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Reduced production of higher alcohols by *Saccharomyces cerevisiae* in red wine fermentation by simultaneously overexpressing *BAT1* and deleting *BAT2*

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Abstract

In red wine, the contents of higher alcohols and ethyl carbamate (EC) are two significant health concerns. To reduce the production of higher alcohols by wine yeast YZ22 with low production of EC, one *BAT2* was replaced by a *BAT1* expression cassette first and then another *BAT2* was deleted to obtain the mutant SYBB3. Real-Time quantitative PCR showed that the relative expression level of *BAT1* in SYBB3 improved 28 times compared with that in YZ22. The yields of isobutanol and 3-methyl-1-butanol produced by mutant SYBB3 reduced by 39.41% and 37.18% compared to those by the original strain YZ22, and the total production of higher alcohols decreased from 463.82 mg/L to 292.83 mg/L in must fermentation of Cabernet Sauvignon. Meanwhile, there were no obvious differences on fermentation characteristics of the mutant and parental strain. This research has suggested an effective strategy for decreasing production of higher alcohols in *Saccharomyces cerevisiae*.

Keywords: higher alcohols, isobutanol, 3-methyl-1-butanol, wine, *Saccharomyces cerevisiae*

Introduction

Red wine, one of the famous healthy alcoholic beverages, is popular all over the world because of its attractive flavor and high nutritive value. In red wine, the contents of ethyl carbamate (EC) and higher alcohols are significant health concerns. EC has been proved to be potentially carcinogenic and mutagenic to humans[1-4] and classified as a group of 2A carcinogen by the World Health Organization's International Agency for Research on Cancer (IARC).[5] Higher alcohols, also known as fusel alcohols, usually including isobutanol, 3-methyl-1-butanol, 2-methyl-1-butanol, n-propanol and β-phenylethanol, are a large group of flavor compounds in various alcoholic beverages and will significantly affect their organoleptic characteristics. An appropriate content of higher alcohols will lead to the alcoholic beverage mellow, soft, plump and coordinate of the bouquet.

Over the past few decades, many studies have been carried out to reduce the content of EC in alcoholic beverages by reducing the accumulation of urea (the precursor of EC). For example, an industrial wine yeast with improved urea degradation was constructed by overexpressing *DUR1,2*, which led to the reduction of EC production by 89% in Chardonnay wine.[6] In addition, overexpressing *DUR3* could also promote the degradation of urea in wine yeast and then reduce the EC production by 81% in Chardonnay wine.[7] In our previous work, in order to reduce the production of urea and EC, an arginase-deficient recombinant strain YZ22 (Δcarl/Δcarl) was constructed from a diploid wine yeast, WY1, by successively deleting two *CAR1* alleles to block the pathway of urea synthesis, which resulted in 77.89% and 73.78%

decline in urea and EC respectively.[8] However, the recombinant strain YZ22 produced 463.82 mg/L of total higher alcohols (THA) in the fermentation of Cabernet Sauvignon grape juice. In general, 300 mg/L of THA is considered acceptable for wine,[9-11] which will make wine mellow, plump and coordinate of the fragrance.[12,13] But when the THA content is above 400 mg/L, it will lead to an unpleasant flavor for the wine and cause a headache and intoxication for the consumers.[9-11] Therefore, to control the content of higher alcohols is important for promoting the quality and popularity of wine.

Higher alcohols in red wine are produced by yeast through two pathways: *de novo synthesis* from carbon source via the Harris pathway and degradation of branched-chain amino acids (BCAAs) via the Ehrlich pathway.[14,15] In *S. cerevisiae*, α-ketoacids involved in both pathways can either be synthesized from pyruvate by the Harris Pathway or from degradation of BCAAs (including L-valine and L-leucine) via the Ehrlich pathway. In the Ehrlich pathway, mitochondrial and cytosolic branched-chain amino acid transferase (BCAATases) encoded by *BAT1* and *BAT2* catalyze the transamination of BCAAs to synthesize α-ketoacids. And then α-ketoacids are converted into higher alcohols via decarboxylation by α-ketoacid decarboxylase and reduction of an aldehyde group by alcohol dehydrogenase.[16] It is reported that mitochondrial BCAATases encoded by *BAT1* catalyzed the biosynthesis of L-valine from α-ketoisovalerate, while cytosolic BCAATases encoded by *BAT2* catalyzed the degradation of BCAAs to α-ketoacids in *S. cerevisiae*.[17-19]

Studies on reducing the production of isobutanol and 3-methyl-1-butanol in *S.*

cerevisiae have been mainly focused on the regulation of the Ehrlich pathway. *BAT1* single-gene-deletion in one haploid yeast strain was reported to have no significant effect on the reduction of higher alcohols in Chinese rice wine fermentation.[20] Additionally, replacing one *ILV2* with *BAT1* or *BAT2* in a commercial wine yeast strain both could lead to the increase of isobutanol production in the fermentation of Colombard grape juice.[21] However, *BAT1* single-gene-deletion in *S. cerevisiae* with mutated *LEU2* led to a 1.8-fold increase in isobutanol production.[16] Therefore, the production of higher alcohols could be regulated through modifying *BAT1* and *BAT2*.

