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FULL PAPER

Isolation and identification of mold and yeast in *medombae*, a rice wine starter culture from Kompong Cham Province, Cambodia

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Abstract

Medombae is a dried starter culture used for traditional rice wine processing in Cambodia. However, studies on the role of mold and yeast present and their efficacy for rice wine fermentation are still limited. Cultural and morphological tests revealed that the isolated representative mold strains were isolated based on the method of identification used as *Mucor* spp and *Rhizopus oryzae*. On the other hand, the biochemical properties of the first yeast isolate using the Vitek 2 identification system and YST Card identification suggests its identity as *Candida tropicalis*. The second yeast strain examined for its morphological and cultural characteristic using agar slide technique, and its protein profile which was compared to the reference and sample protein masses using Biomerieux Vitek MS (MALD-TOF) showed the presence of *Saccharomyces cerevisiae*. The biochemical characteristics and cellular characteristics of the third yeast isolate as described by Lodder (1970) and Kreger-Van Rij (1984) confirmed its identity as *Saccharomycopsis spp*. The DNA test of identification of the isolates should be conducted to further confirm the identity of the isolates.

1. Introduction

A starter culture for rice fermentation is known as *medombae* in Cambodia. Spices, herbs, and a sweetener are ingredients commonly added also for dried starter preparation. Water is also added to the mixture and the previous starter was used as a source of inoculum at the rate of 1 to 2%. After mixing thoroughly, the mixture is being shaped into balls manually and placed on layers of rice husks or dried rice straw for 3 days at room temperature, sun-dried, and used as a starter for the production of alcoholic beverages such as rice wine. This technique of making dried starter culture may have originated in one place and later spread throughout Southeast Asia. On the other hand, milled rice or millet or other starch-based cereals are the main substrates for rice wine fermentation.

One of the major problems faced by commercial brewers of rice wine in Cambodia, as with the brewers of other indigenous beverages, is the variable quality of the product. Variability in quality is strongly correlated with the type of mold and yeast present and quality control in the production of the traditional starter culture. Dung *et al.* (2005) developed a starter culture containing a defined mixed cultures of mold (*Amylomyces rouxii*) and yeast (*Saccharomyces cerevisiae*), and herbal extracts (from fennel and clove). However, starter culture in Cambodia is prepared using the traditional method, not the well-defined culture, and its production is limited only to some families because the recipe is kept secret and handed down from one generation to another. Thus, mold and yeast present in starter culture is unknown. Hence, this investigation isolated and identified dominant and useful mold and yeast in *medombae* from Kompong Cham province, Cambodia.

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2. Materials and methods

2.1 Source of starter culture

Dried, instant starter culture (*medombae*) (Figure 1A) obtained from Kompong Cham province, Cambodia was transported to the University of the Philippines Los Baños (UPLB), Philippines. Isolation and identification of essential mold and yeast were conducted in the Food Microbiology Laboratory, Food Science Cluster, College of Agriculture and The National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB), College, Laguna – 4031, Philippines.

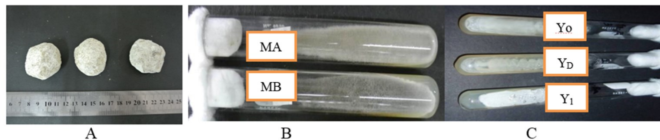

Figure 1. Cambodian dried starter culture (*medombae*) (A), pure culture of mold (B) and yeast (C) isolated from *medombae*

2.2 Isolation of mold and yeast

Isolation of mold and yeast from *medombae* samples was carried out. Ten grams (10g) of the sample was added to 90 mL of 0.85% NaCl solution. Series of dilution was done and 1 mL of appropriate dilution was plated using the standard pour plating technique. Malt Yeast Extract Agar (MYA) medium containing 0.2% sodium propionate for yeast; and Potato Dextrose Agar (PDA) medium containing tartaric acid for mold were used for plating. The petri dishes were incubated upside down at 30°C for 48 hours and then the colonies of yeast and mold were counted and reported as colony forming units/mL (CFU mL^{-1}).

