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Improving flavor metabolism of *Saccharomyces cerevisiae* by mixed culture with *Wickerhamomyces anomalus* for Chinese Baijiu making

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Yeasts are the most important microorganisms in the fermentation of Chinese Baijiu and the interaction of these yeasts could impact the quality of Baijiu. In this study, we initially characterized the Baijiu yeasts and evaluated their fermentation potential. *Wickerhamomyces anomalus* GZ3 and *Saccharomyces cerevisiae* G20 were found to generate high yields of ethyl acetate (2.76 g/L) and 2-phenylethanol, respectively. Results also indicated that the use of *W. anomalus* along with *S. cerevisiae* increased volatile compounds production, the maximum ethyl acetate production was observed in *S. cerevisiae* and *W. anomalus* at 10^6:10^6 ratio and have increased by 33 % compared with single culture of *W. anomalus*. Besides, there was a significant increase of 2-phenylethanol (3.29 g/L) production in single culture of *S. cerevisiae* with the addition of L-phenylalanine. However, the conversion of L-phenylalanine in mixed culture had significant impact on the yeasts interaction and end flavor of Baijiu. Thus, the present study provided new insights into yeasts interactions in Baijiu fermentation and the effect of some primary metabolites on the end flavor and Baijiu quality.

[**Key words:** *Saccharomyces cerevisiae*; *Wickerhamomyces anomalus*; Baijiu; L-Phenylalanine; 2-Phenylethanol; Ethyl acetate]

Chinese Baijiu, which is one of the most famous alcoholic beverages in China, is a traditional indigenous distilled beverage with two thousand years' history (1). Due to the various geographical distributions, climate, manufacturing practices, raw materials, and Daqu, the aroma profiles of various Chinese Baijiu are quite different (2). As a result, Chinese Baijiu could be classified into 11 different flavor groups, with over 1730 compounds reported so far (3).

Ethyl acetate (fruity-like aroma) is one of the most common flavor compounds in Chinese Baijiu, and plays an important role in the liquor style and aroma characteristics of light, Fengxiang, and rice aroma type Baijiu (4). In particular, it is the key aroma compounds of light aroma type Baijiu (5). Besides, 2-phenylethanol (2-PE, rose-like aroma) is also a very important aroma compound in Chinese Baijiu and is known to be the key flavor constituent in rice and Chixiang aroma type Baijiu (4). The processes by which these two key odorants are formed have been well-established and found to be influenced by various yeast species present during fermentation of liquor from cereal and legume materials. Previous studies have shown that *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* are capable of generating ethyl acetate and 2-PE by both *de novo* and biotransformation pathways during fermentation (6,7). Both of these microorganisms are important contributors to liquor quality in solid-state fermentation of Chinese Baijiu. Moreover, the metabolic characteristics of these microorganisms have been extensively studied through detection of the corresponding metabolites in pure cultures (8,9). Although previous studies have provided valuable information on the individual yeast species (10–13), the microbial interactions in mixed culture fermentations and effects of accumulated intermediate metabolites on growth and fermentative behavior of the microorganisms, and their subsequent influence on the microbial community structures and functions have been ignored. In recent years, the interactions between functional microorganisms in Baijiu, such as yeast–yeast (14), yeast–lactic acid bacteria (15,16), and yeast–*Bacillus* spp. (17) have been extensively studied, and these data could be successfully applied to improve the stability and sensory property of fermented liquor.

Chinese Baijiu is mainly prepared by spontaneous fermentation process involving complex communities of microorganisms. The ubiquity of the interaction between the microorganisms could have a significant influence on the quality of Baijiu flavor. However, due to limited knowledge on the relationship between Baijiu flavor and the microbial structure of fermentation liquor, it is necessary to replace spontaneous fermentation with inoculated fermentation and modernize the Chinese Baijiu fermentation process. In the present study, two indigenous yeasts, *W. anomalus* GZ3 and *S. cerevisiae* G20 were selected based on their high capacity to produce ethyl acetate and 2-PE, and their metabolic characteristics and interactions in laboratory-scale mixed culture were investigated and their differences with the addition of specific precursor were analyzed. The results of this study could help in the

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understanding of microbial interaction during Chinese Baijiu fermentation and the variation trends of flavor compounds.

MATERIALS AND METHODS

Strains and medium A total of 47 Baijiu yeasts, belonging to *S. cerevisiae*, *Pichia kudriavzevii*, *Kluyveromyces lactis*, and *W. anomalus*, were isolated from Daqu, soil (around the fermentation room), and pit mud from Shandong Guojing Distillery Co. Ltd. (Gaoqing, Shandong, China). All of the strains (stored at −80°C) had been previously identified by ITS1/ITS4 gene analysis based on their rDNA internal transcribed spacers (18).