It was reported that *BAT1* and *BAT2* affected the production of higher alcohols oppositely.[22] In this study, a new strategy was employed to decrease the production of higher alcohols by replacing one *BAT2* allelic gene with a constructed *BAT1* expression cassette first and then deleting another *BAT2* in diploid wine yeast YZ22. To evaluate the new strategy for decreasing the production of higher alcohols in *S. cerevisiae*, the content of isobutanol and 3-methyl-1-butanol as well as total higher alcohols, fermentation performance and the production of urea and EC were investigated and compared between the mutants and the original strain YZ22

Materials and methods

Strains and plasmids

All the strains and plasmids as well as their relevant genotypes used in this study were listed in Table 1. The diploid wine yeast strain YZ22 with decreased production of urea and EC from WY1 (China General Microbiological Culture Collection Center, CGMCC 3373) and *Escherichia coli* DH5α used in this study were preserved in the

Microbiological Culture Collection Center of Tianjin Industrial Microbiological Key Laboratory, Tianjin University of Science and Technology, China.

Media and culture conditions

E. coli was cultured at 37°C in Luria–Bertani broth, and *S. cerevisiae* was grown at 30°C in yeast extract peptone dextrose (YEPD) medium according to the previous report.[8, 21] G418 (Geneticin, an aminoglycoside antibiotic) and Zeocin (Promega, Madison, USA) were used to screen positive yeast transformants with the *KanMX* and Zeocin-resistant yeast strains, respectively.[21] All solid media contained 2% agar (Difco, USA).

Plasmid construction

Plasmid DNA and genomic DNA of yeast in this study were prepared using Solarbio yeast genome DNA Extraction Kit. Restriction enzymes, DNA ligase, and PrimeSTAR® GXL DNA Polymerase (TaKaRa Biotechnol, Dalian, China) were used in the enzymatic manipulation of DNA, according to the Kit manuals. Primers used in the present study were all designed according to the sequence of *S. cerevisiae* S288c genome (*NCBI*, *http://www.ncbi.nlm. nih.gov/*) and listed in Table 2. BA_1 and BB_1 fragments (the upstream and downstream homologous sequence of *BAT2* respectively) were obtained by PCR using genomic DNA of YZ22 as the template and primers BA_1-U, BA_1-D (containing overlapping sequences used for fusion PCR) and BB_1-U (containing overlapping sequences used for fusion PCR), BB_1-D. Antibiotic gene fragment, *loxP-KanMX-loxP*, for G418 resistance was amplified from plasmid pUG6 using primers K-U and K-D (containing overlapping sequences used for fusion PCR).

The plasmid Yep352-*PGK1-BAT1* was used as the template to amplify sequence of *PGK1*$_P$-*BAT1*-*PGK1*$_T$ using primers PGK-U (containing overlapping sequences used for fusion PCR) and PGK-D. The construction of recombinant plasmid pUC-APTKB with pUC19 as the backbone were carried out as followed: firstly, the primers BA_1-U and PGK-D were used for fusion PCR with a mixture of BA_1 and *PGK1*$_P$-*BAT1*-*PGK1*$_T$ fragments as templates to obtain the fusion PCR product B_1A-*PGK1*$_P$-*BAT1*-*PGK1*$_T$; the fusion PCR product *KanMX*-BB_1 was obtained by the same way; then, BA_1-*PGK1*$_P$-*BAT1*-*PGK1*$_T$ and *KanMX*-BB_1 fragments were inserted sequentially into the pUC19 multiple-cloning site with the same direction by single-enzyme digestion to form the deletion plasmid pUC-APTKB.

Yeast transformation and screening

S. cerevisiae transformation was carried out by using the lithium acetate/PEG method as described previously.[23] The deletion cassette BA_1-*loxP*-*KanMX*-*loxP*-BB_1 from plasmid pUC-B2ABK and BA_1-*PGK1*$_P$-*BAT1*-*PGK1*$_T$-*KanMX*-BB_1 from plasmid pUC-APTKB were amplified and transformed for construction of *BAT2*-deletion mutant SYB1 and SYBB1 via homologous recombination. As wine yeast is diploid, a retractive primer disruption strategy was used to repeat the deletion of *BAT2* efficiently as described previously.[24] The deletion cassette BA_2-*KanMX*-BB_2 obtained by PCR with primers BA_2-U and BA_2-D was used to cut off the second copy of *BAT2* for construction of mutant SYBB2. The positive yeast transformants were selected according to the previous method.[10, 21, 25] The selected cassette with *KanMX* marker in SYBB1 and SYBB2 was removed by *Cre/loxP* recombination system to

147 obtain the recombinants SYBBK and SYBB3 as described previously.[26] The primers
148 used for plasmid construction and verification of recombinant yeast in this study were
149 listed in Table 2.

