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Acetate ester production by Chinese yellow rice wine yeast overexpressing the alcohol acetyltransferase-encoding gene *ATF2*

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ABSTRACT. Acetate ester, which are produced by fermenting yeast cells in an enzyme-catalyzed intracellular reaction, are responsible for the fruity character of fermented alcoholic beverages such as Chinese yellow rice wine. Alcohol acetyltransferase (AATase) is currently believed to be the key enzyme responsible for the production of acetate ester. In order to determine the precise role of the *ATF2* gene in acetate ester production, an *ATF2* gene encoding a type of AATase was overexpressed and the ability of the mutant to form acetate esters (including ethyl acetate, isoamyl acetate, and isobutyl acetate) was investigated. The results showed that after 5 days of fermentation, the concentrations of ethyl acetate, isoamyl acetate, and isobutyl acetate in yellow rice wines fermented with EY2 (pUC-PIA2K) increased to 137.79 mg/L (an approximate 4.9-fold increase relative to the parent cell RY1), 26.68 mg/L, and 7.60 mg/L, respectively. This study confirms that the *ATF2* gene plays an important role in the production of acetate ester production during Chinese yellow rice wine fermentation, thereby

offering prospects for the development of yellow rice wine yeast starter strains with optimized ester-producing capabilities.

Key words: Acetate ester; Alcohol acetyltransferase; ATF2; Yeast; Chinese yellow rice wine

INTRODUCTION

Chinese yellow rice wine is a traditional alcoholic beverage in China. In the brewing of yellow rice wine, rice or glutinous rice starch is saccharified by the glucoamylase of *Aspergillus oryzae*, and the liberated glucose is then fermented to ethanol by *Saccharomyces cerevisiae* (Fukuda et al., 2000); simultaneously, the lactose is transformed to lactic acid by *Lactobacillus*. These 3 processes occur at around the same time. This fermentation system of yellow rice wine brewing is unique compared with other alcoholic fermentation systems throughout the world, resulting in the distinct flavor and certain therapeutic and medicinal values characteristic to this wine. Ester aroma chemicals, which can improve the flavor as well as the quality of yellow rice wine at high concentrations, are the main components that constitute the flavor profiles of the wine. The need to understand and control ester synthesis is motivated by problems commonly encountered in Chinese yellow rice wine brewing procedures, such as high grain consumption, long fermentation periods, low efficiency of production, and high costs. Therefore, a detailed understanding of the metabolism and regulation of ester synthesis would enable better control of ester production.

During fermentation, the yeast produces 2 main categories of volatile esters, which are responsible for the highly desired fruity, candy, and perfume-like aroma characteristics of beverages such as beer and Chinese yellow rice wine (Suomalainen, 1981; Nykanen and Suomalainen, 1983; Nykänen, 1986; Malcorps and Dufour, 1987; Peddie, 1990; Meilgaard, 1991; Debourg, 2000; Cristiani and Monnet, 2001; Pisarnitskii, 2001; Dufour et al., 2002; Aritomi et al., 2004). The first group is the acetate esters such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana flavor), and isobutyl acetate (fruity aroma). The other group is the medium-chain fatty acid ethyl esters, which includes ethyl hexanoate (aniseed, apple-like aroma) and ethyl octanoate (sour apple aroma), among others (Meilgaard, 1975a, b, 2001; Verstrepen et al., 2003a). Of these 2 groups, the acetate esters are produced at much higher levels.

Acetate ester is formed intracellularly in an enzyme-catalyzed condensation reaction. The key enzymes involved in ester synthesis are alcohol acetyltransferases (AATases; EC 2.3.1.84) (Nordström, 1962, 1963, 1964). AATase II, which is encoded by *ATF2*, is 1 of the 2 main AATases in *S. cerevisiae* (Yoshioka and Hashimoto, 1981; Malcorps and Dufour, 1992; Fujii et al., 1994, 1996a,b; Nagasawa et al., 1998; Yoshimoto et al., 1998, 1999). It catalyzes the formation of acetate esters from 2 substrates: an alcohol and an acetyl-CoA. The rate of acetate ester formation is mainly dependent on 2 factors: the concentration of the 2 substrates and the activity of the AATase (Yoshioka and Hashimoto, 1983).

