RESEARCH ARTICLE

Preparation of koji for rice wine fermentation using a mixture of beer lees and rice wine lees

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As the brewing industry continues to expand, the sustainable utilization of by-products, particularly lees, presents a growing challenge. Beyond their traditional use as animal feed and substrates for mushroom cultivation, there is a pressing need to explore new, high-value applications. This study investigated the use of a mixture of rice wine lees and beer lees as a substrate for cultivating specialized fungi for rice wine koji production. Through single-factor and response surface methodology experiments, optimal conditions for two types of rice wine koji, based on *Aspergillus flavus* and *Rhizopus oryzae*, were identified. The highest enzyme activity for *Aspergillus flavus* koji was achieved with beer lees to rice wine lees ratio of 11:6, 33°C, 10% wheat debris, and 50% moisture content. For *Rhizopus oryzae* koji, the optimal conditions included beer lees to rice wine lees ratio of 11:6, 31°C, 15% rice debris, and 75% moisture. In comparative brewing experiments, rice wine produced with the self-made koji demonstrated similar quality to that made with traditional koji with no significant differences in the final product. These results confirmed that wine lees could be effectively recycled within the brewing industry, opening up new avenues for the resource utilization of wine lees.

Keywords: beer lees; rice wine lees; rice wine fermentation; koji; *Aspergillus flavus*; *Rhizopus oryzae*.

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Introduction

Both beer and Chinese rice wine (Huangjiu) are produced from grains through microbial fermentation. These industries consume substantial grain resources and generate significant biomass waste, specifically lees [1, 2]. The beer brewing process yields approximately 0.8 to 1 ton of beer lees (brewer's grains) for every ton of beer produced. Data from 2020 estimated the global annual beer production at 1.82 billion hectoliters, resulting in the production of beer lees at approximately 36.4 million tons [3]. Rice wine, an ancient Chinese beverage popular across East Asia, serves both as an alcoholic drink and a culinary ingredient. It is typically made from rice and other grains, which are fermented by molds and yeasts, leading to rice wine lees that predominantly consist of residual rice components [4]. With the advent of mechanized production, rice wine production has expanded rapidly, generating about 3.5 million tons annually in China with lees accounting for 20-30% of this output [5, 6]. Consequently, the resource consumption and waste generation associated with the rice wine industry have

become increasingly problematic. Utilizing lees for resource recovery is a prominent area of current research with various studies reported. Traditional methods for the utilization of beer and rice wine lees include cross-industry applications such as animal feed [5, 7-9], flavor development for food products [10], mushroom cultivation substrates [11, 12], agricultural fertilizers [13], and bioconversion processes for producing active ingredients [14, 15]. As fermentation residues, beer and rice wine lees retain a wealth of nutrients conducive to microbial growth. The combination of different types of lees could yield new physical and chemical properties, enhancing their value as microbial substrates.

Beer lees, primarily derived from barley, consist of malt husks, sprout leaves, insoluble proteins, hemicellulose, fats, ash, and some unhydrolyzed starch and soluble extracts. They are notably rich in crude protein and trace elements with dry matter comprising 24-28% crude protein, 8-11% crude fat, 55-61% crude fiber, 1-3% sugars, and 3-5% ash [16, 17]. In contrast, rice wine lees, which primarily originate from rice but may also include other grains, exhibit compositional variability depending on the raw materials and brewing processes. Analysis of rice wine lees from a brewery in Wuxi, Jiangsu, China revealed 48% moisture, 30% protein, and 15% starch, while a brewery in Shaoxing, Zhejiang, China showed similar moisture content with 33% starch and 14% protein [18]. These findings highlight the differences in the chemical compositions of beer and rice wine lees, resulting in distinct physical characteristics such as hardness and humidity. When mixed in specific proportions, these lees are expected to yield biomass resources with entirely new physical and chemical properties. However, research in this area remains limited. Given the rich nutrient content in both beer and rice wine lees, their suboptimal utilization in brewing may stem from constraints related to reaction conditions and microbial types. Developing suitable utilization methods could enable their repurposing by microorganisms.

Contrary to previous predominant theories, this research proposed a novel approach to the resourceful utilization of lees by investigating their reuse within the brewing industry. This study mixed rice wine lees and beer lees to enhance their composition and physicochemical properties, facilitating the cultivation of fungi and yeast necessary for rice wine production. This innovative strategy sought to recycle brewing waste back into the brewing process, thereby expanding resource utilization, increasing the value of the lees, and reducing grain consumption in the brewing industry, ultimately offering significant benefits.