Different types of dominant colonies were picked up and transferred to MYA slant for yeast, and PDA slant for mold.

2.3 Purification of cultures

Single colonies of representative isolates were purified following the dilution plating technique in the agar medium specified for a particular type of mold and yeast. Separated colonies were transferred again to the agar slants. Purification was done by streaking on plated agar and repeated two or three times or until pure cultures were obtained, as confirmed by microscopic examination, are obtained.

2.4 Identification of microbial cultures

Purified microbial cultures were identified through morphological, cultural, physiological and biochemical tests following the methods described by Alexopoulus *et al.* (1996), Samson *et al.* (1995), and Frazier and Westhoff (1998) for mold; and Kreger-van Rij (1984) and Lodder (1970) for yeast.

3. Results and discussion

Selection of representative colonies was based on the appearance of growth on PDA medium. Mold were identified through cultural and morphological test using an agar block technique. For yeast, aside from the above tests, physiological and biochemical properties were also examined.

3.1 Identification of mold isolates

Mold were successfully screened using a modification of the screening techniques described by Alexopoulus *et al.* (1996), Samson *et al.* (1995), and Frazier and Westhoff (1998). Two dominant mold strains coded MA and MB (Figure 1B) were chosen for identification.

MA mold isolate was observed white to creamish-yellow cottony mycelia becoming brownish gray with aged; mycelium ≤10 mm in height; no soluble pigments and exudates produced; smooth, white to yellow on reverse side; ≤85 mm colony diameter; non-septated mycelium indicating that it belongs to Class Phycomycetes. Moreover, it has no sporangioles and characterized by the absence of stolon and rhizoids which are typical of *Mucor* spp.

Isolate MB is fast growing on PDA agar with cottony, aerial, white non-septate mycelium that turns grayish-white when aged; produces grayish-black spores and prominently forms rhizoid which is typical of *Rhizopus* spp. Cultural characteristics exhibited on different culture media (Table 1) as well as growth on Potato Dextrose Agar (PDA) at different temperatures were also used as the basis for the identification. Observation of growth was done for 7 days during incubation at 30°C or until fruiting bodies/spores were observed.

Cultural and morphological characteristics of the mold strains using the agar block technique revealed that both MA and MB mold isolates were non-septated which is the typical property of Class Phycomycetes. Further, MA has no sporangioles and stolons and characterized by the absence of rhizoid which is typical of *Mucor* spp. On the other hand, MB strain had a discernible rhizoid. Figure 2 shows the simple key for differentiation of *Mucor* spp and *Rhizopus* spp.

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Table 1. Cultural and morphological characteristics of the mold isolates

Properties	Culture Medium	Isolate Code	
		MA	MB
Colony Characteristics	Potato Dextrose Agar (PDA)	White to creamish-yellow cottony mycelia becoming brownish gray with aged; ≤10 mm in height; no soluble pigments and exudates produces; smooth, white to yellow reverse; ≤85 mm colony diameter	White gray cottony mycelia that becomes dark brown-gray with age; ≤10 mm in height; no exudates and soluble pigments produced; cream to yellow reverse; ≥90 mm colony diameter
	Czapek Dox Agar (CZA)	White to creamish-yellow cottony mycelia becoming brownigh-gray with aged; ≤10 mm in height; no soluble pigments and exudates produced; smooth, white to yellow reverse; ≤85 mm colony diameter	White gray cottony mycelia that becomes dark brown-gray with age; ≤10 mm in height; no exudates and soluble pigments produced; cream to yellow reverse; ≥90 mm colony diameter
	Malt Extract Agar (MEA)	Creamish-yellow cottony mycelia becoming browning-gray with age; ≤10 mm in height; no soluble pigments and exudates produced; smooth, white to yellow reverse; ≤85 mm colony diameter	Dark brown-gray with age; ≤10 mm in height; no exudates and soluble pigments produced; cream to yellow reverse; ≥90 mm colony diameter
Cellular Characteristics		Sporangia and sporagiophores are light-colored and mostly branch; sporangia are globose with the absence of apophysis, 45-50 µm in diameter; chlamydospores are absent; oidia are observed	Sporangia and sporangiophores are dark pigmented, usually dark-brown; mostly unbranched sporangiophores; stolons are smooth or slightly rough, and yellow-brown; rhizoids are brown in color; sporangia may arise directly from stolons without rhizoids; sporangia may be globoso or sub-globose, and are 50-200 µm in diameter; columellae are ovoid or globose, 30-120 µm in diameter; sporangiospores are globose or ovoid, and 4-10 µm in diameter; chlamydospores are present and may be globose, and ellipsoidal or cylindrical, which measure 10-35 µm or 8-13 x 16-24 µm in diameter

