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Enhanced production of ethyl acetate using co-culture of *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae*

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The style and quality of *Baijiu* is greatly influenced by ethyl acetate. Therefore, improving and controlling the ethyl acetate levels in *Baijiu* is important. This study investigated ethyl acetate production using a co-culture of *Saccharomyces cerevisiae* Y3401 and *Wickerhamomyces anomalus* Y3604. More ethyl acetate was produced in mixed fermentations using both yeasts than in single fermentations. The highest ethyl acetate yield was 6.41 g/L using a Y3401/Y3604 ratio of 3:1. Synergistic fermentation using both yeasts not only improved ethyl acetate production, but also increased the contents of other flavor compounds, such as β-phenethyl alcohol and phenethyl acetate. Therefore, the co-culture of *S. cerevisiae* and *W. anomalus* had a positive effect on ethyl acetate production and provides opportunities for altering the aroma and flavor perception of *Baijiu*.

[**Key words:** *Baijiu*; Ethyl acetate; Mixed culture; *Saccharomyces cerevisiae*; *Wickerhamomyces anomalus*]

Baijiu, which is produced by spontaneous mixed-culture solid-state fermentation using a specific saccharifying and fermenting agent called *Daqu*, is generally considered the national alcoholic beverage of China (1). Different raw materials, saccharifying and fermenting agents, and processes generate different compounds in *Baijiu*, which leads to different types of *Baijiu* with special flavors (2). According to these flavor differences, there are four dominant aroma types (strong, light, sauce, and rice flavors) and eight minor aroma types (sesame, feng, herbal, te, fuyu, laobaigan, chi, and mixed flavors) of *Baijiu* found in different regions of China (3). A total of 1870 flavor compounds, including esters, alcohols, aromatics, ketones, heterocyclic compounds, nitrogenous compounds, acids, and aldehydes have been identified in *Baijiu* (3). Ethyl acetate is among the most common flavor components in *Baijiu* (4), and is used as a clear indicator of beverage style and quality in almost all types of *Baijiu* (5). Ethyl acetate is particularly important in the formation of products with strong, light, rice, and feng flavors. Therefore, ethyl acetate, which provides pear-like and banana-like aromas, is a major beneficial ester in *Baijiu* (6). However, in the actual *Baijiu*-making process, the ethyl acetate content of raw *Baijiu* (brewed and distilled liquor that has not been blended) is low, and cannot meet the requirements of the national standard for *Baijiu* quality. This has resulted in a low rate of high-quality production, with some enterprises in pursuit of high profits even adopting illegal methods to add flavors to *Baijiu*. Therefore, to improve the *Baijiu* quality and avoid adding flavors, it is important to improve the ethyl acetate content in *Baijiu* by investigating the whole production process.

In *Baijiu* manufacture, although a small amount of ethyl acetate is produced by the reaction of ethyl alcohol with acetic acid, the main source of ethyl acetate in *Baijiu* is microbial metabolism. In the *Baijiu* fermentation process, many biological strains that can produce ethyl acetate are used, including yeasts, bacteria, and molds (7). Among these strains, studies have shown that *Wickerhamomyces anomalus*, which are non-*Saccharomyces* wild yeasts, are the main strains that produce ethyl acetate (5). *W. anomalus* makes a special contribution to *Baijiu* flavor quality. To date, many strains of *W. anomalus* have been isolated, and the metabolic characteristics of ethyl acetate production in pure cultures have been studied extensively (5,8). In a previous study, our research team had access to a yeast strain of *W. anomalus*, named Y3604, that is reportedly amongst the best strains for producing ethyl acetate (5). Previous studies have shown that the ethyl acetate concentration produced by Y3604 was lower in pure culture when ethanol was not added as a substrate. This indicates that ethanol is an important substrate in the synthesis of ethyl acetate by yeast esterification (5,9). Although Y3604 actually produces some ethanol in the pure culture, the amount is insufficient for ethyl acetate synthesis.

Baijiu fermentation is a spontaneous process involving complex communities of microorganisms (3). The ubiquity of interactions between microbial communities are the main drivers of

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fermentation processes, resulting in *Baijiu* containing many flavor substances (10,11). However, these interactions are poorly understood, leading to an unstable quality level in *Baijiu* produced. In recent years, interactions between functional microorganisms, such as yeast-yeast and yeast-bacteria interactions, have been studied in *Baijiu* brewing (12,13). These results could be useful for improving the stability of *Baijiu* production. As *Saccharomyces cerevisiae* and *W. anomalus* are the most important ethanol-producing strain and most high-yielding ethyl acetate strain, respectively, their interaction must be studied. This investigation will not only aid understanding of microbial interactions in the *Baijiu* brewing process, but also produce various flavor compounds, especially ethyl acetate, because *S. cerevisiae* can provide ethanol as a substrate for *W. anomalus*. The results of this study could improve the ethyl acetate content in the fermentation system and significantly improve *Baijiu* quality. To simplify the study and focus on the interaction between *S. cerevisiae* and *W. anomalus*, liquid fermentation was used, with the aim of tuning the fermentation and enhancing the ethyl acetate content. The results will provide a solid foundation for the application of these strains in *Baijiu* production.