Materials and methods

Preparation of materials

Glutinous rice, wheat, and bran were procured from Hangzhou Dongxin Agricultural Market Co., Ltd. (Hangzhou, Zhejiang, China) and subsequently grounded using a grinder. Rice wine lees were supplied by Jiaxing Brewing Company (Jiaxing, Zhejiang, China), while beer lees were obtained from the Institute of Food and Fermentation Engineering, Zhejiang Shuren University (Hangzhou, Zhejiang, China). Both types of lees were dried at 65°C until a moisture content of 10% being achieved before they were grounded and sieved through a 20-mesh screen. The microorganisms, *Rhizopus oryzae* BY (CICC-40134), *Rhizopus oryzae* Q303 (CICC-3153), *Aspergillus flavus* Su16 (CICC-2384), *Aspergillus oryzae* HN3.402 (CICC-40855), were preserved at the Institute of Food and Fermentation Engineering, Zhejiang Shuren University [19-21]. *Saccharomyces cerevisiae* was purchased from Angel Yeast Co., Ltd. (Yichang, Hubei, China). The potato dextrose agar (PDA) medium was prepared by dissolving 200 g of potato powder, 20 g of dextrose, 20 g of agar, 1.5 g of $MgSO_4 \cdot 7H_2O$, 3 g of KH_2PO_4, 8 mg of Vitamin B_1 in 1,000 mL of water following standard procedures [22]. All ingredients were obtained from Shanghai Bio-way Co., Ltd. (Shanghai, China).

Selection of microorganic strains

Table 1. Design of the single factor optimization experiments.

Experiment	Lees weight (g)	Beer lees to rice wine lees ratio	Humidity (%)	Spore inoculation (mL)	Wheat/Rice debris (g)	Temperature (℃)	Shaking speed (RPM)	Culture time (h)	Response indicators
1	20	3:5, 3:6, 3:7, 1:1, 7:5, 7:6, 11:5, 11:6, 11:7	10	1.0	-	30	150	60	glycase and protease activity
2	20	11:6	10	1.0, 2.0, 3.0, 4.0, 5.0	-	30	150	60	mycelial concentration
3	20	11:6	20, 30, 40, 50, 60, 70, 80, 90.	1.0	-	30	150	60	mycelial concentration
4	20	11:6	10	1.0	0, 1.0, 2.0, 3,0, 4.0 (Wheat for Su16. Rice for Q303)	30	150	60	glycase activity
5	20	11:6	10	1.0	-	29, 31, 33 for Su16 33, 35, 37 for Q303	150	60	glycase activity

The microorganisms were activated by thawing frozen spores, streaking them onto PDA medium plates, incubating the plates at 30°C in an IMH60-S mold incubator (Thermo Fisher Scientific, Waltham, MA, USA) for approximately 5 days until spore formation being observed. Colonies were picked and transferred into 10 mL of sterile water to create spore suspension. 1 mL of each spore suspension was inoculated into 20 g of a mixed lees in 250 mL Erlenmeyer flasks medium with approximately 60% moisture content and a ratio of beer lees to rice wine lees of 3:2. After thoroughly mixing, the mixture was incubated in Wiggens WS-600 shaking incubator (Wiggens, Berlin, Germany) at 30°C, 150 rpm, for 48 to 65 hours.

Single-factor optimization experiments for koji preparation

Five single-factor experiments were conducted for the *Aspergillus flavus* Su16 and the *Rhizopus oryzae* Q303 to optimize the ratio of beer lees to rice wine lees, moisture content of the lees, spore suspension inoculation amount, the addition of wheat debris or rice debris, and incubation temperature (Table 1). Each experiment involved placing the culture in 250 mL Erlenmeyer flasks and incubating them on a shaker for evaluation of mycelial concentration, glycase activity, and protease activity. The determination of glycase activity was carried out using a photometric reagent kit (Sint-Bio Company, Shanghai, China) on an Agilent Cary 60 UV-Vis spectrophotometer (Agilent Technologies Co., Ltd., Santa Clara, CA, USA) following manufacturer's instructions. One enzyme activity unit was defined as the amount of enzyme required to produce 1 mg of glucose per mg of protein per hour at 40℃. The protease activity was determined according to the national standard method based on the Folin method, and the ability to hydrolyze casein to produce 1 mg tyrosine per minute at 40℃ was defined as one enzyme activity unit [23].