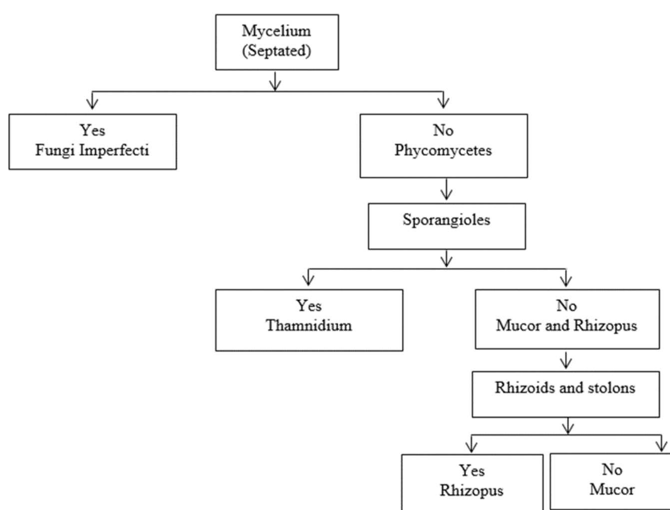

Figure 2. Simple key for differentiation of genera of mold under class Phycomycetes

MB strain is closely related to *R. oligosporus*, *R. stolonifer* and *R. oryzae*. However, chlamydospores of the isolate are not very abundant unlike the *R. oligosporus*, thus this specie was deleted from the choices. Furthermore, incubation of the mold isolates to 37°C showed good growth and this property differentiated the *R. stolonifer* from *R. oryzae*. Thus, the mold MB was identified as *R. oryzae*.

Based on the results of cultural and morphological tests, as summarized in Table 2, the dominant mold strains in *medombae* were identified as *Mucor* spp (coded MA) and *Rhizopus oryzae* (coded MB) (Figure 3). Several previous studies reported the presence of *Mucor spp* and *Rhizopus spp* (particularly *R. oryzae*) in various traditional starter cultures from Southeast Asian countries. Tamang *et al.* (1988), Hesseltine *et al.* (1988) and Thapa and Tamang (2004) reported the presence of mold namely *M. circinelloides* forma circinelloides, *Mucor sp*., *R. chinensis*, *R. stolonifer, Rhizopus* spp in *marcha* starter. Nikkuni *et al.* (1996) and Srestha *et al.* (2002) also stated that *Rhizopus* spp. were present in *mana*. Dwidjoseputro and Wolf (1970), Saono *et al.* (1974), Hadisepoetro *et al.* (1979), Hesseltine *et al.* (1988), Hesseltine and Ray (1988), Ardhana and Fleet