Lilly et al. (2006) showed that the concentrations of ethyl acetate and isoamyl acetate produced by VIN13 overexpressing the *ATF2* gene were 1.3-fold and 1.5-1.8-fold higher, respectively, than those seen for the control. Similarly, Verstrepen et al. (2003b) demonstrated that *ATF2* deletion resulted in an 18% decrease in isoamyl acetate formation, while *ATF2* overexpression resulted in a 6- to 8-fold increase. In consequence, the expression level of *ATF2* in the current study was expected to be a limiting factor for ester synthesis in *S. cerevisiae*, confirming the

hypothesis of Malcorps (Malcorps et al., 1991; Malcorps and Dufour, 1992). However, because different research groups have used different yeast strains and different fermentation conditions in *ATF2* studies, it is difficult to compare results effectively. Furthermore, most overexpression studies were based on multicopy plasmids; therefore, some differences among strains may in fact be due to differences in plasmid copy number. As a result, it is currently unclear as to how meaningful these results are with respect to Chinese yellow rice wine, which is a traditional alcoholic beverage in China and is 1 of the 3 most ancient wines in the world; this wine is characterized by its distinct flavor, high nutritional value, and certain medicinal properties.

In order to determine the precise role of the gene *ATF2* in acetate ester production by Chinese yellow rice wine, we a) overexpressed *ATF2* in a widely used industrial yellow rice wine yeast strain (RY1) through homologous recombination; b) prepared yellow rice wine using the transformed and control strains; and c) analyzed and compared the concentration of acetate ester in yellow rice wines prepared with these host and transformed strains. Our data showed that *ATF2* overexpression could significantly enhance the acetate ester contents of yellow rice wine. These observations can inform the development of new production strategies and new yeast strains of Chinese yellow rice wine, allowing further optimization of Chinese yellow rice wine flavor profiles in order to satisfy different sensory preferences of consumers. Furthermore, the results of this study will contribute to a better understanding of the physiological role of the synthesis of acetate ester.

MATERIAL AND METHODS

Microbial strains, plasmids, media, and culturing conditions

All strains and plasmids used are listed in Table 1. Yeast cultures were routinely cultured at 28°C in a yeast extract peptone dextrose (YEPD) medium [2% glucose (Merck), 2% peptone (Difco), and 1% yeast extract (Difco)] for growth and in wort medium (prepared by treating freshly smashed malt with water at 65°C for 30 min and adjusting the sugar content of the wort to 12°Bx) for the fermentation experiments. For the selection of yeast transformants, 100 mg/mL G418 was added to a final concentration of 240 mg/L. *Escherichia coli* was grown at 37°C in a Luria-Bertani medium containing 1% Bacto tryptone (Difco), 1% NaCl (Merck) and 0.5% yeast extract (Difco). Ampicillin was added to the media at a final concentration of 100 mg/L to select the *E. coli* transformants. All the solid media used in this study contained 2% agar (Difco).

DNA manipulations

Standard procedures for the isolation and manipulation of DNA were used in this study (Ausubel et al., 1994). Restriction enzymes, T4 DNA ligase, and LA Taq DNA polymerase (TaKaRa Biotechnol) were used for enzymatic DNA manipulations as recommended by the supplier. The following primers were used for the amplification of DNA fragments by polymerase chain reaction (PCR): for the *ATF2* open reading frame (ORF), was used *Bgl*II-*ATF2*-ORF-F (GGAAGATCTATGGAAGATATAGAAGGATACG; *Bgl*II restriction site is underlined) and *Bgl*II-*ATF2*-ORF-R (GGAAGATCTTTAAAGCGACGCAAATTCGCCG; *Bgl*II restriction site is underlined); for the *IAH* ORF, was used *Bam*HI-*IAH*-ORF-F (CGCGGATCCTCTTGGAGCCGCATTAGTCAACGAA; *Bam*HI restriction site is underlined) and *Bam*HI-*IAH*-ORF-R (CGCGGATCCCACCTTCCTGTTGAAACGCCT TATT; *Bam*HI site is

underlined); for the *Kan* ORF, was used *Kpn*I-*Kan*-ORF-F (CGGGGTACCCAGCTGAAGC TTCGTACGC; *Kpn*I restriction site is underlined) and *Kpn*I-*Kan*-ORF-R (CGGGGTACCGC ATAGGCCACTAGTGGATCTG; *Kpn*I restriction site is underlined). Genomic DNA from the commercial wine yeast strain, RY1, was used as a template to amplify the coding sequences of the *ATF2* and *IAH* genes, while the plasmid pUG6 was used as a template to amplify the selection marker, *Kan*.