Response surface methodology (RSM) for optimizing reaction conditions

A Box-Behnken response surface experiment was designed for both *Aspergillus flavus* Su16 and *Rhizopus oryzae* Q303, respectively [24]. For Su16, the variables included temperature (X1) at levels of 33, 35, and 37℃; wheat debris (X2) at 1,

2, and 3 g; and humidity (X3) at 40, 50, and 60%. For Q303, the variables were temperature (X1) set at 29, 31, and 33℃; rice debris (X2) at 1, 2, and 3 g; humidity (X3) at 60, 70, and 80%. Glycase activity served as the response variable for both experiments.

Rice wine brewing experiment

The self-prepared koji and starter based on *Rhizopus oryzae* Q303 were evaluated for rice wine brewing. 5 kg of rice were cooked into glutinous rice, to which 1% of either, a traditional pure starter or a self-made starter (self-prepared koji with 1/3 *Saccharomyces cerevisiae*) was added for saccharification and fermentation at 30°C. Once the liquid volume reached 4/5 of the fermentation vessel, 8% of either traditional pure ripe wheat koji or self-made koji along with 110% volume of water was added for tanking and fermentation at 30°C. Samples were collected daily during fermentation to measure alcohol content. After fermentation, total sugar, alcohol content, total acid, and amino nitrogen were analyzed using Fehling reagent - indirect iodometric titration method [25], precision alcohol meter (Zhecheng Technology Co., Ltd., Beijing, China) method [26], acid-base titration method [27], and formaldehyde fixing – acidimeter (Mettler-Toledo Co. Ltd., Zurich, Switzerland) method [28], respectively.

Statistical analysis

Design Expert software, version 11.0.4.0 (Stat-Ease Corp., Chula Vista, CA, USA) was employed for RSM design and subsequent data analysis. SPSS, version 20.0 (IBM, Armonk, NY, USA) with Duncan multiple comparison method was used for statistical analysis.

Results

Selection of microorganisms

The results showed that no visible growth was observed in any strain at 12 hours incubation. Su-16 demonstrated moderate growth (++), while Hu-3.402, Q303, and BY exhibited no growth by 24 hours. At 36 hours, Su-16 displayed substantial growth (+++), while Hu-3.402 and Q303 demonstrated moderate growth (++), and BY began to show initial growth (+). Su-16 exhibited extensive growth (++++), Hu-3.402 maintained moderate growth (++), Q303 showed substantial growth (+++), and BY continued its initial growth (+) at 48 hours. At 60 hours, Su-16 sustained extensive growth (++++), Hu-3.402 remained at moderate growth (++), Q303's growth reached extensive levels (+++), and BY's growth advanced slightly (+). Ultimately, *Aspergillus flavus* Su16 and *Rhizopus oryzae* Q303 were selected for further evaluation.

Optimization of reaction conditions through single-factor experiments

Regarding the optimal ratio of beer lees to rice wine lees, *Aspergillus flavus* Su16 exhibited substantial mycelial growth at ratios of 11:6, 7:5, 11:7, and 3:6, respectively. Enzyme activity measurements indicated that the 11:6 ratio yielded the highest glycase and protease activities (Figure 1-a), identifying it as the optimal condition. For *Rhizopus oryzae*, significant mycelial growth was observed at ratios of 11:5, 11:6, and 7:6, respectively, while enzyme activity measurements showed that the 11:6 ratio resulted in the highest glycase activity (Figure 1-b). However, protease activity was not the highest, but close to the highest group. Thus, the optimal ratio of beer lees to rice wine lees for both microorganisms were both determined to be 11:6. The optimal inoculum for *Aspergillus flavus* Su16 was 4 mL with an estimated spore count of approximately 51,000 spores per mL. For *Rhizopus oryzae* Q303, the optimal inoculum was 3 mL with an estimated spore count of approximately 32,000 spores per mL. The optimal moisture content for mixed lees was 50% with approximately 18 mL of water per 20 g of lees for *Aspergillus flavus* and 70% with approximately 50 mL of water per 20 g of mixed lees for *Rhizopus oryzae*. As for the addition of auxiliary materials, the addition of 2 g of wheat debris per 20 g of mixed lees was optimal for Aspergillus flavus Su16, while 3 g of rice wheat debris per 20 g of mixed lees was optimal for *Rhizopus oryzae* Q303 (Figure 1c). The incubation temperature

