Improvement of flavor profiles in Chinese rice wine by creating fermenting yeast with superior ethanol tolerance and fermentation activity

Yijin Yang, Yongjun Xia, Xiangna Lin, Guangqiang Wang, Hui Zhang, Zhiqiang Xiong, Haiyan Yu, Jianshen Yu, Lianzhong Ai

PII: S0963-9969(18)30212-6
DOI: doi:10.1016/j.foodres.2018.03.036
Reference: FRIN 7473

To appear in: *Food Research International*

Received date: 29 December 2017
Revised date: 9 March 2018
Accepted date: 11 March 2018

Please cite this article as: Yijin Yang, Yongjun Xia, Xiangna Lin, Guangqiang Wang, Hui Zhang, Zhiqiang Xiong, Haiyan Yu, Jianshen Yu, Lianzhong Ai , Improvement of flavor profiles in Chinese rice wine by creating fermenting yeast with superior ethanol tolerance and fermentation activity. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Frin(2018), doi:10.1016/j.foodres.2018.03.036

Improvement of flavor profiles in Chinese rice wine by creating fermenting yeast with superior ethanol tolerance and fermentation activity

Yijin Yang[a], Yongjun Xia[a], Xiangna Lin[a], Guangqiang Wang[a], Hui Zhang[a], Zhiqiang Xiong[a], Haiyan Yu[b], Jianshen Yu[c], Lianzhong Ai[a,*]

[a]Shanghai Engineering Research Center of Food Microbiology, School of Medical Instrument and Food Engineering, University of Shanghai for Science and Technology, Shanghai, 200093, PR China

[b]School of Perfume and Aroma Technology, Shanghai Institute of Technology, Shanghai, 200235, PR China

[c]Shanghai Jinfeng Wine Co., Ltd., Shanghai, 200120, PR China

*****Corresponding author: Lianzhong Ai

Tel: +86-21-55897302

Fax: +86-21-55897302

E-mail address: ailianzhong@hotmail.com

Abstract

Producing alcoholic beverages with novel flavor are desirable for winemakers. We created fermenting yeast with superior ethanol tolerance and fermentation activity to improve the flavor profiles of Chinese rice wine. Strategies of ethanol domestication, ultraviolet mutagenesis (UV) and protoplast fusion were conducted to create yeast hybrids with excellent oenological characteristic. The obtained diploid hybrid F23 showed a cell viability of 6.2% under 25% ethanol, whereas its diploid parental strains could not survive under 20% ethanol. During Chinese rice wine-making, compared to diploid parents, F23 produced 7.07%-12.44% higher yield of ethanol. Flavor analysis indicated that the total content of flavor compounds in F23 wine was 19.99-26.55% higher than that of parent wines. Specifically, F23 exhibited higher capacity in producing 2-phenylethanol, short-chain and long-chain fatty-acid ethyl-ester than diploid parents. Compared to diploid parents, F23 introduced more flavor contributors with odor activity values (OAVs) above one to Chinese rice wine, and those contributors were found with higher OAVs. Based on principal component analysis (PCA), the flavor characteristic of F23 wine was similar to each of parent wine. Additionally, sensory evaluation showed that F23 wine was highly assessed for its intensive levels in fruit-aroma, alcohol-aroma and mouthfeel. Hybrid F23 not only displayed superior flavor production and oenological performance in making Chinese rice wine, but also could act as potential “mixed-like” starter to enrich wine style and differentiation.

Key word: Flavor profiles; Chinese rice wine; ethanol tolerance; fermentation activity; odor activity value; sensory evaluation

1. Introduction

Chinese rice wine has been consumed for long time all over the world and is widely used in medicine in the eastern-south of Asian (Lu et al., 2015). It is produced from rice by simultaneous saccharification with "*Wheat Qu*" (like *koji* used in sake) and fermentation with yeast (*Saccharomyces cerevisiae*) (Li et al., 2015). Chinese rice wine making is generally performed at 28 ºC for 3-5 days (primary fermentation), and 10-15 ºC for 10-20 days (secondary fermentation) under an open environment to enrich its flavor profile. The enological characteristics of different yeast strains have greatly impact on the quality of the resulting wines (Chen and Xu, 2014). Yeast fermentation not only produce ethanol, but also generate a range of minor volatile flavor metabolites, which were resulted in the specific character and style of Chinese rice wine (Ye et al., 2013). As the microbial composition is complex in Chinese rice wine fermentation, in order to avoid deterioration which caused by excessive proliferation of bacteria, purebred yeast starter with growth advantage is generally applied in Chinese rice wine industries (Lv et al., 2016). However, purebred yeast fermentation could result in a less mellow flavor and taste of Chinese rice wine because the structure of microbial community is relatively simple. Mixed yeast starters have been proven that could improve the flavor production and diversity of Chinese rice wine (Yang et al., 2017), although man-made mixed fermentation experiments show dynamic population fluctuations between strains (King et al., 2008). Wine aroma is one of the most important factors governing the sensory perceivable wine quality and consumer preference, therefore, a plenty of researchers have been devoted themselves to improve the flavor profiles in different kinds of wines, including sake (Hirooka et al., 2005; Inoue et al., 2012; Takahashi et al., 2017), beer (Landaud et al., 2008; Pires et al., 2014; Krogerus et al., 2015), cider (Xu et al., 2006; Valles et al., 2007; Ye et al., 2013) and grape wine (Bellon et al., 2011, 2013; Steensels et al., 2014). However, the report for improving flavor profile of Chinese rice wine was still very scarce. As yeast could severely affect flavor profiles of the resulting wine, creating yeast with excellent oenological characteristic would be an effective strategy to improve the flavor profile of Chinese rice wine.

During Chinese rice wine fermentation, due to an accumulation of stressful factors, the fermentation sometimes stuck in the secondary fermentation, thus lower the quality of resultant wine. Though the brewing process of simultaneous saccharification and fermentation could avoid yeast cells exposing to

high concentration of sugar, it also contributes to high ethanol production, which can be 14% - 20% (v/v) in final fermentation mash (Chen and Xu, 2010). Therefore, during secondary fermentation, yeast cells were confronted with so high ethanol concentration that ethanol also became toxic to yeast cells (Snoek et al., 2016). It was a challenge for yeast striving to survive and ferment. Acquiring yeast with high ethanol tolerance was always desirable to winemakers, for which could theoretically result in more complete fermentation and higher production quality of Chinese rice wine (Ciesarova et al., 1996; Dorit, 2011; Steensels and Verstrepen, 2014). It was considered that yeast strains with high ethanol tolerance increased the likelihood of exhibiting high fermentation activity, however, poor correlation between ethanol tolerance and fermentation activity had been reported (Pais et al., 2013). Although some other researchers drew conclusion that yeast with improved ethanol tolerance could get higher fermentation activity (Shi et al., 2008; Yazawa et al., 2007; Nakagawa, 2016), our previous study also suggested that yeasts with superior fermentation activity not always tolerant to high concentration of ethanol (Yang et al., 2015). Hence, creating yeast hybrid, which the genomes of different strains were contained within one cell, to obtain yeast with superior ethanol tolerance and fermentation activity was conducted.

In the present study, fermenting yeast with superior ethanol tolerance and fermentation activity was created to improve the flavor profile in Chinese rice wine. *S.cerevisiae* haploid strain Et20, which could tolerate 20% v/v ethanol, was obtained by ethanol domestication. *S.cerevisiae* haploid strain CTZ30 with high fermentation acitivity was isolated by UV mutagenesis. Haploid strains Et20 and CTZ30 were then combined by protoplast fusion. Yeast hybrids with superior ethanol tolerance and fermentation activity were screened out and then used for Chinese rice wine-making. Subsequently, the flavor profiles in Chinese rice wine fermented by yeast hybrids were investigated. Sensory evaluation was performed to assess the quality of Chinese rice wine.

2. Materials and methods

2.1 Yeast strains, mediums and reagents

Haploid *S.cerevisiae* S288c was kindly gifted by Dr. Siliang Zhang (East China University of Science and Technology). *S.cerevisiae* diploid strain

BR20 could tolerant 18% ethanol and *S.cerevisiae* diploid strain BR30 exhibited superior fermentation activity were preserved in the China General Microbiological Culture Collection with accession number CGMCC 9445 and CGMCC 10378, respectively. Tetraploid strains are not genetically stable because of the high chromosome number (Bisson, 2017). Thus, haploid strains of BR20 and BR30 were generated for protoplast fusion. By performing test of ethanol tolerance and fermentation activity, haploid strain 20-Ha4 (generated from diploid strain BR20) and haploid strain 30-Ha5 (generated from diploid strain BR30) were selected as candidates for ethanol domestication and UV mutagenesis, respectively.

YPD medium (2% glucose, 2% peptone, 1% yeast extract) was used for yeast growth. Clotrimazole (CTZ) + SD medium (2% glucose, 0.67% yeast nitrogen base without amino acid) was used for screening CTZ-resistant strains. For preparing protoplasts, YPD and SD mediums containing 0.6 mol/L KCl were used as regeneration medium. Malt extract broth used for fermentation was adjusted to 12% brix by a hand-held refractometer (ATAGO CO., Ltd; Japan). All reagents and standards were purchased from Sigma Aldrich (Shanghai, China). n-Alkane standards (C_7-C_{40}; Supelco, Bellefonte, PA, USA) were used to determine the retention index.

2.2 Directed domestication of yeast by cultivation under stepwise increase ethanol

The method from previous publications (Sauer, 2001; Novo et al., 2014) were used with some modifications. Haploid strain 20-Ha4 was activated via pre-culturing in YPD at 28 ^{o}C overnight. The cell density was adjusted to OD_{600} around 1.0, then 1 mL of inoculum was inoculated into 9 mL YPD containing 6% v/v ethanol in 50 mL Erlenmeyer flask closed with screw cap with hole and incubated at 28 ^{o}C. Magnetic stirring was used for homogenization. After incubating for 36 hours, the culture was centrifuged, adjusted to the same initial cell density, and transferred into fresh YPD containing the same ethanol concentration. Yeast cells were repetitively cultivated under the same ethanol concentration for three times and then shifted to YPD containing higher ethanol concentration for three times likewise. The stepwise increase ethanol concentrations were set as 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20% (v/v). Finally, the acclimated cultivation was spread on YPD agar plate containing 20% ethanol and incubated at 28 ^{o}C. The biggest

colony was selected and purified on YPD plate containing 20% ethanol for 8 generations to ensure genetic stability. The adapted strain with improved ethanol tolerance was named as Et20.

