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Decreased production of higher alcohols by *Saccharomyces cerevisiae* for Chinese rice wine fermentation by deletion of Bat aminotransferases

Cui-Ying Zhang · Ya-Nan Qi · Hong-Xia Ma · Wei Li · Long-Hai Dai · Dong-Guang Xiao

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Abstract An appropriate level of higher alcohols produced by yeast during the fermentation is one of the most important factors influencing Chinese rice wine quality. In this study, *BAT1* and *BAT2* single- and double-gene-deletion mutant strains were constructed from an industrial yeast strain RY1 to decrease higher alcohols during Chinese rice wine fermentation. The results showed that the *BAT2* single-gene-deletion mutant strain produced best improvement in the production of higher alcohols while remaining showed normal growth and fermentation characteristics. Furthermore, a *BAT2* single-gene-deletion diploid engineered strain RY1-Δ*bat2* was constructed and produced low levels of isobutanol and isoamylol (isoamyl alcohol and active amyl alcohol) in simulated fermentation of Chinese rice wine, 92.40 and 303.31 mg/L, respectively, which were 33.00 and 14.20 % lower than those of the parental strain RY1. The differences in fermentation performance between RY1-Δ*bat2* and RY1 were minor. Therefore, construction of this yeast strain is important in future development in Chinese wine industry and provides insights on generating yeast strains for other fermented alcoholic beverages.

Keywords Chinese rice wine · *Saccharomyces cerevisiae* · Higher alcohol · Ehrlich pathway · Branched-chain amino acid aminotransferase

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Introduction

Higher alcohols, also known as fusel alcohols, are a large group of flavor compounds in many alcoholic beverages [16, 20]. They have a significant effect on the sensorial quality and character of alcoholic beverage for their strong, pungent smell and taste [21]. Alcoholic beverage with appropriate content of higher alcohols is mellow, soft, plump, and coordinate of the bouquet. While the content of higher alcohols is above a certain threshold, alcoholic beverage will have a fusel oil taste and be strongly intoxicating.

Chinese rice wine, which has distinct flavor, high nutrition content, and certain medicinal value, is one of China's national specialities [24]. It belongs to traditional rice wines in East-Asian countries [25] and has recently attracted attention from many researchers [4, 13, 18, 23, 27]. A relatively high content of higher alcohols is present in various Chinese rice wines, which is a common characteristic of traditional rice wines [25]. Higher alcohols at too high concentration in Chinese rice wine are potentially harmful to the human body. Cerebral paralysis, for example, is one of the adverse effects [25]. Taking effective measures to control the content of higher alcohols in Chinese rice wine, so as to cater to the current consumption trend, has a vital significance for improving the position and expanding the market of Chinese rice wine. Yeast is the soul of wine's flavor compounds including higher alcohols, esters, and so on [3, 16]. Therefore, the content of higher alcohols in Chinese rice wine may be decreased by breeding a modified yeast strain to improve its quality and flavor.

Higher alcohols produced by yeast can originate from the biosynthetic route from the carbon source [8] or from the degradation of branched-chain amino acids (BCAAs) via the so-called Ehrlich pathway [7, 12]. Isobutanol,

isoamyl alcohol, and active amyl alcohol are the degradation products of valine, leucine, and isoleucine, respectively [1], so they are also known as branched-chain alcohols. Amino acids are converted to the corresponding α-keto acids via transamination [8, 9]. This transamination reaction is catalyzed by mitochondrial and cytosolic branched-chain amino acid transferases (BCAATases) encoded by *BAT1* and *BAT2* genes, respectively [10]. The following step is the decarboxylation of the α-keto acid into an aldehyde [9], which is catalyzed by a decarboxylase through the removal of one carbon atom [9]. Branched-chain alcohols are then synthesized by the NADH-dependent reduction catalyzed by an alcohol dehydrogenase [8]. Previous work showed that the transaminase reaction of the Ehrlich pathway catalyzed by Bat1p and Bat2p is the main rate-limiting step in the reaction, and deletion of the encoding genes had an impact on the formation of higher alcohols [14, 17, 19, 26].

In this study, we deleted BCAATases encoding genes of an industrial yeast strain to decrease the generation of higher alcohols during Chinese rice wine fermentation. *BAT2* single-gene-deletion diploid mutant strain was successfully constructed and produced significantly less higher alcohols with minor influence on growth and fermentation performance. Therefore, the engineered strain would be more useful in future development in the rice wine industry. The double-gene-deletion mutant strain is of less interest due to growth retardation and reductions in ethanol production and CO_2 weight loss. The results of this study thus lay the foundation for future development of the engineered strain for the production of Chinese rice wine.

