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Identification and characterization of filamentous fungi isolated from fermentation starters for Hong Qu glutinous rice wine brewing

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Hong Qu glutinous rice wine is one of the most popular traditional rice wines in China. Traditionally, this wine is brewed from glutinous rice with the addition of wine fermentation starters (Hong Qu (also called red yeast rice) and White Qu). The objective of this study was to investigate the variability of filamentous fungi associated with traditional fermentation starters through a traditional culture-dependent method and a molecular identification approach. In this study, forty-three filamentous fungi were separated by traditional culture-dependent means (macro- and microscopic characteristics) from 10 fermentation starters and classified into 16 different species based on morphological examination and the internal transcribed spacer (ITS) sequences analysis. It was observed that the genus *Aspergillus* had the highest number (14 isolates) of isolates followed by *Rhizopus* (11 isolates), *Monascus* (5 isolates) and *Penicillium* (4 isolates). The species *R. oryzae*, *A. niger*, *A. flavus* and *M. purpureus* were frequently found in wine starter samples, among which *R. oryzae* was the most frequent species. The enzyme-producing properties (glucoamylase, α -amylase and protease) of all fungal isolates from different starters were also evaluated. *A. flavus*, *R. oryzae* and *M. purpureus* were found to be better glucoamylase producers. *A. flavus*, *R. oryzae* and *A. oryzae* exhibited higher activity of α -amylase. *A. flavus* and *A. oryzae* had higher protease activity. However, some fungal isolates of the same species exhibited a significant variability in the production levels for all determined enzyme activity. This study is the first to identify filamentous fungi associated with the starter of Hong Qu glutinous rice wine using both traditional and molecular methods. The results enrich our knowledge of liquor-related micro-organisms, and can be used to promote the development of the traditional fermentation technology.

Key Words—enzyme-producing properties; fermentation starters; filamentous fungi; Hong Qu glutinous rice wine; molecular identification

Introduction

Chinese yellow rice wine is one of the most ancient wines in the world (Wang and Yi, 2001). Based on the raw materials and starter organisms used, Chinese yellow rice wine can be categorized into Hong Qu glutinous rice wine, Shaoxing rice wine and Shandong Jimo millet wine. Hong Qu glutinous rice wine, a unique kind of Chinese glutinous rice wine, is a well-

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known traditional fermented product in the southeast of China and Southeast Asia. It is likely to become one of the most popular international rice wines, because this wine possesses a bright red color, subtle sweet flavor, and health care functionality due to the addition of Chinese red yeast rice (Hong Qu), which can significantly reduce total cholesterol, LDL cholesterol, and total triacylglycerol concentrations, and also possesses antioxidant activities (Erdoğan and Azirak, 2004; Heber et al., 1999; Que et al., 2006; Taira et al., 2002; Yang et al., 2006). However, like many other traditional fermented foods in China, it is manufactured with uncontrolled fermentation under non-sterile conditions, which is based on empirical knowledge and remains a traditional art in homes, villages and small-scale industries. Thus, the microbial interactions during the fermentation process are neither known nor controlled, which causes uncontrollability of fermentation and instability in the quality of Hong Qu glutinous rice wine.

Traditionally, Chinese yellow rice wine is brewed from glutinous rice with the addition of wine fermentation starters, which are considered to be composed of a large diversity of enzymes and microorganisms including filamentous fungi, yeasts, and bacteria (Rong et al., 2009; Zhang et al., 2008). Most relevant studies have focused on yeasts, particularly *Saccharomyces*, or filamentous fungi, because of their involvement in the transformation of starch into wine during saccharification and alcoholic fermentations, respectively. Of these, the filamentous fungi are the most important ones because they produce α -amylase and glucoamylase, which catalyze the conversion of starch into sugars and manage the stages of liquefaction and saccharification. The sugars produced are subsequently utilized by the yeast to produce alcohol, carbon dioxide and wine flavor. Besides, filamentous fungus also excretes protease and some beneficial metabolites helpful for the quality of rice wine, including the nutritional and biological activity as well as the color and flavor (Que et al., 2006; Taira et al., 2002; Yang et al., 2006; Zhang et al., 2008). Filamentous fungi also greatly influence the health safety of the wine. They include the presence of mycotoxins, like aflatoxin and Ochratoxin A (OTA) (Battilani and Pietri, 2002; Mateo et al., 2011). In recent years, more concern about food safety together with the consumer's demand for safe and healthier products has promoted studies of microorganisms with harmful effects on hu-

man health. Therefore, this issue clearly deserves more attention when evaluating the microbial diversity of traditional fermentation starters for Hong Qu glutinous rice wine, especially the diversity of filamentous fungi.