150 **Real-Time quantitative PCR**

151 mRNA of the yeast pre-cultured in YEPD medium was extracted using the Yeast
152 RNA kit (Omega, Norcross, GA, USA), and the relative expression level of *BAT2* and
153 *BAT1* were assessed via RT-qPCR.[8] The primers used to amplify the small parts of
154 *BAT2* and *BAT1* and the reference gene *ACT1* are listed in Table 1.

155 **Fermentation experiments**

156 Cabernet Sauvignon grape juice (21°Brix, pH 3.33, SO_2 50 mg/L) (Changli,
157 Hebei, China) was used in the fermentation experiments of the mutants and original
158 strain, and the detailed experiment procedures including precultivation of yeast cells
159 and control of the fermentation process was carried out as previously reported.[8] All
160 fermentations were performed in triplicate.

161 **Analytical methods**

162 The cell density and weight loss of the recombinants were measured.[8] The
163 concentrations of residual glucose, ethanol and total acid at the end of fermentation
164 were determined using the methods suggested by the international organization of
165 vine and wine (OIV).[27] The concentrations of higher alcohols and other flavor
166 compounds in the broth at the end of fermentation were measured using a modified
167 gas chromatography (GC) method based on the previous report after the distillation.[21]
168 Agilent 7890B gas chromatograph equipped with an Agilent G4513A autosampler and

a flame ionization detector (FID) (Agilent, USA) was used and the modification was as follows: butyl acetate was used as the internal standard, nitrogen (purity ≥ 99.99%) was used as the carrier gas at a constant flow rate of 0.8 mL/min, the injector temperature was 200°C and the oven temperature program was: initial temperature 50°C for 8 min, followed by an increase to 150°C at 5°C/min for 15 min. The concentration of urea was determined using a high-performance liquid chromatography coupled with fluorescence detection (HPLC-FLD).[28] EC in the samples was quantified using gas chromatograph–mass spectrometry (GC–MS) suggested by OIV after extracted by solid-phase extraction (SPE).[27] The free amino acids (mainly referring to L-valine) were measured using HPLC coupled with ultraviolet detection (HPLC-UV) according to the reported method [29] with a slight modification that 2,4-Dinitrochlorobenzene was used for derivatization.

Statistical analysis

All experiments were conducted in triplicate. Data are represented as the mean ± standard errors. The differences between the transformants and the parental strain were confirmed by Student's *t*-test. Statistical significance was considered when $p < 0.05$.

Results and discussion

Construction of recombinant wine yeast strains

BAT2 on one chromosome of YZ22 was replaced with the deletion cassette BA_1-*loxP*-*KanMX*-*loxP*-BB_1 through homologous recombination to construct recombinant SYB1, while with the deletion cassette

BA_1-*PGK1*$_P$-*BAT1*-*PGK1*$_T$-*KanMX*-BB_1 to construct recombinant SYBB1. PCR validations for recombinant SYB1 and SYBB1 were shown in Figure 1. Amplified fragments with 5536- and 2135-, 1483-, and 1343 bp (shown in lane 2, 5 and 8 respectively) were obtained for SYBB1 by using primers BA_1-U and BB_1-D, 1-U and 1-D, 2-U and 2-D, while bands with 1925- and 1136 bp (shown in lane 11 and 17 respectively) were obtained for SYB1 by using primers 3-U and 3-D, 4-U and 4-D (Table 2). These results indicated that these two deletion cassettes were correctly inserted into the site of *BAT2* to successfully construct recombinant SYB1 with one *BAT2* deleted and SYBB1 with simultaneously one *BAT2* deleted and one *BAT1* overexpressed. Cre recombinase was expressed and then *KanMX* in the recombinants SYB1 and SYBB1 was excised after introducing the plasmid pSH-Zeocin into them to obtain recombinants SYB1K and SYBBK. PCR validations for them were found in lane 12 and 13 in Figure 1 and the results indicated that *KanMX* was successfully removed from SYB1 and SYBB1.

Recombinant SYBB2 was constructed by introducing fragment BA_2-*KanMX*-BA_2 into SYBBK to delete the second *BAT2* allele, and recombinant SYBB3 was then constructed by deleting *KanMX* in SYBB2 using the same method as SYB1K and SYBBK constructions. Bands with 1925- and 1136 bp (shown in lane 14 and 20 respectively) in Figure 1 were obtained for a validation of recombinant SYBB2. In lane 15 and 21, no PCR products were found, which indicated that *KanMX* was successfully removed from SYBB2.

Relative expression levels of *BAT2* and *BAT1*

The relative expression levels of *BAT1* and *BAT2* measured by RT-qPCR were shown in Figure 2. Results showed that relative expression level of *BAT1* in mutant strains SYB1K (one *BAT2* was deleted), SYBBK (one *BAT2* was deleted and *BAT1* was overexpressed) and SYBB3 (two *BAT2* were deleted and *BAT1* was overexpressed) increased by 3.3, 19 and 28 folds compared to that in the original strain YZ22 ($P<0.05$), while the relative expression level of *BAT2* in SYB1K and SYBBK decreased by 20% and 60% compared to that in YZ22 ($P<0.05$).

