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Screening and evaluation of *Monascus purpureus* FJMR24 for enhancing the raw material utilization rate in rice wine brewing

Runing title: Effect of fungus strain on URRM

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Abstract

BACKGROUND: The rapid development of the rice wine industry has increased the demand for raw materials worldwide. A fungal strain with good adaptability to rice wine brewing conditions that can also enhance the utilization rate of raw materials (URRM), thus increasing the production efficiency, was sought in the present research.

RESULTS: The strain FJMR24 was successfully isolated and screened from 35 fermentation starters and exhibited high amylase activity (2200.9 ± 18.5 U g^{-1}) and high glucoamylase activity (2330.4 ± 31.9 U g^{-1}). Based on morphological examination and sequence analysis of the internal transcribed spacer (ITS) gene and *β-tubulin* gene, FJMR24 was identified as *Monascus purpureus*, which is an edible and versatile fungus that plays a dominant role in the processing of Hong Qu. A moderate pH of 5–6 under incubation at 35 °C for 5–6 d was favorable for the growth and enzyme production of FJMR24. The strain could also tolerate the extreme conditions of 15–45 °C, 18% ethanol (v/v), and an acidity of pH 2. The excellent fermentation adaptability of FJMR24 might enable it to retain high enzyme activity during rice wine brewing. As a result of the action of FJMR24, the URRM of the base liquor increased by around 26% due to increased starch hydrolysis efficiency, which was mainly due to the high unit enzyme activity of FJMR24.

CONCLUSION: This study provides perspectives for the application of a *M. purpureus* strain with high starch hydrolysis activity for enhancing the URRM in traditional rice wine brewing.

Keywords: rice wine; Hong Qu; raw materials; *Monascus purpureus*; enzyme activity; fermentation adaptability

INTRODUCTION

Rice wine (also called “Huangjiu” in China, or yellow wine because of its yellowish color) is a very popular alcoholic beverage in China and East Asia.[1] Hong Qu rice wine using Hong Qu as the fermentation starter is a quintessential representative of rice wine.[2] It is known as “liquid cake” due to its bright red color, subtle sweet flavor, unique aroma, low alcohol content, and healthcare functions.[3] Recently, the production of Hong Qu rice wine rice has been increasing rapidly due to increased consumer demand. This has led to the increasing demand for raw materials, thus provoking grain shortages worldwide.[4] Strategies to reduce the demand for raw materials without affecting the demand of customers are needed. Glutinous rice, the main raw material of rice wine in China, is rich in starch, 98% of which is amylopectin. However, the low starch hydrolysis rate (65–67%) in the traditional rice wine process causes 20–30% residual starch in the vinasse.[5] Therefore, enhancing the utilization rate of raw materials (URRM) might constitute an effective strategy for improving the vinasse.

A series of new technologies and combined techniques using materials and fermentation processes have been developed in an attempt to improve the URRM and decrease the ratio of vinasse. For instance, liquefaction technology, extrusion technique, roasting method, and brewing method of uncooked material have achieved improved starch degradation and enhanced fermentation efficiency.[6,7] However, these techniques present some challenges, such as declines in nutritional value, a lighter flavor, and higher cost,[8] which limit their practical application.

Microbial properties are the principal internal factors affecting fermentation efficiency. The highly diverse microorganisms in Hong Qu derived from the traditional fermentation process provide abundant enzymes, facilitating the transformation of substances such as starch, protein, and

fat into nutrients, flavors, and functional secondary metabolites.[9] Notably, filamentous fungi in Hong Qu were reported to be the most important microorganisms converting starch to low-molecular substances based on the performance of amylase (liquefying power) and glucoamylase (saccharifying power).[10] Thus, the activities of amylase and glucoamylase in filamentous fungi are the key to enhancing the URRM in rice wine brewing. However, traditional Hong Qu is still made under nonsterile conditions based on empirical practice, causing an unclear microbial profile and inconsistent wine quality.[11] In this situation, it would be difficult to clarify and regulate the function of filamentous fungi within Hong Qu. Therefore, a solution was proposed in the present study, namely to screen the strains responsible for the high utilization of starch and then produce a defined starter. This would not only enhance the URRM but also improve the wine quality.