Numerous studies have been conducted to describe the microbial diversity of rice wine fermentation starters used (Han et al., 2009; Rong et al., 2009; Shi et al., 2009; Xie et al., 2007; Zhang et al., 2008). It has recently been reported that *Monilia*, *Mucor*, *Rhizopus*, *Absidia*, *Monascus*, *Aspergillus* and *Penicillium* are the most common filamentous fungi in Chinese yellow rice wine starters (Rong et al., 2009). Sixteen kinds of filamentous fungi were separated from wheat Qu, a starter of Shaoxing rice wine, and then identified by internal transcribed spacer sequencing technology (Cao et al., 2008; Lu et al., 2008). These studies helped us to understand how a fermentation product was made and the relationship between the microbial diversity, its dynamic change and its special characteristics. However, the literature concerning the diversity of filamentous fungi and the comparative functionality of various fungal species associated with fermentation starters for Hong Qu glutinous rice wine brewing are still limited and remains to be documented.

The diversity of filamentous fungi on fermentation starters for Hong Qu glutinous rice wine brewing have not been extensively investigated at the species level. In this study, some dominant filamentous fungi were separated from traditional Hong Qu glutinous rice wine starters, collected in Fujian Province of China. They were identified on the basis of their macro- and microscopic characteristics and by internal transcribed spacer sequencing technology. Besides, the α -amylase, glucoamylase and protease-producing ability of these isolated fungi were compared.

Materials and Methods

Sample collection. Ten samples (5 Hong Qu and 5 White Qu) of dry commercial traditional rice wine starters were obtained from a local market or from the small-scale factories located in the northern, southern and western part of Fujian, China. Samples were stored at 4°C immediately after collection prior to testing.

Isolation and purification of microorganisms. Ten grams of powdered wine fermentation starter sample was homogenized in 90 ml of 0.85% w/v sterile physi-

ological saline. Then a series of decimal dilution was made. 1.0 ml each from the dilutions of 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} was poured onto Czapek-Dox agar (Difco, Detroit, Michigan, USA) and Potato Dextrose Agar (Difco) supplemented with ampicillin (100 ng/ μ l, Merck, Darmstadt, Germany) and incubated at 28°C for 3 days. All the colonies isolated were identified with traditional methods including a macroscopic level (surface color, reverse side color, spores and diameter) and microscopic characteristics such as conidiophores, conidia, and fertile hyphae (Pitt and Hocking, 1997; Samson et al., 1996).

The selected colonies were sub-cultured on new Czapek-Dox agar plates (Difco) and purified by repeated streaking. The purified isolates were obtained and maintained on potato dextrose agar (Difco) slants at 4°C.

DNA extraction of fungal isolates. All fungal strains were grown on potato-dextrose broth (Difco, Fisher Bioblock Scientific, Illkirch, France) at 28°C for 5 days. Cultures were centrifuged to collect the mycelium which was frozen in liquid nitrogen and ground to a fine powder. About 150–200 mg of frozen mycelium was used for DNA extraction using the commercial EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA) according to the manufacturer's instructions.

PCR amplification of the ribosomal internal transcribed spacers (ITS) and the 5.8S rDNA region. The 5.8S-ITS region was amplified by PCR using universal fungal primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). PCR reactions were performed in 50 μ l of 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 μ M of each primer, 0.025 U of *Taq* polymerase (Promega Corp., USA) and 10–30 ng of fungal DNA. Reactions were performed on a MyCycler thermal cycler (BIO RAD, Hercules, USA) using a program described by Phalip et al. (2004).