From Figure 2, under the same cultivation conditions, the expression level of *BAT1* in SYB1K increased obviously when one *BAT*2 was deleted from the original strain YZ22, and that in SYBB3 with the second *BAT*2 was deleted also increased obviously compared to that in SYBBK. On the other hand, the expression level of *BAT2* decreased in recombinant SYBBK when *BAT1* was overexpressed in SYB1K. These results have been not reported in previous studies and the possible explanation is that Bap2p encoded by *BAT2* may be the transcription repressors of *BAT1*. Therefore, more detailed researches should be carried out to clarify the authentic relationship between *BAT2* and *BAT*1 on the transcriptional level.

Effects of *BAT2* deletion and *BAT1* overexpression on growth and fermentation performances of the wine yeast

To investigate the effect of *BAT2* deletion and *BAT1* overexpression on the growth performances, growth curves of the original strain and its recombinants were determined and results (Figure 3) showed that recombinants had almost the same growth rate and the final cell density as the original strain YZ22 except SYBB3. The

growth of mutant SYBB3 was slower compared to that of YZ22，and the possible reason was that the *BAT2* double-gene-deletion would block the catabolism of BCAAs and then lead to the defective mitochondrial activity for cell growth.[30] However, there were no obvious differences in the final cell density between SYBB3 and YZ22.

Small-scale grape must fermentation was conducted to determine the effects of *BAT2* deletion and *BAT1* overexpression on the fermentation performance of parental strain YZ22. Weight loss during the fermentation process of the original strain and its recombinants were shown in Figure 4. The same trend of weight loss suggested that YZ22, SYB1K, SYBBK and SYBB3 had similar fermentation rates. The concentrations of ethanol, residual sugar and total acid as well as pH value were listed in Table 3. No significant differences in fermentation behavior between the parental strain and its mutants were observed, which indicated that deletion of *BAT2* combined with overexpression of *BAT1* had no apparent effects on the fermentation performance of YZ22.

Effects of *BAT2* deletion and *BAT1* overexpression on the production of higher alcohols

In synthesis of higher alcohols via Ehrlich pathway in *S. cerevisiae*, *BAT1* and *BAT2* are two key genes encoding mitochondrial and cytosolic BCAATases (Bat1p and Bat2p) respectively.[14,30] Therefore, modification of *BAT1* and *BAT2* would regulate the synthesis of higher alcohols in *S. cerevisiae*.[20,32]

In this study, a series of recombinants were genetically engineered from

arginase-deficient recombinant YZ22 by *BAT2* deletion or *BAT1* overexpression combined with simultaneous *BAT2* deletion. Figure 5A showed strong reduction in the concentrations of isobutanol, 3-methyl-1-butanol, β-phenylethanol and n-propanol in the wine of the grape must fermentation with the recombinants than that with the original strain YZ22. In addition, the concentration of the individual higher alcohol in YZ22, SYB1K, SYBBK and SYBB3 decreased sequentially except that of the n-propanol. The concentrations of isobutanol and 3-methyl-1-butanol produced by SYBBK were 65.91 mg/L and 234.73 mg/L, which were reduced by 30.47% and 24.11% respectively compared with those produced by YZ22. And those produced by SYBB3 were further reduced to 57.43 mg/L and 196.89 mg/L with a reduction of 39.41% and 37.18% respectively compared to those produced by YZ22. Finally, the content of THA produced by SYBB3 reduced to 292.83 mg/L from 463.82 mg/L. These results indicated that deleting *BAT2* or overexpressing *BAT1* both could decrease the production of higher alcohols and the combination of these two strategies could decrease the production of higher alcohols to a greater extent. It was reported that the *BAT1* single-gene-deletion in *S. cerevisiae* with mutated *LEU2* led to a 1.8-fold increase in isobutanol production.[16] Therefore, overexpression of *BAT1* combined with simultaneous deletion of *BAT2* is an effective way for reducing the synthesis of higher alcohols in *S. cerevisiae*.

On the other hand, the other flavor compounds and L-valine in wine were also quantified and the results were shown in Figure 5B. There was no obvious difference in the content of ethyl acetate produced by the mutants and the original strain.

However, the contents of ethyl butyrate and isoamyl acetate produced by YZ22 were slightly higher than those produced by the mutants, while the contents of acetic acid produced by the mutants all increased slightly compared with that produced by YZ22. Some flavor compounds such as ethyl caproate, butyl acetate, n-caproic acid, n-butanol, sec-butanol, formic acid, ethyl enanthate, ethyl lactate and ethyl caprylate were not detected in the broth at the end of fermentation in this study. Additionally, the contents of L-valine produced by YZ22 and SYB1K were 1.99 mg/L and 1.98 mg/L respectively, which were less than that produced by SYBBK (2.51 mg/L) and SYBB3 (3.33 mg/L), which also suggested that *BAT2* and *BAT1* played different roles in metabolism of BCAAs.