With respect to the screening and application of a pure strain, many studies have analyzed the microbial diversity and screened and characterized the dominant species of Hong Qu during the rice wine brewing process.[12,13] These results would provide theoretical guidance for screening high starch-hydrolysis strains. In addition, previous studies on the technique of multi-strain co-fermentation have preliminarily proved the feasibility of using pure strains to govern wine quality.[14] However, its application in rice wine brewing remains tenuous.

The main objectives of the present study were to (1) screen a fungal strain that could efficiently hydrolyze starch; (2) evaluate the adaptability of the screened strain under fermentation environments such as temperature, pH, and alcohol based on its growth profiles; and (3) investigate the effects of the screened strain on the physicochemical properties and quality of the rice wine, validating the potential of the new strain for application in rice wine production.

MATERIALS AND METHODS

Sample collection and isolation of fungal strains

Thirty-five samples of traditional rice wine fermentation starters (19 Hong Qu, 14 Wuyi Hong Qu, and 2 Yao Qu) were collected from different geographical areas across Fujian province, China (26.510°N, 118.090°E). Samples were stored at 4 °C immediately until further processing.

Fungi isolation was performed using a program described by Lv *et al.*[10] The media of Czapek-Dox Agar (CDA) (Guangdong Huankai Microbial Sci. & Tech. Co., Ltd., Guangzhou, China) and Potato Dextrose Agar (PDA) (Guangdong Huankai Microbial Sci. & Tech. Co., Ltd., Guangzhou, China) were used for isolation, and the isolates were maintained on PDA slants at 4 °C.

Screening strains with high starch hydrolysis efficiency

Spore suspensions were prepared as follows: all the isolated strains were activated by two successive transfers to PDA slants and incubated at 30 °C for 7 d under aerobic conditions. Spores were harvested by flooding the surface of the agar with sterile distilled water. The concentration of spore suspension was adjusted to 10^6 spores mL^{-1} by cell-counting method with a haemocytometer.[15] The spore suspensions were stored at 4 °C before further use.

Preliminary screening was performed as follows: the isolated strains were preliminarily screened using the transparent circle method.[16] Each spore suspension (1 mL) was serially diluted, and 0.2 mL of 10^{-5} dilution was spread on an agar plate containing 20 mL of 5 g kg^{-1} peptone, 5 g kg^{-1} yeast extract, 30 g kg^{-1} soluble starch, 0.5 g kg^{-1} calcium chloride, 0.5 g kg^{-1} manganese chloride, 0.5 g kg^{-1} magnesium sulfate, 1 g kg^{-1} monopotassium phosphate, and 20 g kg^{-1} agar. After incubation at 30°C for 2–3 d, iodine solution was spread on the plate, following which transparent circles formed around the single colonies. The diameters of the transparent circles (H) and the

corresponding colonies (C) were measured by a caliper, and H/C was used to evaluate the ability of the strains to hydrolyze starch.

Re-screening was performed as follows: the top 10 isolated strains with higher H/C values were used to perform a re-screening by comparing their enzyme-producing abilities in liquid culture. To prepare the inocula, 10% of each spore suspension was inoculated into 50 mL of seed medium (20 g kg^{-1} peptone, 60 g kg^{-1} glucose, 2 g kg^{-1} sodium nitrate, 2 g kg^{-1} monopotassium phosphate, and 1 g kg^{-1} magnesium sulfate) in a 250 mL conical flask with a cotton plug and incubated in a thermostatic shaker at 30 °C for 3 d. After incubation, four layers of sterile gauze were used to remove the mycelia from the conical flask. The remaining fermentation broth was adjusted with sterile distilled water to 10^6 spores mL^{-1}. Ten percent of such inoculum was then inoculated into a 500 mL conical flask filled with 150 mL medium (same as mentioned in the "preliminary screening" but without agar) and covered with eight layers of gauze. All flasks were incubated in a thermostatic shaker at 30 °C for 6 d. After incubation, the mycelia and the fermentation broth were separated through ultrafiltration, and the weight of the mycelia was determined by gravimetric analysis.[17] The fermentation broth was used to determine the activities of amylase and glucoamylase according to the methods described by Liu *et al.*[2] One unit (U) of amylase activity was defined as the quantity of enzyme that was required by 1 g mycelium to hydrolyze 1 mg soluble starch per hour, at 40 °C and pH 6. One unit (U) of glucoamylase activity was defined as the amount of enzyme that was required by 1 g mycelia to release 1 mg glucose per hour from soluble starch, at 40 °C and pH 4.6. The target strain showing the highest enzyme activity would be screened.