Materials and methods

Microbial strains, plasmids, media, and culture conditions

All strains and plasmids used in this study are listed in Table 1. *Saccharomyces cerevisiae* cells were grown at 30 °C in yeast extract peptone dextrose (YEPD) medium (2 % glucose, 2 % peptone, and 1 % yeast extract). G418 (Geneticin, an aminoglycoside antibiotic) was added into the media at a final concentration of 600 μg/mL to select the yeast transformants. Yeast cells were also cultured in a rich medium, wort medium (prepared by treating freshly mashed malt with water at 65 °C for 2 h and adjusting the sugar content of wort to 12 °Bx) for the fermentation

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Reference or source
Escherichia coli DH5α	Host of plasmid	Stratagene
Saccharomyces cerevisiae		
Industrial strains		
RY1	Commercial rice wine yeast strain	Angel Yeast, China
RY1-a1	Haploid yeast strain from RY1, a mating type	Zhang [27]
RY1-α3	Haploid yeast strain from RY1, α mating type	Zhang [27]
Transformants		
RY-a1-Δ*bat1*	MATa Δ*BAT1*:: *loxP-kanMX-loxP*, haploid yeast strain	This study
RY-α3-Δ*bat1*	MATα Δ*BAT1*:: *loxP-kanMX-loxP*, haploid yeast strain	This study
RY-a1-Δ*bat2*	MATa Δ*BAT2*:: *loxP-kanMX-loxP*, haploid yeast strain	This study
RY-α3-Δ*bat2*	MATα Δ*BAT2*:: *loxP-kanMX-loxP*, haploid yeast strain	This study
RY-a1-Δ*bat2*(*kanMX*$^-$)	MATa Δ*BAT2*:: *loxP*, haploid yeast strain	This study
RY-α3-Δ*bat2*(*kanMX*$^-$)	MATα Δ*BAT2*:: *loxP*, haploid yeast strain	This study
RY-a1-Δ*bat1*Δ*bat2*	MATa Δ*BAT2* :: *loxP*Δ*BAT1*:: *loxP-kanMX-loxP*, haploid yeast strain	This study
RY-α3-Δ*bat1*Δ*bat2*	MATα Δ*BAT2*:: *loxP* Δ*BAT1*:: *loxP-kanMX-loxP*, haploid yeast strain	This study
RY1-Δ*bat2*	Δ*BAT2*:: *loxP*, diploid yeast strain	This study
Plasmids		
pUG6	Kan^r, containing *loxP-kanMX-loxP* gene disruption cassette	Hegemann JH, Heinrich-Heine-University Düsseldorf
pUC19	Ap^r, cloning vector	Stratagene
pUC-B_1ABK	Ap^r, Kan^r, containing B_1A-*loxP-KanMX-loxP*-B_1B	This study
pUC-B_2ABK	Ap^r, Kan^r, containing B_2A-*loxP-KanMX-loxP*-B_2B	This study

experiments. *Escherichia coli* cells were grown at 37 °C in a Luria–Bertani medium (1 % Bacto tryptone, 1 % NaCl, and 0.5 % yeast extract). Ampicillin was added into the media at a final concentration of 100 μg/mL to select the *E. coli* transformants. All solid media contained 2 % agar.

Recombinant DNA methods and plasmid constructions

Plasmid DNA and genomic DNA of yeast in this study were prepared as described by Ausubel et al. [2]. Restriction enzymes, T4 DNA ligase, and LA Taq DNA polymerase (TaKaRa Biotechnol, Dalian, China) were used in the enzymatic manipulation of DNA, according to the instruction manuals of the supplier.

The primers used for plasmid construction and verification of recombinant yeast in this study are listed in Table 2. Genomic DNA from the commercial rice wine yeast strain, RY1-a1, was used as the template for PCR to amplify the flanking sequences of the *BAT1* and *BAT2*. The plasmid pUG6 was used as the template to amplify antibiotic gene (*KanMX* gene) for G418 resistance. The plasmid vector pUC19 was used as the backbone for recombinant plasmid construction. The B_1A (upstream fragment of *BAT1*), *KanMX* fragment, and B_1B (downstream fragment of *BAT1*) were inserted sequentially into the pUC19 multiple cloning site with the same direction to form the deletion plasmid pUC-B_1ABK, so is the construction of the deletion plasmid pUC-B_2ABK.

Yeast transformation and screening

Gene deletion cassettes amplified via PCR from the deletion plasmids were transformed to yeast haploid RY1-a1 and RY1-α3 using the previously described lithium acetate procedure [11], respectively. After transformation, the yeast cells were then immediately plated out on YEPD medium containing 600 μg/mL of G418 and incubated at 30 °C for at least 3 days.

Growth curve determination

Yeast cells were precultured in 5 mL of YEPD medium at 30 °C for 12 h and then transferred into fresh YEPD medium and incubated at 30 °C for 18 h. Optical density (OD_{600}) was determined using a Bioscreen Automated Growth Curves analysis system (OY Growth Curves Ab Ltd., Helsinki, Finland) every 1 h.