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic	Reference or source
Escherichia coli DH5α	F' *endA1 hsdR17* (rk-mk+) *supE44 thi-1 recA1gyrA* (Nalr) *relA1*_(*laclZYA-argF*)*U169 deoR*[F80d*lac* DE(*lacZ*)M15]	Dongguang Xiao, Tianjin University of Science and Technology, Tianjin, China
Saccharomyces cerevisiae		
RY1	Commercial yellow rice wine yeast strain	Angel Yeast, China
RY1-α1	Haploid yeast strain from RY1, a mating type	This study
RY1-α3	Haploid yeast strain from RY1, α mating type	This study
EY2-α1 (pUC-PIA2K)	*IAH1*::pUC-PIA2K, haploid yeast strain, a mating type	This study
EY2-α1 (pUC-PIA2K)	*IAH1*::pUC-PIA2K, haploid yeast strain, α mating type	This study
EY2 (pUC-PIA2K)	*IAH1*::pUC-PIA2K, diploid yeast strain	This study
Plasmids		
pPGK1	Ap^r, containing the $PGK1_p$-$PGK1_t$ expression cassette	Ref. Lilly et al., 2000; Bauer F, University of Stellenbosch
pUC19	Ap^r, cloning vector	Dongguang Xiao, Tianjin University of Science and Technology, Tianjin, China
pUG6	Kan^r, containing *loxP-kanMX-loxP* gene disruption cassette	Hegemann JH, Heinrich-Heine-University Düsseldorf
pUC-PIA2K	Ap^r, Kan^r, recombinant plasmid with partial *IAH1* gene, and containing $PGK1_p$-*ATF2*-$PGK1_T$ gene expression cassette	This study

The plasmid pUC-PIA2K was constructed by insertion of the *ATF2* ORF into the *Bgl*II restriction site in the yeast phosphoglycerate kinase I gene *PGK1*. The gene *IAH* was used as the homologous arm, while the gene *Kan* was used as a marker for the selection of yeast transformants.

Yeast transformation and screening

The plasmid pUC-PIA2K was linearized by *Bpu*1102I, and integrated into the genome of the haploids (a- and α-type) of the yellow rice wine yeast strain RY1. Yeast transformation was carried out by the lithium acetate procedure as described previously (Gietz and Schiestl, 1995). The recombinant diploid was obtained after the fusion of the purified a- and α-type haploid recombinants. The resulting recombinants were verified via PCR using the primer pair *IA2K1*'F (TAGTCTGTTTGAGCAGTCCTACCCT) and *IA2K1*'R (GAACCTCAGTG GCAAATCCTAACCT), and the primer pair *IA2K2*'F (TCGGCCTAAACGTTTGCTCCACA) and *IA2K2*'R (TGGTTTGGAGGAGAAGATAACGACG).

Real-time PCR (RT-PCR)

In order to determine the expression levels of *ATF2*, the *GAPDH* (*GPD1*) gene was selected as the internal control gene, and changes in gene expression were assessed by RT-PCR using the following primers: TTGCCCCGTATCTGTAGC (*GPD1* forward), AGCACCAA CTTCAAAACCC (*GPD1* reverse), CGCCTATCTAATCTCCTC (*ATF2* forward), and AGTG GTCACCGTTGTCGT (*ATF2* reverse). The strains were cultured in YEPD medium for 24 h and

used for RNA extraction. RNA was extracted using the Yeast RNA Kit (OMEGA) and cDNA was synthesized using the Ultra SYBR Two Step qRT-PCR kit (with ROX) (CWBIO China).

Enzyme assay

The *ATF2*-encoded AATase activity was measured by a modification of the procedure described in Fujii et al. (1996b). A reaction mixture (1 mL) containing 100 mM Tris-HCl, pH 7.5, 0.6 M ethanol and 1 mM acetyl-CoA was used. The reactions were stopped by the addition of 5.5 μM H_2SO_4 to lower the pH to 3.0 and 3 mM (final concentration) of amyl acetate was added as an internal standard. The concentration of ethyl acetate produced was measured by gas chromatography (GC). One unit of AATase activity was defined as the amount of ethyl acetate (mmol) produced by a certain weight of centrifuged yeast cells (1 g) per hour at 25°C.