Identification of the target strain

Colony morphology of the target strain grown on Malt Extract Agar (MEA) (Guangdong Huankai

Microbial Sci. & Tech. Co., Ltd., Guangzhou, China) plates at 30 °C for 7 d was evaluated. The microstructure was determined by both an optical microscope (CI-L, Nikon, Japan) and scanning electron microscope (SEM) (JSM-6380/LV, Electron Optics Laboratory Co., Ltd., Japan). The ITS rDNA and *β*-tubulin genes of the strain were partially sequenced using the primers ITS1/ITS4 (TCCGTAGGTGAACCTGCGG/TCCTCCGCTTATTGATATGC) and *β*-tubulin-F/*β*-tubulin-R (CAACTGGGCTAAGGGTCATT/GTGAACTCCATCTCGTCCATA), as described by Park *et al.*[18] Each 25-μL PCR reaction mixture consisted of 1 μL DNA template, 2.5 μL dNTPs (2.5 mmol L^{-1}) (Tiangen Biotech Co., Ltd. Beijing, China), 2.5 μL 10×PCR buffer (containing $MgCl_2$) (Tiangen Biotech Co., Ltd. Beijing, China), 1 μL primer, and 0.5 μL Taq polymerase (5 U μ^{-1}) (Tiangen Biotech Co., Ltd. Beijing, China), and ddH_2O was added to a final volume of 25 μL. The PCR was conducted under the following conditions: for ITS rDNA, initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 90 s, followed by a final extension cycle at 72 °C for 5 min prior to maintaining the mixture at 4 °C; for *β*-tubulin genes: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 2 min, extension at 72 °C for 2 min, followed by a final extension cycle at 72 °C for 5 min prior to maintaining the mixture at 4 °C. Sequences were analyzed using Blast at NCBI (http://www.ncbi.nlm.nih.gov/). Sequence alignment and phylogenetic analyses were performed using the Clustal X program with MEGA 5.0 software.[19]

Adaptability of the target strain to the fermentation test

The effects of three fermentation conditions (temperature, alcohol, and pH) on the target strain were investigated through both solid and fluid media. For the temperature test, sterile circular filter papers (φ 6 mm) were placed in the middle of PDA plates, and 10 μL of spore suspension of the target

strain was inoculated on each circular filter paper and then incubated at temperatures ranging from 15 to 45 °C for 4 d. For the alcohol and pH tests, the PDA plates were prepared with ethanol (Huankai Microbial Sci Co., Ltd. China) concentrations ranging from 8 to 18% (v/v) or pH ranging from 4 to 8, followed by the same process as above, and incubated at 30 °C for 4 d. The morphology and diameter of the colonies were evaluated as growth parameters throughout this solid culture. Meanwhile, tests on fluid media were carried out. Seed medium (as mentioned in "Re-screening") was freshly prepared and inoculated with 10% spore suspension and then incubated at temperatures ranging from 15 to 45°C for 6 d. Similarly, seed medium was adjusted to pH 2–8 or supplemented with 8–18% ethanol and inoculated with 10% spore suspension, and then incubated at 30 °C for 6 d. The weight of the mycelia obtained from each treatment was determined by gravimetric analysis.[17]

URRM in rice wine brewing

The preparation procedures of the rice wine fermentation starters were as follows: a traditional Hong Qu (sample no. HQM23, the traditional strain *M. purpureus* FM23 was its dominant *Monascus* spp.) that had been commercially applied in a rice wine factory in Fujian province was used as the control. The target strain FJMR24 was used to produce a new Hong Qu (sample no. HQR24) according to the traditional Hong Qu production procedures (Supplementary Figure S1).[20] All base liquor samples were brewed according to the method of Lv *et al.*[21] with slight modifications: 100.0 g of Hong Qu was placed into a steam-sterilized pottery cylinder jar and soaked with purified water overnight. Two kilograms of cooled steamed glutinous rice and 2.0 kg of sterilized water were added into the jar and mixed thoroughly. After 30 d of fermentation at 25 to 30 °C and filter pressing, the base liquor was collected into ceramic wine bottles and pasteurization in a water bath for 30 min at