2.3 Isolation of CTZ-resistant mutants by UV mutagenesis

The pre-cultivated haploid strain Et20 and haploid strain 30-Ha5 ($1x10^8$ cfu/mL) were centrifuged, resuspended into sterilized water, serially diluted from 10^{-1} to 10^{-6}, separately spread on SD mediums containing 0, 2, 4, 6, 8, 10, 12, 14, 16 and 18 mg/L CTZ, and incubated at 28 °C for 2-5 days. Survival rates of yeast strains under different concentration of CTZ were determined by counting colonies grown on mediums with and without CTZ.

Optimal UV treatment conditions were determined as follows. Haploid strain 30-Ha5 ($1x10^8$ cfu/mL) was put into magnetic stirring apparatus and mutagenized by treatment with UV at the power of 30 W and the irradiation distance of 30 cm for 0, 30, 60, 90, 120, 150 and 180 s. Cells were washed twice with sterilized water, serially diluted from 10^{-1}-10^{-6}, spread on SD medium and incubated in dark at 28 °C. Lethality rates of different UV treatment conditions were determined by counting colonies grown on SD mediums treated with and without UV.

Finally, 30-Ha5 cell mutagenized with optimal UV condition would be spread on SD medium containing lethal CTZ concentration and incubated in dark at 28 °C for 7-10 days. Colonies growing on the medium were selected as CTZ-resistant mutants. Those CTZ-resistant mutants were purified on SD medium containing lethal concentration of CTZ for 8 generations to ensure genetic stability. After performing fermentation activity test, the best CTZ-resistant strain used for further study was named as CTZ30.

2.4 Protoplast preparation and fusion

For protoplast preparation, the method described by Curran and Bugeja (1996) was applied. Haploid strains Et20 and CTZ30 were pre-activated and inoculated into 50 mL YPD medium in 250 mL Erlenmeyer flasks for 16 h at 28 °C in a rotary shaker (200 r/min). Suspensions ($1x10^8$ cfu/mL) of the two

stains were centrifuged with 3000xg for 5 min at room temperature and washed twice in sterile cold water. Cells were resuspended in 10 mL of protoplast solution (CPB: 0.6 M KCl, 0.1 M citric acid, 0.2 M Na_2HPO_4, pH6.0) with 50 uL β-mercapto-ethanol, and incubated for 15 min in a rotary shaker (150 r/min) at 28 ºC. Cells were then washed, resuspended in 10 mL of CPB containing with 150 U lyticase (from *Arthrobacter Luteus*, ≥2000 U/mg, sigma Aldrich, USA) and incubated in water bath at 30 ºC. Protoplasts were washed and suspended in CPB for further use.

The method described by Pérez-Través et al. (2012) was applied with some modifications for fusion. The lethal CTZ concentrations were 10 mg/L of Et20, while CTZ30 resist to 14 mg/L of CTZ. Before fusion, protoplast CTZ30 was inactived by heating at 60 ºC for 20 min. For protoplast fusion, equivalent amounts ($1x10^8$ cfu/mL) of heat-inactivated protoplast CTZ30 and protoplast Et20 were mixed, centrifuged and treated with 2 mL of 35% polyethylene-glycol (molecular weight 6000) and 100 mM $CaCl_2$ for 30 min in a rotary shaker (150 r/min) at 28 ºC. Cells were centrifuged (3000×g during 5 min at room temperature) and washed twice with CPB. Appropriate dilutions of cells in CPB were spread onto SD medium containing osmotic stabilizer and 14 mg/L of CTZ, which was totally lethal for Et20. After incubating at 28 ºC for 2-7 days, the observed colonies were purified on the same medium.

2.5 Ploidy determination of yeast

Flow cytometry (Guava EasyCyte plus cytometry, Merk Millipore, USA) was used for assessing the DNA contents of yeast strains following the method described previously (Pérez-Través et al. 2012). DNA content was assessed on the basis of the fluorescence intensity compared with haploid *S.cerevisiae* S288c reference strain.

2.6 Ethanol tolerance assay and determination of fermentation activity

To evaluate ethanol tolerance of yeast strains, growth ability, cell viability and spot assay under the presence of ethanol were performed (Shi et al., 2008; Torre-González et al., 2016). Pre-cultivated yeast cells ($1x10^8$ cfu/mL) were recovered by centrifugation, washed with sterilized water and resuspend

in YPD consisting ethanol. Cell growth at 28 ^{o}C for 48 h was monitored by Bioscreen C MBR apparatus (Oy Growth Curves Ab Ltd, Finland). After culturing in YPD consisting ethanol for 24 h, cell viability was expressed as percentage of colony-forming units of the ethanol treatment with an untreated control. Spot assay was performed by spotting 5 μL of 10-fold serially diluted suspension onto YPD agar containing ethanol. The plates were incubated at 28 ^{o}C for 48-72 h.

Fermentation activity was determined using the method described in previous papers (Nabais et al., 1988; Meijnen et al., 2016) with a few modifications. Pre-cultured cell suspension (1x10^{8} cfu/mL) were inoculated into 250 mL Erlenmeyer flask containing 45 mL malt extract broth at ration of 10% and incubated at 28 ^{o}C. Fermentative activity was monitored via measuring reduction weight of malt medium, which could represent CO_2 production. Reduction weight of malt extract broth was determined every 24 hours. When reduction weight was lower than 0.2 g, the fermentation was considered to be over.

2.7 Chinese rice wine brewing

Chinese rice wine was brewing according our previous study (Yang et al., 2017). Yeast was inoculated into malt extract broth (12% brix) and used as starter culture. The fermentation mash was incubated at 28 ^{o}C for 5 days and then migrated to incubate at 15 ^{o}C for 10 days. After filter pressing, clarification and sterilization, young Chinese rice wine was obtained. Wine samples were collected every 3 days during fermentation, and sample at the fifth day (end of the primary fermentation) was also collected for further analysis.

2.7.1 Oenological properties of fermented Chinese rice wines

Reducing sugar content in wine samples were determined by the dinitrosalicylic acid method (Miller 1959). Total acidity (in terms of lactic acid) was measured by a China official method (GB/T 5517-2010). Ethanol contents were determined by the method described in (Di Egidio et al., 2010). It was

carried out using high performance liquid chromatograph (Waters e2695-Empower system, Waters CO. Ltd, USA) equipped with an exclusion column (Carbomix H-NP, 300 mm×7.8 mm, Sepax Technologies, Inc., USA) at 55 ºC with a mobile phase of sulfuric acid (2.5 mM) at a flow rate of 0.6 mL/min.

2.7.2 Determination of volatile flavor compounds

Volatile flavor compounds in the Chinese rice wine were analyzed by solid-phase microextraction (SPME) coupled with GC-MS as described in (Yang et al., 2017). Briefly, each of wine sample was diluted to a final concentration of 6% ethanol (v/v) and then added into 20 mL headspace glass vials with 2 g of sodium chloride and 10 μL internal standard of 4-methyl-2-pentanol (250 μg/mL in absolute ethanol). The fiber (50 μm DVB/CAR/PDMS) headed into a solid phase microextraction (SPME) device (SUPELCO Co., Bellefonte, PA, USA) was inserted into the vials, then the vails were shaken at 50 °C for 30 min to extract volatile compounds. The volatile compounds were then desorbed at 250 °C in 7 min. A GC-MS system (SCIONSQ-456, Bruker Daltonics Inc., USA) equipped with a DB-WAX column (60 m × 0.25 mm × 0.25 μm, Agilent Technologies, USA) and programmed from 40 °C (holding for 3 min) to 210 °C at 6 °C/min, then 210 °C to 230 °C at 8 °C/min (holding for 15 min). Helium was delivered at a flow rate of 1 mL/min as the carrier gas. The mass detector was operated in electron impact mode at an ionizing voltage of 70 eV. The ion source temperature was set at 220 °C. The extracted volatile compounds were identified by comparing the spectra with a mass spectrum library search (NIST 1.6 and Wiley 6.0). The Kovats index (KI) of unknown compounds was determined by sample injection with a homologous series of alkanes (C_7-C_{30}). The quantity of volatile compounds was determined by using internal standard method.

2.7.3 Determination of Odor activity value

OAVs of volatile flavor compounds were calculated according to:

$$OAV_i = \frac{C_i}{OT_i}$$

where C_i is the concentration of the compound *i* in the sample and OT_i is its odor detection threshold concentration which is found in the literature.

2.7.4 Sensory evaluation

Descriptive sensory evaluation of the 3 final Chinese rice wines were conducted by a panel of 8 judges (4 males and 4 females between 20-50 years old). The judges were previously selected and trained according to ISO 8586-1 (ISO, 1993). A total of 11 sensory attributes of appearance (color and turbidity), aroma (alcohol, fruit and cereal), taste (sweet, sour and bitter) and mouthfeel (astringency, continuation and full body) were chosen to characterize the sensory properties of Chinese rice wine samples (GB/T 13662-2008). During training, the judges were instructed to read the definitions (Table1) of sensory attributes (Jung et al., 2014). Subsequently, the reference materials of sensory attributes were subjected to the panelists for a ranking test and scored on a 9-pointed linear scale from 0 (very weak) to 9 (9: very strong) (dos Santos Navarro et al., 2012). There were three reference scales to illustrate the minimum, the medium and the maximum intensity of each attribute, prepared as described in Table 1. The training was terminated when the intensities and characteristics of sensory attribute references reached an agreement by all the panelists. Then the panelists proceeded to formal wine-tasting test.

Sensory evaluation was conducted in a room with uniform source of lightening, absence of noise and distracting stimuli at 20 ^{o}C. The 15 mL-wine samples and duplicates were presented in tulip-shaped glasses (250 mL) marked with a random order and covered with petri dishes to the panelists. Drinking water was provided to the panelists for cleansing their palate between samples. An index card which contained all sensory attributes with their definitions and the 9-point scales (from 0 to 9) was used in the wine-tasting test. The panelists then scored each sensory attribute of the wine samples at individual speed.