Table 2 Primers used in this study

Primer names	Sequence[a]	Restriction site
For plasmid construction		
B_1A-U	5′-CCGGAATTCATACCGGCTGTCGCTATTATTACTG-3′	*Eco*RI
B_1A-D	5′-CGCGGATCCCAACTTCAAGGAATGTCTCTGCAAC -3′	*Bam*HI
B_1B-U	5′-CGCGGATCCGTAACTGGTCAAAAACTGTTGCCGA-3′	*Bam*HI
B_1B-D	5′-TGCACTGCAGCAGCGAGATACCTTGGCAACTAAAT-3′	*Pst*I
B_2A-U	5′-AACTGCAGTCCTTTCCAAACATCTTCGAACGTG-3′	*Pst*I
B_2A-D	5′-AACTGCAGAAAACTCGTGGAGATGCTTTCCCTTA-3′	*Pst*I
B_2B-U	5′-CGAGCTCGCGTTTTTTCTACTGAGTTAAGGGGTC-3′	*Sac*I
B_2B-D	5′-CGAGCTCGCCCTCTAAAGATTCATCGGCTACT-3′	*Sac*I
Kan-U	5′-CGCGGATCCCAGCTGAAGC TTCGTACGC-3′	*Bam*HI
Kan-D	5′-CGCGGATCCGCATAGGCCA CTAGTGGATCTG-3′	*Bam*HI
For PCR verification		
P1	5′- CATGTTGCAGAGACATTCCTTG-3′	None
P2	5′- CATTAGTTCAAGTCGGCAACAG-3′	None
P3	5′-CATCTAAGCCAAAACCGAACAGTGA-3′	None
P4	5′-CCAATTGCCATGCTCAGTCTCGCCA-3′	None
P5	5′-GCATATCTGCTCAAGTAGACAAGG-3′	None
P6	5′-CGGATAAAATGCTTGATGGTCGGA-3′	None
P7	5′-CGGTTGCATTCGATTCCTGTTTGT-3′	None
P8	5′-CTGTATGCCACGATGATGGAAGG-3′	None
P9	5′-ACAGACAATCTCCTACCACCAGTTT-3′	None
P10	5′-CCTTTTATATTTCTCTACAGGGGCG-3′	None
P11	5′-TAGGTTGTATTGATGTTGGACGAGT-3′	None
P12	5′-GTTGTGATTCCTGATTGGACATTCT-3′	None

[a] Restriction sites are underlined

Fermentation experiments

Yeast cells were precultured in 5 mL of wort medium at 30 °C for 12 h and then transferred into 50 mL of wort medium and incubated for 24 h. A total of 100 g of rice was dipped in water at 25–30 °C for 72 h. The dipped rice was washed, cooked, and then cooled at room temperature. Fermentation experiments were performed in Erlenmeyer flasks (500 mL) containing the cooked rice, 10 g of mature wheat koji, 105 mL of water (consisting of 60 mL of clean water and 45 mL of rice seriflux) and 30 mL of yeast cells. The mixture was fermented at 30 °C for 6 days. The fermentation performance of biomass, weight loss of CO_2, residual reducing sugar, and ethanol production, and production of volatile flavor compounds including higher alcohols, esters and volatile acidity were determined using a microscope, analytical balance, a Brix hydrometer, an oenometer, and gas chromatographic (GC) analysis, respectively. All fermentations were performed in triplicate.

Gas chromatographic analysis

Samples from fermentation were distilled for GC analysis. The analysis of volatile compounds was carried out on an Agilent 7890B gas chromatograph equipped with an Agilent G4513A autosampler and injector and a flame ionization detector (FID). Amyl acetate was used as the internal standard. The column used for separation was a HP-INNOWax polyethylene glycol (higher limit temperature 260 °C; LabAlliance), which is an organic-coated fused silica capillary column with 30 m × 320 μm i.d. and a 0.5 μm coating thickness. Nitrogen was used as the carrier gas at a constant flow rate of 2 mL/min. The injector temperature was 230 °C, the split ratio was 25:1, the split flow rate was 50 mL/min, and the injection volume was 1.0 μL. The FID was operated at 250 °C. The oven temperature program was as follows: 50 °C for 0 min, followed by an increase to 62 °C at 2 °C/min, and then increasing to 200 °C (0 min) at 15 °C/min for 0 min. For each of the compounds measured, an internal calibration curve was constructed by using known amounts of authentic standards. The internal standard and the chemicals were sourced from Merck.

Statistical analysis

Statistical differences between the results for rice wines produced by the wild-type strain and the modified yeasts were determined through standard ANOVA. The significant differences between the GC results were determined using a two-tailed test.

Results

Construction of haploid yeast recombinant strains

BAT1 and *BAT2* genes were replaced with the constructed cassettes B_1A-*loxP*-*KanMX*-*loxP*-B_1B and B_2A-*loxP*-*KanMX*-*loxP*-B_2B to delete the two genes in yeast strains, respectively. G418-resistant colonies were confirmed via PCR with primers and subsequent nucleotide sequencing for verifications of recombinant *BAT1* or *BAT2* deletion yeast strains (Fig. 1a–c). 1.9- and 1.2-kb of the expected bands were produced from *BAT1* deletion yeast strains using the primer pairs P5/P6 and P7/P8, respectively. PCR amplification from *BAT2* deletion yeast strains using the primer pairs P9/P10 and P11/P12 produced 1.4- and 1.9-kb of the expected bands. Therefore, we have successfully obtained engineered haploids, RY-a1-Δ*bat1*, RY-α3-Δ*bat1*, RY-a1-Δ*bat2*, and RY-α3-Δ*bat2*.