The PCR products were separated on a 4.5% polyacrylamide (29 : 1 acrylamide : bisacrylamide) 10 cm $\times$ 10 cm gel. The electrophoresis was run at 70 V for 2 h. After electrophoresis, gels were stained with ethidium bromide, and documented by the Gel Doc 2000 (BioRad, California, USA). Band sizes were estimated by comparison against DNA ladders using Quantity One version 4.5.1 software (BioRad).

Sequencing and comparison of the ITS1-5.8S-ITS2 amplicons. Most amplicons were sequenced by cycle extension in an automated 3100 Genetic Analyser at Shanghai Sangon Biological Engineering Technol-

ogy & Services Co., Ltd. The Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used for database searches. The identities of the isolates were determined based on the highest score basis.

Nucleotide sequence accession numbers. The sequences of the ribosomal internal transcribed spacers (ITS) and the 5.8S rDNA region obtained in this study have been deposited in the GenBank database under accession numbers JN561250 to JN561292.

Analysis of microbial community composition of filamentous fungi. The similarity between fungal community profiles was investigated by constructing non-metric multidimensional scaling (nMDS) scatter plots (Hammer et al., 2001). nMDS analysis on the microbe presence/absence matrix (provided by the software) was performed using the free software package PAST (Palaeontology Statistics, <http://folk.uio.no/ohammer/past/>).

Comparison of enzyme-producing ability of fungal isolates based on steamed bran substrate. To examine the α -amylase, glucoamylase and protease ability of the isolated filamentous fungi, steamed bran was used as the substrate. Before the experiment, starter organisms were activated by two successive transfers to PDA (Difco) slants and incubated at 30°C for 3 days. To prepare the inocula, the activated culture was inoculated into PDA and incubated at 30°C for 3 days. Spores of the test organism were harvested by flooding the surface of the agar with sterile distilled water containing 0.1% Tween 80 (Lin et al., 2006). The spore suspension was adjusted with sterile distilled water to a concentration of ca. 10^6 /ml and served as inocula for the fermentation of steamed bran.

To perform fermentation, 10 g of bran and 10 ml of distilled water were placed in a 250 ml conical flask covered by a cotton plug and steamed in an autoclave for 30 min at 121°C. After cooling to 35–40°C, the culture medium was inoculated with the spore suspension of test organism at a level of 10^6 spores/g of steamed bran, and incubated for 3 days at 30°C. Then enzyme activity was measured.

Physical and chemical analysis methods. Samples of the fermented products were extracted with water (1 : 10, w/v) on a shaking table at a constant temperature of 30°C for 1 h. After filtering through Whatman No. 1 filter paper, the extract was used for determination of enzymes activity and reducing sugar content.

Glucoamylase activity was determined as described by Bernfeld (1955). An enzyme activity unit (U) was defined as the amount of enzyme that liberated 1 mg of glucose per hour under assay conditions.

The method used to determine α -amylase activity of the samples was as described by De Moraes et al., (1995). One unit (U) of α -amylase activity was defined as the quantity of enzyme required to hydrolyze 1 mg starch in 10 min at 40°C. At least three measurements were made for each condition and the reported values are an average of these results.

Protease activity was determined according to the method described by Farley and Ikasari (1992). One unit of protease activity was defined as the amount of enzyme that liberated 1 μ g tyrosine per min under assay conditions.

Statistical analysis. The mean value and standard deviation was calculated from the data obtained from the three separate experiments. Significant differences were determined using Duncan's multiple range test (SPSS 11.0).

Results and Discussion

Morphotypic identification of the fungal isolates

A total of 43 fungal strains were isolated from ten varieties of rice wine starters (5 Hong Qu and 5 White Qu) and identified on the basis of their macro- and microscopic characteristics from PDA and Czapek-Dox agar media. The analyses allowed the 43 fungal isolates to be classified as belonging to different genera (Table 1), including *Mucor* spp., *Rhizopus* spp., *Paecilomyces* spp., *Penicillium* spp., *Aspergillus* spp., *Monascus* spp. and an unknown genus. It was observed that the genus *Aspergillus* had the highest number (14) of isolates followed by *Rhizopus* (11 isolates), *Monascus* (5 isolates), *Penicillium* (4 isolates), *Paecilomyces* and *Mucor* both had 2 isolates, while there were also five fungal isolates which could not be classified precisely through morphotypic identification. Similar research was carried on as to microorganisms in Daqu, a starter culture of Chinese Maotai-flavor liquor, and results revealed that the fungal isolates could be classified into five genera: *Aspergillus*, *Mucor*, *Rhizopus*, *Trichoderma* and *Monascus* as discriminated by phenotypic and conventional biochemical taxonomic methods (Wang et al., 2008).