2.8 Statistical analysis

Principal component analysis (PCA) using a correlation matrix with no rotation was performed to investigate the relationships between different brewing stages of Chinese rice wines and volatile compounds. SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) and Origin version 9.0 (Origin Lab Inc.,

Hampton, MS, USA) were used to analyze data. All of the GC-MS data had been conveyed by the diluted factor.

3. Results and discussion

3.1 Directed domestication of yeast strain by stepwise increase of ethanol

The approach of evolutionary engineering is considered to be useful for acquiring microorganism with desirable phenotypes (Ma et al., 2017). By the strategy of repetitive cultivation in the same ethanol concentration and stepwise elevation of ethanol concentration, yeast strain could acquire remarkable adaptability in culture containing higher ethanol. The adapted strain with improved ethanol tolerance was named as Et20. In order to avoid self-hybrid had occurred on Et20, flow cytometry was applied to measure nuclear acid content, and haploid strain S288c was set as reference strain. The results were showed in Fig 1a, 1b. Nuclear acid content of strain Et20 was almost the same with that of strain S288c, illustrating strain Et20 was haploid.

Cell growth of haploid strain Et20 and its initial strain 20-Ha4 cultured in the presence of 5%, 10% and 20% (v/v) ethanol was showed in Fig 1c. Under low ethanol level (5%), haploid strains Et20 and 20-Ha4 presented similar lag and log phase of growth. When the culture contained 10% ethanol, the lag phase of both strain was extended, while strain Et20 experienced shorter lag phase time than 20-Ha4. Strain Et20 exhibited much higher growth rate than 20-Ha4. What's more, strain Et 20 was able to grow under the presence of 20% ethanol, whereas 20-Ha could never grow at all.

3.2 Screening of CTZ-resistant mutants

UV induced random mutagenesis was applied to acquire yeast strain with high-level tolerance towards inhibitors and improve fermentative activity. It had been reported that CTZ-resistant mutant of sake yeast showed enhanced fermentative activity than the parent strain during sake fermentation (Mizoguchi et al., 2002). Therefore, CTZ was used for screening strains with improved fermentative activity. The optimal UV mutagenesis condition and lethal

concentration of CTZ were pre-tested. The lethality was 19.89, 54.5, 75.9, 98.79, 99 and 99.53% under 30, 60, 90, 120, 150 and 180 s of UV treatment, respectively. The optimal UV treatment time was set as 90 s, as high lethality led high cell death and low lethality would result in low mutation rate. The lethal CTZ concentrations were 10 mg/L of haploid strain Et20 and 14 mg/L of haploid strain 30-Ha5. After 90 s of UV mutagenesis, 30-Ha5 cells were serial dilutions and spread on SD plates containing 14 mg/L CTZ and incubated at 28 ºC in dark for 2-7 days. A total of 44 mutants were isolated. By performing 8 generations of culturing onto the SD + 14 mg/L CTZ agar medium and fermentation activity test, we found 17 mutants whose total CO_2 production during 7 days were higher than that of the original strain 30-Ha5. Haploid strain CTZ30 exhibited the best fermentation activity was selected as candidate for protoplast fusion.

3.3 Protoplast fusion and screening of hybrids

The protoplast Et20 and heat-inactivated protoplast CTZ30 were fused under the optimal conditions, finally spread onto SD regeneration medium containing 14 mg/L CTZ, which was lethal for haploid strain Et20. No haploid protoplasts of Et20, diploid protoplasts of Et20, haploid protoplasts of CTZ30, and diploid protoplasts of CTZ30 could survive on the regeneration medium containing 14 mg/L CTZ. However, if haploid protoplasts Et20 and CTZ30 were successfully fused, the nuclear fusion and genetic recombination could repair the lethal damage (Peberdy, 1980). Therefore, diploid hybrids of Et20 and CTZ30 could grow on the regeneration medium. Fifty-two colonies were alive on the regeneration medium. To ensure hereditary stability, those colonies were purified onto SD agar medium containing 14 mg/L CTZ for 10 generations. As haploid yeast strains were not applied in commercial wine fermentation, original diploid strains BR20 and BR30 were used as control. After culturing in fermentation medium, seven diploid hybrids (F1, F3, F5, F8, F11, F21 and F23) showed better fermentation activity than original diploid strains BR20 and BR30 (Fig 2a). Diploid strain F23 produced the maximum content of CO_2 in the first two days, followed by F5 and F11. Though strain BR30 fermented fast in the first two days, its fermentation rate dropped rapidly from the third day. From the third day, all of the 7 hybrids showed higher fermentation activity than strains BR20 and BR30, and strains

F23, F21, F11and F5 showed superior post-fermentation activity. Thus, the four hybrids were selected for small scale of Chinese rice wine brewing.

Reducing sugar, total acidity and ethanol content results of Chinese rice wine fermented by selected hybrids during brewing were presented in Fig 2b, 2c, 2d. As Chinese rice wine was fermented in opened environment, it required yeast strain must grow fast to produce ethanol at initial stage to restrain excessive growth of other bacteria and avoid deterioration (Wu et al., 2015). Thus, total contents of reducing sugar were sharply decreased in initial-fermentation and then decreased slowly, ranging from 12.84-1.59 g/L. Whereas, ethanol contents showed an opposite trend, ranging from 47.20-134.70 g/L. As for total acidity, great rises were observed in initial-fermentation because of vigorous growth of microorganisms. Even though similar trends were shown in all the evolution curves, different strains exhibited different oenological properties. In the final Chinese rice wines, reducing sugar was utilized the most thoroughly by diploid hybrid F23, meanwhile, F23 wine produced the highest contents of ethanol and acidity. Ethanol content in final F23 wine (134.70 g/L) was respectively about 7.07% and 12.44% higher than BR30 (125.80 g/L) and BR20 (119.8 g/L) wines. Cell viabilities of diploid hybrids in the presence of different ethanol concentration were shown in Fig 2e. In contrast to hybrid strains, no colonies were found on the parental strains plate under ethanol stress above 20%. However, all of the 4 hybrid strains could survive well under 21% ethanol. Under 22% ethanol, cell viability of F23 was higher than 30% and the other three hybrids were lower than 30%. When the culture contained 25% ethanol, F23 showed a viability of 6.2%, whereas other hybrids could not survive. Spot assay (Fig 2f) showed strains F23 and F11 were more tolerant to 24% (v/v) ethanol, while strains of F5 and F21 were relatively sensitive, which were in consistent with the cell viability results. The significantly improved ethanol tolerance and oenological properties of diploid hybrid F23 demonstrated that united application of adaptive evolution, UV mutagenesis and protoplast fusion was an efficient approach to get superior industrial yeast strains.

3.4 Analysis of volatile flavor compounds

The volatile compounds in Chinese rice wines fermented by diploid strains F23, BR20 and BR30 produced at the third day, the twelfth day, and final wines (after sterilization) were listed in Table 2. No statistical difference of flavor compounds was observed between samples of the third day and the fifth day, and wine samples collected at the twelfth day and the fifteenth day exhibited similar numbers and contents of flavor compounds (data was showed in Fig S1). Thus, wine samples collected at the third day and the twelfth day were respectively selected as representative for the primary fermentation and secondary fermentation.

A total of 43 volatile compounds were identified, which included 9 alcohols, 21 esters, 6 aldehydes, 3 ketones, and 4 acids. Among which, benzyl alcohol, 2-phenethyl acetate and 2-heptanone were specific in F23 and BR30 wines, while decanal was only detected in F23 wine. During fermentation, the content of flavor compounds increased rapidly for the vigorous metabolisms of microorganisms. After sterilization, a great loss of flavor compounds was occurred in the three wines. Comparing with BR20 and BR30 wines, F23 wine showed noteworthy increase in the contents of 3-methyl-1-butanol, 2-phenylethanol, ethyl acetate, isoamyl acetate, ethyl-2-hydroxypropanoate, ethyl octanoate, ethyl hexadecanoate, ethyl (9E)-9-octadecenoate, linoleic acid ethyl ester, benzaldehyde and 2-octanone during fermentation. Hybrid F23 experienced more vigorous metabolism than BR20 and BR30, which might be resulted in the improvement of ethanol tolerance and fermentation activity.

The total contents of flavor compounds in final F23 wine (98.13 mg/L) was significantly higher than that of BR20 (81.78mg/L) and BR30 (77.54mg/L) wines. The flavor production in F23 wine was increased by 19.66-26.55% relative to the parent wines. Except for volatile acids, the highest contents of alcohols, esters, aldehydes and ketones were all observed in F23 wine, indicating F23 exhibited higher flavor producing capacity than the diploid parents. Though the total content of flavor compounds were similar in BR20 and BR30 wines, the content of individual flavor compound was different between them. No significant different was observed in esters concentration between final BR20 and BR30 wines, while the contents of alcohols and ketones in BR20 wine were significantly higher than that in BR30 wine. Alcohols and esters were generally considered as the major flavor compounds, which were closely related to yeast metabolism (Ubeda et al., 2016). In terms of alcohols, F23 wine (53.14 mg/L) showed respectively 25.57% and 13.64% higher

concentration of alcohol than that in BR30 and BR20 wines. The high concentration of 2- phenylethanol in F23 wine was responsible for the differentiation. As for esters, the total ester concentration in F23 wine was 30.14 mg/L, while that in BR20 and BR30 wines were 22.88 mg/L and 23.04 mg/L, respectively. F23 wine exhibited significant higher levels of ethyl acetate, isoamyl acetate, ethyl butanoate, ethyl-2-hydroxypropanoate, ethyl octanoate, ethyl hexadecanoate, ethyl (9E)-9-octadecenoate and linoleic acid ethyl ester than the other two wines. Hybrid F23 produced especially higher quantity in short-chain and long-chain fatty acid ethyl ester than BR20 and BR30.

