains and Chinese Baijiu fermentation

Different results on flavor compounds were obtained by α 5, α 5-BAT2, α 5+ATF1, α 5-IAH1+ATF1, α 5-BAT2+ATF1, and α 5-IAH1-BAT2+ATF1. Then, a-type haploid recombinants were constructed. The engineered strains a8-BAT2, a8-IAH1+ATF1, a8-BAT2+ATF1, and a8-IAH1-BAT2+ATF1 were obtained. Recombinant diploid yeast strain was constructed by hybridizing a-type and α -type haploid recombinants. The engineered diploid strains AY15-BAT2, AY15-IAH1+ATF1, AY15-BAT2+ATF1, and AY15-IAH1-BAT2+ATF1 were obtained successfully. In addition, the engineered strains a8+ATF1 and AY15+ATF1 were constructed. The recombinant yeast strains showed slightly weaker growth abilities than their parental strains (Fig. 3b, c). The data of volatile flavor compounds produced by these engineered strains during the liquid fermentation of corn hydrolysate are presented in Fig. 4.

We investigated the acetate ester and higher alcohol productions of the obtained recombinant diploid strains and the parental strain AY15 in Chinese Baijiu fermentation with natural mixed culture starter Daqu. The data of fermentation samples analyzed by GC are presented in Fig. 5. Compared with the liquid fermentation of corn hydrolysate, similar results of the difference between the obtained mutants and the parental strain were measured for the Chinese Baijiu fermentation. The concentrations of ethyl acetate produced by AY15+ATF1, AY15-IAH1+ATF1, AY15-BAT2+ATF1, and AY15-IAH1-BAT2+ATF1 were 3.29-, 3.48-, 3.89-, and 3.91-fold higher than that produced by AY15, respectively. The concentrations of higher alcohols produced by AY15-BAT2, AY15+ATF1, AY15-IAH1+ATF1, AY15-BAT2+ATF1, and AY15-IAH1-BAT2+ATF1 were 27.35, 17, 18.01, 38.22, and 39.41% less than that by AY15, respectively. Moreover, the productions of isoamyl acetate by the *ATF1* overexpression mutants increased to 2.14–6.23 mg/L.