Simulated rice wine fermentation experiments

Yeast cells were pre-cultured in 5 mL wort medium at 28°C for 12 h. One milliliter yeast pre-culture was transferred into 25 mL fresh wort medium and incubated until for 24 h. One hundred grams rice was dipped in the water for 72 h at 28°C, washed, cooked for 25 min, cooled, and finally put into a 500 mL flask. Ten grams mature wheat koji, 105 mL water (including 60 mL clean water and 45 mL serofluid), and a 25 mL second pre-culture of yellow rice wine yeast was added to the flask.

The mixtures were separately subjected to pre-fermentation at 28°C for 5 days, at which point the temperature of the rice wine broth was set to 16°C and post-fermentation continued for 30 days. The weight loss, alcohol content, and glucose content of each tested strain were determined separately after the 2 fermentation periods (pre-fermentation over 5 days and the total fermentation including the 5 days of pre-fermentation and the 30 days of post-fermentation). All fermentations were performed in triplicate.

GC analysis

GC was used to measure ethyl acetate, isoamyl acetate, and isobutyl acetate concentrations. The samples were filtered and distilled for GC analysis after fermentation. The analysis was performed on an Agilent 7890C GC. Amyl acetate was used as the internal standard. The column used was a HP-INNOWax polyethylene glycol (higher limit temperature of 260°C; LabAlliance), which is an organic coated fused silica capillary column with a 30 m x 320 μm inside diameter and a 0.5 μm coating thickness. Nitrogen was used as the carrier gas and the temperature of the flame ionization detector was adjusted to 250°C. The injector temperature was 230°C, the split ratio was 25:1, and the injection volume was 1.0 μL. The oven temperature program was as follows: 52°C for 0 min, an increase by 2°C/min to 70°C for 0 min, an increase by 4°C/min to 90°C for 0 min, then an increase by 10°C/min to 200°C for 0 min.

Statistical analysis

Statistical differences between the results for wines produced by the control yeasts and those for wines produced by the modified yeasts were determined by applying standard

analysis of variance methods to the data. Significant differences between mean values were determined by two-tailed tests.

RESULTS

Construction of the ATF2 overexpressing mutant

The plasmid pUC-PIA2K was linearized and transformed into haploids (RY1-α1 and RY1-α3) of the Chinese yellow rice wine yeast RY1. The resulting recombinants were verified via PCR using the primer pair *IA2K1*'F and *IA2K1*'R, and the primer pair *IA2K2*'F and *IA2K2*'R, which produced 1213- and 1250-bp fragments, respectively (Figure 1). This merely confirmed that the transformation was successful. The RT-PCR results (Figure 2) and the tests of *ATF2*-encoded AATase activity (Figure 3) further confirmed the overexpression of the gene *ATF2* in the haploids. The *ATF2* overexpressing mutant, EY2 (pUC-PIA2K), was obtained after the fusion of the a- and α-type integrated haploid recombinants [EY2-α1 (pUC-PIA2K) and EY2-a1 (pUC-PIA2K)], which were also verified by PCR, RT-PCR, and the enzyme assay (data not shown).