The OAVs of determined flavor compounds were calculated and list in Table S1. It has been accepted that volatile compound with its concentration above its odor threshold (OAV>1) could significantly contribute its flavor to the overall aroma of wine (Noguerol-Pato et al. 2012). A total of 17 compounds were found with OAV>1 in F23 wine, whereas BR20 wine contained 15 compounds and BR30 wine contained 14. The OAVs of volatile compounds were significantly different in the 3 wines, except for ethyl decanoate, ethyl benzoate and 2-nonanone. Isoamyl acetate exhibited the highest OAV among all the flavor compounds, while the highest OAV was found in F23 wine (OAV=1020). The following substances with high OAVs were ethyl octanoate, benzeneacetaldehyde and nonanal, and their highest OAVs were still observed in F23 wine. The different numbers and OAVs of flavor compounds could be greatly responsible for the differentiation in the flavor profile of Chinese rice wine. Hybrid F23 produced flavor compounds with higher OAVs and more flavor contributors with OAV>1 relative to its diploid parents, suggesting that hybrid F23 introduce more flavor diversity to Chinese rice wine.

3.5 PCA of volatile flavor compounds during Chinese rice wine brewing

PCA was conducted using the concentrations of 43 volatile flavor compounds in BR20, BR30 and F23 wines during brewing as analytical variables. The association between the 43 compounds in three wines during different brewing stages was presented in Fig 3.

The results in Fig 3a revealed loading plots, whereby the first, the second and the third principal components (PC) respectively explained 44.69%, 16.92% and 14.17% of the total variance, which reflected 75.78% of the total flavor compounds information. Thirty-nine compounds positively loaded on

PC 1, containing high positive loadings for 1-butanol, ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl hexadecanoate, linoleic acid ethyl ester and benzeneacetaldehyde. In addition, those high loading compounds on PC1 also exhibited high OAVs, except for 1-butanol and linoleic acid ethyl ester.

The PC scores of BR20, BR30 and F23 wines during brewing were shown in Fig 3b. A good separation of different wines could be observed, and 3 groups were well-defined. The flavor characteristic of Chinese rice wines after sterilization were different with wines during fermentation. Through sterilization, the great loss of flavor compounds might account for the difference. Group 1 was formed by final F23 and BR20 wines which were distinctly differentiated from other wines for the large negative value on PC 3. It was mainly affected by the presence of 1-butanol, 2-heptanol, 2-furanmethanol, ethyl decanoate, γ-nonanolactone and 2-nonanone. Final BR30 wine characterized by 2-methyl-1-propanol, 1-butano, diethyl succinate, ethyl dodecanoate, furfural and 2-octanone was alone in group 2 for it highly positive positioned on PC 3. All the 3 wines during fermentation could be contained in group 3, which mean the 3 wines had similar flavor characteristic. The predominant flavor compounds of the 3 wines during fermentation were esters. It is important to note that the flavor characteristics of Chinese rice wines fermented by hybrid F23 and yeast parents exist correlations, though each yeast produced its own unique characteristic. The flavor characteristic of Chinese rice wine fermented 12 days by F23 scored the highest (data not shown), which mean the flavor characteristic exhibited in F23 wine could well present flavor characteristic of wines fermented by each diploid parent. Hybrid F23 might be used as a “mixed-like” starter to introduce more flavor complexity and aroma to Chinese rice wine.

3.6 Sensory evaluation

In consideration of consumer preferences, sensory evaluation of Chinese rice wines produced by diploid hybrid F23 and diploid parents was conducted. The results of sensory evaluation in final BR20, BR30 and F23 wines were presented in Fig 4. The three wines exhibited similar appearance of yellowness and turbidity. Comparing with BR30 wine, F23 wine exhibited high levels in alcohol-aroma, fruit-aroma, full body and continuation mouthfeel, while BR20 wine was intense in sweet, bitter and astringency taste. The high intensity in aroma of alcohol and fruit in F23 wine was in agreement with the high alcohols

amounts and OAVs of major contributors presented in Table 2, S1. The yeast parents performed poorly in the alcohol-aroma, fruit-aroma, continuation and full body of mouthfeel (relative popular for consumers) relative to hybrid F23. Although different wines exhibited its own flavor characteristic, the yeast parents performed poorly in the alcohol-aroma, fruit-aroma, continuation and full body of mouthfeel (relative popular for consumers) relative to hybrid F23. The F23 wine was the most highly assessed wine in total mouthfeel, followed by BR30 and BR20 wines.

4. Conclusions

In order to produce quality Chinese rice wine under high ethanol accumulation and open environment, fermenting yeast with superior ethanol tolerance and fermentation activity was created. Strategies of adaptive evolution, UV mutagenesis and protoplast fusion were conducted to obtain yeast diploid hybrids with excellent oenological characteristic. The flavor profiles in Chinese rice wine were improved by using yeast diploid hybrid F23, which exhibited higher ethanol tolerance and fermentation activity than the original diploid parents. The total content of flavor compounds in F23 was 19.99-26.55% higher than that of parent wines. Compared to parent strains, F23 introduced more flavor contributors with OAVs above one to Chinese rice wine, and those contributors were found with higher OAVs. The results indicating F23 could introduce more flavor diversity to Chinese rice wine than its parents. Flavor characteristic of F23 wine was similar to wine fermented by each parent by PCA. Additionally, F23 wine was highly assessed for its intensive levels in fruit-aroma, alcohol-aroma and mouthfeel, which were pleasant for customer. The acquired hybrid F23 not only displayed superior flavor production and oenological performance, but also could be potentially used as “mixed-like” starter to enrich wine style and differentiation. As the problem in degeneration of fermenting yeast in some wineries, the strategy could be applied to enhance flavor profiles and yield of other alcoholic beverages. In addition, the mechanism of hybrid F23 introduce improved flavor profiles to Chinese rice wine will be studied in our future work.

Acknowledgement

This work was supported by the Capacity building project of Shanghai (15150502400); the Project of industry transformation and upgrading of Shanghai (CXY-2016-016); the Shanghai Natural Science Fund (15ZR1428900); Key project of special development fund in national self-innovative pilot area (Zhangjiang, Shanghai) (201705-PD-LJZ-B2074-007); and the Graduate student innovation fund project of Shanghai (JWCXSL1402). The authors acknowledge to Shanghai Jinfeng wine Co., Ltd. for the cooperation in Enterprise-University-Research.

References

Bellon, J., Eglinton, J., Siebert, T., Pollnitz, A., Rose, L., de Barros Lopes, M., Chambers, P. (2011). Newly generated interspecific wine yeast hybrids introduce flavour and aroma diversity to wines. *Applied Microbiology and Biotechnology, 91(3)*, 603-612.

Bellon, J., Yang, F., Day, M., Inglis, D., Chambers, P. (2015). Designing and creating *Saccharomyces* interspecific hybrids for improved, industry relevant, phenotypes. *Applied Microbiology and Biotechnology, 99(20)*, 8597-8609.

Bisson, L.F. (2017). Yeast hybrids in winemaking. *Catalyst, 1*, 27-34.

Ciesarova, Z., Šmogrovičová, D., Dömény, Z. (1996). Enhancement of yeast ethanol tolerance by calcium and magnesium. *Folia Microbiology, 41(6)*, 485-488.

Curran, B.P.G., Bugeja, V.C. (1996). Protoplast fusion in *Saccharomyces cerevisiae*. In Evans, I. (Ed.), *Yeast protocols* (pp.45-49). New Jersey: Humana Press Inc.

Chen, S., Xu, Y. (2010). The influence of yeast strains on the volatile flavour compounds of Chinese rice wine. *Journal of the Institute of Brewing, 116(2)*, 190-196.

Chen, S., Xu, Y. (2014). Adaptive evolution of *Saccharomyces cerevisiae* with enhanced ethanol tolerance for Chinese rice wine fermentation. *Applied Biochemistry and Biotechnology, 173(7)*, 1940-1954.

dos Santos Navarro, R. D. C., Minim, V. P. R., Simiqueli, A. A., da Silva Moraes, L. E., Gomide, A. I., Minim, L. A. (2012). Optimized descriptive profile:

A rapid methodology for sensory description. *Food Quality and Preference, 24(1),* 190-200.

Di Egidio, V., Sinelli, N., Giovanelli, G., Moles, A., Casiraghi, E. (2010). NIR and MIR spectroscopy as rapid methods to monitor red wine fermentation. *European Food Research Technology, 230(6),* 947-955.

Dorit, S. (2011). Better yeast for better wine-genetic improvement of *saccharomyces cerevisiae* wine strains. In Rai, M. (Ed.), *Progress in mycology* (pp.1-49). German: Springer Science and Business Media.

De la Torre-González, F.J., Narváez-Zapata, J.A., López-y-López, V.E., Larralde-Corona, C.P. (2016). Ethanol tolerance is decreased by fructose in *Saccharomyces* and non-*Saccharomyces* yeasts. *LWT-Food Science and Technology, 67,* 1-7.

GB/T 13662-2008. Official standards. China: Chinese rice wine.

GB/T 5517-2010. Official methods of analysis. China: Determination of total acidity.

Hirooka, K., Yamamoto, Y., Tsutsui, N., Tanaka, T. (2005). Improved production of isoamyl acetate by a sake yeast mutant resistant to an isoprenoid analog and its dependence on alcohol acetyltransferase activity, but not on isoamyl alcohol production. *Journal of Bioscience and Bioengineering, 99(2),* 125-9.

Inoue, T., Iefuji, H., Katsumata, H. (2012). Characterization and isolation of mutants producing increased amounts of isoamyl acetate derived from hygromycin B-resistant sake yeast. *Bioscience Biotechnology and Biochemistry, 76(1),* 60-66.

ISO (1993). Sensory analysis—General guidance for selection, training and monitoring of assessors. Switzerland: International Organization for Standardization Geneva.

Jung, H., Lee, S., Lim, J., Kim, B., Park, K. (2014). Chemical and sensory profiles of makgeolli, Korean commercial rice wine, from descriptive, chemical, and volatile compound analyses. *Food Chemistry, 152,* 624-632.

King, E., Swiegers, J., Travis, B., Francis, I., Bastian, S., Pretorius, I. (2008). Coinoculated fermentations using *Saccharomyces* yeasts affect the volatile composition and sensory properties of Vitis vinifera *L.cv. Sauvignon blanc* wines. *Journal of Agriculture and Food Chemistry, 56(22),* 10829-10837.

Krogerus, K., Magalhães, F., Vidgren, V., Gibson, B. (2015). New lager yeast strains generated by interspecific hybridization. *J of Indusrial Microbiology and Biotechnology, 42(5)*, 769-78.