80 °C. The liquor was cooled down, sealed, and stored at 20–25 °C. Three pottery cylinder jars of the base liquor were brewed for each Hong Qu sample. The base liquors brewed using HQR24 and HQM23 were labelled as BLR24 and BLM23, respectively.

The fermentation performance indexes of Hong Qu and the physicochemical indexes of the base liquors were detected. The alcoholic strength (percentage of alcohol by volume), total acidity, pH, total sugars, and non-sugar solids were measured according to the previously reported methods.[22] The total phenol content was determined by standard curves prepared from a standard solution of gallic acid using the Folin-phenol method.[23] The URRM was expressed as follows: URRM (%) = liquor output/the weight of steamed glutinous rice × 100. The liquor yield was expressed as follows: liquor yield (%) = (liquor output/the weight of glutinous rice) × (alcoholic strength/15%) × 100. The red pigment concentration (color value) of Hong Qu and base liquor was measured following the method of Xu *et al*.[24] The color value of Hong Qu (u g^{-1}) = A × N × 100/m, where A is the absorbance value at 505nm, N is the dilution factor, and m is the weight of dry Hong Qu. And the color value of base liquor was defined as the absorbance value at 505nm. HPLC was used to determine the concentration of citrinin as previously described.[25] The number of microbes in the two Hong Qu samples was measured in terms of colony forming units (CFU).[26] The amylase activity and glucoamylase activity of Hong Qu were measured according to the method mentioned in “Re-screening” part. One unit (U) of amylase activity was defined as the quantity of enzyme that was required by 1 g Hong Qu to hydrolyze 1 mg soluble starch per hour, at 40 °C and pH 6. One unit (U) of glucoamylase activity was defined as the amount of enzyme that was required by 1 g Hong Qu to release 1 mg glucose per hour from soluble starch, at 40 °C and pH 4.6.

Statistical analysis

The experiments were conducted in triplicate, and the results were presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed across multiple groups to test significance ($p < 0.05$) using SPSS 23.0 software (SPSS Inc., Chicago, USA). A histogram was generated using Origin 2018 software (OriginLab, Northampton, Massachusetts, USA).

RESULTS

The enzyme-producing properties of the different fungal isolates

A total of 67 fungal isolates were initially classified from 35 varieties of traditional fermentation starters. We screened these strains using a combination of qualitative and quantitative detection. The qualitative detection, as a preliminary screening, yielded 10 strains with relatively high H/C values, which were used for re-screening through quantitative analysis. As shown in Figure 1, the activity of amylase and glucoamylase varied among the isolates, and strain FJMR24 showed the highest amylase activity and glucoamylase activity of 2200.9 ± 18.5 U g^{-1} and 2330.4 ± 31.9 U g^{-1}, respectively. Therefore, this strain was selected for further study.

Morphology and molecular identification of strain FJMR24

The macro-characteristics of strain FJMR24 were presented by the colonies on an MEA plate (Figure 2A and B). The front of the colonies was orange, round, or ellipse, with a raised center, regular edge, layered line, and villous texture. The back of the colonies had a bright red center and orange periphery, without fluid exudation or soluble pigment. These colonies were 28–30 mm in diameter.

The micro-characteristics of strain FJMR24 were analyzed by an optical microscope (Figure 2C and D) and SEM (Figure 2E and F). The transparent hyphae were 3–5 μm in width, irregularly

branched, and with visible oil droplets and septa. The conidia were an inverted pear or sphere and were arranged in bunches or singly and grew on the end of the hypha or branch, with a diameter of 3.0–5.0 μm. The ascospores were oval or ovoid, with a smooth wall and were 4.0–6.0 μm × 3.0–4.5 μm in size. There were many brown cleistothecia with diameters of 20–50 μm.