Effects of *BAT2* deletion and *BAT1* overexpression on production of urea and EC

To reduce its potential carcinogenic risk to human, the contents of EC in red wine must be controlled. The concentration of EC produced by YZ22 was already lower than the safe dosage (10 μg/L).[8,33] In this work, the concentrations of EC and urea produced by the mutants and the original strain in must fermentation of Cabernet Sauvignons were measured and the results were listed in Table 4. No obvious differences were observed in the contents of urea and EC produced by the mutants and the original strain, and their concentrations were in the range of 0.47-0.49 mg/L and 7.29-7.43 μg/L, respectively.

As an important precursor of EC in wine, urea produced by yeast was mainly involved in urea cycle, in which urea was from the decomposion of arginine catalyzed by arginase (encoded by the *CAR1*).[34] In the previous research, [8] deletion of *CAR1*

alleles caused the higher production of higher alcohols in YZ22, and the possible reason might be that arginine could not be utilized after deleting CAR1 which caused YZ22 to utilize other BCAAs as the nitrogen source. As the synthesis of higher alcohols through the Ehrlich pathway was mainly regulated by *BAT2* and *BAT1* which were involved in the degradation of BCAAs,[35] the production of higher alcohols will be affected after the deletion of *CAR1* alleles. Meanwhile, arginine is the precursor of urea synthesis and it will not influence the urea synthesis because arginine cannot be decomposited and utilized in YZ22 with *CAR1* deleted. Therefore, the modification of *BAT1* and *BAT2* in this work could not affect the production of urea and EC. Our results showed that *BAT1* overexpression combined with *BAT2* deletion is an effective strategy to regulate the production of higher alcohols in *S. cerevisiae* with low production of EC in wine fermentation.

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Notes

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Supporting Information Available

Figure 1s Sequence comparison and sequencing results of the integration sites (A) and the target gene *BAT1* in *PGK1*$_P$-*BAT1*-*PGK1*$_T$-*KanMX* (B). Only 20 bp of the integration sites BA_1 (upstream homologous sequence of *BAT2*) and BB_1 (downstream homologous sequence of *BAT2*) in the fragment BA_1-*PGK1*$_P$-*BAT1*-*PGK1*$_T$-*KanMX*-BB_1 in mutant SYBB1 were shown in Figure 1sA, and the whole sequence of *BAT1* (1182 bp) in *PGK1*$_P$-*BAT1*-*PGK1*$_T$-*KanMX* in mutant SYBB1was compared with that of the original sequence of *BAT1* in Figure 1sB. Sequence comparison and sequencing results indicated that there was no mutation in the sequence of BA_1-*PGK1*$_P$-*BAT1*-*PGK1*$_T$-*KanMX*-BB_1 and *BAT1* was successfully integrated and overexpressed in mutant SYBB1.

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466 **Figure Captions**

467 **Figure 1. PCR verification of the recombinants**.

468 M: DL5000 DNA Marker; lane 1-3: PCR amplification results from YZ22, SYBB1

469 and SYBBK genome using the primers BA_1-U and BB_1-D; lane 4-6: PCR

470 amplification results from YZ22, SYBB1 and SYBBK genome using the primers 1-U

471 and 1-D; lane 7-9: PCR amplification results from the original strain YZ22, SYBB1

472 and SYBBK genome using the primers 2-U and 2-D; lane 10-15: PCR amplification

473 results from YZ22, SYB1, SYB1K, SYBBK, SYBB2 and SYBB3 genome using the

474 primers 3-U and 3-D; lane 16-21: PCR amplification results from YZ22, SYB1,

475 SYB1K, SYBBK, SYBB2 and SYBB3 genome using the primers 4-U and 4-D.

476 **Figure 2. Relative expression levels of *BAT2* and *BAT1* in the original strain**

YZ22 and its recombinants.

The total RNA of mutants and the original strain were isolated and used as the template to obtain the cDNA of *BAT1* and *BAT2*, which was used to determinate the relative expression level of corresponding gene by RT-qPCR. Error bars represented SD of the biological triplicates ($P < 0.05$).

Figure 3. Cell growth of the original strain YZ22 and its recombinants.

The yeast cell was inoculated into the YEPD media and incubated at 30°C, and the optical density (OD_{600}) of the fermentation broth at different time was determined. The growth curves were plotted by a Bioscreen Automated Growth Curves analysis system. Error bars represented SD of the biological triplicates ($P > 0.05$).

Figure 4. Comparison of fermentation rates of the original strain YZ22 and its recombinants.

The mutants and original strain were inoculated into Cabernet Sauvignon grape juice for alcoholic fermentation and the weight loss was monitored every 12 h to evaluate the fermentation rates of the original strain YZ22 and its mutants. Error bars represented SD of the biological triplicates ($P > 0.05$).

Figure 5. Contents of higher alcohols and other flavor compounds produced by the original strain YZ22 and its recombinants.

(A) Contents of higher alcohols produced by mutants and the original strain YZ22; (B) Contents other flavor compounds including ethyl acetate, isoamyl acetate, ethyl butyrate, acetic acid produced by mutants and the original strain YZ22. Error bars represented SD of the biological triplicates ($P < 0.05$).