Landaud, S., Helinck, S., Bonnarme, P. (2008). Formation of volatile sulfur compounds and metabolism of methionine and other sulfur compounds in fermented food. *Applied Microbiology and Biotechnology, 77*, 1191-1205.

Li, X.M., Shen, C., Wu, D.H., Lu, J., Chen, J., Xie, G.F. (2015). Enhancement of urea uptake in Chinese rice wine yeast strain N85 by the constitutive expression of DUR3 for ethyl carbamate elimination. *Journal of the Institute of Brewing, 121(2)*, 257-261.

Lu, Q.Y., Lee, R.P., Huang, J.J., Yang, J.P., Henning, S.M., Hong, X.T., Heber, D., Li, Z.P. (2015). Quantification of bioactive constituents and antioxidant activity of Chinese yellow wine. *Journal of Food Composition and Analysis, 44*, 86-92.

Lv, X.C., Jia, R.B., Li, Y., Chen, F., Chen, Z.C., Liu, B., Chen, S.J., Rao, P.F., Ni, L. (2016). Characterization of the dominant bacterial communities of traditional fermentation starters for Hong Qu glutinous rice wine by means of MALDI-TOF mass spectrometry fingerprinting, 16S rRNA gene sequencing and species-specific PCRs. *Food Control, 67*, 292-302.

Miller, G. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31(3)*, 426-428.

Mizoguchi, H., Yamauchi, T., Watanabe, M., Yamanaka, H., Nishimura, A., Hanamoto, H. (2002). Different missense mutations in PDR1 and PDR3 genes from clotrimazole-resistant sake yeast are responsible for pleiotropic drug resistance and improved fermentative activity. *Journal of Bioscience and Bioengineering, 93(2)*, 221-227.

Meijnen, J.P., Randazzo, P., Foulquié-Moreno, M.R., den Brink, J., Vandecruys, P., Stojiljkovic, M., Dumortier, F., Zalar, P., Boekhout, T., Gunde-Cimerman, N., Kokošar, J. (2016). Polygenic analysis and targeted improvement of the complex trait of high acetic acid tolerance in the yeast *Saccharomyces cerevisiae*. *Biotechnology for Biofuels, 9(1)*, 5.

Ma, K.D., He, M.X., You, H.Y., Pan, L.W., Hu, G.Q., Cui, Y.B., Maeda, T. (2017). Enhanced fuel ethanol production from rice straw hydrolysate by an

inhibitor-tolerant mutant strain of Scheffersomyces stipitis. *RSC Advance, 7(50),* 31180-31188.

Nabais, R. C., Sá-Correia, I., Viegas, C. A., Novais, J. M. (1988). Influence of calcium ion on ethanol tolerance of *Saccharomyces bayanus* and alcoholic fermentation by yeasts. *Applied Environment and Microbiology, 54(10),* 2439-2446.

Noguerol-Pato, R., González-Álvarez, M., González-Barreiro, C., Cancho-Grande, B., Simal-Gándara, J. (2012). Aroma profile of Garnacha Tintorera-based sweet wines by chromatographic and sensorial analyses. *Food Chemistry, 134(4),* 2313-2325.

Novo, M., Gonzalez, R., Bertran, E., Martínez, M., Yuste, M., Morales, P. (2014). Improved fermentation kinetics by wine yeast strains evolved under ethanol stress. *LWT-Food Science and Technology, 58(1),* 166-172.

Nakagawa, K., Katayama, T., Yamamoto, T., Maeda, K. (2016). Alcoholic fermentation by yeast is improved by immobilization in freeze-dried poly (vinyl alcohol) foam. *Journal of Chemical Engineering of Japan, 49(7),* 707-713.

Pais, T.M., Foulquié-Moreno, M.R., Hubmann, G., Duitama, J., Swinnen, S., Goovaerts, A., Yang, Y.D., Dumortier, F., Thevelein, J.M. (2013). Comparative polygenic analysis of maximal ethanol accumulation capacity and tolerance to high ethanol levels of cell proliferation in yeast. *PLoS Genetics, 9(6),* p.e 1003548.

Peberdy, J.F. (1980). Protoplast fusion-a tool for genetic manipulation and breeding in industrial microorganisms. *Enzyme and microbial technology*, 2, 23-29.

Pérez-Través, L., Lopes, C., Barrio, E., Querol, A. (2012). Evaluation of different genetic procedures for the generation of artificial hybrids in Saccharomyces genus for winemaking. *International Journal of Food Microbiology, 156(2),* 102-111.

Pires, E., Teixeira, J., Branyik, T., Vicente, A. (2014). Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. *Applied Microbiology and Biotechnology, 98,* 1937-1949.

Sauer, U. (2001). Evolutionary engineering of industrially important microbial phenotypes. *Advances in Biochemical Engineering-Biotechnology, 73*, 129-

170.

Shi, D.J., Wang, C.L., Wang, K.M. (2008). Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. *Journal of Industrial Microbiology and Biotechnology, 36(1)*, 139-147.

Steensels, J., Meersman, E., Snoek, T., Saels, V., Verstrepen, K. (2014). Large-Scale selection and breeding to generate industrial yeasts with superior aroma production. *Applied Environment and Microbiology, 80(22)*, 6965-6975.

Steensels, J., Verstrepen, K.J. (2014). Taming wild yeast: potential of conventional and nonconventional yeasts in industrial fermentations. *Annual Review of Microbiology, 68*, 61–80.

Snoek, T., Verstrepen, K., Voordeckers, K. (2016). How do yeast cells become tolerant to high ethanol concentrations? *Current Genetics, 62(3)*, 475-480.

Takahashi, T., Ohara, Y., Sueno, K. (2017). Breeding of a sake yeast mutant with enhanced ethyl caproate productivity in sake brewing using rice milled at a high polishing ratio. *Journal of Bioscience and Bioengineering, 123(6)*, 707-713.

Ubeda, C., Callejón, R.M., Troncoso, A.M., Peña-Neira, A., Morales, M.L. (2016). Volatile profile characterisation of Chilean sparkling wines produced by traditional and Charmat methods via sequential stir bar sorptive extraction. *Food Chemistry, 207*, 261-271.

Valles, B.S., Bedriñana, R.P., Tascón, N.F., Simón, A.Q., Madrera, R.R. (2007). Yeast species associated with the spontaneous fermentation of cider. *Food Microbiology, 24(1)*, 25-31.

Wu, Z.Z., Xu, E.B., Long, J., Zhang, Y.J., Wang, F., Xu, X.M., Jin, Z.Y., Jiao, A.Q. (2015). Monitoring of fermentation process parameters of Chinese rice wine using attenuated total reflectance mid-infrared spectroscopy. *Food Control, 50*, 405-412.

Xu, Y., Zhao, G.A., Wang, L.P. (2006). Controlled formation of volatile components in cider making using a combination of *Saccharomyces cerevisiae* and *Hanseniaspora valbyensis* yeast species. *Journal of Industrial Microbiology and Biotechnology, 33(3)*, 192-196.

Yazawa, H., Iwahashi, H. and Uemura, H. (2007). Disruption of URA7 and GAL6 improves the ethanol tolerance and fermentation capacity of

Saccharomyces cerevisiae. *Yeast, 24(7)*, 551-560.

Ye, M.Q., Yue, T.L., Yuan, Y.H., Wang, L.S. (2013). Production of yeast hybrids for improvement of cider by protoplast electrofusion. *Biochemical Engineering Journal, 81*, 162-169.

Yang, Y.J, Yu, J.S., Xia, Y.J. Fang, Y.Q., Ni, B., Wang, G.Q., Ai, L.Z. (2015). Screening for quality strains of yeast from yellow rice wine and stress resistance analysis. *Modern Food Science and Technology, 31(8)*, 261-267.

Yang, Y.J., Xia, Y.J., Wang, G.Q., Yu, J.S., Ai, L.Z. (2017). Effect of mixed yeast starter on volatile flavor compounds in Chinese rice wine during different brewing stages. *LWT - Food Science and Technology, 78*, 373-381.

Zhang, C., Zong, H., Zhuge, B., Lu, X.Y., Fang, H.Y, Zhu, J.L., Zhuge, J. (2016). Protoplast preparation and polyethylene glycol (PEG)-mediated transformation of Candida glycerinogenes. *Biotechnology and Bioprocess Engineering, 21(1)*, 95-102.

Table 1 Definitions and reference scales of sensory attributes for Chinese rice wine.

Attributes	Definitions	Reference scales
Appearance		
Color	Color observed	No standards
Turbidity	Degree of haziness	No standards
Aroma		
Alcohol-aroma	Smell related to alcohol	Minimum: Water; Medium: 12.5% (w/v) Ethanol; Maximum: 25% (w/v) Ethanol.
Fruit-aroma	From fruit aroma (e.g. Banana)	Minimum: Water; Medium: 7.5 g Crushed banana/100 mL distilled water; Maximum: 15 g Crushed banana/100 mL distilled water.
Cereal-aroma	Smell related to rice or barley	Minimum: Water; Medium: 2 g Crushed unpolished rice or barley/20 mL distilled water; Maximum: 4 g Crushed unpolished rice or barley/20 mL distilled water.
Taste		
Sweet	Sucrose as typical	Minimum: Drinking water; Medium: 3% (w/v) Sucrose; Maximum: 6% (w/v) Sucrose.
Sour	Vinegar taste	Minimum: Drinking water; Medium: 0.1 mL Vinegar/100 mL distilled water; Maximum: 0.2 mL Vinegar/100 mL distilled water.
Bitter	Caffeine as typical	Minimum: Drinking water; Medium: 0.05% (w/v) Caffeine; Maximum: 0.1% (w/v) Caffeine.
Mouthfeel		
Astringency	Dryness mouthfeel	Minimum: Chinese rice wine aged for 8 years (Shanghai Jinfeng Wine Co., Ltd., Shanghai, China); Medium: Chinese rice wine aged for 4 years (Shanghai Jinfeng Wine Co., Ltd.); Maximum: Young Chinese rice wine (Shanghai Jinfeng Wine Co., Ltd.).
Continuation	Feeling of continuing taste	Minimum: Young Chinese rice wine; Medium: Chinese rice wine aged for 4 years; Maximum: Chinese rice wine aged for 8 years.
Full body	General feeling while tasting	