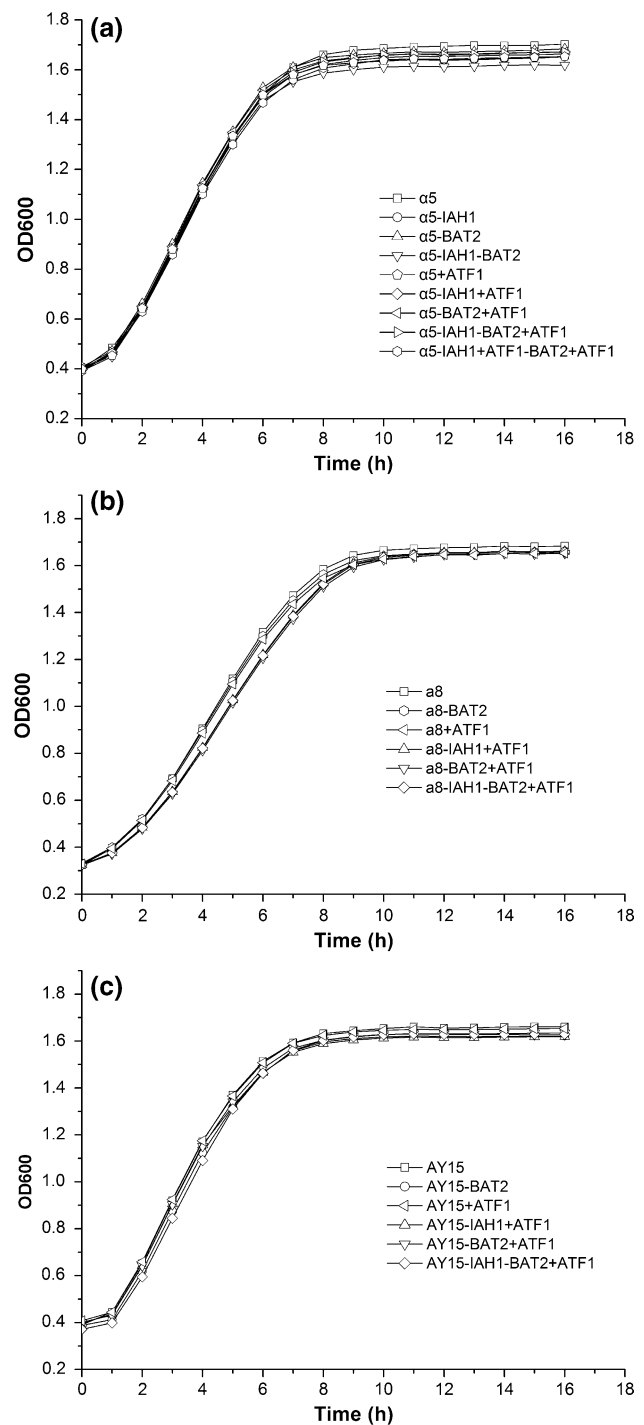


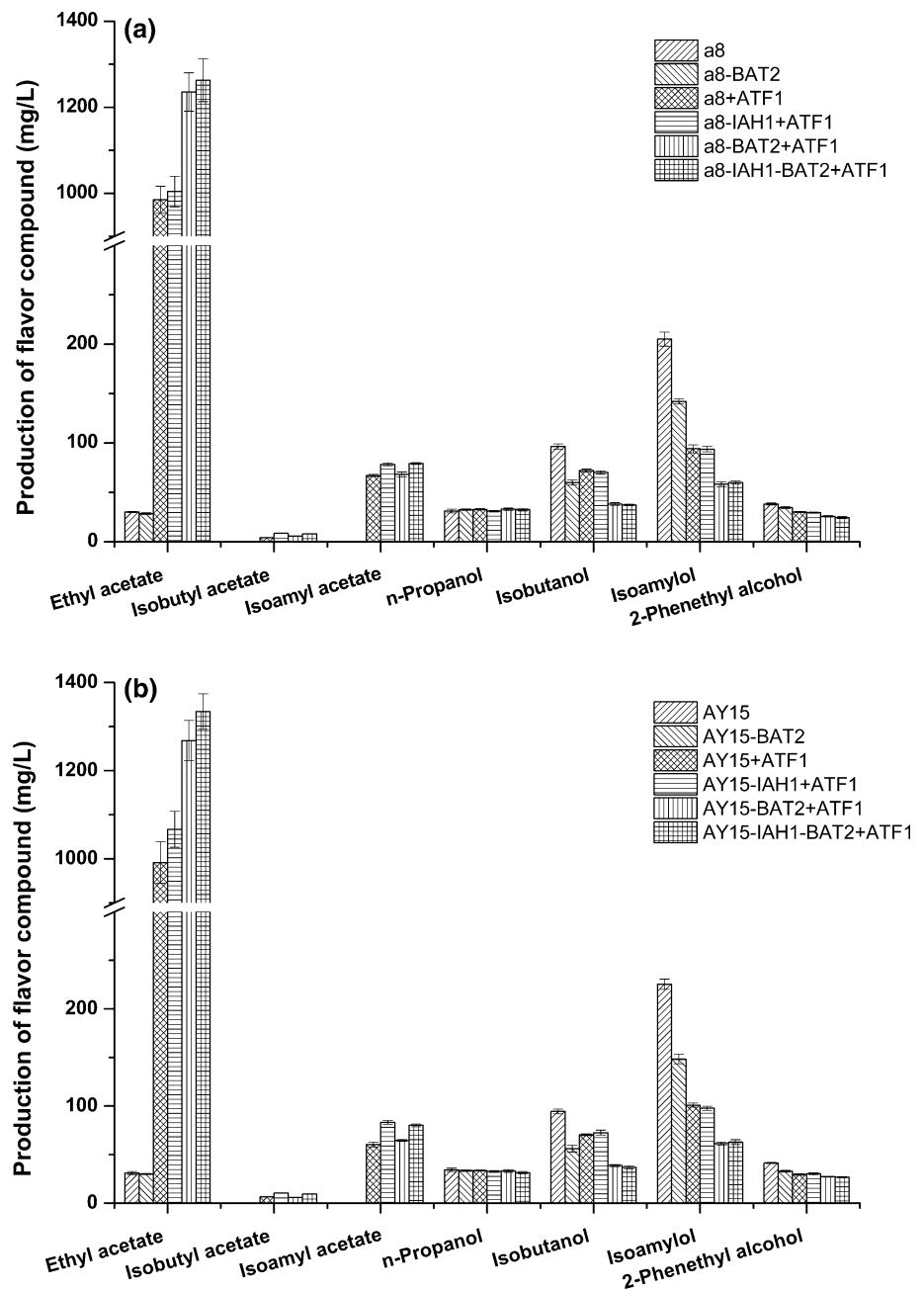
Fig. 3 Growth curves of recombinant strains and their parental strains. **a** The growth curves of α mating-type mutant strains and α 5; **b** the growth curves of a mating-type mutant strains and a8; **c** the growth curves of diploid mutant strains and AY15. Growth curves (in triplicate) were monitored at 30 °C by measuring the optical density (OD600) of the cultures every 1 h