MATERIALS AND METHODS

Yeasts and medium *S. cerevisiae* Y3401 and *W. anomalus* Y3604 were isolated from *Daqu* and were deposited in the China General Microbiological Culture Collection Center (CGMCC) under accession no. 14828 and 13103, respectively. They all stored on yeast extract peptone dextrose (YPD) medium (glucose 20 g, peptone 20 g, yeast extract 10 g, agar powder 20 g, and ddH_2O 1000 mL, pH 6.0–6.2, autoclaved at 115°C for 20 min) in slant tubes and numbered in our laboratory.

The fermentation medium (Sorghum hydrolyzate medium, SHM) was prepared according to the methods described by Fan et al. (5). The mixture of 250 g of sorghum flour and 1000 mL of ddH_2O was liquefied by α-amylase (Novozymes, Denmark) at 90°C for 1 h. Then the mixture was saccharified by amyloglucosidase (Novozymes) at 60°C for 2 h. After filtered using gauze, the sugar content of the liquid medium was adjusted to 10°Brix, pH 6.8–7.0, and autoclaved at 115°C for 20 min.

Effect of inoculation method on ethyl acetate content To examine the effect of inoculation method on ethyl acetate content, three types of mixed fermentations, including simultaneous and sequential mixed fermentations, were carried out with *S. cerevisiae* Y3401 and *W. anomalus* Y3604 at inoculum ratio of 1:1. Simultaneous mixed fermentation (SMF) was performed by inoculating Y3401 and Y3604 at the same time, and the initial inoculation of both is 1×10^6 CFU/mL. One sequential mixed fermentation (SW) used an initial inoculation of 1×10^6 CFU/mL of Y3401, followed 12 h later with 1×10^6 CFU/mL of Y3604 to give the yeast mixture. The other sequential mixed fermentation (WS) reversed this process to form the mixture. Single-culture fermentations by Y3401 (S) and Y3604 (W) were also carried out under the same conditions with an initial inoculation of 1×10^6 CFU/mL. All of the above-described fermentations were carried out in 250-mL Erlenmeyer flasks containing 50 mL of SHM at 30°C and 180 rpm for 6 days after inoculation with precultures of Y3401 or Y3604. Throughout the fermentation process, three flasks for each inoculation method were randomly selected each day to detect the remaining reducing sugars, fermenting property, ethanol content, ethyl acetate content, and flavor compounds (end of fermentation).

Effect of inoculation ratio on ethyl acetate content Mixed-culture fermentations with Y3401:Y3604 at different inoculation ratios (1:1, 3:1, 6:1, 1:2, 1:3, and 0:1) were tested. Fermentations were performed by inoculating simultaneously of Y3401 and Y3604 into SHM and the cell population of Y3604 was 1×10^6 CFU/mL at the beginning of the fermentation. All of these fermentations were also carried out as the above-described method. The remaining reducing sugars, fermenting property, ethanol content, ethyl acetate content, and flavor compounds (end of fermentation) were determined.

Analytical methods Yeast cell growth during fermentation was obtained by viable cell counts using YPD agar plates according to the method described previously (14). Samples were taken aseptically throughout the fermentations and diluted appropriately with saline, then the viable counts of Y3401 and Y3604 were enumerated on YPD agar plates. The fermenting property was measured using the carbon dioxide (CO_2) weight loss method, monitored by weighing the fermentation flasks (15). The weight loss was monitored every day and the quantity (g) of CO_2 produced was used to express strain fermenting property, expressed as grams of produced from 100 mL of fermentation medium. The pH was measured using a pH meter (FiveEazy Plus, Mettler Toledo Instruments Co. Ltd., Shanghai, China). Remaining reducing sugars and ethanol content were measured by the dinitrosalicylic acid (DNS) assay and by high-performance liquid chromatography (HPLC), respectively, according to the method of Meng et al. (11). A BioRad 87H column (0.01 mmol/L H_2SO_4 as a mobile phase, flow rate of 0.5 mL/min, column temperature of 25°C, and injection volume of 10 μL) and a refractive index detector (Varian 355 RI) were used for HPLC. Ethyl acetate content was measured by gas chromatography–mass spectrometry (GC–MS) and flavor compounds were analyzed by headspace solid-phase microextraction gas chromatography–mass spectrometry (HS-SPME GC–MS), following previously described methods (5,16). The GC–MS conditions were as follows: DB-WAX capillary column (30 m × 0.25 mm × 0.25 μm) was used and the carrier gas was high-purity helium with a flow rate of 1.0 mL/min. The inlet temperature was 250°C, the oven temperature was 50°C for 2 min, then 10°C/min up to 180°C held for 2 min, and finally raised to 230°C at a rate of 5°C/min for 2 min. The vaporization chamber temperature was 250°C, and split mode was adopted, the split ratio 37:1 and the injection volume was 1 μL. The electron impact energy was 70 eV, and the ion source temperature was set at 230°C. The electron impact mass spectra ranged from 30 amu to 550 amu. The GC grade ethyl acetate (Sigma–Aldrich Chemical Co., St. Louis, MO, USA) was prepared to set up the calibration curve. The HS-SPME conditions were as follows: the 50/30- μm divinylbenzene/carboxen on polydimethylsiloxane (DVB/CAR on PDMS)-coated fibers (Supelco, Inc., Bellefonte, PA, USA) were used for volatile compound extraction. Approximately 5 mL of fermentation sample with saturated NaCl and 50 μL of 0.5 mg/L methyl octanoate (chromatographically pure, Sigma–Aldrich Chemical Co.) solution were placed into a 15-mL headspace vial, and heated in a water bath with 50°C for 10 min, then an SPME needle was inserted for 30 min at 50°C to extract volatile flavor compounds. The extraction head was removed and analyzed through GC–MS. The mass spectra of volatile components were identified with those from the National Institute of Standards and Technology Library (NIST). The average value of the identified compound was calculated as the ratio of the mass concentration of methyl octanoate to the mass concentration of volatile components.