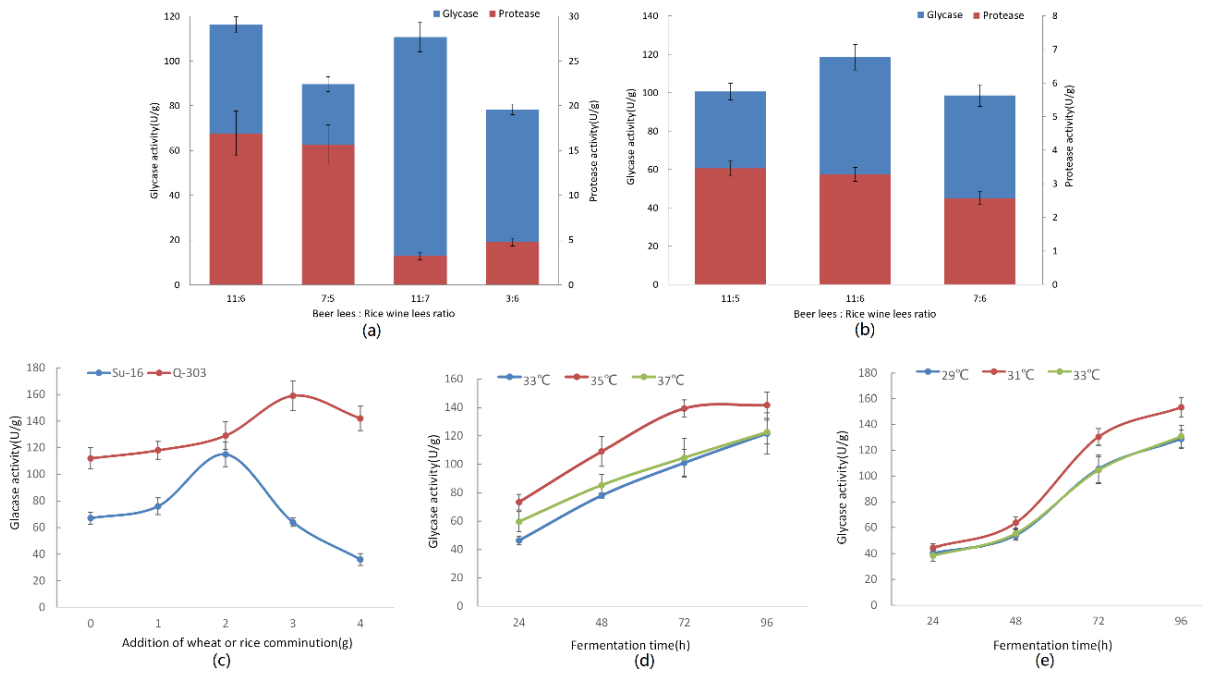

Figure 1. The influence of excipients and temperature on the enzymatic activity of two microorganisms. **(a)** the effects of beer lees to rice wine lees ratio on the enzyme activity of Su16. **(b)** the effects of beer lees to rice wine lees ratio on the enzyme activity of Q303. **(c)** the effects of wheat and rice additives on the enzyme activity of Su16 and Q303. **(d)** the effects of temperature on the enzyme activity of Su16. **(e)** the effects of temperature on the enzyme activity of Q303.

experiments showed that *Aspergillus flavus* Su16 had the highest glycase activity at 35°C (Figure 1d), while *Rhizopus oryzae* Q303 had the highest glycase activity at 31°C (Figure 1e).

Response surface experiments

(1) Aspergillus flavus Su16

The variance analysis results of the RSM experiment showed that the regression model was highly significant ($P < 0.001$) with a Lack of Fit P value larger than 0.05 and an R^2 of 0.9839, indicating a good model fit (Table 2). According to the regression equation, the factors affecting enzyme activity in descending order of significance were B^2, A^2, C^2, C, and B, while the other variables were not significant. Interaction effects demonstrated the strongest interaction between AB followed by BC with AC being the weakest one (Figures 2a-2c). The regression equation for enzyme activity and variables was as follows.

$$Y = 117.92 + 1.90A + 6.24B + 7.69C - 2.22AB - 0.5447AC - 1.47BC - 25.14A^2 - 43.16B^2 - 17.93C^2$$

The optimal conditions determined from the response surface analysis were temperature 35.064°C, wheat debris 2.068 g/L, humidity 52.105% with a glycase activity of 118.976 U/g, and desirability of 0.958.