Table 2 Identification and relative contents of volatile flavor compounds in Chinese rice wine fermented by hybrid F23 and its diploid parents BR20 and BR30 (n=3)

Compounds	Code	Identification*	KI**	Relative content (mg/L)								
				3d[d]	12d[d]	STE[d]	3d[e]	12d[e]	STE[e]	3d[f]	12d[f]	STE[f]
Alcohols												
2-Methyl-1-propanol	al1	MS, RI	1094	3.93±0.05[a]	3.25±0.08[b]	3.17±0.10[c]	4.06±0.09[a]	3.72±0.12[b]	3.23±0.06[c]	3.42±0.08[b]	3.90±0.17[a]	3.10±0.09[c]
1-Butanol	al2	MS, RI	1158	0.06±0.002[b]	0.16±0.003[a]	0.17±0.003[a]	0.03±0.001[b]	0.11±0.004[a]	0.10±0.008[a]	0.09±0.004[c]	0.27±0.011[b]	0.35±0.022[a]
3-Methyl-1-butanol	al3	MS, RI	1212	21.15±0.81[b]	26.12±0.72[a]	19.54±0.87[c]	22.17±0.62[b]	25.71±0.92[a]	19.18±0.52[c]	26.54±0.67[b]	26.08±0.70[a]	21.99±0.64[c]
2-Heptanol	al4	MS, RI	1330	0.10±0.004[a]	0.12±0.008[a]	0.11±0.012[a]	0.16±0.028[b]	0.19±0.020[a]	0.19±0.022[a]	0.11±0.016[c]	0.17±0.015[b]	0.22±0.013[a]
1-Hexanol	al5	MS, RI	1342	0.48±0.017[c]	0.66±0.028[a]	0.57±0.011[b]	0.10±0.007[c]	0.38±0.012[a]	0.21±0.009[b]	0.40±0.022[c]	0.61±0.037[a]	0.48±0.026[b]
2-Furanmethanol	al6	MS, RIL	1661	0.14±0.008[a]	0.11±0.011[b]	0.10±0.015[b]	0.17±0.024[b]	0.14±0.004[b]	0.18±0.006[a]	0.11±0.005[b]	0.13±0.013[a]	0.11±0.021[b]
Citronellol	al7	MS, RIL	1772	ND	ND	ND	0.042±0.002[a]	0.044±0.001[a]	0.037±0.001[b]	0.059±0.001[a]	0.057±0.001[b]	0.057±0.001[b]
Benzyl alcohol	al8	MS, RI	1876	0.02±0.001[c]	0.31±0.004[a]	0.11±0.008[b]	0.11±0.019[b]	0.17±0.025[a]	0.10±0.023[b]	0.046±0.027[a]	0.09±0.005[b]	0.08±0.005[b]
2-Phenylethanol	al9	MS, RI	1915	19.86±0.66[c]	28.09±0.73[a]	23.00±0.56[b]	19.80±0.78[b]	25.77±0.96[a]	19.10±0.65[c]	23.45±0.83[c]	32.81±0.97[a]	26.76±0.72[b]
Total				**45.74**	**58.82**	**46.76**	**46.64**	**56.23**	**42.32**	**54.22**	**64.12**	**53.14**
Esters												
Ethyl acetate	et1	MS, RI	894	2.83±0.12[c]	4.76±0.24[a]	3.08±0.17[b]	2.89±0.009[b]	3.67±0.11[a]	2.22±0.17[c]	2.86±0.10[c]	5.44±0.32[a]	3.94±0.21[b]
Ethyl butanoate	et2	MS, RI	1049	0.16±0.002[b]	0.46±0.010[a]	0.17±0.004[b]	0.12±0.002[b]	0.68±0.008[a]	0.26±0.005[b]	0.53±0.010[c]	1.04±0.027[a]	0.72±0.008[b]
Isoamyl acetate	et3	MS, RI	113	2.12±0.07	2.64±0.10	2.15±0.08	2.21±0.11[c]	2.68±0.13[a]	2.34±0.07[b]	2.59±0.09[c]	3.24±0.11[a]	3.06±0.15[b]

			5	b	a	b						
Ethyl hexanoate	et4	MS, RI	1242	0.12±0.003[b]	0.16±0.002[a]	0.17±0.003[a]	0.12±0.004 b	0.18±0.002 a	0.10±0.005 b	0.10±0.008 b	0.21±0.013 b	0.19±0.007 a
Ethyl-2-hydroxypropanoate	et5	MS, RI	1357	0.77±0.05 c	1.69±0.09 a	1.54±0.02 b	0.91±0.008 b	1.83±0.02[a]	1.87±0.07[a]	1.28±0.09[c]	2.33±0.02[a]	2.17±0.07[b]
Ethyl octanoate	et6	MS, RI	1447	1.48±0.16 c	1.75±0.11 b	1.82±0.10 a	2.05±0.08[b]	2.30±0.12[a]	1.97±0.09[c]	2.05±0.11[c]	2.73±0.14[b]	2.86±0.12[a]
Ethyl nonanoate	et7	MS, RI	1561	0.10±0.002[a]	0.11±0.002[a]	0.10±0.003[a]	0.10±0.002 a	0.12±0.002 a	0.11±0.004 a	0.10±0.002 b	0.10±0.002 b	0.15±0.002 a
Ethyl 2-hydroxy-4-methylvalerate	et8	MS, RIL	1572	0.14±0.003[b]	0.18±0.004[a]	0.17±0.002[a]	0.17±0.003 a	0.15±0.004 b	0.12±0.001 c	0.16±0.002 b	0.13±0.001 c	0.34±0.003 a
Ethyl decanoate	et9	MS, RI	1644	0.51±0.06 c	0.63±0.05 a	0.56±0.09 b	0.69±0.10[c]	0.85±0.07[a]	0.81±0.05[b]	0.68±0.02[c]	0.93±0.01[a]	0.81±0.02[b]
Ethyl benzoate	et10	MS, RI	1671	1.14±0.11 c	1.92±0.09 a	1.49±0.12 b	1.57±0.08[b]	1.90±0.05[a]	1.45±0.01[c]	1.49±0.04[c]	1.80±0.08[a]	1.66±0.05[b]
Diethyl succinate	et11	MS, RI	1688	0.16±0.003[c]	0.47±0.007[a]	0.22±0.005[b]	0.31±0.007 b	0.38±0.008 a	0.19±0.005 c	0.27±0.004 c	0.52±0.008 a	0.43±0.009 b
Ethyl 4-hydroxybutanoate	et12	MS, RI	1804	0.13±0.002[b]	0.14±0.002[b]	0.17±0.002[a]	0.19±0.001 a	0.14±0.004 b	0.13±0.001 b	0.14±0.002 c	0.29±0.004 b	0.33±0.007 a
2-Phenethyl acetate	et13	MS, RI	1823	ND	ND	ND	0.37±0.07[a]	0.38±0.04[a]	0.19±0.01[b]	0.25±0.03[b]	0.49±0.05[a]	0.46±0.02[a]
Ethyl dodecanoate	et14	MS, RI	1837	0.07±0.001[c]	0.11±0.002[b]	0.34±0.006[a]	0. 18±0.004[a]	0.17±0.008 a	0.14±0.004 b	0.10±0.003 c	0.36±0.010 b	0.43±0.007 a
γ-Nonanolactone	et15	MS, RIL	2016	0.12±0.002[a]	0.13±0.002[a]	0.13±0.002[a]	0.18±0.006 a	0.15±0.004 b	0.10±0.001 c	0.19±0.001 a	0.16±0.004 b	0.15±0.007 b
Ethyl tetradecanoate	et16	MS, RI	2038	0.28±0.05 c	0.42±0.03 b	0.71±0.07 a	0.34±0.01[c]	0.63±0.06[a]	0.56±0.07[b]	0.66±0.01[b]	0.78±0.03[a]	0.62±0.02[c]
Ethyl	et17	MS, RIL	214	0.19±0.00	0.23±0.01	0.24±0.00	0.17±0.004	0.19±0.012	0.17±0.004	0.26±0.003	0.35±0.007	0.32±0.011

pentadecanoate			6	6[b]	0[a]	7[a]	[b]	[a]	[b]	[c]	[a]	[b]
Ethyl hexadecanoate	et18	MS, RI	2254	6.56±0.22[c]	7.28±0.27[a]	6.61±0.19[b]	6.68±0.14[c]	7.16±0.20[a]	6.82±0.13[b]	6.83±0.21[c]	8.90±0.18[a]	7.54±0.13[b]
Ethyl (9E)-9-octadecenoate	et19	MS, RIL	2470	0.91±0.13[c]	1.14±0.09[b]	1.26±0.04[a]	0.82±0.02[c]	1.27±0.06[b]	1.44±0.01[a]	0.97±0.08[c]	1.73±0.03[b]	2.11±0.09[a]
Ethyl-(9Z, 12Z)-9,12-Octadecadienoate	et20	MS, RI	2486	0.27±0.007[b]	0.33±0.005[a]	0.32±0.004[a]	0.24±0.006[b]	0.28±0.004[a]	0.28±0.008[a]	0.29±0.006[c]	0.45±0.007[a]	0.42±0.006[b]
Linoleic acid ethyl ester	et21	MS, RIL	2521	1.12±0.03[c]	1.51±0.04[b]	1.63±0.07[a]	0.95±0.12[c]	1.50±0.15[b]	1.77±0.11[a]	0.93±0.08[c]	1.71±0.06[b]	1.93±0.06[a]
Total				**19.18**	**26.06**	**22.88**	**21.08**	**26.61**	**23.04**	**22.73**	**33.69**	**30.64**
Aldehydes												
Nonanal	ad1	MS, RIL	1389	0.20±0.003[c]	0.68±0.008[a]	0.31±0.006[b]	0.31±0.003[b]	0.74±0.008[a]	0.29±0.004[b]	0.18±0.002[c]	0.62±0.006[a]	0.24±0.004[b]
Furfural	ad2	MS, RI	1463	ND	0.32±0.004[b]	0.51±0.004[a]	0.24±0.002[c]	0.61±0.008[a]	0.28±0.004[b]	0.37±0.005[b]	0.79±0.007[a]	0.36±0.004[b]
Decanal	ad3	MS, RI	1501	ND	ND	ND	ND	ND	ND	0.10±0.002[c]	0.27±0.002[a]	0.18±0.003[b]
Benzaldehyde	ad4	MS, RI	1518	2.74±0.07[c]	4.84±0.15[a]	3.34±0.13[b]	2.46±0.10[c]	4.78±0.17[a]	4.12±0.11[b]	3.23±0.09[c]	5.42±0.18[a]	4.74±0.17[b]
Benzeneacetaldehyde	ad5	MS, RI	1640	1.64±0.09[a]	1.32±0.04[c]	1.45±0.02[b]	1.42±0.06[a]	1.20±0.05[c]	1.34±0.11[b]	1.83±0.05[b]	1.86±0.06[a]	1.47±0.07[c]
4-Methyl-benzaldehyde	ad6	MS, RI	1647	0.11±0.002[b]	0.15±0.002[a]	0.14±0.003[a]	0.38±0.006[a]	0.27±0.004[b]	0.11±0.002[c]	0.30±0.003[c]	0.60±0.009[a]	0.41±0.008[b]
Total				**4.69**	**7.31**	**5.75**	**4.81**	**7.6**	**6.14**	**6.01**	**9.56**	**7.4**
Ketones												
2-Heptanone	kt1	MS, RI	1184	ND	ND	ND	0.15±0.003[a]	0.12±0.002[c]	0.13±0.001[b]	0.14±0.001[a]	0.13±0.002[a]	0.12±0.002[a]