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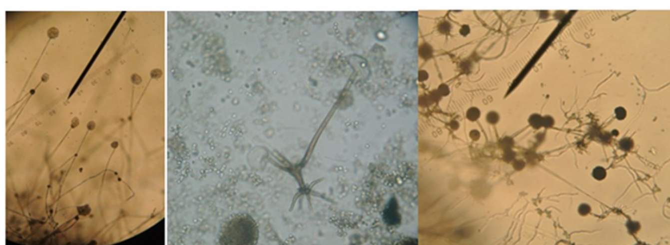
(1989), Yokotsuka (1991) and Elegado and Fujio (1993) confirmed the presence of *Mucor spp* and *Rhizopus spp* in ragi starter from Indonesia. *Rhizopus* spp. and *Mucor* spp. were also found in *bubod* starter from the Philippines (Kozaki and Uchimura 1990; Hesseltine and Kurtzman 1990). *Loogpang* also contained *Mucor* and *Rhizopus* (Dhamcharee 1982; Uchinura *et al.* 1991). *Rhizopus* spp. was also found in *nuruk* starter from Korea (Kim 1968). Dung (2004), Dung *et al.* (2005, 2006, 2007), Lee and Fujio (1999) and Thanh *et al.* (2008) revealed that *Rhizopus oryzae* was isolated in *banh men* starter from Vietnam. *Rhizopus* was also found in *chiu-yueh* for *lao-chao*, a fermented rice product (Wei and Jong, 1983). Recently, Dizon *et al.* (2009, 2013) identified the dominant mold strains in *bubod* from the Philippines as *Mucor* spp. and *R. oryzae*.

Table 2. Summary of cultural and morphological characteristics of selected mold strains

Test	Characteristics	Strain Code	
		MA	MB
Cultural	Form of growth	Cottony	Cottony
	Colony color	Brownish-gray	Grayish
Morphological	Mycelium	Non-septated	Non-septated
	Color of Fruiting body	White to light brown	Greyish to black
	Spore	Sporangiospores	Sporangiospores
Special Structure		No rhizoid	Rhizoids present
Identification		*Mucor spp.*	*Rhizopus oryzae*



Mucor spp *Rhizopus oryzae*

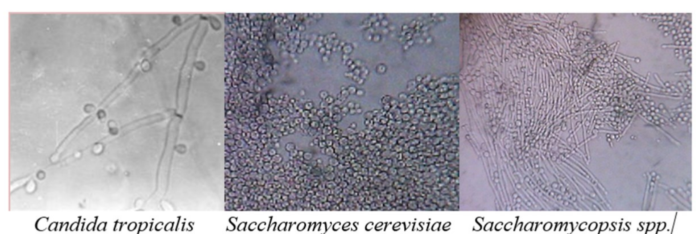
Figure 3. Photomicrograph of identified strains of mold from *medombae*

3.2 Identification of yeast isolates

Three yeast isolates, coded as Yo, Y_D and Y_1 (Figure 1C), were chosen for identification based on their cultural, morphological, and physiological properties following the methods described by Lodder (1970) and Kreger-Van Rij (1984). The colony and cellular characteristics of yeast are presented in Table 3.

The biochemical characteristics of Yo isolate were determined using the Vitek 2 identification system, and YST Card identification (Appendix A) and results revealed the identity as *Candida tropicalis*. On the other hand, the identity of Y_D was determined through its morphological and cultural characteristic using agar technique (Appendix B). Moreover, its protein profile was compared to the reference and sample protein masses using BiomerieuxVitek MS (MALD-TOF) showing that Y_D is a *Saccharomyces cerevisiae*. The biochemical and cellular characteristics of Y_1 isolate as described by Lodder (1970) and Kreger-Van Rij (1984) confirmed its identity as *Saccharomycopsis* spp. (Appendix C).