Table 2 Fermentation performances of the α mating-type strains in the liquid fermentation of corn hydrolysate

Yeast strains	Weight loss of CO ₂ (g)	Ethanol (% v/v, 20 °C)	Residual reducing sugars (g/100 mL)
$\alpha 5$	23.13 ± 1.28	14.50 ± 0.68	0.23 ± 0.03
$\alpha 5$ -IAH1	23.58 ± 1.13	14.64 ± 1.13	0.45 ± 0.01
$\alpha 5$ -BAT2	23.61 ± 0.94	14.38 ± 0.29	0.58 ± 0.03
$\alpha 5$ -IAH1-BAT2	23.34 ± 0.67	14.12 ± 0.36	0.43 ± 0.03
$\alpha 5$ +ATF1	23.49 ± 0.95	13.37 ± 0.68	0.40 ± 0.03
$\alpha 5$ -IAH1+ATF1	23.81 ± 0.49	13.77 ± 0.60	0.35 ± 0.01
$\alpha 5$ -BAT2+ATF1	23.46 ± 0.76	13.46 ± 0.43	0.57 ± 0.03
$\alpha 5$ -IAH1-BAT2+ATF1	22.76 ± 1.49	13.52 ± 0.47	0.48 ± 0.02
$\alpha 5$ -IAH1+ATF1-BAT2+ATF1	23.31 ± 0.61	13.45 ± 0.32	0.61 ± 0.02

Values are means ± standard deviations from at least three independent tests

Fig. 4 Flavor compound productions of mutant and parental strains in the liquid fermentation of corn hydrolysate. **a** Flavor compound productions of α mating-type strains and a8; **b** flavor compound productions of diploid mutant strains and AY15. Data are average values and standard deviations from at least three independent tests



By contrast, no isoamyl acetate was detected in the fermentation sample of AY15.

Discussion

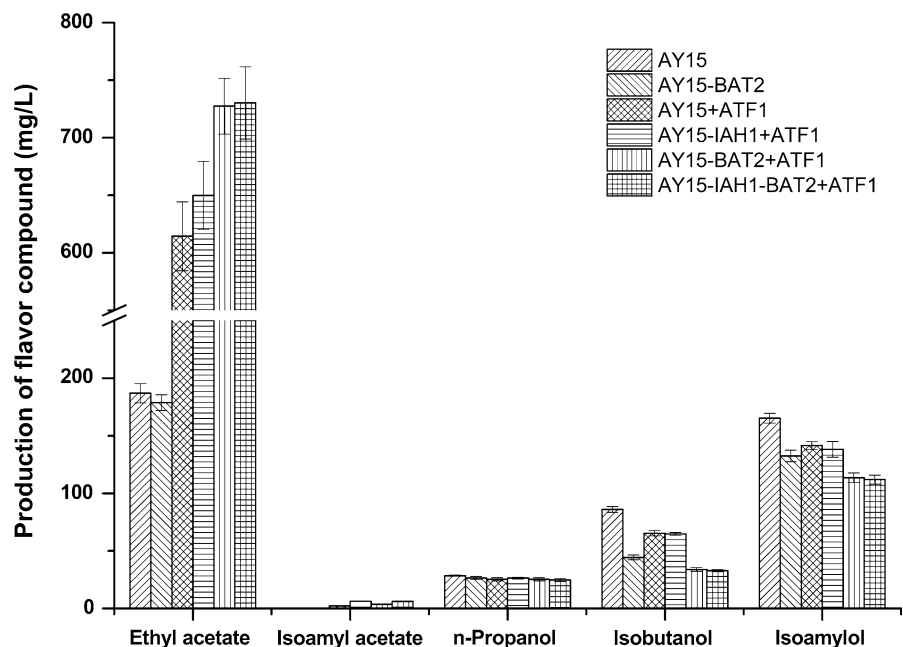
A yeast strain capable of producing high amounts of esters and low concentration of higher alcohols is very important and necessary in the Chinese Baijiu industry. In the present study, we showed for the first time that overexpression of *ATF1* and double-gene deletion of *BAT2* and *IAH1* in yeast cells could further increase the ratio of ester to higher alcohol. Moreover, the interactions of *ATF1* overexpression, *BAT2* deletion, and *IAH1* deletion were investigated. Mutants with varying abilities of producing acetate esters and higher alcohols were constructed.