Molecular characterization of the fungal isolates

The ITS1 and ITS4 primers described by White et al. (1990) were used to amplify the ribosomal region, which includes the non-coding ITS1 and ITS2 and the 5.8S rRNA gene and which shows low intraspecific polymorphism and high interspecific variability (Lee and Taylor, 1992).

The size of all amplified PCR products was estimated to be around 625 bp for *Mucor* spp., 581 bp for *Monascus* spp. 606 bp for *Paecilomyces* spp. and 587 bp for *Penicillium* spp. (Table 1). However, the sizes of PCR products varied from 610 bp to 695 bp for fungal isolates belonging to *Rhizopus* spp. and 568 bp to 600 bp for *Aspergillus* spp., perhaps due to these fungal isolates belonging to different species although they were of the same genus. The size of the amplified PCR products differs between genera and allows us to distinguish, for example, a strain of the genus *Aspergillus* (approximately 600 bp) from a strain of the genus *Monascus* (approximately 581 bp). However, a single PCR based on the size of the amplified products was unable to discriminate between some genera, for example between *Aspergillus* and *Penicillium*. A single PCR was also unable to distinguish between some species within a genus, for example *A. flavus* and *A. oryzae*, due to the length of the amplified region being very similar.

Thus, the ITS1-5.8S-ITS2 gene sequences from different fungal isolates were compared directly with those of type strains from GenBank in order to accurately determine which species they were. The relative identification and its accession numbers obtained by alignment in GenBank are shown in Table 1. All sequences exhibited higher than 99% identity with sequences in the GenBank databases. For example, of the fungal isolates belonging to *Rhizopus* spp., strain F1-02, F2-01, F3-02, F5-01, F7-05, F8-04, F9-04 and F10-03 were identified as *R. oryzae*-EU484274 (100% identity) by 5.8S-ITS region sequence analysis, F2-02 and F6-03 as *R. microsporus*-DQ119009 (99% identity), and F4-06 as *R. arrhizus*-AF543520 (99% identity).

Fungal community composition in the various rice wine starters

One important goal of this study was to compare the microbial diversity of various rice wine starters. As shown in Fig. 1, the main filamentous fungi detected differed from sample to sample. The fungal communities were closely related to the wine starter categories.

Table 1. Origin of filamentous fungi isolated from wine starter samples, and their morphotypic, genetic identification profiles.