The fermentation medium was prepared according to a previous report (17) with minor modifications. In brief, 1000 g of milled sorghum powder were added to 4 L of deionized water containing moderate thermostable α-amylase solution (1×10^4 U/L). The mixture was boiled for 1 h, followed by saccharification at 60°C for 4 h with the addition of glucoamylase (2.5×10^4 U/L). Subsequently, the mixture was centrifuged for 6 min at 8000 ×*g* and the supernatant was collected as sorghum extract. The sugar content of the sorghum extract was measured using a Leica refractometer (Thermo Fisher Scientific, Pittsburg, PA, USA), and the extract was appropriately diluted with water to a final sugar concentration of 10°Bx (75 ± 5 g/L) and then sterilized at 121°C for 15 min before use. In addition, L-phenylalanine (5 g/L) was prepared as precursor compound for 2-PE, and was purified by filtration, and mixed with the sterilized sorghum-extract medium.

Fermentation In order to evaluate the capability to produce ethyl acetate and 2-PE, all of the 47 yeasts were respectively precultured in YPD liquid medium (20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract) at 30°C for 18 h and inoculated into the sorghum extract at a concentration of 1×10^6 cfu/mL. Fermentation was performed in 250-mL flasks containing 50 mL of sterile sorghum extract at 30°C and 150 rpm. The yeasts interaction was carried out for the single cultures of *S. cerevisiae* and *W. anomalus*, respectively, as well as for their mixed cultures with different inoculation ratios. Mixed culture fermentation was conducted with *S. cerevisiae* and *W. anomalus* at inoculum ratios of 10^6:10^7, 10^6:10^6, and 10^7:10^6, respectively. To enumerate viable cell counts and analyze the fermentation characteristics, samples were taken from each flask throughout the fermentation process, and the fermented liquid was centrifuged (6000 ×*g*, 5 min) and the supernatant was collected for the analysis of flavor compounds (ethyl acetate and 2-PE). Each experiment was performed in triplicate.

Determination of fermentation characteristics and flavor compounds The pH values of the fermented liquid were measured using a pHFE20 pH meter (Mettler Toledo Instruments Co. Ltd., Shanghai, China). The viable counts of *S. cerevisiae* and *W. anomalus* were enumerated on YPD agar plates according to the method described previously (19). Fermenting cultures (2 mL) were sampled every 12 h and centrifuged for 5 min at 6000 rpm, and the supernatant was collected and filtered through a 0.45-μm membrane and stored at −80°C until further analysis using high-performance liquid chromatography (HPLC).

The concentrations of ethanol, acetic acid, ethyl acetate, 2-PE, and L-phenylalanine in the samples were determined using HPLC (Shimadzu LC-20A, Shimadzu, Kyoto, Japan). The ethanol and acetic acid contents in the samples were analyzed by concatenating refractive index detector and ultraviolet detector in a Bio-Rad 87H column (0.05 mM H_2SO_4 as a mobile phase, flow rate of 0.6 mL/min, column temperature of 60°C, and injection volume of 10 μL) according to the procedure described previously with minor modification (17). The content of ethyl acetate was assayed by HPLC equipped with ultraviolet detector and Inertsil ODS-SP column (Shimadzu). The mobile phase was methanol−0.1 M KH_2PO_4 solution (50:50, v/v) and the operation conditions were as follows: flow rate of 1 mL/min at 35°C, detection at 210 nm, and injection volume of 5 μL. In addition, standards for ethyl acetate were dissolved in ethanol−water (60:40, v/v) solution, and the samples were mixed with ethanol (final content of ethanol, 60%, v/v). The concentrations of 2-PE and L-phenylalanine were detected by HPLC according to the method described by the previous report (20).

Statistical analysis Analysis of variance was conducted using an ANOVA Tukey's test to determine any significant difference between samples. The statistical level of significance was set at $P < 0.05$. All the experiments were repeated at least thrice, and the results were expressed as mean ± standard deviation (n = 3). Statistical analyses were performed with SPSS 9.0 software.

RESULTS AND DISCUSSION

Screening of strains producing high contents of ethyl acetate or 2-phenylethanol The initial screening of 47 Baijiu yeasts belonging to *S. cerevisiae*, *P. kudriavzevii*, *W. anomalus*, and *K. lactis* was performed based on the production of ethyl acetate and 2-PE after fermentation for 48 h. Table 1 shows the profile of ethyl acetate and 2-PE produced by different yeast strains, and most of the strains were able to generate both these compounds. Six of the yeast strains produced considerable amount of ethyl acetate (>1 g/L), and were attributed to *W. anomalus* (2 strains) and *P. kudriavzevii* (4 strains). Among them, *W. anomalus* GZ3 produced the highest amount of ethyl acetate (2.76 g/L). It has been reported that *W. anomalus* WS15 produced about 335.07 mg/L ethyl acetate during apple cider fermentation (21), which is rather lower than that observed in the present study.