2-Octanone	kt2	MS, RI	1312	1.12±0.04^{c}	2.06±0.07^{b}	2.35±0.06^{a}	1.24±0.01^{c}	1.43±0.04^{b}	1.67±0.03^{a}	1.66±0.09^{b}	2.38±0.06^{a}	2.85±0.04^{a}
2-Nonanone	kt3	MS, RI	1390	0.14±0.003^{c}	0.23±0.005^{a}	0.18±0.003^{b}	0.13±0.002^{a}	0.11±0.001^{a}	0.13±0.002^{a}	0.12±0.002^{a}	0.14±0.001^{a}	0.13±0.002^{a}
Total				**1.26**	**2.29**	**2.53**	**1.52**	**1.66**	**1.93**	**1.94**	**2.65**	**3.1**
Acids												
Acetic acid	ac1	MS, RI	1426	3.21±0.06^{b}	5.66±0.14^{a}	2.81±0.09^{c}	3.03±0.07^{c}	5.09±0.16^{a}	3.16±0.11^{b}	3.53±0.07^{b}	5.60±0.12^{a}	3.06±0.10^{c}
Isobutyric acid	ac2	MS, RI	1584	0.82±0.04^{b}	1.49±0.01^{a}	0.69±0.02^{c}	0.86±0.03^{b}	1.23±0.01^{a}	0.63±0.04^{c}	0.56±0.02^{b}	1.10±0.05^{a}	0.52±0.04^{c}
Hexanoic acid	ac3	MS, RI	1853	0.12±0.002^{b}	0.11±0.001^{b}	0.16±0.002^{a}	0.09±0.001^{c}	0.42±0.007^{a}	0.20±0.005^{b}	0.10±0.003^{b}	0.12±0.002^{a}	0.10±0.002^{b}
Octanoic acid	ac4	MS, RI	2056	0.08±0.001^{c}	0.11±0.002^{b}	0.20±0.003^{a}	0.06±0.001^{b}	0.05±0.001^{b}	0.12±0.001^{a}	0.09±0.001^{b}	0.15±0.002^{a}	0.17±0.003^{a}
Total				**4.23**	**7.37**	**3.86**	**4.04**	**6.89**	**4.11**	**4.28**	**6.97**	**3.85**

Note: “ND”, not detected. “3d” represents wines ferment for 3 days; “12d” represents wines ferment for 12 days; “STE” represents final wines after sterilization.

Different letters in each row indicate significant differences (*p<0.05, Duncan's tests*) between Chinese rice wine samples fermented by different yeast starters during brewing.

[*] MS, compounds were identified by MS spectra. RI, compounds were identified by comparison to pure standard. RIL, compounds were identified by comparison with RI from http://webbook.nist.gov/.

[**] KI is the retention index calculated by the Kovats method.

[d] The relative contents (mg/L) of flavor compounds in BR20 wine during different brewing stages.

[e] The relative contents (mg/L) of flavor compounds in BR30 wine during different brewing stages.

[f] The relative contents (mg/L) of flavor compounds in F23 wine during different brewing stages.

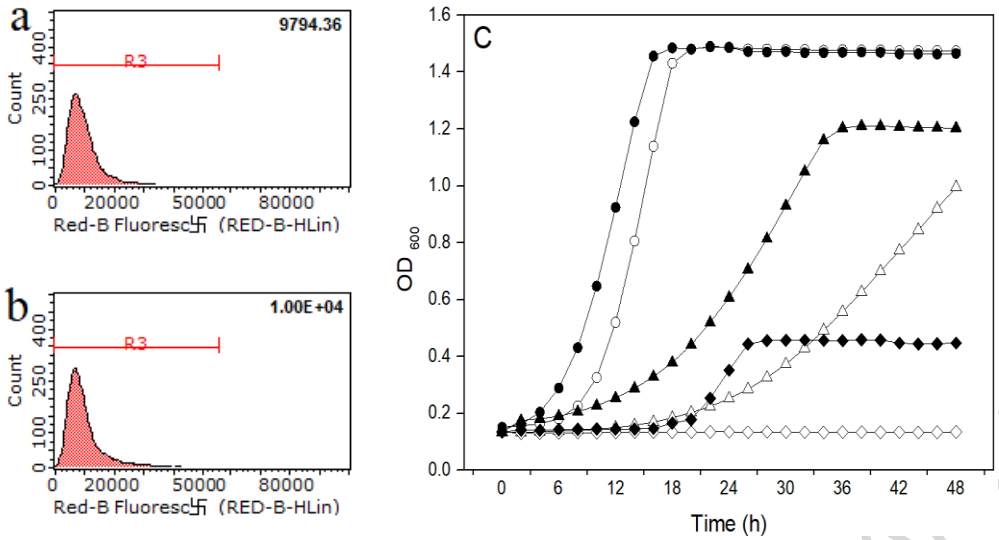
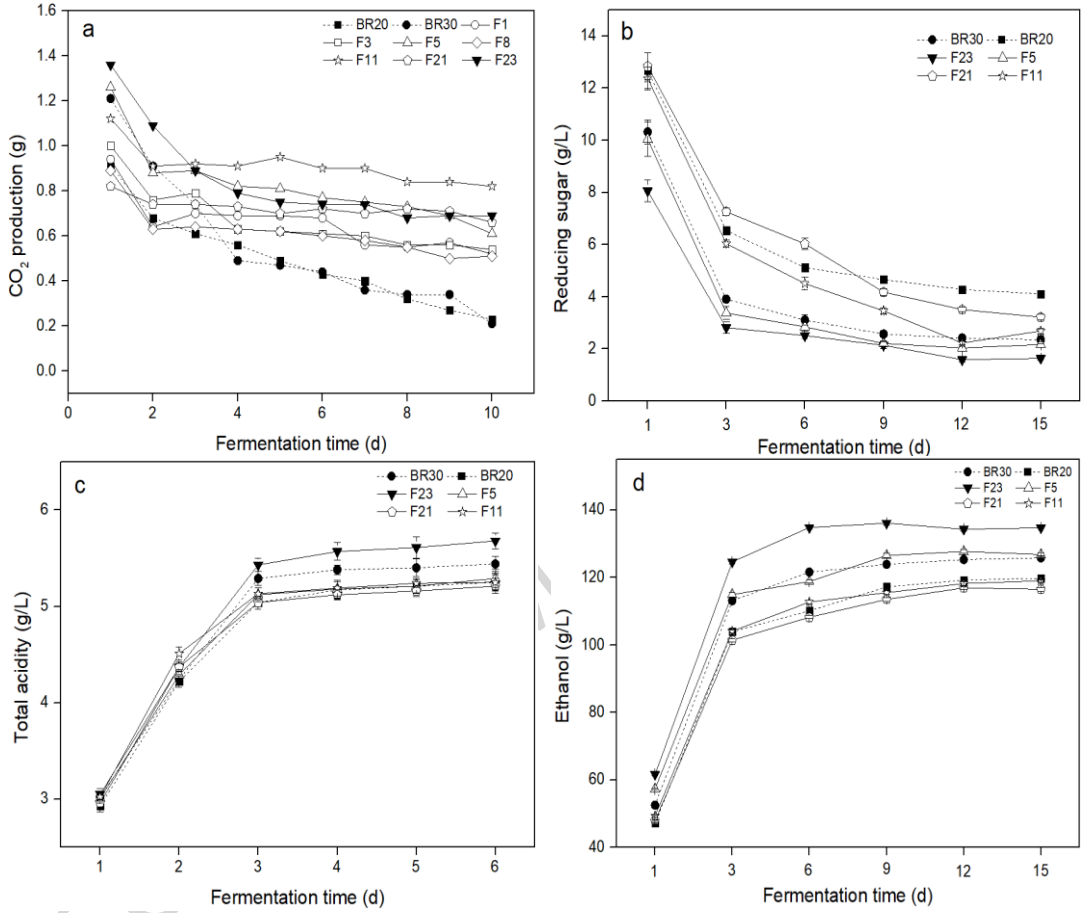


Fig.1 Flow cytometry analysis and cell growth of yeast strains. **(a)** DNA content of strain Et20. **(b)** DNA content of reference strain S288c. **(c)** Cell growth of initial strain 20-Ha4 and ethanol domesticated strain Et20 growing in YPD containing 5% (circles), 10% (triangles) and 20% (diamonds) ethanol. Open symbols, 20-Ha4; closed symbols, Et 20.