To construct the strain with both *BAT1* and *BAT2* double-gene deletion, the *KanMX* marker gene was removed from the *BAT2* deletion yeast strains using the Cre/*loxP* recombination system and verified via PCR analysis to produce the mutant strains RY-a1-Δ*bat2*(*kanMX*$^-$) and RY-α3-Δ*bat2*(*kanMX*$^-$) (Fig. 1d). The transformation fragment B_1A-*loxP*-*KanMX*-*loxP*-B_1B was then transformed into the RY-a1-Δ*bat2*(*kanMX*$^-$) and RY-α3-Δ*bat2*(*kanMX*$^-$) strains, respectively. The resulting recombinants RY-a1-Δ*bat1*Δ*bat2* and RY-α3-Δ*bat1*Δ*bat2* were identified to be the correct transformants via PCR analysis and nucleotide sequencing (data not shown).

Effects of *BAT1* and *BAT2* gene deletions on the production of higher alcohols

Simulated fermentations of Chinese rice wine were carried out using the recombinant strains RY-a1-Δ*bat1*, RY-α3-Δ*bat1*, RY-a1-Δ*bat2*, RY-α3-Δ*bat2*, RY-a1-Δ*bat1*Δ*bat2*, and RY-α3-Δ*bat1*Δ*bat2*, and the parental strains RY1-a1 and RY1-α3 at 30 °C. The rice wines were filtered and distilled after 6 days of fermentation. The concentration of the higher alcohols in the samples was then determined through GC analysis (Table 3). The data presented in Table 3 suggested that *BAT1* single-gene deletion had no significant effect on the production of higher alcohols, while *BAT2* single-gene deletion showed obvious decrease in the levels of isobutanol, isoamyl alcohol, and active amyl alcohol compared to the parental strain. The content of isobutanol and isoamylol (isoamyl alcohol and active amyl alcohol) produced by RY-a1-Δ*bat2* were 96.73 and 308.02 mg/L, respectively, which were 38.48 and 12.43 % less than those of the parental haploid RY1-a1. The recombinant RY-α3-Δ*bat2* produced isobutanol of 100.08 mg/L and isoamyl alcohol and active amyl alcohol of