Wine starter samples	Representative isolates	Morphotypic identification	PCR band size (bp)	Sequencing of ITS1-4 gene-accession number* (% identity)
White Qu	I F1-01	<i>Mucor</i> spp.	641	<i>M. circinelloides</i> -DQ118991 (99%)
	F1-02	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	II F2-01	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	F2-02	<i>Rhizopus</i> spp.	697	<i>R. microsporus</i> -DQ119009 (99%)
	F2-03	<i>Paecilomyces</i> spp.	606	<i>Paecilomyces</i> sp.-AB217858 (99%)
	F2-04	... **	548	<i>Ascomycota</i> sp.-GQ141705 (99%)
	III F3-01	...	655	Uncultured eukaryote clone-FJ176538 (99%)
	F3-02	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	IV F4-01	...	655	Uncultured eukaryote clone-FJ176538 (99%)
	F4-02	<i>Penicillium</i> spp.	587	<i>P. chrysogenum</i> -AY213669 (99%)
	F4-03	<i>Paecilomyces</i> spp.	606	<i>Paecilomyces</i> sp.-AB217858 (99%)
	F4-04	<i>Aspergillus</i> spp.	595	<i>A. tubingensis</i> -GU723439 (99%)
	F4-05	...	548	<i>Ascomycota</i> sp.-GQ141705 (99%)
	F4-06	<i>Rhizopus</i> spp.	620	<i>R. arrhizus</i> -AF543520 (99%)
	V F5-01	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	F5-02	<i>Mucor</i> spp.	625	<i>M. indicus</i> -DQ118994 (99%)
	F5-03	<i>Aspergillus</i> spp.	596	<i>A. oryzae</i> -EU409806 (99%)
	F5-04	<i>Aspergillus</i> spp.	597	<i>A. flavus</i> -EF661566 (100%)
	F5-05	<i>Aspergillus</i> spp.	568	<i>E. nidulans</i> -EF652458 (100%)
Hong Qu	VI F6-01	<i>Aspergillus</i> spp.	601	<i>A. niger</i> -FJ717685 (100%)
	F6-02	<i>Monascus</i> spp.	581	<i>M. purpureus</i> -AY498579(100%)
	F6-03	<i>Rhizopus</i> spp.	697	<i>R. microsporus</i> -DQ119009 (99%)
	F6-04	<i>Aspergillus</i> spp.	597	<i>A. flavus</i> -EF661566 (100%)
	VII F7-01	...	857	<i>Lichtheimia ramosa</i> -FJ719390 (99%)
	F7-02	<i>Aspergillus</i> spp.	601	<i>A. niger</i> -FJ717685 (100%)
	F7-03	<i>Penicillium</i> spp.	587	<i>P. chrysogenum</i> -AY213669 (99%)
	F7-04	<i>Monascus</i> spp.	581	<i>M. purpureus</i> -AY498579(100%)
	F7-05	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	F7-06	<i>Aspergillus</i> spp.	597	<i>A. flavus</i> -EF661566 (100%)
	VIII F8-01	<i>Aspergillus</i> spp.	601	<i>A. niger</i> -EU709774 (99%)
	F8-02	<i>Penicillium</i> spp.	587	<i>P. chrysogenum</i> -AY213669 (99%)
	F8-03	<i>Monascus</i> spp.	581	<i>M. purpureus</i> -AY498579(100%)
	F8-04	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	F8-05	<i>Aspergillus</i> spp.	597	<i>A. flavus</i> -EF661566 (100%)
	IX F9-01	<i>Aspergillus</i> spp.	601	<i>A. niger</i> -FJ668837 (99%)
	F9-02	<i>Penicillium</i> spp.	587	<i>P. chrysogenum</i> -AY213669 (99%)
	F9-03	<i>Monascus</i> spp.	581	<i>M. purpureus</i> -AY498579(100%)
	F9-04	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	F9-05	<i>Aspergillus</i> spp.	597	<i>A. flavus</i> -EF661566 (100%)
	X F10-01	<i>Aspergillus</i> spp.	601	<i>A. niger</i> -FJ717685 (100%)
	F10-02	<i>Monascus</i> spp.	581	<i>M. purpureus</i> -AY498579(100%)
	F10-03	<i>Rhizopus</i> spp.	629	<i>R. oryzae</i> -EU484274 (100%)
	F10-04	<i>Aspergillus</i> spp.	597	<i>A. flavus</i> -EF661566 (100%)

* Assession number of the sequence of the closest relative found by BLAST search.

** Cannot be classified precisely through morphotypic identification.

Species (number)	White Qu					Red Qu				
	I	II	III	IV	V	VI	VII	VIII	IX	X
<i>Mucor circinelloides</i> (1)	■									
Uncultured eukaryote clone (2)			■	■						
<i>Lichtheimia ramosa</i> (1)						■	■			
<i>Aspergillus niger</i> (5)						■	■	■	■	■
<i>P. chrysogenum</i> (4)				■						■
<i>Monascus</i> sp. (5)						■	■	■	■	■
<i>Rhizopus oryzae</i> (8)	■	■	■	■	■	■	■	■	■	■
<i>Rhizopus microsporus</i> (2)	■	■				■				
<i>Paecilomyces</i> sp. (2)		■		■						
<i>Aspergillus tubingensis</i> (1)				■						
<i>Mucor indicus</i> (1)					■					
<i>Aspergillus oryzae</i> (1)					■					
<i>Aspergillus flavus</i> (6)					■	■	■	■	■	■
<i>Ascomycota</i> sp. (2)		■		■						
<i>Rhizopus arrhizus</i> (1)				■						
<i>Emerella nidulans</i> (1)					■					

Fig. 1. Fungal species composition of various rice wine starters.