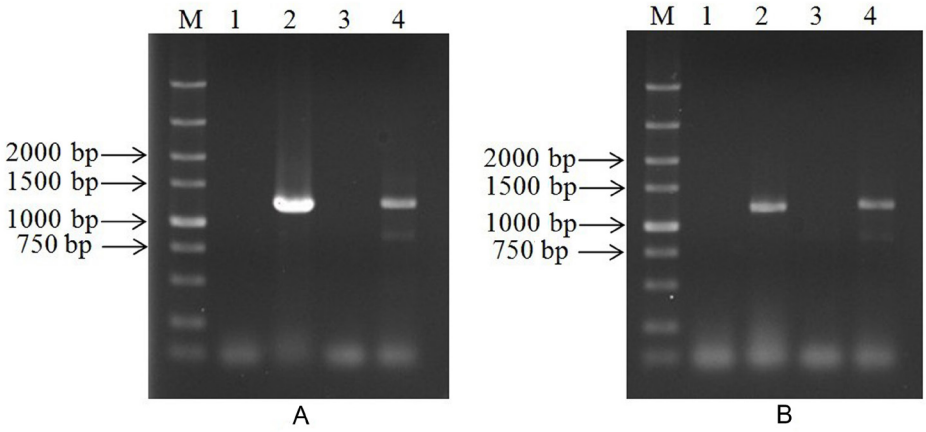

Figure 1. PCR verification results of genetic engineering haploids. **A**. Verification results of a-type haploid recombinants; **B**. verification results of α-type haploid recombinants. *Lane M* = 5000-bp DNA ladder marker, *lane 1* = PCR amplification result from the haploid receptors (a/α) genome by using the forward primer (*IA2K1*'F) and reverse primer (*IA2K1*'R), *lane 2* = PCR amplification result from the haploid recombinants (a/α) genome by using the forward primer (*IA2K1*'F) and reverse primer (*IA2K1*'R), *lane 3* = PCR amplification result from the haploid receptors (a/α) genome by using the forward primer (*IA2K2*'F) and reverse primer (*IA2K2*'R), *lane 4* = PCR amplification result from the haploid recombinants (a/α) genome by using the forward primer (*IA2K2*'F) and reverse primer (*IA2K2*'R).

Effects of ATF2 overexpression on fermentation performance of rice wine yeast

Six yellow rice wine yeast strains, RY1, RY1-α1, RY1-α3, EY2 (pUC-PIA2K), EY2-α1 (pUC-PIA2K), and EY2-α1 (pUC-PIA2K), were separately subjected to simulated rice wine fermentation (as described in Material and Methods). The fermentation performance results (Table 2) revealed no obvious differences in the fermentation characteristics among the tested strains.

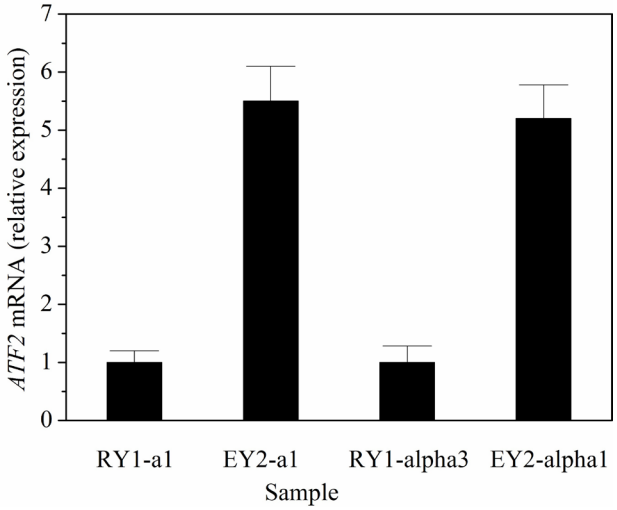

Figure 2. RT-PCR results of genetic engineering haploids. *ATF2* mRNA levels were analyzed by RT-PCR. The *GAPDH* (*GPD1*) gene was used as the internal control genes. Error bars indicate the means ± SE. from the experiments performed in duplicate, and the experiments were repeated three times.

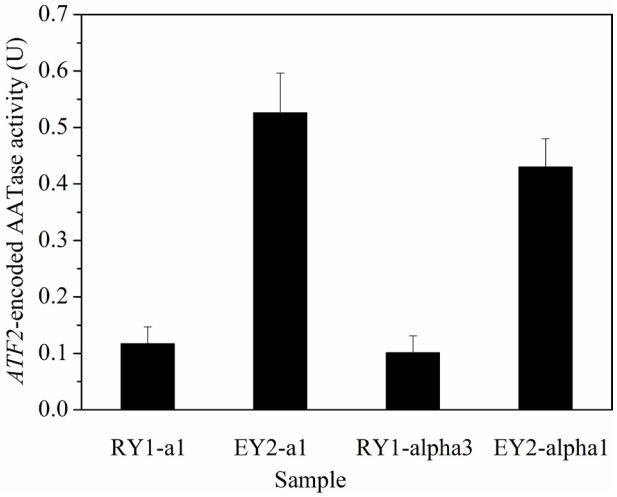

Figure 3. *ATF2*-encoded AATase activity of genetic engineering haploids. Error bars indicate as the means ± S.E. from the experiments performed in duplicate, and the experiments were repeated three times.

GC analyses of Chinese yellow rice wines

In order to evaluate the acetate ester production ability of the *ATF2* overexpressing mutant, the concentrations of acetate esters were determined for the distillates of Chinese yellow rice wines by GC analysis (Table 3). The esters production of the cells transformed with the plasmid pUC-PIA2K was significantly increased relative to that of control cells. After 5

Table 2. Fermentation performance of each strain.