Statistical analysis Analysis of variance (ANOVA) was performed by Duncan's multiple range tests to determine any significant difference between samples. Differences at $P < 0.05$ were considered significant. Each treatment was performed in triplicate, and the results were expressed as mean ± standard deviation. All statistical analyses were performed with OriginPro 9.1 (OriginLab, Northampton, MA, USA) and SPSS16.0 (SPSS, Chicago, IL, USA). The consumption or production rates of reducing sugars, the CO_2 weight loss, and the ethanol and ethyl acetate concentrations were calculated using the following equation:

$$R = (C_i - C_0)/i \quad (1)$$

where R is the consumption or production rate, C_i is the concentration on the *i*th day ($i = 1$–6), and C_0 is the initial concentration.

RESULTS AND DISCUSSION

Effect of inoculation method on ethyl acetate content Reducing sugar utilization is an important index for judging yeast growth (17). As shown in Fig. 1A, the reducing sugar consumption of the single and mixed fermentations was significantly different (Fig. 1A). For single cultures, the reducing sugar consumption rate of Y3401 was higher than that of Y3604. Meanwhile, for mixed cultures, the reducing sugar consumption rate of SW was higher than that of SMF, while that of WS was lowest. Interestingly, the reducing sugar consumption rates of all mixed cultures were lower than that of S, but higher than that of W. This indicated that earlier Y3401 inoculation resulted in faster sugar utilization in the mixed fermentation system, with *S. cerevisiae* showing the highest fermentability among single cultures, in accordance with previous reports (17,18). Furthermore, the reducing sugars took 4 days to fall below 10 g/L in the mixed fermentation system and Y3401 single fermentation. Nearly a third of the reducing sugars remained in the Y3604 single fermentation after 6 days. Similar results for other non-*Saccharomyces* have been reported previously (17–19). Two possible explanations were proposed: (i) Y3401 growth was faster than that of Y3604 (single-culture fermentations using Y3401 and Y3604 reached maximum viable cell counts of 1.3×10^8 cfu/mL and 7.7×10^7 cfu/mL within 3 days, respectively); (ii) Y3604 might have little or no effect on Y3401 growth (maximum viable cell count was 8.9×10^7 cfu/mL within 3 days in SMF). This inference was consistent with previous