It is worth noting that the highest 2-PE-producing yeasts (>80 mg/L) were *S. cerevisiae*, although some of the *S. cerevisiae* strains were substandard, indicating that the ability to produce 2-PE was strain-specific (22). Previous reports have demonstrated that yeasts are capable of producing 2-PE by *de novo* synthesis, and that the final concentration of 2-PE in the culture broth of selected strains generally remains very low (23,24). In the present study, *W. anomalus* GZ3 and *S. cerevisiae* G20 were selected on the basis of

TABLE 1. The formation of ethyl acetate and 2-phenylethanol by different wine yeasts.

Strain	Species	Ethyl acetate (g/L)	2-Phenylethanol (mg/L)	Strain	Species	Ethyl acetate (g/L)	2-Phenylethanol (mg/L)
GZ10-2	*P. kudriavzevii*	1.06 ± 0.11	24.07 ± 1.2	G1	*S. cerevisiae*	0.18 ± 0.04	35.56 ± 1.53
GZ15-1	*P. kudriavzevii*	0.97 ± 0.1	14.56 ± 0.93	G2	*S. cerevisiae*	/	85.03 ± 2.96
GZ19	*P. kudriavzevii*	0.74 ± 0.06	21.22 ± 1.12	G3	*S. cerevisiae*	/	83.77 ± 2.92
GJ16	*P. kudriavzevii*	0.48 ± 0.01	14.45 ± 0.92	G4	*S. cerevisiae*	/	10.56 ± 0.81
GN10	*P. kudriavzevii*	0.33 ± 0.01	13.28 ± 0.89	G5	*S. cerevisiae*	/	85.86 ± 2.16
GN20	*P. kudriavzevii*	0.29 ± 0.02	12.8 ± 0.88	G6	*S. cerevisiae*	0.13 ± 0.05	11.42 ± 0.84
GZ2	*P. kudriavzevii*	0.25 ± 0.03	10.82 ± 0.82	G7	*K. lactis*	0.2 ± 0.04	5.93 ± 0.68
GF21	*W. anomalus*	0.29 ± 0.02	14.22 ± 0.92	G8	*S. cerevisiae*	0.15 ± 0.04	11.07 ± 0.83
GJ11-1	*P. kudriavzevii*	0.31 ± 0.02	13.84 ± 0.91	G9	*S. cerevisiae*	/	76.82 ± 2.72
GZ3	*W. anomalus*	2.76 ± 0.46	28.92 ± 1.34	G10	*S. cerevisiae*	/	77.01 ± 0.83
GN16	*W. anomalus*	0.98 ± 0.09	16.17 ± 0.97	G11	*S. cerevisiae*	0.13 ± 0.03	10.47 ± 0.81
GN13	*P. kudriavzevii*	0.43	13.16 ± 0.89	G12	*S. cerevisiae*	/	87.79 ± 0.88
GN22	*P. kudriavzevii*	0.97 ± 0.1	23.26 ± 1.18	G13	*S. cerevisiae*	/	6.29 ± 0.69
GZ22-3	*W. anomalus*	0.27 ± 0.02	7.07 ± 0.71	G14	*S. cerevisiae*	0.15 ± 0.04	9.69 ± 0.79
GZ9-2	*P. kudriavzevii*	/	11.75 ± 0.85	G15	*S. cerevisiae*	0.18 ± 0.04	38.27 ± 1.61
GJ17	*P. kudriavzevii*	0.34 ± 0.01	12.12 ± 0.86	G16	*S. cerevisiae*	/	10.46 ± 0.81
GZ1	*P. kudriavzevii*	0.82 ± 0.07	25.13 ± 1.42	G17	*S. cerevisiae*	0.16 ± 0.04	9.81 ± 0.79
GJ3	*W. anomalus*	2.44 ± 0.37	20.99 ± 1.11	G18	*S. cerevisiae*	0.14 ± 0.05	10.51 ± 0.81
GJ4-1	*P. kudriavzevii*	1.12 ± 0.12	23.94 ± 1.2	G19	*S. cerevisiae*	/	59.7 ± 2.23
GJ5-1	*P. kudriavzevii*	1.50 ± 0.14	20.51 ± 1.1	G20	*S. cerevisiae*	/	98.38 ± 3.34
GJ5-2	*P. kudriavzevii*	0.72 ± 0.05	22.47 ± 1.15	G21	*S. cerevisiae*	0.18 ± 0.04	39.92 ± 1.66
GJ10	*P. kudriavzevii*	1.14 ± 0.13	27.82 ± 1.31	G22	*S. cerevisiae*	0.2 ± 0.04	33.64 ± 1.48
GJ22-41	*P. kudriavzevii*	0.35 ± 0.01	12.26 ± 0.86	G23	*S. cerevisiae*	0.15 ± 0.04	39.29 ± 1.64
GJ22-42	*P. kudriavzevii*	0.34 ± 0.01	15.24 ± 0.95				

Value represent means ± S.E.M. (n = 3).

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