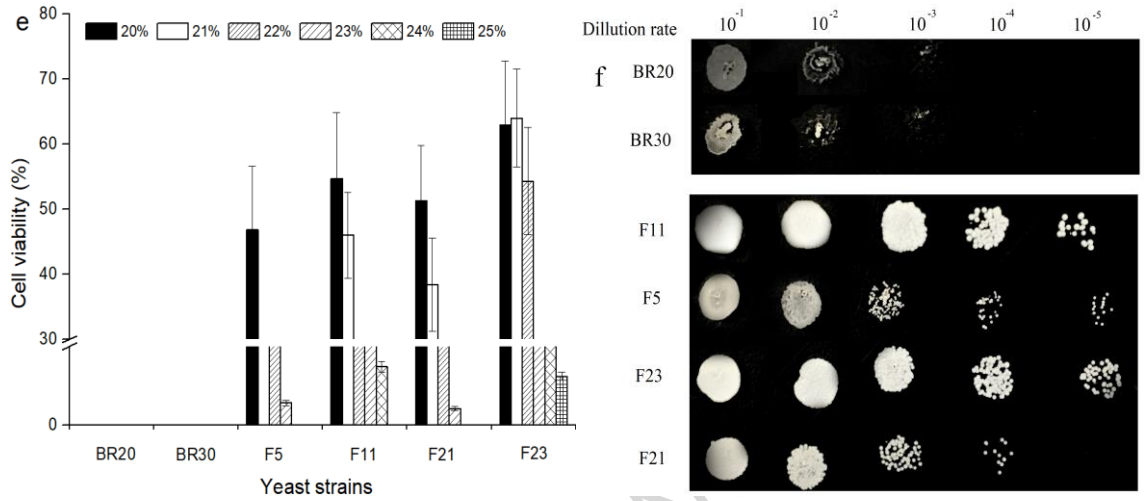


Fig.2 Results of fermentation performance and ethanol tolerance. **(a)** Time course of CO_2 evolution during fermentation by hybrids and diploid parental strains. Contents of reducing sugar **(b)**, total acidity **(c)** and ethanol **(d)** of Chinese rice wine fermented by different strains. Strain F1, open circles; F3, open squares; F5, open triangles; F8, open diamonds; F11, open stars; F21, open pentagons; F23, closed triangles; BR20, closed squares; BR30, closed circles. **(e)** Cell viabilities of different hybrid strains under different ethanol stress. 20%, 21%, 22%, 23%, 24% and 25% ethanol stress were respectively symbolled by black, none filled, dense, medium, sparse and dense with cross stripe. **(f)** Spot assay of hybrids and parental strains on YPD medium containing 24% ethanol.

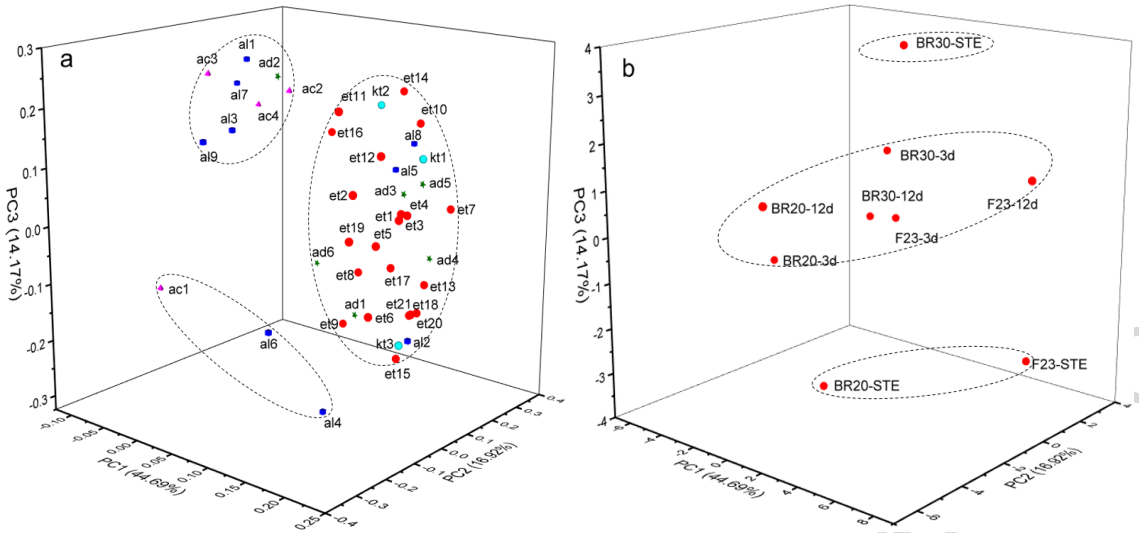


Fig.3 Viable plot for forty-three volatile flavor compounds **(a)** and scores plot **(b)** for Chinese rice wines fermented by hybrid F23, diploid parental strains BR20 and BR30 during brewing. Flavor compounds were numbered in Table 1; "STE" means young Chinese rice wine after sterilization.

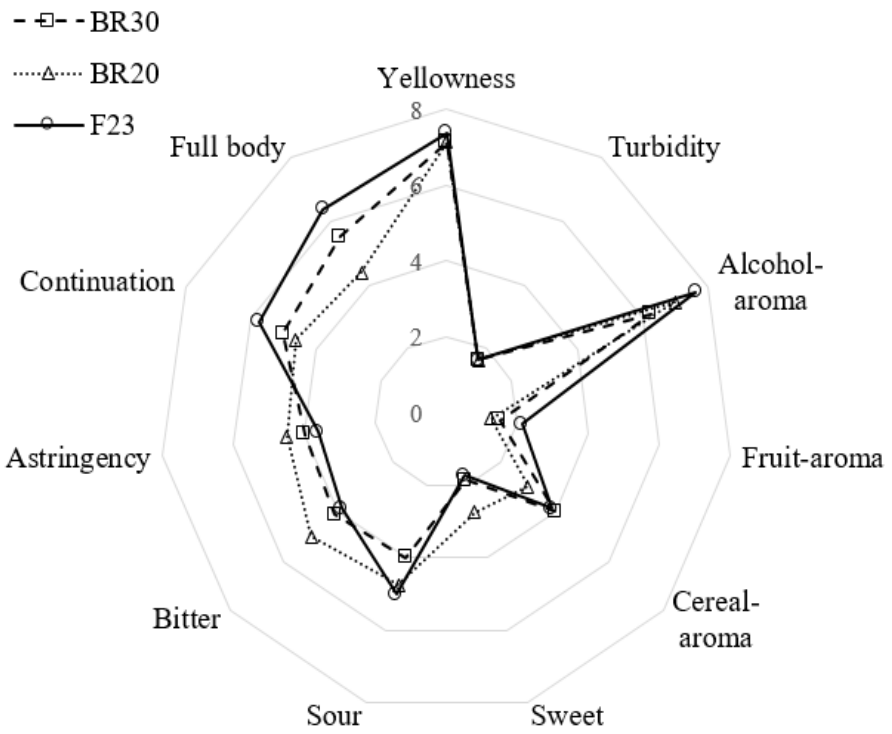
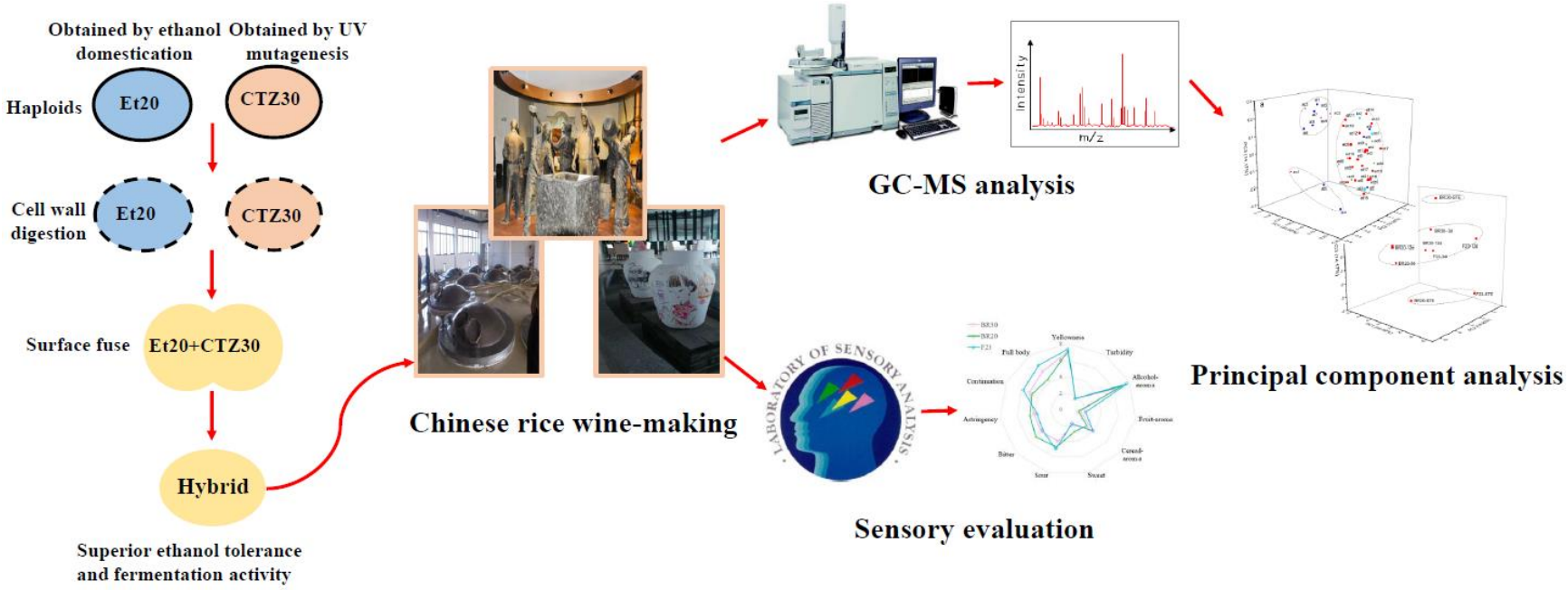


Fig.4 Sensory scores of organoleptic attributes for Chinese rice wines fermented by hybrid F23, diploid parental strains BR20 and BR30.



Graphical abstract

Highlights

- Adaptive evolution, UV mutagenesis and protoplast fusion were used for creating yeast hybrid.
- Hybrid F23 produced more flavor compounds with OAVs >1 than its diploid parents.
- Flavor characteristic of F23 wine was similar to the parent wines by PCA.
- Flavor profiles of Chinese rice wine could be enriched by applying “mixed-like” starter.