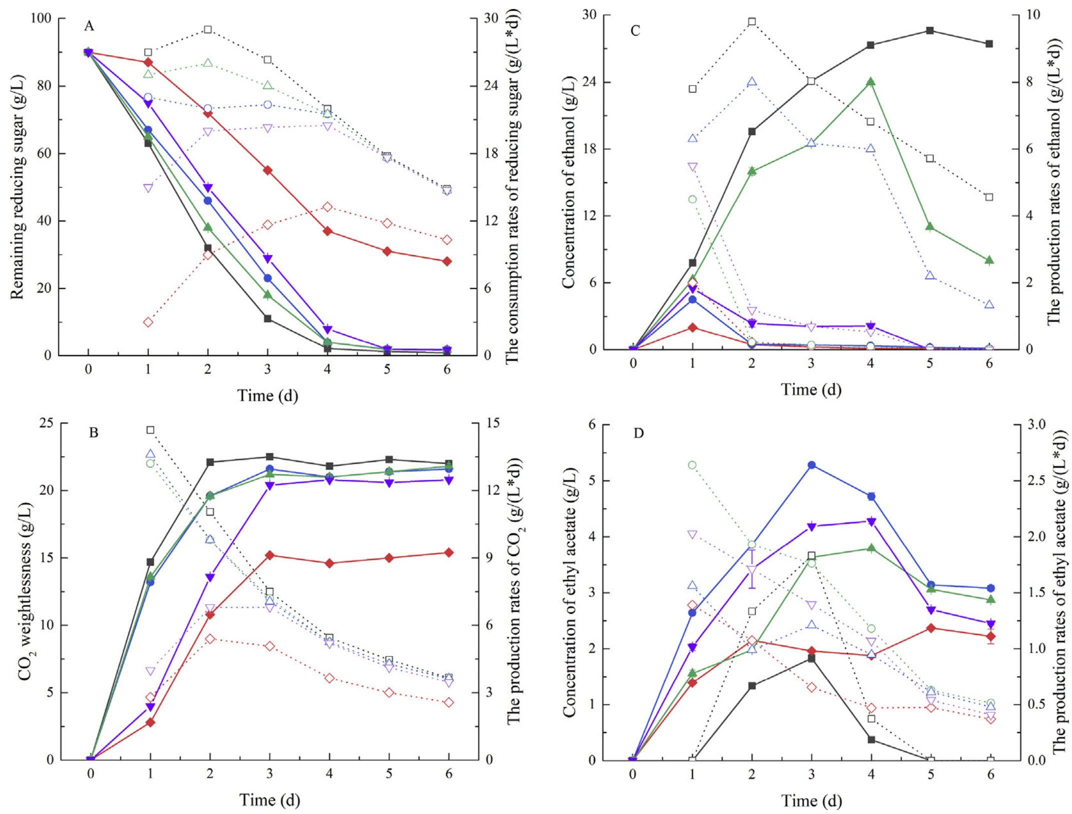

FIG. 1. Changes in remaining reducing sugars (A), fermenting properties (B), ethanol concentration (C), and ethyl acetate concentration (D) in different fermentations using different inoculation methods. Squares, single-culture fermentation by *S. cerevisiae* Y3401 (S); diamonds, single-culture fermentation by *W. anomalus* Y3604 (W); circles, simultaneous mixed fermentation performed by inoculating 1×10^6 CFU/mL each of *S. cerevisiae* Y3401 and *W. anomalus* Y3604 (SMF); regular triangles, mixed fermentation performed by initially inoculating 1×10^6 CFU/mL of *S. cerevisiae* Y3401 for 12 h, followed by 1×10^6 CFU/mL of *W. anomalus* Y3604 (SW); inverted triangles, mixed fermentation performed by initially inoculating 1×10^6 CFU/mL of *W. anomalus* Y3604 for 12 h, followed by 1×10^6 CFU/mL of *S. cerevisiae* Y3401 (WS). Closed symbols and solid line, the changes of remaining reducing sugars (A), fermenting properties (B), ethanol concentration (C), and ethyl acetate concentration (D); open symbols and dot line, the consumption or production rates of reducing sugars (A), fermenting properties (B), ethanol (C), and ethyl acetate (D). Results are averages and bars denote SD.

studies, which showed that the growth and survival of *S. cerevisiae* were initially influenced by *W. anomalus*, but remained stable after 36 h, irrespective of the initial inoculum ratio (13).

Carbon dioxide weight loss is commonly used to determine yeast fermenting properties in fermentation (20). From fermentation analysis, although the fermenting property curve profile was opposite to that of the reducing sugars, the intrinsic conclusion provided by both curves was essentially consistent (Fig. 1B). In detail, the fermenting property of the Y3401 single system was superior to that of the mixed fermentation system. Furthermore, the fermenting property of the mixed fermentation system was related to the Y3401 inoculation time. Therefore, earlier Y3401 inoculation afforded a higher fermentation capacity (especially in the first 2 days), and the fermenting property was higher than that of the single system with only *W. anomalus*. These results were in good agreement with the results of previous reports (17,21). The results also indicated that the fermenting property of Y3401 was superior to that of Y3604, which was consistent with the results of Medina et al. (22). Notably, in our study, some effects were observed in the early stage of co-culture compared with that using Y3401 alone. However, in the late stage of fermentation, the fermenting property of the co-culture system was gradually restored to that of Y3401 in the single culture owing to an the increase in the number of *S. cerevisiae*, in accordance with the observations of *S. cerevisiae* and *Klockera apiculate* co-culture (23). Previous reports have shown that the biomass of *S. cerevisiae* in co-culture with *W. anomalus* can be similar to that of *S. cerevisiae* in a single culture at the later stage (13). Therefore, over the whole fermentation process, it can be inferred that Y3604 had little effect on the fermenting property of Y3401, and that Y3401 improved the fermenting property of the mixed culture. In this respect, it is feasible to produce more ethyl acetate in a mixed fermentation system using Y3604, because Y3401 provides more ethanol as the ethyl acetate precursor for Y3604. Previous reports generally considered that using *S. cerevisiae* with a non-*Saccharomyces* yeast would result in sluggish fermentation compared with using pure cultures of *S. cerevisiae*, indicating that non-*Saccharomyces* yeast can inhibit the fermentation property of *S. cerevisiae* (22,24). However, this might be caused by the strong consumption of nutrients (especially reducing sugars) and accumulation of metabolites (such as ethyl alcohol and ethyl acetate) in the fermentation system, resulting in decreased cell viability and the fermentation property of all fermentation systems decreasing gradually with time and becoming very low after 3 days (25).

The ethanol analysis results are shown in Fig. 1C. Ethanol production varied among the different fermentation systems. The fermentation systems with a single culture of Y3401 showed the highest ethanol production, followed by the SW mixed culture. The amount of ethanol produced by the other two mixed cultures (WS and SMF) was lower, but higher than that using a single culture of Y3604. The results implied that Y3401 increased the amount of ethanol in the fermentation system, in agreement with other literature reports. Generally, the lowest ethanol levels were observed in the non-*Saccharomyces* pure cultures, while ethanol production was significantly increased with *S. cerevisiae* inoculation in mixed fermentations (18,21,26). Furthermore, with the exception of SMF and WS, for which the ethanol levels were reversed, earlier Y3401 inoculation resulted in higher ethanol yields, in agreement with some previous reports (21). Similar to reports on other non-*Saccharomyces* yeasts, Y3604 was able to produce ethanol, but with a lower yield, which explained why a larger amount of ethyl acetate was not produced by Y3604 without adding of ethanol (27,28). Ethanol was possibly reused or converted into other metabolites (such as ethyl acetate) by yeast. Meanwhile, the ethanol content decreased in the later stages of fermentation, especially in the fermentation system with a single culture of Y3604 and all mixed cultures, which was consistent with previous reports (13). In summary, regarding ethanol production, Y3401 was confirmed to afford ethanol, an ethyl acetate precursor, allowing Y3604 to produce more ethyl acetate. This has also been confirmed in other reports; for example, Medina et al. (22) reported a significant increase in flavor compounds, such as ethyl acetate, by co-fermentation of *S. cerevisiae* with non-*Saccharomyces* yeast in wine. Furthermore, Zha et al. (13) reported that ethyl acetate production in a mixed-culture fermentation with *S. cerevisiae* was approximately 33% higher than that in a single-culture fermentation with *W. anomalus*.