Parameters	After 5 days pre-fermentation						After 5 days pre-fermentation and 30 days post-fermentation					
	RY1-*a*1	EY2-*a*1 (pUC-PIA2K)	RY1-*a*3	EY2-*a*1 (pUC-PIA2K)	RY1	EY2 (pUC-PIA2K)	RY1-*a*1	EY2-*a*1 (pUC-PIA2K)	RY1-*a*3	EY2-*a*1 (pUC-PIA2K)	RY1	EY2 (pUC-PIA2K)
Weight loss (g)	27.4 ± 0.20	27.6 ± 0.24	28.2 ± 0.31	29.2 ± 0.27	28.5 ± 0.35	27.8 ± 0.24	33.32 ± 0.29	33.26 ± 0.26	34.97 ± 0.39	35.75 ± 0.42	34.38 ± 0.32	33.67 ± 0.23
Glucose (%)	0.85 ± 0.01	0.86 ± 0.01	0.82 ± 0.01	0.86 ± 0.01	0.81 ± 0.01	0.88 ± 0.01	0.35 ± 0.01	0.39 ± 0.01	0.35 ± 0.01	0.38 ± 0.01	0.32 ± 0.01	0.37 ± 0.01
Ethanol (%, v/v, 20°C)	13.8 ± 0.07	14.0 ± 0.14	14.2 ± 0.14	14.3 ± 0.21	14.2 ± 0.07	14.2 ± 0.07	17.9 ± 0.21	18.2 ± 0.07	18.0 ± 0.21	17.6 ± 0.14	18.0 ± 0.07	18.3 ± 0.21

Results are averages from three parallel independent experiments. Values are reported as means ± standard deviations from three different tests.

Table 3. Gas chromatography measurement of acetate esters produced by each strain.

Acetate esters	After 5 days pre-fermentation					After 5 days pre-fermentation and 30 days post-fermentation				
	RY1-α1	EY2-α1 (pUC-PIA2K)	RY1-α3	EY2-α1 (pUC-PIA2K)	RY1	EY2 (pUC-PIA2K)	RY1-α1	EY2-α1 (pUC-PIA2K)	RY1-α3	EY2-α1 (pUC-PIA2K)	RY1	EY2 (pUC-PIA2K)
Ethyl acetate (mg/L)	25.59 ± 0.23	150.33 ± 0.83	23.55 ± 0.31	107.18 ± 0.50	28.30 ± 0.28	137.79 ± 0.63	45.76 ± 0.45	151.35 ± 0.72	40.33 ± 0.43	108.64 ± 0.61	39.25 ± 0.34	140.57 ± 0.67
Isoamyl acetate (mg/L)	-	31.31 ± 0.22	-	20.04 ± 0.33	-	26.68 ± 0.34	-	22.93 ± 0.28	-	13.87 ± 0.25	-	18.49 ± 0.21
Isobutyl acetate (mg/L)	-	6.72 ± 0.24	-	4.75 ± 0.08	-	7.60 ± 0.25	-	5.19 ± 0.13	-	4.10 ± 0.07	-	5.63 ± 0.16

Results are averages from three parallel independent experiments. Values are reported as means ± standard deviations from three different tests. (-) = no detected. GC results were statistically evaluated by a two-tailed test. The results indicate that the values obtained for all the yellow rice wines for ethyl acetate, isoamyl acetate and isobutyl acetate differed significantly ($P \leq 0.05$).

days of pre-fermentation, the concentration of ethyl acetate in the yellow rice wines fermented with EY2-α1 (pUC-PIA2K), EY2-α1 (pUC-PIA2K), and EY2 (pUC-PIA2K) increased to 150.33, 107.18, and 137.79 mg/L (approximately 4- to 6-fold higher than that of the control cells, RY1-α1, RY1-α3, and RY1), respectively. Similarly, isoamyl acetate and isobutyl acetate also increased to 20-32 mg/L and 4-8 mg/L, respectively. In contrast, overexpression of the *ATF2* gene had a major effect on acetate ester production.