Results of various tests for identification of three yeast strains (Y_o, Y_D, and Y_1) isolated from *medombae* suggest that they are *Candida tropicalis*, *Saccharomyces cerevisiae* and *Saccharomycopsis spp*. based on the method of identification used, respectively (Figure 4). This study agreed with Tsuyoshi *et al.* (2005) and Thapa and Tamang (2004) who also identified the presence of *S. cerevisiae* in *marcha*. *S. cerevisiae* has been selected for the production of defined granulated starters for the production of high-quality Vietnamese rice wine (Dung 2004; Dung *et al.* 2005). In addition, *Sm. fibuligera* was also found as the most dominant yeast in *marcha* (Tamang and Sarkar 1995). Thapa and Tamang (2004) reported that saccharifying activities are mostly shown by *Rhizopus* spp. and *Sm. fibuligera*, whereas liquefying activities are shown by *Sm. fibuligera* and *S. cerevisiae.* Uchimura *et al.* (1990) isolated *Saccharomycopsis* in *poo* or *phab* (*marcha* of Bhutan). Yeast associated with *ragi* was *Saccharomycopsis* (Dwidjoseputro and Wolf 1970; Saono *et al.,* 1974; Hadisepoetro *et al.,* 1979; Hesseltine *et al.,* 1988; Hesseltine and Ray, 1988; Ardhana and Fleet, 1989; Yokotsuka, 1991). *S. cerevisiae and Sm. fibuligera* have also been reported to be present in *bubod* (Kozaki and Uchimura, 1990; Dizon *et al.,* 2009, 2013); however, *Sm. fibuligera* is the dominant amylolytic yeast in *bubod* (Hesseltine and Kurtzman, 1990). *Loogpang* is an ethnic amylolytic starter from Thailand, which is commoly used to prepare alcoholic drinks and vinegar. Species of yeast present in *loogpang* are *Sm. fibuligera*



Candida tropicalis *Saccharomyces cerevisiae* *Saccharomycopsis spp.*

Figure 4. Photomicrograph of isolated yeast strains from *medombae*

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Table 3. Morphological characteristics of the yeast isolates

Isolate code	Colony Characteristics	Cellular Characteristics
Y_O	White, circular, smooth, dull, opaque, convex to umbonate, entire margin; 3.0 mm colony diameter	Sub-globose to globose cells arranged in singles, pairs, and cluster; exhibits unipolar and bipolar budding; 3.0-6.0 µm diameter
Y_D	White, circular, rough, dull, opaque, convex to umbonate, entire to erose margin; 2-3 mm colony diameter	Globose to sub-globose cells arranged in singles, pairs, and clusters; exhibits unipolar budding; 3-6 µm diameter
Y_1	White, circular, rough, dull, opaque, convex to umbonate, entire to erose margin; 3.0 mm colony diameter	Sub-globose to cylindrical cells arranged in singles, and clusters; exhibits unipolar budding; 2.0->30.0 µm length; 2.0-5.0 µm width

and *Saccharomyces* (Dhamcharee, 1982; Uchinura *et al.,* 1991). *Sm. fibuligera* of *loogpang* showed high glucoamylase activity (Sukhumavasi *et al.,* 1975). *Sm. fibuligera, S. cerevisiae* and *Candida tropicalis* have been isolated in *men* (Dung *et al.,* 2005, 2006, 2007). *Sm. fibuligera* and *S. cerevisiae* were also present in *banh men* (Thanh *et al*., 2008).

4. Conclusion and recommendation

Identified dominant mold and yeast from *medombae* are *Rhizopus oryzae* and *Mucor spp.* for mold; *Candida tropicalis*, *Saccharomyces cerevisiae* and *Saccharomycopsis spp*. for yeast. Mold strains (*R. oryzae, Mucor spp*) and one strain of special yeast (*Saccharomycopsis spp*) are known for their starch saccharification capability while yeast strain, *S. cerevisiae* for its alcohol production. It is however recommended that DNA test be done in the future studies to confirm the identity of the isolates.

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Appendix A. Biochemical characteristics of the yeast isolate