The strain FJMR24 was identified by BLAST sequences of the ITS rDNA gene (501 bp) and by mapping the strain phylogeny, which showed 100% homology with *M. purpureus* ATCC 16379 (AY498573) and *Monascus aurantiacus* CICC 5014 (AY629435) (Figure 3A). This result was further confirmed by analysis of *β*-tubulin gene (901 bp), which had 100% homology with *M. purpureus* ATCC 16379 (AY498573) (Figure 3B). Combining morphological and genetic characteristics, strain FJMR24 was identified as *M. purpureus* (code for FJMR24, same as below). This strain has been recorded in the China Center for Type Culture Collection (CCTCC) under the number M2016192, and the ITS rDNA sequence has been deposited in the GenBank database under accession number MT525241.

The growth characteristics of FJMR24 under different fermentation conditions

Figure 4A shows significant differences in the response of FJMR24 to a temperature gradient. The colony size and biomass of FJMR24 showed relatively consistent results. The growth trend of FJMR24 was characterized by a gentle increase from 15 to 35 °C and then followed by a rapid decrease, suggesting that FJMR24 was very sensitive to high temperature (above 35 °C). FJMR24 could still survive at both low (15 °C) and high temperatures (45 °C), with a biomass of 2.05 ± 0.32 g L^{-1} and 4.03 ± 0.20 g L^{-1}, respectively. However, the colonies on the PDA plates were not big enough to be measured at these two temperatures. It indicated that 15 °C and 45 °C were the limit temperature of FJMR24.

As expected, the strain colony diameter and biomass declined markedly with an increase in ethanol from 8 to 18% (v/v) (Figure 4B). At a concentration of 18% ethanol, though the colony was undetected on solid PDA, the biomass was determined as 1.89 ± 0.21 g L^{-1} in fluid medium, suggesting that 18% ethanol was the tolerable limit of FJMR24.

The growth of FJMR24 in fluid medium at pH 2–8 reflected a wide pH adaptability (Figure 4C). The colony diameter and biomass of FJMR24 increased but then decreased with the increasing pH, as observed with temperature. In accordance with this tendency, FJMR24 presented the biggest colony on PDA plates at pH 6, while its biomass reached the maximum at pH 5. However, there was no significant difference in growth capacity between these two pH values, demonstrating that pH 5–6 was the optimum pH condition, suggesting that a relatively high acidity environment was more conducive for spore germination or mycelium reproduction. In addition, FJMR24 could survive at pH 2, with a biomass of 2.41 ± 0.23 g L^{-1}, but no colonies were obtained, as the agar did not solidify at pH < 4.

Liquor yield

The fermentation performance of Hong Qu is shown in Table 1. In terms of biomass, the number of *Monascus* or yeast within HQR24 and HQM23 (control) was in the same order of magnitude. Compared to HQM23, HQR24 showed significantly higher glucoamylase activity and amylase activity ($p < 0.05$), followed by a significantly higher URRM and liquor yield ($p < 0.05$), which increased by 26% and 14%, respectively. The color value of HQR24, although much lower than that of HQM23 ($p < 0.05$), reached primary standard (≥ 660 u g^{-1}, 505nm) described by the Local Standard of Hong Qu DBS 002-2017. Similarly, the citrinin produced by HQM24 was much lower than that of HQR23 ($p < 0.05$), and also lower than the limit of quantitation (80 μg kg^{-1}) defined by

the National Food Safety Standard of the People's Republic of China GB 5009.222-2016.

Table 2 presents the physicochemical indexes of the base liquors brewed by HQR24 and HQM23. A significantly higher alcoholic strength and lower color value were obtained in BLR24 compared to BLM23 (control) ($p < 0.05$). While there was no significant difference between the other indexes ($p > 0.05$). Total sugars and non-sugar solids within BLR24 were 2.48 ± 0.13 g L^{-1} and 30.41 ± 1.64 g L^{-1}, respectively. They reached the demands of excellent dry rice wine described by both the Standard of the People's Republic of China QB/T 13662-2018 and the Local Standard of Food Safety DBS35/003-2017. And total phenol within BLR24 was 721.35 ± 12.32 mg L^{-1}, which met the requirements of AAA rice wine (≥ 400 mg L^{-1}).