(2) Rhizopus oryzae Q303

The RSM variance analysis results for *Rhizopus oryzae* Q303 showed that the regression model was highly significant ($P < 0.001$) with a Lack of Fit P value larger than 0.05 and an R^2 of 0.9550, indicating a good model fit (Table 3). The factors influencing enzyme activity based on the regression equation were B^2, A^2, and C^2. Interaction effects revealed AC as the strongest followed by BC with AB as the weakest, though none of those interactions were significant (Figures 2d-2e). The regression equation for enzyme activity and variables was as below.

$$Y = 134.51 - 0.1273A + 5.76B - 4.62C - 8.12AB - 11.63AC + 10.08BC - 26.09A^2 - 56.64B^2 - 17.23C^2$$

The optimal conditions obtained were

Table 2. Variance analysis in RSM experiments of *Aspergillus flavus* Su16.

Source	Sum of squares	df	Mean square	*F* value	*P* value
Model	13,856.71	9	1,539.634	47.389	< 0.001***
Temperature (A)	28.736	1	28.736	0.884	0.378
Wheatmeal (B)	311.014	1	311.014	9.573	0.017*
Humidity (C)	472.735	1	472.735	14.551	0.007**
AB	19.638	1	19.638	0.604	0.462
AC	1.187	1	1.187	0.037	0.854
BC	8.585	1	8.585	0.264	0.623
A^2	2,661.506	1	2,661.506	81.920	< 0.001***
B^2	7,842.217	1	7,842.217	241.381	< 0.001***
C^2	1,354.300	1	1,354.3	41.685	< 0.001***
Residual	227.423	7	32.489		
Lack of Fit	104.136	3	34.712	1.126	0.438
Pure Error	123.286	4	30.822		
Cor Total	14,084.130	16			

Notes: *: $P < 0.05$. **: $P < 0.01$. ***: $P < 0.001$.

Table 3. Variance analysis in RSM experiments of *Rhizopus oryzae* Q303.

Source	Sum of squares	df	Mean square	*F* value	*P* value
Model	20,739.560	9	2,304.396	16.522	< 0.001***
Temperature (A)	0.130	1	0.130	0.001	0.977
Rice debris (B)	265.455	1	265.455	1.903	0.210
Humidity (C)	170.598	1	170.598	1.223	0.305
AB	263.608	1	263.608	1.890	0.216
AC	540.795	1	540.795	3.877	0.090
BC	406.567	1	406.567	2.915	0.132
A^2	2,866.874	1	2,866.874	20.554	0.003*
B^2	13,505.830	1	13,505.830	96.832	< 0.001***
C^2	1,249.842	1	1,249.842	8.961	0.020*
Residual	976.342	7	139.478		
Lack of Fit	804.927	3	268.309	6.261	0.054
Pure Error	171.416	4	42.854		
Cor Total	21,715.910	16			

Notes: *: $P < 0.05$. **: $P < 0.01$. ***: $P < 0.001$.

temperature 31.041°C, rice debris 2.038%, humidity 68.704% with a glycase activity of 134.915 U/g and desirability of 0.910.

Brewing experiment

Regarding saccharification, rice wine brewed with self-made starters and koji after 2 days of fermentation showed a higher degree of saccharification than those using traditional products. The alcohol content curves for rice wine brewed with traditional starters and koji, self-made starters and koji, and their cross-combinations were similar, indicating that all methods were viable for rice wine brewing. Rice wine brewed with self-made starters and koji showed slightly higher alcohol content than the others, demonstrating the quality of the self-made koji (Figure 3). Rice wine brewed with self-made starters and koji had higher total sugar content, indicating better saccharification compared to traditional products ($P < 0.05$). Additionally, the alcohol content and amino