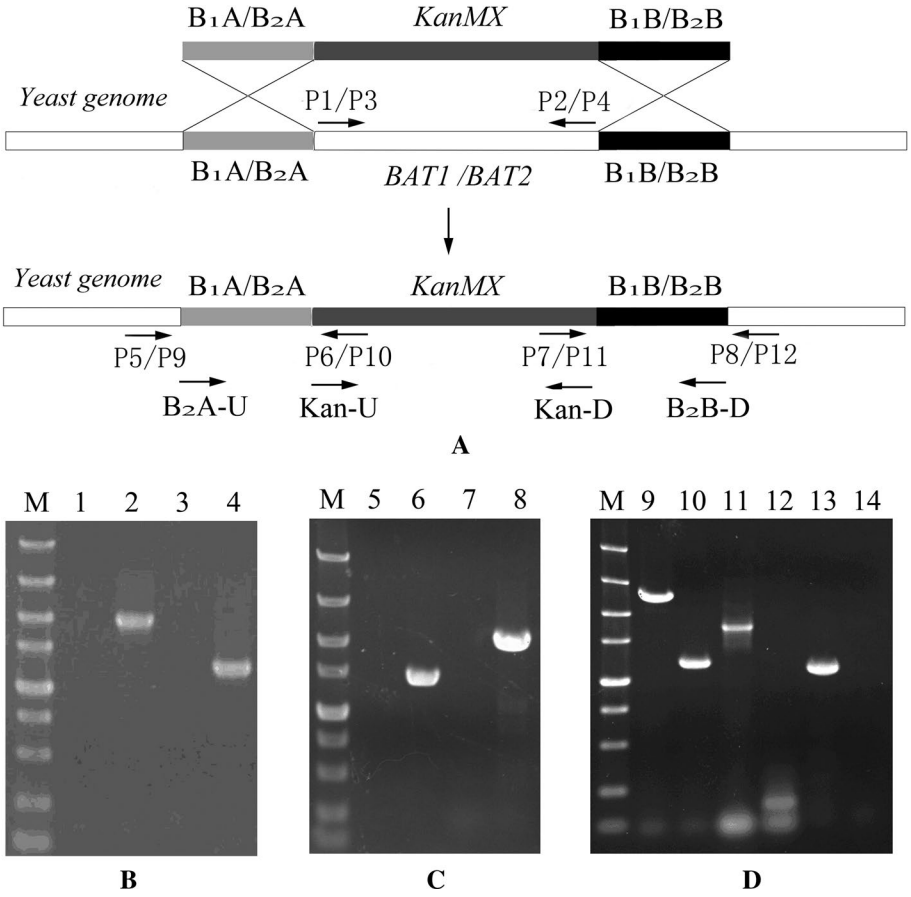

Fig. 1 Homologous recombination of *BAT1* and *BAT2* genes in recombinant yeast strains (**a**) and PCR verification of the recombinants (**b** RY-a1-Δ*bat1*; **c** RY-a1-Δ*bat2*) and the mutant strain [**d** RY-a1-Δ*bat2*(*kanMX*$^-$)]. *M* DL5000 DNA marker; *lanes 1* and *2* PCR amplification results from the parental strain (RY1-a1) genome and the recombinant (RY-a1-Δ*bat1*) genome, respectively, obtained using the primers P5 and P6; *lanes 3* and *4* PCR amplification results from the parental strain (RY1-a1) genome and the recombinant (RY-a1-Δ*bat1*) genome, respectively, obtained using the primers P7 and P8; *lanes 5* and *6* PCR amplification results from the parental strain (RY1-a1) genome and the recombinant (RY-a1-Δ*bat2*) genome, respectively, obtained using the primers P9 and P10; *lanes 7* and *8* PCR amplification results from the parental strain (RY1-a1) genome and the recombinant (RY-a1-Δ*bat2*) genome, respectively, obtained using the primers P11 and P12; *lanes 9* and *10*, PCR amplification results from the recombinant (RY-a1-Δ*bat2*) genome and the mutant strain [RY-a1-Δ*bat2*(*kanMX*$^-$)] genome, respectively, obtained using the primers B_2A-U and B_2B-D; *lanes 11* and *12*, PCR amplification results from the recombinant (RY-a1-Δ*bat2*) genome and the mutant strain [RY-a1-Δ*bat2*(*kanMX*$^-$)] genome, respectively, obtained using the primers Kan-U and Kan-D; *lanes 13* and *14*, PCR amplification results from the parental strain (RY1-a1) genome and the mutant strain [RY-a1-Δ*bat2*(*kanMX*$^-$)] genome, respectively, obtained using the primers P3 and P4

Table 3 Higher alcohol production of the mutant strains and the parental strains

Yeast strains	*n*-Propanol (mg/L)	Isobutanol (mg/L)	Isoamylol (isoamyl alcohol and active amyl alcohol) (mg/L)
RY1-a1	100.15 ± 1.51	157.24 ± 3.86	351.73 ± 1.01
RY1-α3	98.49 ± 2.64	152.39 ± 3.24	347.14 ± 3.99
RY-a1-*Δbat1*	96.78 ± 3.11	156.68 ± 2.08	352.51 ± 4.03
RY-α3-*Δbat1*	101.12 ± 2.25	150.85 ± 4.20	346.13 ± 4.36
RY-a1-*Δbat2*	101.44 ± 3.16	96.73 ± 3.42**	308.02 ± 3.94**
RY-α3-*Δbat2*	97.89 ± 3.22	100.08 ± 2.96**	311.22 ± 0.92**
RY-a1-*Δbat1Δbat2*	87.77 ± 2.14**	72.35 ± 3.19**	258.82 ± 3.20**
RY-α3-*Δbat1Δbat2*	90.07 ± 1.78**	75.69 ± 2.27**	263.12 ± 1.15**

The results are the average of three replicates ± the standard deviation

** Significant differences of the constructed strains from their parental strains were confirmed by Student's *t* test ($P < 0.01$, $n = 3$)

Table 4 Fermentation performances of the rice wine yeast strains

Yeast strains	Weight loss of CO_2 (g)	Ethanol (%, v/v, 20 °C)	Residual reducing sugars (g/100 mL)
RY1-a1	30.1 ± 0.1	14.1 ± 0.1	0.95 ± 0.12
RY1-α3	30.0 ± 0.2	14.0 ± 0.1	0.83 ± 0.25
RY-a1-*Δbat1*	30.1 ± 0.1	13.9 ± 0.2	0.91 ± 0.15
RY-α3-*Δbat1*	30.2 ± 0.2	14.1 ± 0.2	0.77 ± 0.24
RY-a1-*Δbat2*	30.3 ± 0.1	14.2 ± 0.1	0.72 ± 0.15
RY-α3-*Δbat2*	30.2 ± 0.1	14.0 ± 0.1	0.85 ± 0.16
RY-a1-*Δbat1Δbat2*	27.5 ± 0.1	12.7 ± 0.2	0.79 ± 0.21
RY-α3-*Δbat1Δbat2*	27.2 ± 0.2	12.5 ± 0.1	0.82 ± 0.28
RY1	30.4 ± 0.2	13.8 ± 0.2	0.57 ± 0.14
RY1-*Δbat2*	30.5 ± 0.2	13.7 ± 0.2	0.56 ± 0.12