As shown in Fig. 1D, ethyl acetate production was different among the different culture fermentation systems in our study. In general, the amount of ethyl acetate first increased and then decreased with time in all cultures. Notably, mixed fermentation systems showed higher ethyl acetate production than single fermentation systems, suggesting that ethyl acetate production was attributed to yeast synergy (13). Y3604 or Y3401 produced comparatively less ethyl acetate in single-culture fermentations, which was consistent with previous reports (5). Interestingly, the ethyl acetate yield behavior was the opposite to that of ethanol in the mixed-fermentation systems, possibly because ethanol was converted to ethyl acetate by Y3604. Previous reports found that *S. cerevisiae* was able to continue to produce plenty of ethanol for *W. anomalus* to form ethyl acetate during mixed fermentation (13). Specifically, among the mixed fermentations, ethyl acetate production was higher in mixed fermentation systems with simultaneous inoculation than in sequential mixed fermentation systems. In SMF, Y3604 probably converted ethanol produced by Y3401 into ethyl acetate, with the ethyl acetate yield reaching 5.28 g/L, which was 122%, 39%, and 23% higher than those achieved by the Y3604 single culture (2.37 g/L), SW (3.79 g/L), and WS (4.28 g/L) systems, respectively. In SW, nutrient competition and metabolite inhibition from Y3401 was thought to impede the conversion of ethanol to ethyl acetate by Y3604, leading to ethanol the accumulation (13,27). Therefore, the *W. anomalus* population declined in SW when *S. cerevisiae* was the dominant species, similar to the co-culture of non-*Saccharomyces* yeasts and *S. cerevisiae* (13). Although there are few reports of optimal conditions for increasing the ethyl acetate yield in mixed-fermentation systems, the use of a non-*Saccharomyces*–*S. cerevisiae* couple has been reported to significantly boost the production of most detected compounds, particularly higher alcohols, total esters, acids, and terpenes (21,26,29–32). Therefore, the application of mixed fermentation has potential value for enhancing flavor compounds in *Baijiu*.

Table 1 shows the results of flavor fractions in different culture systems. Significant differences were observed between culture types for most aroma compounds analyzed. The flavor compound contents were higher in the mixed fermentations than in the single fermentation by Y3604, especially for ethyl acetate and β-phenethyl alcohol. Owing to Y3401 inoculation, the ethanol content in the mixed fermentations was slightly higher than in the single fermentation by Y3604. This could lead to a significant rise in ethanol conversion to ethyl acetate in mixed fermentations. Therefore, compared with single fermentation by Y3604, there was a significant increase in the ethyl acetate content in the mixed fermentations due to the presence of *S. cerevisiae*, which was consistent with other reports (18). Furthermore, except for SW, levels of β-phenethyl alcohol and the corresponding phenethyl acetate, with a rose-like odor, were higher in mixed fermentations than in single fermentations. These results indicated that *S. cerevisiae* and *W. anomalus* had synergistic effects that increased the β-phenethyl alcohol or phenethyl acetate contents, as demonstrated in previous reports (18). For isoamyl acetate, the existence of *S. cerevisiae* might have a negative effect. The isoamyl acetate contents under mixed-culture conditions using different inoculation methods were lower than that of the single culture of *W. anomalus*, which might be due to the change in *W. anomalus* metabolism caused by *S. cerevisiae* or its products. Previous reports have shown that volatile compound production in the mixed fermentations would be affected by interactions between *S. cerevisiae* and *W. anomalus* (18,33). Table 1 shows that a few flavor compounds were not produced in single cultures of both yeasts, but were produced in the mixed culture. A mixed culture and a suitable

TABLE 1. Some of the main volatile compounds present after different fermentations using different inoculation methods (g/L).