BAT2, *ATF1*, and *IAH1* are key genes in the metabolic pathways related to higher alcohols and acetate esters. The amino acid aminotransferase encoded by *BAT2* converts BCAAs to α -keto acids, which are precursors of branched-chain alcohols in the Ehrlich pathway [2–4, 14]. Alcohols and acetyl-coenzyme A are catalyzed to acetate esters by *ATF1*-encoded alcohol acetyltransferase [21–23]. Several acetate esters, such as isoamyl acetate, are hydrolyzed to corresponding alcohols by *IAH1*-encoded esterase [19]. The overexpression of *ATF1* gene under the control of the *PGK1* promoter in *S. cerevisiae* could significantly improve the production of acetate esters (ethyl acetate, isobutyl acetate, and isoamyl acetate) in Chinese Baijiu fermentation. The result is consistent with those of previous

studies [15–17, 26] and our previous studies in other alcoholic beverages [24, 34]. The *IAH1* expression level was markedly upregulated because of the overexpression of *ATF1* (Fig. 2c). Hence, the deletion of *IAH1* in *ATF1* overexpression strains may effectively increase the productions of isobutyl acetate and isoamyl acetate by reducing the hydrolysis of acetate esters. The results support the hypothesis that the expression of *ATF1* and *IAH1* encoding opposite enzymatic activities is finely regulated [8]. Furthermore, higher alcohol production in the *ATF1* overexpression strains with *BAT2* deletion decreased due to the interaction of *ATF1* overexpression and *BAT2* deletion. The production of isobutanol was affected more obviously by the deletion of *BAT2*, and the overexpression of *ATF1* contributed more in the reduction of isoamylol and β -phenylethanol productions. The overexpression of *ATF1* with the deletion of *BAT2* could further decrease the production of isobutanol and isoamylol. Moreover, no obvious interaction between *BAT2* and *IAH1* was observed in the study.

Studies on the interactions of the three genes introduce methods for future optimization of yeast strains. Different genetic engineering strategies can be applied based on the distinct requirements on the productions of acetate esters and higher alcohols. In addition, the studies provide novel ideas to investigate the metabolism of esters and higher alcohols. Similar to *IAH1*-encoded esterase, additional other related enzymes involved in the hydrolysis of acetate esters may be more produced because of *ATF1* overexpression, since it was proved that the two insertions of *PGK1_p-ATF1-PGK1_T* cassettes showed no obvious difference with one insertion in the production of esters (Figs. 1a, 2a). Further

Fig. 5 Volatile flavor compound productions of diploid yeast strains in Chinese Baijiu fermentation. Data are average values and standard deviations from at least three independent tests



increase in acetate ester production may be derived from the deletion of these ester hydrolytic enzymes.

Stable performance of the industrial strain is significant for fermentation application. In this study, industrial strains were used as parent strains for genetic engineering. We constructed diploid mutants with varying abilities of producing acetate esters and higher alcohols. The recombinant strains showed slightly weaker growth abilities and similar fermentation properties to the parental strain in Chinese Baijiu fermentation (Fig. 3c). These strains with satisfactory performance exhibit broad application prospects in industrial production.

In Chinese Baijiu industry, the contradiction between increasing liquor yield and enriching flavor substance is particularly prominent. A yeast strain capable of producing high amounts of acetate esters and low concentration of higher alcohols is highly important and necessary to simplify the traditional technological process, shorten the traditional fermentation period and reduce costs. The results of this study introduced a method for future optimization of yeast strains that produce high amounts of acetate esters and low concentration of higher alcohols in Chinese Baijiu production. Over-expression of *ATF1* and double-gene deletion of *BAT2* and *IAH1* in yeast cells could further increase the ratio of ester to higher alcohol. Moreover, the interactions of *ATF1* over-expression, *BAT2* deletion, and *IAH1* deletion were preliminarily investigated. These interactions are significant in the investigation of the metabolic mechanism of esters and higher alcohols. Hence, these recombinant yeast strains with varying abilities of producing acetate esters and higher alcohols can be remarkably applied to industrial application.

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Compliance with ethical standards

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

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

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