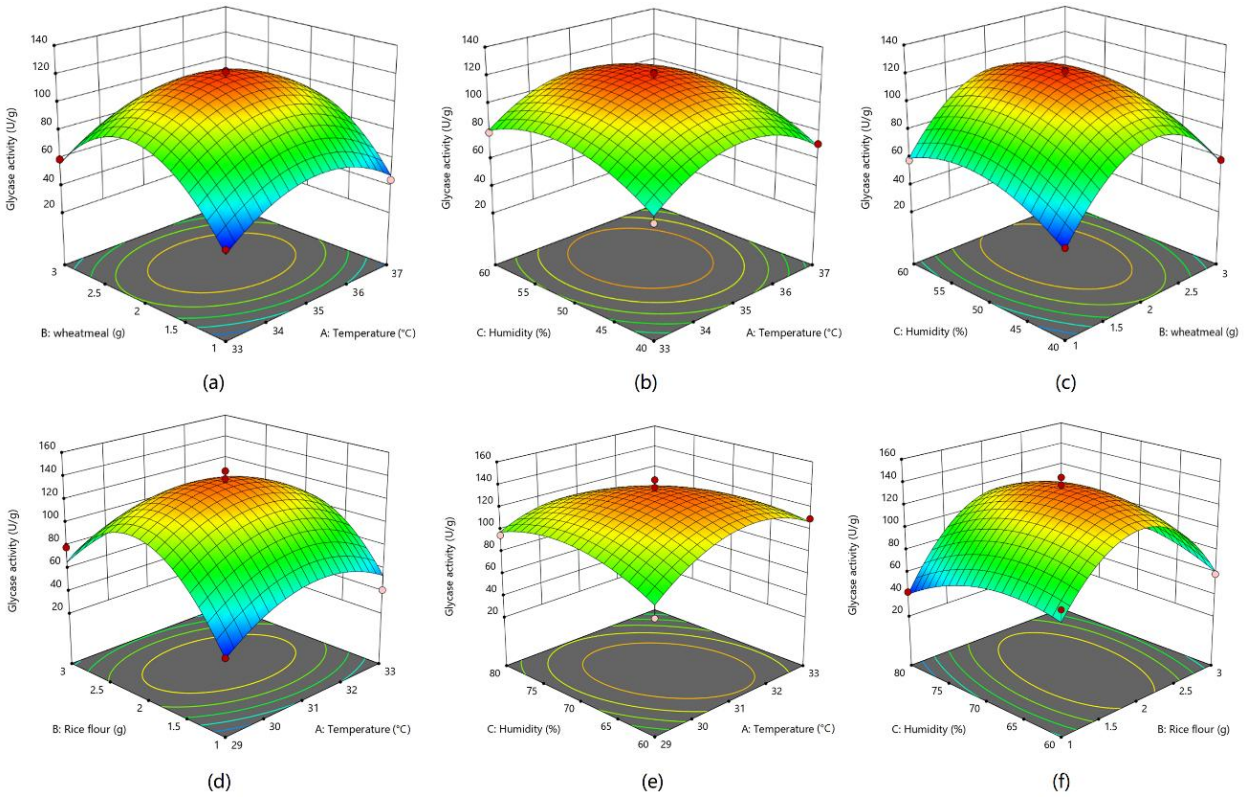

Figure 2. Variable interaction effects in RSM experiments. **(a) - (c):** *Aspergillus flavus* Su16. **(d) - (f):** *Rhizopus oryzae* Q303.

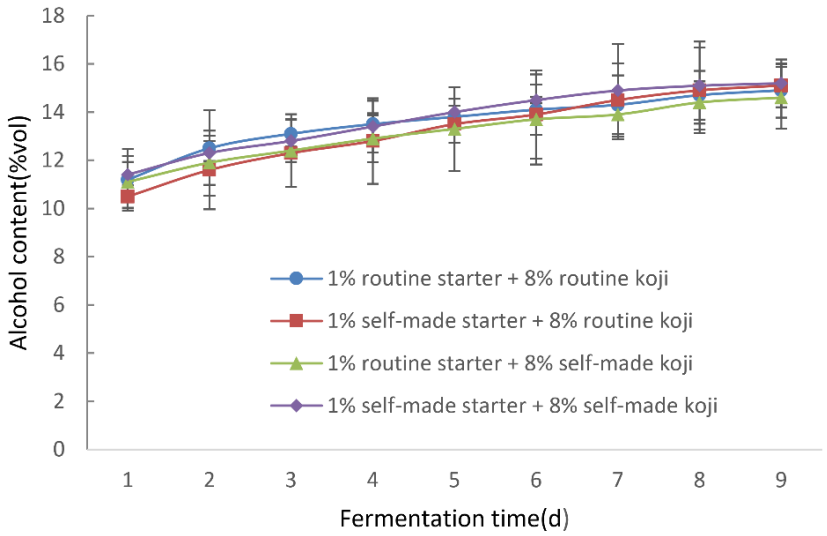

Figure 3. The variation curve of alcohol content in the fermentation broth during the brewing process.

nitrogen levels were slightly higher, while total acid content was slightly lower than traditional products. Overall, rice wine brewed with self-made starters and koji achieved similar quality to traditional methods with even better performance in total sugar and amino nitrogen (Table 4).