Volatile compounds	SHM	S	W	SMF	SW	WS
Ethanol	–	2.42 ± 0.14^{c}	0.05 ± 0.03^{a}	0.09 ± 0.02^{ab}	0.18 ± 0.04^{b}	0.07 ± 0.05^{ab}
β-Phenethyl alcohol	–	1.90 ± 0.07^{b}	0.72 ± 0.13^{a}	5.23 ± 0.04^{d}	4.72 ± 0.09^{c}	5.86 ± 0.02^{e}
Isoamylol	–	0.43 ± 0.03^{a}	0.86 ± 0.13^{bc}	0.82 ± 0.03^{b}	1.17 ± 0.04^{d}	1.06 ± 0.05^{c}
Isobutanol	–	–	0.12 ± 0.04^{a}	0.08 ± 0.03^{a}	0.14 ± 0.06^{a}	0.12 ± 0.03^{a}
Σ Higher alcohols	–	2.33	1.70	6.13	6.03	7.04
Ethyl acetate	–	–	1.58 ± 0.05^{a}	2.40 ± 0.11^{d}	1.85 ± 0.04^{c}	1.79 ± 0.03^{b}
Phenethyl acetate	–	0.22 ± 0.04^{a}	1.22 ± 0.17^{b}	3.65 ± 0.26^{d}	0.24 ± 0.03^{a}	1.86 ± 0.12^{c}
Isoamyl acetate	–	–	3.56 ± 0.12^{d}	2.36 ± 0.02^{c}	0.10 ± 0.04^{a}	1.47 ± 0.06^{b}
Ethyl caprylate	–	–	–	–	–	0.08 ± 0.09
Σ Esters	–	0.22	6.36	8.41	2.19	5.20
Acetic acid	–	–	0.02 ± 0.08^{a}	–	–	0.02 ± 0.02^{a}
Isobutyric acid	–	–	0.11 ± 0.02^{a}	0.10 ± 0.02^{a}	0.16 ± 0.05^{a}	–
Methylbutanoic acid	–	–	–	0.25 ± 0.08^{a}	0.23 ± 0.07^{a}	–
Octanoic acid	–	–	–	–	–	0.02 ± 0.01
Caproic acid	–	–	–	–	–	–
Σ Acids	–	–	0.13	0.35	0.39	0.04
Vinyl guaiacol	0.02 ± 0.01^{a}	–	0.10 ± 0.07^{b}	0.14 ± 0.03^{b}	0.14 ± 0.07^{b}	0.16 ± 0.12^{b}
Sum	0.02	4.97	8.34	15.12	9.73	12.51

SHM, sorghum hydrolyzate medium; S, single-culture fermentation by *S. cerevisiae* Y3401; W, single-culture fermentation by *W. anomalus* Y3604; SMF, simultaneous mixed fermentation performed by inoculating 1×10^6 CFU/mL each of *S. cerevisiae* Y3401 and *W. anomalus* Y3604; SW, mixed fermentation performed by initially inoculating 1×10^6 CFU/mL of *S. cerevisiae* Y3401 for 12 h, followed by 1×10^6 CFU/mL of *W. anomalus* Y3604; WS, mixed fermentation performed by initially inoculating 1×10^6 CFU/mL of *W. anomalus* Y3604 for 12 h, followed by 1×10^6 CFU/mL of *S. cerevisiae* Y3401. Data are averages of three replicates ± standard deviations; –, not detected; Same lowercase letters in each column indicate no significantly difference at the 5% probability level using Duncan's multiple range tests.

inoculation method are required to produce the flavor substances not produced by single cultures. For example, ethyl caprylate and octanoic acid were only produced by WS, while methylbutanoic acid was only produced by SMF and SW. Therefore, the types and contents of flavor substances in fermentation products can be adjusted by changing the two-yeast inoculation method, allowing the regulation of *Baijiu* quality to be realized using functional microorganisms. In previous reports, mixed fermentations of *S. cerevisiae* and *W. anomalus* also showed interesting enological properties and provided favorable combinations for the production of compounds such as esters and linear alcohols (31).

Effect of inoculation ratio on ethyl acetate content Overall, the consumption rate of reducing sugars increased with an increasing proportion of Y3401 (Fig. 2A). The reducing sugars were almost fully consumed within 4–5 days in the mixed fermentations. Furthermore, the reducing sugars consumption rate was similar or fastest when the inoculation ratio of Y3401 ≥ Y3604. When the Y3401/Y3604 inoculation ratio was 1:2 and 1:3, the utilization of reducing sugars was similar and higher, respectively, than that of the Y3604 single culture. Therefore, when the proportion of *S. cerevisiae* was higher, the reducing sugars consumption rate was increased, which was consistent with a previous report (23). Furthermore, these results indicated that the growth rate of Y3401 was higher than that of Y3604, in accordance with the above results.

The fermenting property trend is shown in Fig. 2B. From these cultivation ratios, the fermenting property was better when Y3401 was dominant than when Y3604 was dominant, including the single culture of Y3604. Among the mixed cultures, the fermenting property was highest when the inoculation ratio of Y3401/Y3604 was 3:1. Notably, the lowest fermenting property was observed for a Y3401/Y3604 ratio of 1:1, which was somewhat different from the reducing sugar consumption trend. The interaction between the yeasts was most obvious in this case, and some nutrients, including reducing sugars, were perhaps consumed and converted to antimicrobial metabolites during the course of this interaction (28,34,35).

As shown in Fig. 2C, ethanol production increased with an increasing proportion of Y3401. Furthermore, the ethanol yield was usually highest on the first or second day, with the ethanol content decreasing in the later stages of fermentation, owing to the rate of ethanol consumption and conversion into other substances becoming faster than the rate of ethanol production. Generally, the ethanol content increased as the inoculation ratio of Y3401 increased, and the ethanol content was higher when Y3401 was co-cultured with Y3604 compared with Y3604 single fermentation. These results indicated that a large number of precursors for the synthesis of ethyl acetate were introduced when Y3401 was added, in accordance with other reports (17,21). From the perspective of producing ethanol precursors, a higher proportion of *S. cerevisiae* in the mixture culture resulted in a higher ethanol content.