Blank: fungus not found in wine starter; Black: fungus found in wine starter.

Three fungi species (*A. niger*, *M. purpureus* and *A. flavus*) were widely found in Hong Qu, of which, *A. niger* and *M. purpureus* were only detected in Hong Qu. The microbial diversity of White Qu was more complex than that of Hong Qu, suggesting that universality of *M. purpureus* in Hong Qu would have obvious effects on the microbial diversity. Some fungal species could only be detected in White Qu, for instance, *Mucor circinelloides*, *Paecilomyces* sp., *Ascomycota* sp. and *R. arrhizus* etc. This may be due to the antimicrobial substances produced by *Monascus* sp., creating conditions unfavorable for the growth of other micro-organisms.

The microbial diversity of various rice wine starters was further analyzed to estimate the extent of species variation and assess their similarity across samples. Non-metric MDA, based on the calculation of the Dice similarity coefficient between fungal community composition profiles, revealed the occurrence of two main clusters, suggesting that the data set could be broadly divided into two groups (shown in Fig. 2). Marked variability was also observed across samples from different rice wine starters, especially for White Qu. Of note was the substantial similarity in fungal community structure among different samples of Hong Qu, except

Fig. 2. Non Metric Multidimensional Scaling analysis of fungal community composition in the various rice wine starters.

The presence-absence of fungal species was provided by the image analysis software and the analysis was performed in PAST as described in MATERIALS AND METHODS.

for Hong Qu VI. This is not surprising since cereals used for the production of these two types of fermented starters are different. Traditionally, Hong Qu is fermented from polished round-grained rice by inoculating *Monascus* sp. strains under non-sterile conditions. By contrast, White Qu is manufactured from starchy substrates originating from common agricultural areas based on empirical knowledge, and sometimes a different Chinese native medicine ingredients was added in a certain amount. This is why the species variability of White Qu was larger than that of Hong Qu.

R. oryzae, a strong amylase producer frequently found in amylolytic Asian fermentation starters, was the most frequent species detected in both White Qu and Hong Qu, having been identified in 8 out of 10 tested starter samples. We speculate that it may play an important role in Hong Qu glutinous rice wine brewing. The genus *Rhizopus* has been considered as one dominant mould in rice wine starters, and also occurred frequently in Chinese Daqu. They can produce volatile compounds during fermentation, such as ethanol, 2-methyl-1-butanol and 3-methyl-1-butanol, which were the most abundant volatile compounds released from grains (Bramorski et al., 1998; Christen et al.,

2000; Feng et al., 2007). *Aspergillus*, a dominant genus existing in Daqu, also occurred frequently in White Qu and Hong Qu in this study, probably due to the abundance of this genus in nature. The genus *Aspergillus* has the ability to secrete large amounts of a wide range of different enzymes into its environment (Oda et al., 2006), which may contribute to the saccharification of the remaining starch. *A. oryzae*, included in the genus *Aspergillus*, has been used for more than a thousand years in the making of such traditional fermented food products such as *miso* (Japanese fermented soybean paste), soy sauce and *sake* (Japanese rice wine) (Machida et al., 2008; Wang et al., 2004). *Monascus* species were also found frequently in Hong Qu; by their production of esterase, they have the ability to hydrolyze and synthesize the ester. The strains could esterify the mixed acids and ethanol to produce esters such as ethyl caproate in the ripening period, which can also contribute to the aroma of the Hong Qu liquor.