DISCUSSION

New biotechnologies, such as gene modification and strain mutations, have often been applied in *Monascus* spp. to enhance or decrease the production of some metabolites.[27] However, these approaches are still not widely accepted by consumers and are also limited by legal restrictions, especially in the food industry. Traditional methods or adaptive evolutionary approaches are claimed to be safe and practical for the selection of strains with specific phenotypes.[28] In the present study, FJMR24 with high amylase and glucoamylase activity was successfully isolated and screened from traditional wine fermentation starters representative of the typical characteristics of various areas of Fujian province (Figure 1). Its superior enzyme activity indicated that its use in enhancing raw material utilization would be more efficient than other strains. FJMR24 was identified as *M. purpureus* (Figure 3A and B), which is an edible and versatile fungus that has a dominant role in Hong Qu.[29] In addition, the citrinin produced by FJMR24 was much lower than the limit of quantitation 80 μg kg^{-1}, suggesting that it could be safely applied in rice wine brewing (Table 1).

Hong Qu is considered as the “the bone of wine,” and the growth and metabolism of strains within Hong Qu are greatly affected by the key parameters (temperature, pH, and alcohol) during rice wine brewing, thus affecting the quality of the wine.[30] In practice, traditional rice wine is often made around December (room temperature is about 15 °C) each year in Fujian province to avoid the high temperatures that would result in fast fermentation and undesirable strain contamination (one reason being excessive acidification).[31] However, it is typically difficult for strains to begin fermentation in cold December. Some insulation measures are generally used in the initial stage to maintain the desired temperature of 25–28 °C for fermentation,[32] which is time-consuming and costly. As discussed above, FJMR24 survived from 15 to 45°C (Figure 4A), suggesting that it could initiate the fermentation without any insulation measures and remain functional with the increased temperature in the middle and late fermentation stages. Additionally, the optimum growth temperature of FJMR24 at 35 °C provides a theoretical basis for the setting of stirring temperature (35–37 °C) to maintain suitable fermentation conditions, as excessive temperature would accelerate the cellular aging that would reduce the accumulation of metabolites.[32] The traditional brewing of rice wine is a typical simultaneous saccharification, alcoholization, and acidification process, which is also known as trilateral fermentation.[8] Additionally, molds, yeasts, and *Lactobacillus* are involved in this process, structuring a complex microbial ecosystem during rice wine brewing. In this study, a medium pH 5.0–6.0 was found to be favorable for the growth of FJMR24 (Figure 4C), while the optimal pH for *Lactobacillus* and yeast growth is 6.0–7.2 and 4.0–5.0, respectively.[20] These differences would require an adjustment of the acidity to control *Lactobacillus* and ensure the rapid growth of molds or yeasts in Hong Qu making and rice wine brewing. In practice, most *Monascus* strains can tolerate 10–15% alcohol, but the level of alcohol often reaches 20% or above in the

fermentation mash without distillation, threatening the growth of these strains.[22] The ability of FJMR24 to survive at 18% ethanol could enable it to have high activity during alcoholic fermentation and supply sufficient carbohydrates for yeasts in trilateral fermentation (Figure 4B).

Physical and chemical factors affecting protein functions will affect the activity of enzymes; for example, high temperatures, strong acids, and strong base can cause a loss of catalytic ability. Studies on other strains have shown that increased temperatures and other stresses (for example, ethanol stress) could lead to a number of changes, including protein denaturation, DNA damage, cell cycle arrest, and changes in the fluidity of the membrane.[33] One of the most deleterious factors in stressed cells is the disturbance (unfolding) of the protein structure that leads to protein aggregation, thus affecting the functionality.[34] Rice wine brewing is a process associated with increasing temperature, alcohol, and acidity as fermentation proceeds. Thus, FJMR24 was stressed by these three factors simultaneously. However, some strains can tolerate the stress as a result of their stress response systems. For instance, the heat shock proteins (HSPs) and stress proteins (GPs) generated under heat stress can repair injured protein and mitigate cell membrane damage so as to maintain normal metabolism and functioning of the strain.[35,36] In this study, FJMR24 might also have a similar stress response system to cope with the complex fermentation environment and maintain a high enzyme activity. Previous research has mainly focused on the synthesis conditions and applications of functional substances produced by *Monascus* spp.[37,38] However, limited information is available on the tolerance mechanism of *Monascus* spp. to temperature, alcohol, and acid. Moreover, morphological studies have shown that ascospores are more heat resistant than mycelia and conidia, and there is a positive correlation between the number of ascospores and the thermotolerance of filamentous fungi.[39] In the present study, the large number of cleistothecia (a