Fig. 2D shows that the amount of ethyl acetate initially increased and then decreased with time for the different inoculation ratios. The amount of ethyl acetate was higher when Y3401 was dominant, which was inconsistent with some previous reports (13). Furthermore, the highest ethyl acetate yield of 6.41 g/L was obtained with a Y3401/Y3604 ratio of 3:1. Unlike the fermenting property, the ethyl acetate yield was also relatively high with a 1:1 inoculation ratio, which was higher than when Y3604 was dominant. Therefore, except for a Y3401/Y3604 ratio of 6:1, ethyl acetate production

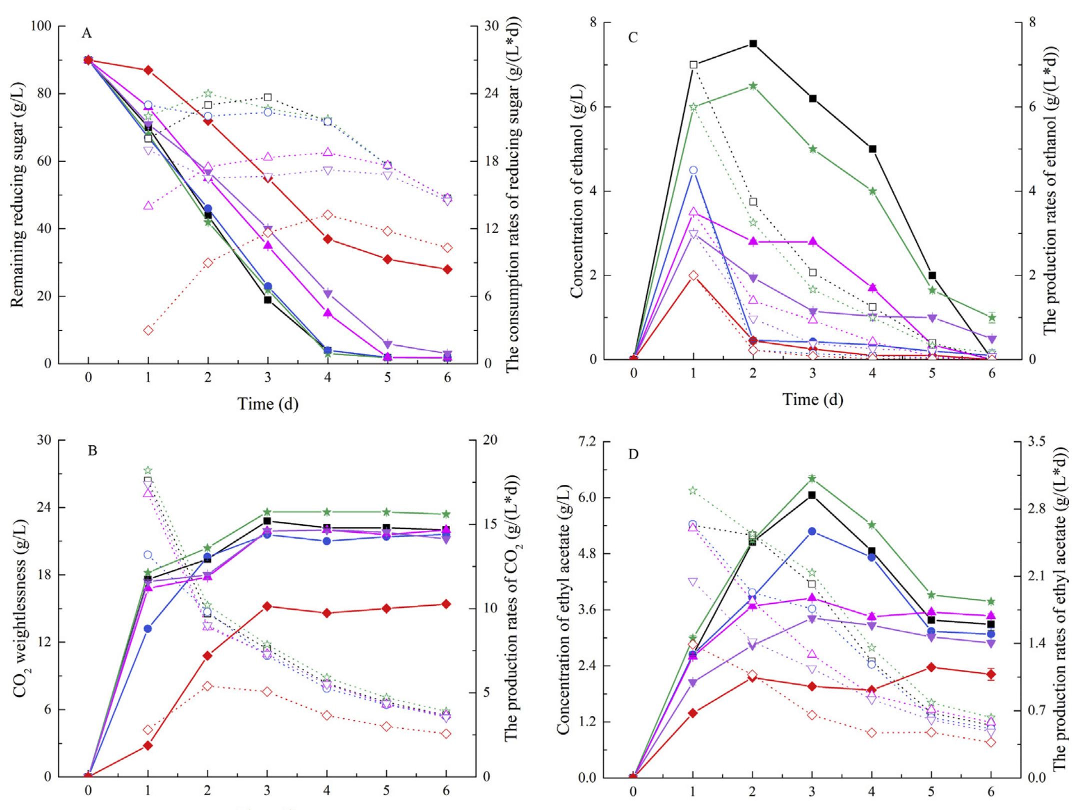

FIG. 2. Changes in remaining reducing sugars (A), fermenting properties (B), ethanol concentration (C), and ethyl acetate concentration (D) in different fermentations with different inoculation ratios. Squares, *S. cerevisiae* Y3401/*W. anomalus* Y3604 inoculation ratio of 6:1; stars, Y3401/Y3604 inoculation ratio of 3:1; circles, Y3401/Y3604 inoculation ratio of 1:1; regular triangles, Y3401/Y3604 inoculation ratio of 1:2; inverted triangles, Y3401/Y3604 inoculation ratio of 1:3; diamonds, single-culture fermentation by *W. anomalus* Y3604. Closed symbols and solid line, the changes of remaining reducing sugars (A), fermenting properties (B), ethanol concentration (C), and ethyl acetate concentration (D); open symbols and dot line, the consumption or production rates of reducing sugars (A), fermenting properties (B), ethanol (C), and ethyl acetate(D). Results are averages and bars denote SD.

TABLE 2. Some of the main volatile compounds present after different fermentations using different inoculation ratios (g/L).