The results are the average of three replicates ± the standard deviation

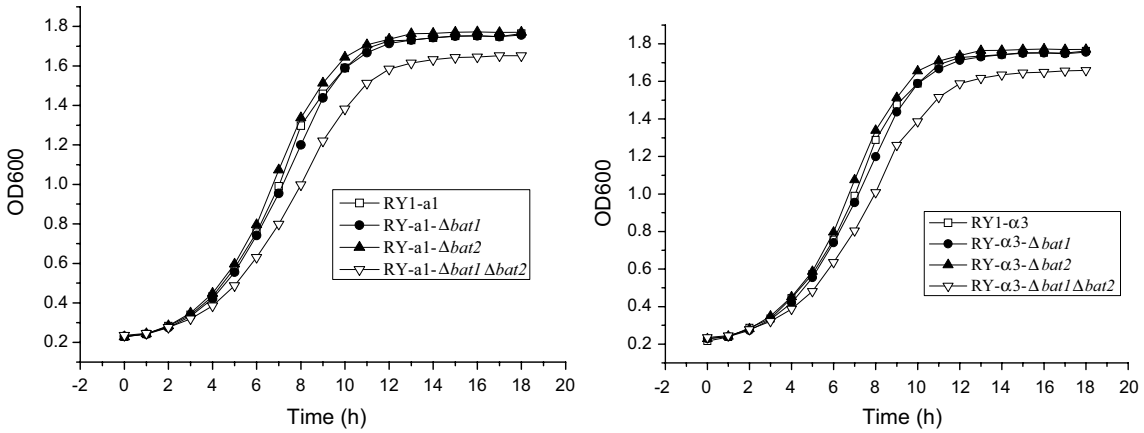

Fig. 2 Growth curve of haploid yeast recombinant strains and their parental strains. Growth curves (in triplicate) were monitored at 30 °C by measuring the optical density (OD600) of the cultures every 1 h in a Bioscreen Automated Growth Curves analysis system (OY Growth Curves Ab Ltd., Helsinki, Finland)

311.22 mg/L, respectively, which were 34.33 and 10.35 % less than those produced by the parental haploid RY1-α3. The production of the above four higher alcohols was also dramatically decreased by the double-gene-deletion mutation. The contents of *n*-propanol, isobutanol, and isoamylol (isoamyl alcohol and active amyl alcohol) produced by RY-a1-*Δbat1Δbat2* were 87.77, 72.35, and 258.82 mg/L, respectively, which were 12.36, 53.99, and 26.42 % less than those of the parental haploid RY1-a1. The recombinant RY-α3-*Δbat1Δbat2* produced 90.07 mg/L *n*-propanol, 75.69 mg/L isobutanol, and 263.12 mg/L isoamyl alcohol and active amyl alcohol, which were 8.55, 50.33, and 24.20 % less than those produced by the parental haploid RY1-α3, respectively.

Effects of *BAT1* and *BAT2* gene deletions on growth and fermentation properties of the rice wine yeast

After fermentation at 30 °C for 6 days, total loss of CO_2, ethanol production, and residual reducing sugar were measured (Table 4). Deletion of either *BAT1* or *BAT2* showed similar growth abilities to their parental strains (Fig. 2). Only the double-gene-deletion mutant strains had negative impacts on fermentation performances (total loss of CO_2 and ethanol production). By comparison, the final cell densities of the double-gene-deletion mutant strains were less, although they reached the stationary phases after the similar growth time compared with their parental strains (Fig. 2).

Construction of diploid yeast engineered strain

The recombinant diploid strain was obtained after the fusion of the purified a- and α-type haploid recombinants RY-a1-*Δbat2* and RY-α3-*Δbat2*, and then verified using a spore formation experiment (data not shown). The *KanMX* marker gene was removed from the recombinant diploid using the Cre/*loxP* recombination system to yield the engineered diploid strain RY1-*Δbat2*.

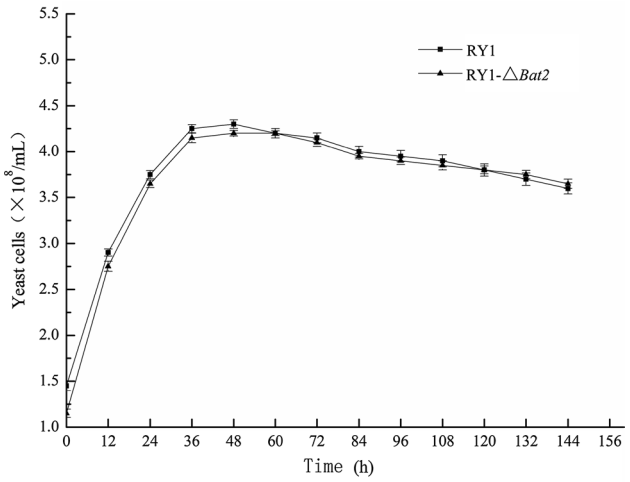

Fig. 3 Biomass of the engineered strain RY1-Δ*bat2* and the parental strain RY1 during the simulated fermentation of Chinese rice wine