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Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant characteristic	Reference or source
Strains		
WY-1	Diploid wine yeast	Guo et al., 2016
E. coli DH5α	Host of plasmid	Stratagene
YZ22	*Δcar1/Δcar1::loxP::loxP*	Guo et al., 2016
SYB1	YZ22 *BAT2/Δbat2:: loxP-KanMX-loxP*	This study
SYB1K	YZ22 *BAT2/Δbat2:: loxP::loxP*	This study
SYBB1	YZ22 *BAT2/Δbat2::PGK1$_P$-BAT1-PGK1$_T$-loxP-KanMX-loxP*	This study
SYBBK	YZ22 *BAT2/Δbat2::PGK1$_P$-BAT1-PGK1$_T$-loxP*	This study
SYBB2	YZ22 *Δbat2/Δbat2::PGK1$_P$-BAT1-PGK1$_T$-loxP-KanMX-loxP*	This study
SYBB3	YZ22 *Δbat2/Δbat2::PGK1$_P$-BAT1-PGK1$_T$-loxP::loxP*	This study
Plasmids		
Yep352-*PGK 1-BAT1*	Ap^r, Kan^r, containing the *PGK1$_P$-BAT1-PGK1$_T$* gene expression cassette	This study
pUC-B$_2$ABK	Ap^r, Kan^r, recombinant plasmid with *BAT2* disruption cassette	Zhang et al., 2015
pUG6	Kan^r, containing *loxP-KanMX-loxP* disruption cassette	Güldener et al., 2002
pSH-Zeocin	Zeo^r, Cre recombinant enzyme expression vector	Lu et al., 2012
pUC-APTKB	Ap^r, Kan^r, recombinant plasmid with *BAT2* flanking fragments, and containing *PGK1$_P$-BAT1-PGK1$_T$* expression cassette	This study
pUC19	Ap^r, cloning vector	Invitrogen

Table 2 Primers used in this study

Primers	Sequence（5’ ⟶ 3’）	Restriction site
For plasmid construction		
BA_1-U	TCCCCCGGGTAACGCTCCTTTCCAAACATC	*SmaI*
BA_1 -D	GTTTTGGATAGATCAGTTAGAATCGTTCTTAAAACTCGTGG	
BB_1-U	GATCCACTAGTGGCCTATGCAGTATCGCTATTGCTACGTAA AGT	
BB_1 -D	CATGCATGCCTTTCTGAAGTCTAAGTGGGAT	*SphI*
PGK-U	CACGAGTTTTAAGAACGATTCTAACTGATCTATCCAAAACT GA	
PGK-D	TCCCCCGGGGCGTACGAAGCTTCAGCTGTAACGAACGCA GAATTTTC	*SmaI*
K-U	CATGCATGCGAAAATTCTGCGTTCGTTACAGCTGAAGCTTC GTACGC	*SphI*
K-D	ACGTAGCAATAGCGATACTGCATAGGCCACTAGTGGATCTG	
For delection of second copy of *BAT2*		
BA_2-U	CCTAGACGCCTCCAAAGTTAAGATAACTACCACACAACA TGCATCTAAGCCAAAACCGAACAGTGAGTTACAGCTGAA GCTTCGTACGC	
BA_2-D	AATCAGTAACAACCCTTGACCAATTGCCATGCTCAGTCT CGCCATATTGGATTCCATTAATCCATTGGCATAGGCCACTA GTGGATCTG	
For PCR verification		
1-U	TCTTTTCGTCACCGTGTCGC	
1-D	AATATCTGTGCGTCTTGAGTTG	
2-U	CGCAATCACGAATGAATAACGGT	
2-D	TTCGCCCAGTTAGCTGTTTG	
3-U	ACAATCTCCTACCACCAG	
3-D	GTATTTCGTCTCGCTCAG	
4-U	ATGCGTCAATCGTATGTG	
4-D	TTTCCTCTATGTCCTCCC	
For real-time qPCR		

BAT1-F	CAAGCCAAGACCAAATGA
BAT1-R	TGGAATACACAAGCAGAT
BAT2-F	GCCAAAACCGAACAGTGA
BAT2-R	CGGAAGGGTCTAAAGACA
ACT1-F	CGTCTGGATTGGTGGTTCTA
ACT1-R	GTGGTGAACGATAGATGGAC

Table 3 Fermentation performances of the original strain YZ22 and its recombinants

Strains	Ethanol (% v/v, 20°C)	Residual sugar (g/L)	Total acid (g/L)	pH value
YZ22	11.42 ± 0.26	1.78 ± 0.20	6.56 ± 0.21	3.18 ± 0.01
SYB1K	11.40 ± 0.31	1.81 ± 0.17	6.58 ± 0.20	3.17 ± 0.01
SYBBK	11.33 ± 0.25	1.85 ± 0.09	6.78 ± 0.15	3.23 ± 0.02
SYBB3	11.40 ± 0.17	1.80 ± 0.15	6.48 ± 0.16	3.19 ± 0.02