Volatile compounds	*S. cerevisiae* Y3401:*W. anomalus* Y3604					
	6:1	3:1	1:1	1:2	1:3	0:1
Ethanol	0.06 ± 0.01[ab]	0.22 ± 0.05[c]	0.09 ± 0.02[b]	0.04 ± 0.02[a]	0.1 ± 0.03[b]	0.05 ± 0.03[ab]
β-Phenethyl alcohol	4.97 ± 0.22[b]	5.48 ± 0.16[d]	5.23 ± 0.04[c]	4.83 ± 0.18[b]	5.02 ± 0.11[b]	0.72 ± 0.13[a]
Isoamylol	0.84 ± 0.10[a]	0.93 ± 0.09[a]	0.82 ± 0.03[a]	0.73 ± 0.12[a]	1.02 ± 0.17[a]	0.86 ± 0.13[a]
Isobutanol	–	–	0.08 ± 0.03[a]	0.12 ± 0.02[a]	0.11 ± 0.03[a]	0.12 ± 0.04[a]
Σ Higher alcohols	5.81	6.41	6.13	5.68	6.15	1.70
Ethyl acetate	2.47 ± 0.14[c]	2.67 ± 0.21[c]	2.40 ± 0.11[c]	2.53 ± 0.18[c]	1.84 ± 0.09[b]	1.58 ± 0.05[a]
Phenethyl acetate	4.23 ± 0.22[c]	4.76 ± 0.18[d]	3.65 ± 0.26[b]	3.89 ± 0.12[bc]	3.75 ± 0.11[b]	1.22 ± 0.17[a]
Isoamyl acetate	3.79 ± 0.13[d]	3.74 ± 0.17[d]	2.36 ± 0.02[a]	2.84 ± 0.08[b]	3.08 ± 0.11[c]	3.56 ± 0.12[d]
Σ Esters	10.49	11.17	8.41	9.26	8.67	6.36
Acetic acid	0.04 ± 0.01[a]	0.03 ± 0.02[a]	–	0.06 ± 0.01[a]	0.03 ± 0.02[a]	0.02 ± 0.08[a]
Isobutyric acid	0.02 ± 0.01[a]	–	0.10 ± 0.02[b]	0.02 ± 0.02[a]	0.02 ± 0.00[a]	0.11 ± 0.02[b]
Methylbutanoic acid	–	–	0.25 ± 0.08	–	–	–
Octanoic acid	0.02 ± 0.01[a]	0.07 ± 0.02[b]	–	0.03 ± 0.00[a]	0.02 ± 0.02[a]	–
Caproic acid	–	0.02 ± 0.01	–	–	–	–
Σ Acids	0.08	0.12	0.35	0.11	0.07	0.13
Vinyl guaiacol	0.06 ± 0.02[a]	0.06 ± 0.01[a]	0.14 ± 0.03[b]	0.08 ± 0.03[ab]	0.07 ± 0.02[a]	0.10 ± 0.07[ab]
Sum	16.50	17.98	15.12	15.17	15.06	8.34

Data are averages of three replicates ± standard deviations; –, not detected; Same lowercase letters in each column denote no significant difference at the 5% probability level using Duncan's multiple range tests.

showed the same tendency as ethanol production, with the content increasing as the inoculation ratio of Y3401 increased. Although the concentration of ethanol, the ethyl acetate precursor, was highest with a Y3401/Y3604 ratio of 6:1, the ethyl acetate yield was lower than that with a Y3401/Y3604 ratio of 3:1 (Fig. 2C and D). The results also showed that the amount of ethyl acetate produced was relatively high compared with other mixed cultures (at Y3401/Y3604 ratios of 1:1, 1:2, and 1:3) and the single culture of Y3604. These results were essentially in line with expectations and might be explained by the interaction between yeasts. Firstly, although Y3604 had a high ethanol tolerance, as reported previously, its growth and normal metabolism would always be suppressed by a high ethanol concentration and the metabolites of Y3401 (5,18,19,23,25,36). Therefore, when the proportion of Y3401 was too high (Y3401/Y3604 ration of 6:1 in our results), the synthesis of ethyl acetate by Y3604 was affected, despite more ethanol being generated. Secondly, when the inoculation of Y3604 was too high, it had an inevitable effect on Y3401, influencing its ability to produce ethanol, as a precursor of ethyl acetate (18,36).

The levels of volatile compounds using different inoculation ratios are shown in Table 2. Similar to the inoculation method, the types and contents of flavor substances were also affected by the inoculation ratio. Under different inoculation ratios, the types and contents of flavor substances in the fermentation products that can affect the quality of *Baijiu* were different, and can be changed by adjusting the inoculation ratios of functional microorganisms. Compared with the single culture of *W. anomalus*, the total flavor contents, especially the alcohol and total ester contents, were higher in all tests with different inoculation ratios. Notably, the ethyl acetate, β-phenethyl alcohol, and phenethyl acetate contents were higher in all mixed cultures than in the single cultures, in agreement with the above results. Determining whether there is a correlation between these compounds in the synthetic pathway requires further study, but the synergistic effect of Y3401 and Y3604 clearly improved the esterification and enhanced the flavor of *Baijiu*. The isoamyl acetate content was not consistent with previous results, in which the content decreased in mixed fermentations compared with the single culture of *W. anomalus* (Table 1). With a high proportion of *S. cerevisiae* in the mixed fermentation, the isoamyl acetate content was higher than in the single culture of *W. anomalus*. However, previous results showed that *S. cerevisiae* did not produce isoamyl acetate itself (Table 1). This increase in isoamyl acetate content in the presence of a high *S. cerevisiae* concentration needs to be further studied. Furthermore, methylbutanoic acid was only produced under co-culture conditions at a Y3401/Y3604 ratio of 1:1. Meanwhile, octanoic acid was produced under all co-culture conditions except at a Y3401/Y3604 ratio of 1:1. Furthermore, caproic acid was produced only at a Y3401/Y3604 ratio of 3:1, with the vinyl guaiacol content in the mixed fermentation culture decreasing except when the Y3401/Y3604 inoculation ratio was 1:1. These results indicated that the type and content of flavor substances in *Baijiu* could be attributed to microbial synergy.

In conclusion, ethyl acetate production was enhanced by the mixed culture of *S. cerevisiae* and *W. anomalus* under suitable culture conditions, compared with using a single culture of *W. anomalus*. When the Y3401/Y3604 inoculation ratio was 3:1 by SMF, the highest ethyl acetate yield was 6.41 g/L. The results of this study could aid further understanding of interactions between two functional yeasts, and help improve the application of both yeasts in *Baijiu* production.

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