A simulated fermentation of Chinese rice wine was carried out using the engineered strain RY1-Δ*bat2* and the parental strain RY1 as the control, and the results were shown in Table 4 and Figs. 3 and 4. No obvious distinction in fermentation characteristics (biomass, residual sugar, weight loss of CO_2, and ethanol production) (Fig. 3; Table 4) between the engineered strain RY1-Δ*bat2* and the parental strain RY1 was observed. The engineered strain RY1-Δ*bat2* showed obvious decrease in the levels of isobutanol, isoamyl alcohol, and active amyl alcohol compared to the parental strain RY1 (Fig. 4). Isobutanol and isoamylol (isoamyl alcohol and active amyl alcohol) produced by RY1-Δ*bat2* were decreased to 92.40 and 303.31 mg/L, respectively, which were 33.00 and 14.20 % lower than those of the parental strain RY1. The productions of *n*-butanol and butyric acid by the engineered strain RY1-Δ*bat2* were also influenced by *BAT2* gene deletion (Fig. 4). It produced 4.67 mg/L *n*-butanol and 12.63 mg/L butyric acid, which were 33.42 and 46.86 % greater than those produced by the parental strain RY1. However, effects of the relatively low concentration of *n*-butanol and butyric acid in Chinese rice wine on wine flavor and quality were negligible. No obvious distinction in the production of *n*-propanol, β-phenylethanol, formic acid, acetic acid, and ethyl acetate between the engineered strain RY1-Δ*bat2* and the parental strain RY1 was obtained (Fig. 4). The production of *sec*-butanol, *n*-caproic acid, isobutyl acetate, isoamyl acetate, ethyl caproate, ethyl lactate, ethyl caprylate and ethyl oenanthate was not detected in the simulated fermentation of Chinese rice wine in this study (Fig. 4).

Discussion

Higher alcohols are one of the most important aroma-active substances produced by fermenting yeast due to their essential aroma impressions [20]. Great advances have been

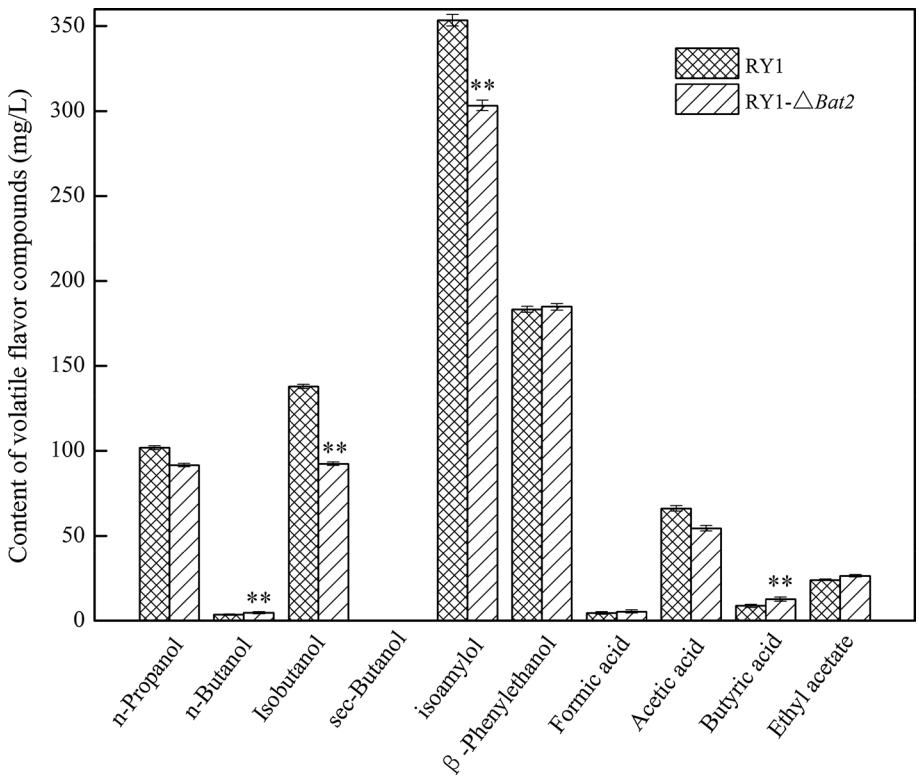

Fig. 4 Production of volatile flavor compounds by the engineered strain RY1-Δ*bat2* and the parental strain RY1 in the simulated fermentation of Chinese rice wine. Isoamylol includes isoamyl alcohol and active amyl alcohol. The production of *sec*-butanol, *n*-caproic acid, isobutyl acetate, isoamyl acetate, ethyl caproate, ethyl lactate, ethyl caprylate, and ethyl oenanthate was not detected in this experiment. The data of these volatile flavor compounds except *sec*-butanol were not shown in this figure. **Significant differences of the engineered strain from the parental strain were confirmed by Student's *t* test ($P < 0.01$, $n = 3$)