Table 4. Comparison of key indicators in brewing experiments.

	Total sugar (g/L)	Ethanol (%, v/v)	Total acid (g/L)	Amino nitrogen (g/L)
1% routine starter + 8% routine koji	2.79 ± 0.33[a]	14.88 ± 0.17	5.31 ± 0.14	0.80 ± 0.04
1% self-made starter + 8% routine koji	7.62 ± 0.08[b]	15.11 ± 0.21	5.69 ± 0.06	1.14 ± 0.11
1% routine starter + 8% self-made koji	8.02 ± 0.23[b]	14.63 ± 0.13	6.56 ± 0.18	1.13 ± 0.07
1% self-made starter + 8% self-made koji	9.02 ± 0.14[b]	15.20 ± 0.18	5.03 ± 0.07	1.23 ± 0.05

Note: Different letters meant significant differences ($P < 0.05$).

Discussion

This study investigated the combination of beer lees and rice wine lees, which possessed entirely different properties, for resource utilization. By optimizing key parameters such as blending ratio and water addition, a nutrient-rich mixed lees that was conducive to the growth of brewing microorganisms in terms of humidity and fluffiness was obtained. Two classic fungal strains, Su16 and Q303, were employed in rice wine brewing to successfully prepare two types of koji through careful optimization of specific reaction conditions. A small-scale brewing trial indicated that the primary indicators of rice wine produced using self-made koji were strikingly similar to those achieved through traditional techniques, which confirmed that the production waste generated by the brewing industry could be effectively recycled within the sector, thus reducing solid waste emissions. This study contributed to the broader field of waste utilization by presenting a viable method for recycling brewing by-products within the brewing industry itself. By converting beer lees and rice wine lees into valuable substrates for koji production, this approach promoted a circular economy and mitigated the environmental impact associated with brewing waste. Furthermore, the successful application of mixed lees in rice wine brewing implied that similar strategies could be explored for other types of brewing and fermentation processes, potentially leading to more sustainable practices across the food and beverage industry. The utilization of mixed beer and rice wine lees to produce starter and koji in rice wine brewing represented a significant advancement in the valorization of brewing by-products. This study not only offered a practical solution for waste management but also enhanced the sustainability and efficiency of rice wine production. Future research could investigate the scalability of this approach and explore its application in other fermentation-based industries.

The rising number of lees produced in the brewing industry poses significant challenges for environmental protection, making their resource utilization a critical research focus. Taking rice wine lees as an example, solid-state fermentation and pressing are vital processes in Chinese rice wine production. During fermentation, starch and protein are converted into alcohols and esters. After pressing, the remaining solid, known as lees, contains substantial amounts of biomass, including protein, starch, fiber, and phenolic compounds. Rice wine lees are difficult to preserve due to their high-water content and low pH. Without efficient treatment, decomposing lees can lead to environmental issues such as odors and pollution of water and soil. To mitigate this, research is concentrating on resourceful applications of lees including feed production, food additives, vinegar brewing, edible fungi cultivation, and the extraction of high-value compounds. While substituting for livestock feed has historically been the primary use, modern farms increasingly prefer standardized animal feed, resulting in a rapid decline in demand for rice wine lees [29]. Therefore, it is crucial to identify new application areas for rice wine lees. Recent advancements have explored more innovative applications, such as the extraction of bioactive compounds, production of biofuels, and incorporation into functional foods. There is also a notable surge in developing techniques for

recovering compounds derived from food waste and by-products [30]. However, the extraction of small amounts of components often still results in significant waste generation. Beer lees are primarily composed of malt husks, leaf buds, insoluble proteins, hemicellulose, fats, ash, and a small amount of undecomposed starch and unwashed soluble extracts. The variations in raw materials and fermentation processes employed in beer production lead to different components in beer lees. Therefore, analyzing their composition is necessary for effective utilization. Overall, beer lees contain abundant crude protein and trace elements, providing high nutritional value and can be utilized in various ways including production of enzymes, animal feed, biogas, and protein extraction. Growing research has increasingly focused on their applications within the food industry, while their low-value utilization in the feed industry remains extensive [29]. Consequently, it is also necessary to identify new high-value utilization methods for beer lees. The investigation into using beer lees to prepare rice wine koji has not been previously reported, making it a worthwhile area for exploration.