To determine the effect of bottle aging on the ester concentrations, Chinese yellow rice wines were bottle-aged continuously for 30 days at 16°C and then filtered, distilled, and subjected to GC analysis (Table 3). After 5 days of pre-fermentation and 30 days of post-fermentation, the concentration of ethyl acetate in the yellow rice wines fermented with the control strains (RY1-α1, RY1-α3, and RY1) drastically increased to 45.76, 40.33, and 39.25 mg/L, respectively, during the storage period. In contrast, the difference in the concentration of ethyl acetate produced by the constructed strains, EY2-α1 (pUC-PIA2K), EY2-α1 (pUC-PIA2K), and EY2 (pUC-PIA2K), between the pre-fermentation and post-fermentation periods was not significant. However, the ethyl acetate concentration nonetheless remained 2.6- to 3.6-fold higher in the yellow rice wines fermented with the recombinant strains than in the control yellow rice wines. Although the isoamyl acetate and isobutyl acetate content decreased to 13-23 and 4-6 mg/L, respectively, as a result of fermentation, their concentrations were also still higher than those observed in the control yellow rice wines.

The GC results were statistically evaluated by a two-tailed test. The results indicated that the values obtained for ethyl acetate, isoamyl acetate, and isobutyl acetate differed significantly ($P \leq 0.05$).

DISCUSSION

Volatile esters constitute the largest and most important class of flavor compounds produced by fermenting yeast cells. Their aromas are essential for the fruity character of alcoholic beverages, including beer and Chinese yellow rice wine. Acetate esters, such as ethyl acetate and isoamyl acetate, are the most influential flavor components. When absorbed by the human body, these esters lead to stress release through the activation of the GABA receptor, thereby effectively reducing the negative side effects of drinking alcohol. Therefore, the synthesis of volatile esters by yeast is of major industrial interest.

Detailed understanding of the molecular basis of ester synthesis would enable better control of ester production. In 1962, Nordström demonstrated that esters are formed via an intracellular process catalyzed by alcohol acetyltransferase from acetyl coenzyme A and the corresponding alcohols. There are 3 types of AATase, namely, AATases, AATase I, and AATase II, which are encoded by *ATF1*, *Lg-ATF1*, and *ATF2*, respectively (Lilly et al., 2006).

Several studies have suggested that the expression level of *ATF1* is an important limiting factor for ester synthesis. Our previous study also strongly suggested that the content of acetate esters is significantly elevated on *ATF1* overexpression. After 5 days of fermentation, the concentrations of ethyl acetate in the yellow rice wines fermented with EY1 (pUC-PIAK) increased by 22-fold compared to that of the parent cell, RY1. However, the ester levels were still too high to be potential useful for industrial applications. Verstrepen et al. (2003b) confirmed that overexpression of *ATF2* caused smaller increases in ester formation compared to overexpression of *ATF1* in the commercial lager strain CMBS33. The smaller effect of *ATF2*

overexpression on ester production may be an advantage for commercial production, since the dramatic effects of *ATF1* overexpression might be undesirable in industrial applications. However, the strains, fermentation medium, and set-up used for our investigations of Chinese yellow rice wine differ substantially from those of other studies related to beer and wines.

In this study, $PGK1_P$ and $PGK1_T$ were used as the promoter and the terminator, respectively, to overexpress the *ATF2*-encoded alcohol acetyltransferase in Chinese yellow rice wine yeast. The results showed that *ATF2* overexpression significantly improved the acetate ester content of Chinese yellow rice wine. In the same manner, the expression level of *ATF2* in the current study was expected to be an important limiting factor for ester synthesis in *S. cerevisiae*, confirming the results of Lilly et al. (2006) and Verstrepen et al. (2003b). These observations can inform the development of new production strategies and new yeast strains, allowing further optimization of rice wine flavor profiles in order to satisfy different sensory preferences of consumers, as well as contribute to a better understanding of the physiological role of acetate ester synthesis.

Although isoamyl acetate and isobutyl acetate contents were not detected in yellow rice wine fermented by the parent strains, this does not necessarily imply that the parent strains do not produce isoamyl acetate or isobutyl acetate. Instead, we suggest that this lack of detection was due to their relatively low contents in the wine. In industrial yellow rice wine, the contents of isoamyl acetate and isobutyl acetate are approximately 0.1-0.3 mg/L and 0.4-0.6 mg/L, respectively. Since our fermentation conditions simulated those of industrial fermentation practices, these concentrations are likely reflective of those in our study but are too low to be detected by GC analysis.

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