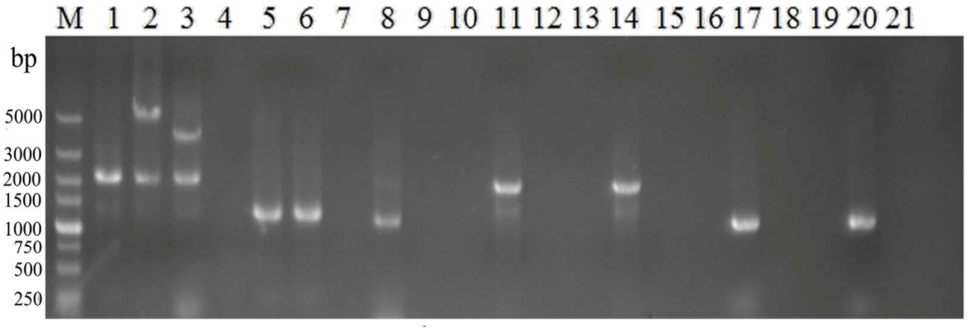
Data are the average of three biological triplicates ± the standard deviation ($P > 0.05$)

Table 4 Production of urea and EC in wine fermentation with the original strain YZ22 and its recombinants

Strains	YZ22	SYB1K	SYBBK	SYBB3
Urea (mg/L)	0.47 ± 0.018	0.48 ± 0.035	0.49 ± 0.057	0.48 ± 0.045
EC (μg/L)	7.43 ± 0.240	7.38 ± 0.420	7.35 ± 0.450	7.29 ± 0.330

Data are the average of three biological triplicates ± the standard deviation ($P > 0.05$)

Figure 1


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
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3000
2000
1500
1000
750
500
250

Figure 2

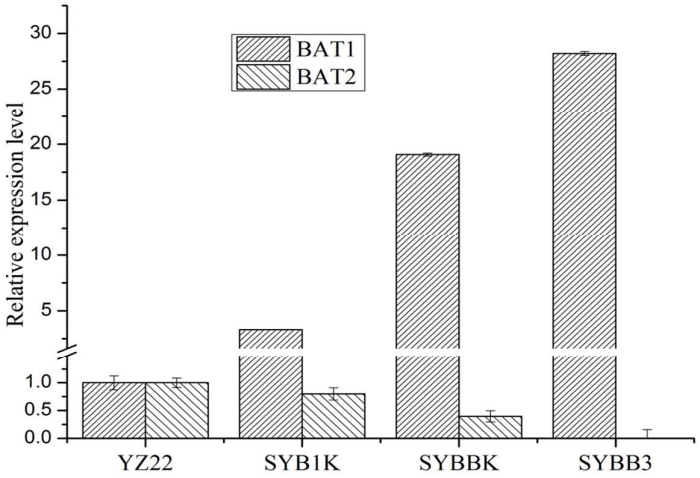

BAT1
BAT2
Relative expression level
30
25
20
15
10
5
1.0
0.5
0.0
YZ22
SYB1K
SYBBK
SYBB3

Figure 3

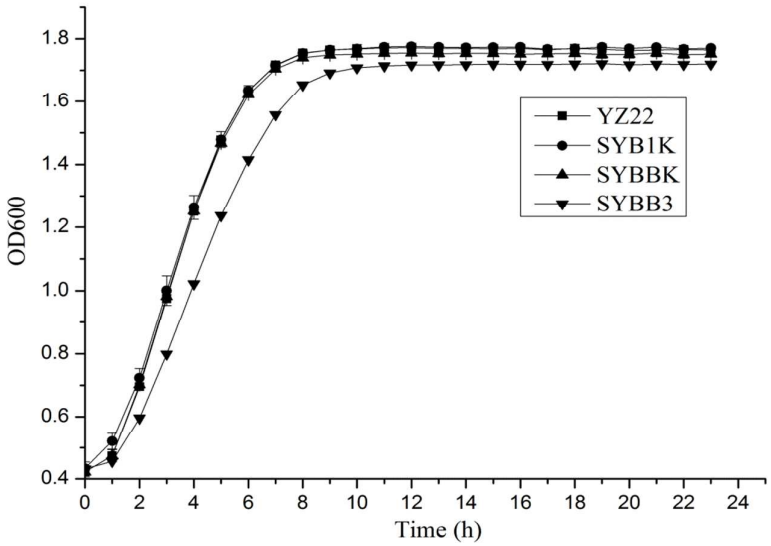

OD600
Time (h)
YZ22
SYB1K
SYBBK
SYBB3
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure 4