The *Aspergillus* and *Rhizopus* fungi used in rice wine koji primarily serve to provide highly active enzymes for the degradation and transformation of macromolecular substances, establishing favorable conditions for subsequent yeast fermentation. Consequently, the enzyme production capacity and activity of *Aspergillus* and *Rhizopus* warrant significant attention. *Aspergillus flavus* is a strain known for producing a complex array of enzymes. In addition to proteases, it can generate amylase, glucoamylase, cellulase, phytase, and pectinase. Notably, *Aspergillus flavus* does not produce aflatoxins and is the predominant strain utilized in the traditional production of foods such as soy sauce and miso in China. In alcohol production, it acts as a saccharifying agent and is also a producer of kojic acid [31]. *Rhizopus oryzae*, a potent saprophytic and pathogenic fungus, can produce a diverse range of metabolites including enzymes, esters, organic acids, volatile materials, polymers, and bioalcohols. Various strains of *Rhizopus oryzae* synthesize numerous extracellular and intracellular enzymes, such as cellulases, hemicellulases, pectinases, tannase, phytase, amylase, lipase, protease, and other enzymes of considerable industrial importance [32]. The nutrients necessary for the growth of *Aspergillus* and *Rhizopus* primarily consist of carbon compounds commonly referred to as carbon sources, nitrogen compounds generally referred to as nitrogen sources, inorganic salts, water, and growth factors. However, different strains often exhibit distinct preferences, which can result in variations in their growth abilities under identical conditions [33, 34]. The selection of *Aspergillus flavus* Su16 and *Rhizopus oryzae* Q303 as the optimal strains for this study underscored their robust growth and enzyme production capabilities on the mixed lees substrate. This adaptability suggested that these strains could thrive on the nutrient profiles provided by the combination of beer lees and rice wine lees. The successful application of these strains indicated that mixed lees could serve as a suitable and effective medium for microbial cultivation in brewing applications. The single-factor experiments yielded valuable insights into the optimal conditions for maximizing enzyme activity during koji production. The identification of the optimal ratio of beer lees to rice wine lees (11:6) and the precise adjustment of inoculation amounts, moisture content, and auxiliary material additions highlighted the significance of meticulous control over fermentation parameters. These optimizations were essential for enhancing microbial activity and enzyme production, both critical for high-quality rice wine brewing. The response surface methodology (RSM) further refined these conditions, illustrating the value of a systematic and statistical approach to fermentation optimization. The high R^2 values obtained in the RSM analysis for both *Aspergillus flavus* and *Rhizopus oryzae* strains indicated a strong correlation between the predicted and observed enzyme activities, confirming the reliability of the optimized conditions. The brewing experiments demonstrated that rice wine produced with the

self-made starters and koji exhibited comparable, if not superior, qualities to those brewed with commercially available products. The slightly elevated alcohol content and improved saccharification observed with the self-made koji suggested that the mixed lees not only supported effective microbial growth but might also enhance the overall fermentation process [35, 36], which underscored the potential for improving the efficiency and quality of rice wine production through the integration of mixed lees into the fermentation process.

Conclusion

This study confirmed for the first time that a specific ratio of rice wine lees and beer lees could be used as a culture medium for brewing microorganisms. The results demonstrated the potential for recycling and reusing lees in the brewing industry, opening new avenues for the resource utilization of brewing lees. The research identified two rice wine microorganisms, *Rhizopus oryzae* Q303 and *Aspergillus oryzae* Su16, as suitable for mixed substrates of rice wine and beer lees. Through detailed optimization of cultivation conditions, both strains were shown to produce rice wine koji with high protease and amylase activity. In a brewing experiment using *Rhizopus*, rice wine produced with self-made koji and starter demonstrated key quality indicators comparable to those of traditional koji, suggesting that the results of this study had promising industrial application prospects.

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