Similar research was conducted on the microbial diversity of traditional Vietnamese alcohol fermentation starters (*banh men*), and moulds isolated from *banh men* were identified as *R. oryzae*, *M. indicus*, *M. circinelloides*, *R. microsporus*, *Absidia corymbifera* and *Amy. rouxii*. These species are strong amylase producers and are frequently found in amylolytic Asian

fermentation starters (Haard et al., 1999; Hesseltine, 1983; Hesseltine et al., 1988; Lee and Fujio, 1999; Thanh et al., 2008). *R. oryzae*, *A. oryzae*, *E. nidulans*, *A. niger*, and *R. microsporus*, which were found in our wine starter samples, have also been described in Shaoxing Rice Wine Wheat Qu through comparison of ITS sequences of excised RISA bands and recovered pure isolates with those in GenBank database (Xie et al., 2007).

The enzyme-producing properties of different fungal isolates and fungal species

The ability to produce α -amylase, glucoamylase and protease vary with the filamentous fungi. Therefore, enzyme-producing properties exerted by fungal isolates obtained from wine starters were investigated in this study.

Different studies have shown that the production of certain metabolites depends on the fungal species. Here we have grouped enzyme production by different fungal species and carried out a statistical analysis of the data as shown in Table 2. Interestingly, there are some significant differences ($p < 0.05$) among fungal species. Results show the species *R. oryzae* to be the best glucoamylase producer, which stands out given the production of glucoamylase with $3,609.30 \pm 830.15$ U/g. Similar results were reported in

Table 2. Mean values and standard deviations of glucoamylase, α -amylase and protease activities (U/g) produced by different fungal species obtained from different wine starters.*

Species (number of strains)	Glucoamylase activity	α -Amylase activity	Protease activity
<i>A. flavus</i> (6)	3,314.92 $\pm$ 1,657.03 ^{ab}	9,155.17 $\pm$ 4,866.04 ^a	227.36 $\pm$ 91.74 ^b
<i>A. oryzae</i> (1)	1,982.67 $\pm$ 149.55 ^{bcd}	2,461.55 $\pm$ 213.63 ^b	307.41 $\pm$ 4.22 ^a
<i>A. tubingensis</i> (1)	1,544.01 $\pm$ 38.77 ^{cdefg}	443.36 $\pm$ 9.62 ^b	1.68 $\pm$ 2.37 ^e
<i>Ascomycota</i> sp. (2)	766.34 $\pm$ 35.33 ^{fg}	n.d	26.51 $\pm$ 2.68 ^{de}
<i>A. niger</i> (5)	1,425.07 $\pm$ 169.00 ^{cdefg}	386.71 $\pm$ 45.70 ^b	0.30 $\pm$ 0.83 ^e
<i>E. nidulans</i> (1)	939.88 $\pm$ 18.00 ^{fg}	598.46 $\pm$ 27.76 ^b	280.73 $\pm$ 0.26 ^{ab}
<i>Lichtheimia ramosa</i> (1)	1,564.58 $\pm$ 23.54 ^{cdefg}	839.34 $\pm$ 49.73 ^b	35.63 $\pm$ 2.90 ^{de}
<i>M. circinelloides</i> (1)	267.22 $\pm$ 47.08 ^g	n.d	n.d
<i>Monascus</i> sp. (5)	2,437.24 $\pm$ 379.17 ^{abcde}	322.10 $\pm$ 36.30 ^b	82.55 $\pm$ 11.78 ^{cde}
<i>M. indicus</i> (1)	379.82 $\pm$ 15.23 ^g	n.d	n.d
<i>P. chrysogenum</i> (4)	939.78 $\pm$ 268.77 ^{fg}	330.08 $\pm$ 209.32 ^b	121.01 $\pm$ 85.62 ^c
<i>Paecilomyces</i> sp. (2)	2,613.72 $\pm$ 206.48 ^{abc}	1,718.91 $\pm$ 484.43 ^b	39.08 $\pm$ 18.00 ^{de}
<i>R. microsporus</i> (2)	1,117.11 $\pm$ 226.76 ^{defg}	247.68 $\pm$ 38.54 ^b	44.67 $\pm$ 5.30 ^{cde}
<i>R. oryzae</i> (8)	3,609.30 $\pm$ 830.15 ^a	2,592.75 $\pm$ 727.56 ^b	98.33 $\pm$ 10.81 ^{cd}
<i>R. arrhizus</i> (1)	2,504.55 $\pm$ 18.00 ^{abcd}	1,951.69 $\pm$ 163.03 ^b	89.72 $\pm$ 2.37 ^{cd}
Uncultured eukaryote clone (2)	1,058.13 $\pm$ 29.87 ^{efg}	273.80 $\pm$ 12.30 ^b	37.23 $\pm$ 1.91 ^{de}

n.d — not detected because the enzyme produced by fungal isolates was too low.