made in elucidating their biochemical pathways and their regulatory mechanisms [16]. During fermentation, the higher alcohols produced by yeast strains can originate from the biosynthetic route from the carbon source [8] or from the degradation of BCAAs via the Ehrlich pathway [7, 12]. BCAAs are key substrates in the formation of higher alcohols. In the Ehrlich pathway, BCAAs are first processed by transamination to form their respective α-keto acids, which are then processed into higher alcohols by decarboxylation and reduction [8]. It has been well documented that transamination is catalyzed by mitochondrial and cytosolic branched-chain amino acid aminotransferase encoded by *BAT1* gene and *BAT2* gene, respectively [14, 17, 19, 26].

In this study, industrial rice wine yeast strains of single- and double-gene deletions of *BAT1* and *BAT2* genes encoding BCAATases were constructed. The results of simulated fermentation assays of Chinese rice wine showed that *BAT1* single-gene deletion did not impair the production of higher alcohols in rice wine yeast strain, while *BAT2* single-gene deletion strains showed an obvious decrease in the level of isobutanol, isoamyl alcohol, and active amyl alcohol compared with the parental strains (Table 3), which suggested that the roles of the two BCAATases in producing higher alcohols in rice wine yeast strain were different and only *BAT2* gene was likely to be involved in the production of these higher alcohols. Recently, Picotti et al. [15] mapped almost entirely the proteome of *S. cerevisiae* and highlighted a protein module consisting of Bat1, Bat2, Rpn11, Hsp60, and Ilv2 (which they termed the 'B1B2 module'). The B1B2 module is functionally related to the metabolism of the BCAAs and the core of this module is composed of Bat1p and Bat2p. Importantly, they showed that Bat1p favors the reverse metabolic reaction catalyzed by Bat2p, that is, Bat1p is mainly involved in the anabolism of BCAAs (amination of α-keto acids), whereas Bat2p is almost exclusively involved in the catabolism of BCAAs (deamination of BCAAs) [15]. This conclusion is consistent with our results in this work and potentially explains why the production of higher alcohols in rice wine yeast strain was not affected by *BAT1* single-gene deletion.

General growth and fermentation properties of all deletion mutant strains constructed in this work were monitored (Fig. 2; Table 4). The results demonstrated that the single-gene deletions of *BAT1* and *BAT2* made no changes in growth and fermentation performances in comparison with the parental strains, while the double-gene-deletion mutant had a distinctly negative impact on growth and fermentation performances. One possible explanation is that *BAT2* is also responsible for the anabolism of BCAAs in the *BAT1* single-gene-deletion mutant strains. Therefore, *BAT1* single-gene-deletion mutant strains could possess similar growth and fermentation abilities to the parental strains, whereas when both *BAT* genes were deleted, anabolism of BCAAs was blocked, resulting in defects of their growth and fermentation abilities. Another possible explanation is that *BAT* genes are possibly required for some cellular activities involved in cell growth. Recently, it was reported that the BCAA metabolism is linked to mitochondrial activity and aging [6]. Moreover, the production of four higher alcohols investigated in this work was dramatically decreased by the double-gene-deletion mutation, possibly due to the reduced growth rate of the double-gene-deletion mutant strain.

Chinese rice wine, popular in local place among East-Asian countries, belongs to traditional rice wines [25]. The traditional fermentation process used in wine making produced high levels of higher alcohols in various Chinese rice wines [5, 25]. Some wine making techniques have been developed to control the content of higher alcohols in Chinese rice wine, such as removing the saccharified liquid from wine mash constantly during the wine making process of shanlan rice wine [25]. Nevertheless, yeast strain is the soul of wine's flavor compounds [3, 16, 22], and higher alcohols are almost always produced by yeast strains during the fermentation process. Therefore, breeding an excellent yeast strain producing less higher alcohols would be an effective method to control the content of higher alcohols in Chinese rice wine. Our work suggested that the *BAT2* single-gene-deletion mutant strain is an excellent strain for Chinese rice wine making to produce less higher alcohols.

Diploid strains are frequently used in industrial fermentation because of their stable fermentation performance. We further fused *BAT2* single-gene-deletion haploid mutant strains and removed the *KanMX* marker gene by using the Cre/*loxP* system to construct the engineered diploid strain RY1-Δ*bat2*. This engineered strain would be safe for industrial fermentation. Although the excision leaves behind a copy of the heterologous "*loxP*" direct repeat and may increase the likelihood of chromosomal rearrangements between these and cryptic *loxP* sites in engineered strain, the engineered diploid strain RY1-Δ*bat2* possessed the same growth and fermentation characteristics as the parental strain and produced less higher alcohols, which makes this strain useful in quality improvement and future development of the rice wine. These features can significantly improve the technology and market competitiveness of Chinese rice wine industry. Also, the results pave the way for improving yeast strains for other wine production.

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