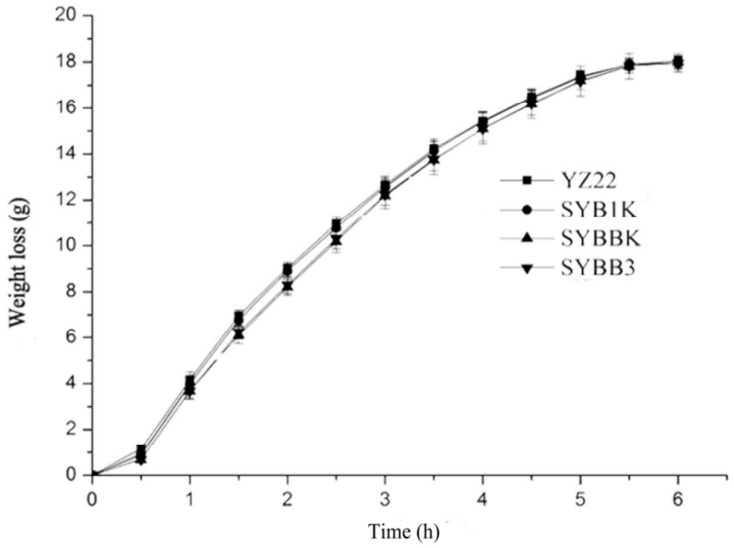
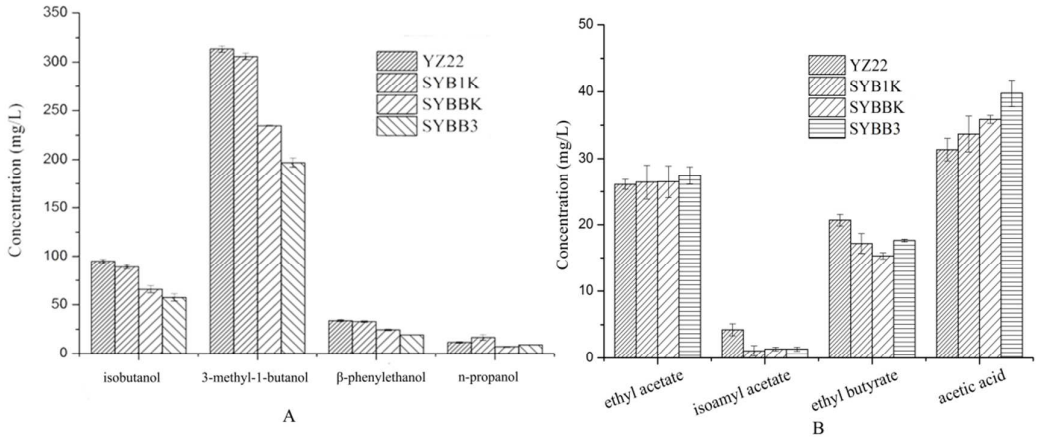
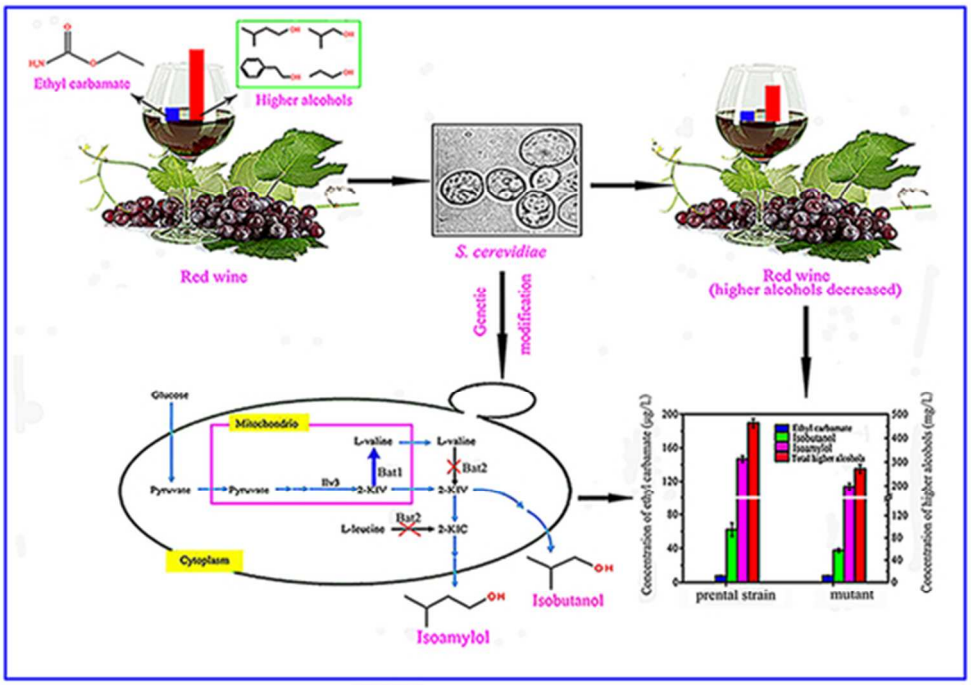

Weight loss (g)
Time (h)
YZ22
SYB1K
SYBBK
SYBB3
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5
6

Figure 5


Concentration (mg/L)
350
300
250
200
150
100
50
0
YZ22
SYB1K
SYBBK
SYBB3
isobutanol
3-methyl-1-butanol
β-phenylethanol
n-propanol
A
Concentration (mg/L)
50
40
30
20
10
0
YZ22
SYB1K
SYBBK
SYBB3
ethyl acetate
isoamyl acetate
ethyl butyrate
acetic acid
B



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