* Mean values for three independent experiments. Values are presented as means $\pm$ SD ($n = 3$), values within the same column in each characteristic with different letters are significantly different by Duncan's multiple range test ($p < 0.05$).

other work (Dung et al., 2007), where reported mould isolates with excellent functionality (glucoamylase producing property) were identified as *R. oryzae*. The species *A. flavus* showed similar ($p > 0.05$) glucoamylase activity levels ($3,314.92 \pm 1,657.03$ U/g) to *R. oryzae* and was the best producer of α -amylase. There were no significant differences ($p > 0.05$) among the species *A. flavus*, *M. purpureus*, *Paecilomyces*, *R. oryzae*, and *R. arrhizus* for glucoamylase production, all of which exhibited high glucoamylase activities. With respect to α -amylase activity, the species *A. flavus* was the best producer ($9,155.17 \pm 4,866.04$ U/g), followed by *R. oryzae* ($2,592.75 \pm 727.56$ U/g) and *A. oryzae* ($2,461.55 \pm 213.63$ U/g). In present study, *Monascus* sp. showed high glucoamylase activity ($2,437.24 \pm 379.17$ U/g) but low α -amylase activity (322.10 ± 36.30 U/g), indicating *Monascus* sp. to be one of the best glucoamylase producers, but not a good α -amylase producer. *Ascomycota* sp., *M. circinelloides* and *M. indicus* did not produce α -amylase although they showed a little glucoamylase activity. With respect to protease activities, *A. oryzae* was the best producer (307.41 ± 4.22 U/g), followed by *E. nidulans* (280.73 ± 0.26 U/g) and *A. flavus* (227.36 ± 91.74 U/g). There were no significant differences among the species *R. oryzae*, *R. arrhizus* and *Monascus* sp. for protease production, all of which showed a moderate level of protease activities.

Some fungal isolates of the same species exhibited a significant variability in the production levels for all determined enzyme activity (Table 2), in particular the species of *A. flavus* and *R. oryzae*. On the other hand, a very low variability was found in *Monascus* sp. and *A. niger*. *A. flavus*, classified as a separate species but genetically almost identical to *A. oryzae*, is not used for commercial applications mainly due to its capability of producing aflatoxins (Scheidegger and Payne, 2003). However, certain non-aflatoxigenic *A. flavus* isolates obtained from the field have shown characteristics of *A. oryzae* as reported previously by various investigators (Sanchez-Hervas et al., 2008; Yin et al., 2009). In the present study, all the isolates identified as *A. flavus* were measured to be non-aflatoxin producers through yeast extract agar to which methylated β -cyclodextrin derivative plus sodium desoxycholate had been added (data not shown) (Jaimez Ordaz et al., 2003). Therefore, all the isolates belonging to *A. flavus* in this study can be employed for yellow rice wine brewing.

The unique composition of microbial consortia cer-

tainly plays a very important role in liquor making, facilitating sugar utilization and ethanol production. This study is the first joint application of culture-dependent and molecular methods to characterize the distribution and diversity of filamentous fungi in the fermentation starters for Hong Qu glutinous rice wine. Having gained this important insight into the microbial structure of filamentous fungi in the starter, we potentially have the knowledge to make substantial improvements to the Hong Qu glutinous rice wine fermentation process, such as improving the selection of beneficial strains and their composition for modern fermentation or the optimization of the storage conditions of the starters in order to accelerate the process of making liquor. In further study, the compatibility of moulds and yeasts is an important aspect for the production of starters with good quality, because both of them play an important role in producing protease, wine flavor and other substances with biological activity.

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