Tests using the Vitek 2 identification system, YST Card for Yo

Substrates for tests	Yo		Yo
L-Lysine Arylamidase	-	D-Sorbitol Assimilation	+
L-Malate Assimilation	+	Saccharose/Sucrose Assimilation	+
Leucine Arylamidase	+	Urease	-
Arginine	+	Alpha-Glucosidase	+
Erythritol Assimilation	-	D-Turanose Assimilation	+
Glycerol Assimilation	-	D-Trehalose Assimilation	+
Tyrosine Arylamidase	-	Nitrate Assimilation	-
Beta-N-Acetyl-Glucosaminidase	-	L-Aracturonate Assimilation	-
Arbutin Assimilation	-	Esculin Hydrolysis	+
Amygdalin Assimilation	-	L-Glutamate Assimilation	-
D-Galactose Assimilation	+	D-Xylose Assimilation	+
Lactose Assimilation	-	DL-Lactate Assimilation	+
Methyl-Alpha-D-Glucopyranoside Assimilation	+	Acetate Assimilation	-
D-Cellobiose Assimilation	-	Citrate (sodium salt) Assimilation	+
Gamma-Glutamyl-Transferase	-	Glucuronate Assimilation	+
D-Maltose Assimilation	+	L-Proline Assimilation	+
D-Raffinose Assimilation	-	2-Keto-D-Gluconate Assimilation	+
PNP-N-Acetyl-Beta-D-Galactosaminidase 1	-	N-Acetyl-Glucosamine Assimilation	+
D-Mannose Assimilation	+	D-Gluconate Assimilation	+
D-Melibiose Assimilation	-	L-Rhamnose Assimilation	-
D-Melezitose Assimilation	+	Xylitol Assimilation	-
L-Sorbose Assimilation	-		

Appendix B. Comparison of the reference and sample protein masses for Y_D

superspectrum_Saccharomyces_cerevisiae_7_ <=> UPLB_000_0049_1F4[c]

superspectrum_Sa	UPLB_000_0049_	Error
3103.8	3105.1	0.0406
3505.7	3505.4	0.0077
3661.2	3662.8	0.0442
3874.9	3873.6	0.0333
4229.7	4230.4	0.0175
4393.2	4392.9	0.0080
4400.9	4400.0	0.0205
5803.7	5802.9	0.0146
6016.8	6015.9	0.0153
6212.0	6211.4	0.0098
6310.3	6313.7	0.0539
6408.4	6412.2	0.0588
6534.2	6531.0	0.0492
6534.2	6538.7	0.0693
6598.1	6597.7	0.0065
6690.4	6689.3	0.0170
6803.2	6801.5	0.0254
6986.5	6988.7	0.0318
7325.1	7323.6	0.0199
7385.7	7382.2	0.0481
7635.3	7633.8	0.0195
8460.4	8459.7	0.0079
8788.4	8786.0	0.0275
9655.4	9657.7	0.0238
9697.4	9702.0	0.0476
9932.0	9931.2	0.0079
11604.8	11605.1	0.0027

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Appendix C. Tests using the Vitek 2 identification system, YST Card for Y1

Substrates for tests	Y1		
L-Lysine Arylamidase	-	L-Sorbose Assimilation	-
L-Malate Assimilation	+	L-Rhamnose Assimilation	-
Leucine Arylamidase	+	Xylitol Assimilation	-
Arginine	-	D-Sorbitol Assimilation	+
Erythritol Assimilation	-	Saccharose/Sucrose Assimilation	+
Glycerol Assimilation	-	Urease	-
Tyrosine Arylamidase	+	Alpha-Glucosidase	(+)
Beta-N-Acetyl-Glucosaminidase	-	D-Turanose Assimilation	+
Arbutin Assimilation	-	D-Trehalose Assimilation	-
Amygdalin Assimilation	+	Nitrate Assimilation	-
D-Galactose Assimilation	-	L-Aracturonate Assimilation	-
Gentiobise Assimilation	+	Esculin Hydrolysis	-
D-Glucose Assimilation	+	L-Glutamate Assimilation	-
Lactose Assimilation	-	D-Xylose Assimilation	-
Methyl-Alpha-D-Glucopyranoside Assimilation	-	DL-Lactate Assimilation	-
D-Cellobiose Assimilation	-	Acetate Assimilation	+
Gamma-Glutamyl-Transferase	-	Citrate (sodium salt) Assimilation	+
D-Maltose Assimilation	+	Glucuronate Assimilation	-
D-Raffinose Assimilation	-	L-Proline Assimilation	-
PNP-N-Acetyl-Beta-D-Galactosaminidase 1	-	2-Keto-D-Gluconate Assimilation	-
D-Mannose Assimilation	+	N-Acetyl-Glucosamine Assimilation	-
D-Melibiose Assimilation	-	D-Gluconate Assimilation	-
D-Melezitose Assimilation	-		

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