structure that produces ascospores) of FJMR24 (Figure 2D and F) would contribute to the thermotolerance of FJMR24. In addition, the high level of stress proteins induced by one stress event could improve the adaptability of a strain to other stresses,[40] indicating that an adaptive response may initiate cross-protective mechanisms.[41] According to these concepts, the thermo-resistance of FJMR24 would help improve its adaptability to high alcohol and acid, and *vice versa*.

As mentioned above, filamentous fungi play an important part in metabolizing starch into low-molecular sugars for yeast and bacteria to use in the saccharification and liquefaction stage of rice wine. [12] In the processing procedure of HQR24 and HQM23, pure strains FJMR24 and FM23 were inoculated respectively after an expanding culture (Supplementary Figure S1), and the fermentation conditions were adjusted according to the growth characteristics of *Monascus*. Under these conditions, FJMR24 and FM23 would be the dominant microorganism within HQR24 and HQM23, respectively. Therefore, the significant enhancement of URRM within HQR24 (Table 1) might be attributed to the high amylase and glucoamylase activity of strain FJMR24. Additionally, Table 1 shows the similarity in the amount of biomass but significantly different enzyme activity between HQR24 and HQM23, indicating that the higher enzyme activity of HQR24 was not mainly due to the quicker growth but to the higher unit enzyme activity of FJMR24. However, not all *Monascus* species exhibit high amylase and glycosylase activity at the same time. Lv *et al.*[10] reported that the variability of filamentous fungi was associated with traditional fermentation starters and that *Monascus* spp. possessed high glucoamylase activity but low α-amylase activity, indicating that they could be good glucoamylase producers rather than α-amylase producers. These studies demonstrated the diversity of enzyme production capacity among strains.

According to the quality evaluation of two base liquors, there was no significant difference

between BLR24 and BLM23, except for the color value (Table 2). In practice, the technique of multi-strain co-fermentation should be applied to offset the defect of FJMR24. And further research is needed to screen a strain that could form a symbiotic relationship with FJMR24 and produce high *Monascus* pigment.

CONCLUSION

In conclusion, FJMR24 with high amylase and glycosylase activities was screened from traditional Hong Qu samples. The rice wine-making tests confirmed that FJMR24 could significantly enhance the URRM and liquor yield. The tolerance of FJMR24 to extreme temperatures, high alcohol, and low pH endows it with high vitality during rice wine brewing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Figure 1. The activity of amylase and glucoamylase of the isolated strains. Error bars indicate the standard deviation (SD) of the means (n = 3), the lowercase letters and the capital letters represent significant differences between amylase activities and glucoamylase activities within isolated strains respectively ($p < 0.05$).

Figure 2. The colony characteristics of the front (A) and back (B) of strain FJMR24 grown on an MEA plate. The individual morphological characteristics of strain FJMR24 by optical microscopy (C, D) and scanning electron microscopy (SEM) (E, F).

Figure 3. The phylogenetic trees of strain FJMR24 and the related species based on ITS rDNA sequence (A) and β-tubulin sequence (B).

Figure 4. The colony diameter and biomass of strain FJMR24 grown at different temperatures (A), ethanol (B), and pH (C). Error bars indicate the SD of the means (n = 3). N.A means no colonies were applied for the detection, as they were not big enough to be observed (A, B) or PDA plates cannot be made when pH < 4 (C). The lowercase letters and capital letters represent significant differences between the biomass and the colony diameters of FJMR24 respectively ($p < 0.05$).

Supplementary Figure S1. The traditional production process of Hong Qu HQR24.

Supplementary Figure S2. The appearance of ground Hong Qu samples HQM23 and HQR24.