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.
2. Materials and methods

2.1 Source of starter culture

Dried, instant starter culture \textit{(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.

2.2 Isolation of mold and yeast

Isolation of mold and yeast from \textit{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 \textit{et al.} (1996), Samson \textit{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 \textit{et al.} (1996), Samson \textit{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 \textit{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 \textit{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 \textit{Mucor} spp. On the other hand, MB strain had a discernible rhizoid. Figure 2 shows the simple key for differentiation of \textit{Mucor} spp and \textit{Rhizopus} spp.
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 2). 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), Hadisepuro et al. (1979), Hesseltine et al. (1988), Hesseltine and Ray (1988), Ardhana and Fleet

![Figure 2. Simple key for differentiation of genera of mold under class Phycomycetes](Image)

**Table 1. Cultural and morphological characteristics of the mold isolates**

| Properties                  | Culture Medium           | MA                                                                 | Isolate Code                                                                 |
|-----------------------------|--------------------------|-------------------------------------------------------------------|----------------------------------------------------------------------------|
| Colony Characteristics      | Potato Dextrose Agar     | White to creamish-yellow cottony mycelia becoming brownish 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 |
|                             | Czapek Dox Agar          | White to creamish-yellow cottony mycelia becoming brownish-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        | Creamish-yellow cottony mycelia becoming brownish-gray with aged; ≤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 sporangiophores are light-colored and mostly branch; sporangia are globose with the absence of apophysys, 45-50 µm in diameter; chlamydosporases 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-globoso, 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; chlamydosporases are present and may be globose, and ellipsoidal or cylindrical, which measure 10-35 µm or 8-13 x 16-24 µm in diameter |

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| Properties                  | Culture Medium           | MA                                                                 | Isolate Code                                                                 |
|-----------------------------|--------------------------|-------------------------------------------------------------------|----------------------------------------------------------------------------|
| Colony Characteristics      | Potato Dextrose Agar     | White to creamish-yellow cottony mycelia becoming brownish 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 |
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|                             | Malt Extract Agar        | Creamish-yellow cottony mycelia becoming brownish-gray with aged; ≤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 |
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**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, chlamydosporases of the isolate are not very abundant unlike the *R. oligosporus*, thus this specie was deleted from the
(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; Uchimura 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*.

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 YD 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 YD is a *Saccharomyces cerevisiae*. The biochemical and cellular characteristics of Y1 isolate as described by Lodder (1970) and Kregers-Van Rij (1984) confirmed its identity as *Saccharomycopsis* spp. (Appendix C).

Results of various tests for identification of three yeast strains (Yo, YD, and Y1) isolated from *medombae* suggest that they are *Candida tropicalis*, *Saccharomycopsis 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; Hadisoepetro 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 commonly used to prepare alcoholic drinks and vinegar. Species of yeast present in *loogpang* are *Sm. fibuligera*.

3.2 Identification of yeast isolates

Three yeast isolates, coded as Yo, YD and Y1 (Figure 1C), were chosen for identification based on their cultural, morphological, and physiological properties following the methods described by Lodder (1970) and Kregers-Van Rij (1984). The colony and cellular characteristics of yeast are presented in Table 3.

The characteristics of selected mold strains are presented in Table 3. Table 2. Summary of cultural and morphological characteristics of selected mold strains

| Test          | Characteristics | Strain Code | MA   | MB   |
|---------------|-----------------|-------------|------|------|
| Cultural      | Form of growth  | Cotton      | Cotton |      |
|               | Colony color    | Brownish-gray | Grayish |      |
| Morphologic   | 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 | Rhizoids present |      |
| Identification |                 | *Mucor spp.* | *Rhizopus oryzae* |      |

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

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

| Masses | Error |
|--------|-------|
| 3103.8 | 0.0406 |
| 3505.7 | 0.0077 |
| 3661.2 | 0.0442 |
| 3874.9 | 0.0333 |
| 4229.7 | 0.0175 |
| 4331.2 | 0.0080 |
| 4400.9 | 0.0205 |
| 5803.7 | 0.0146 |
| 6016.8 | 0.0153 |
| 6212.0 | 0.0098 |
| 6310.3 | 0.0539 |
| 6408.4 | 0.0588 |
| 6532.4 | 0.0492 |
| 6532.4 | 0.0693 |
| 6598.1 | 0.0065 |
| 6690.4 | 0.0170 |
| 6803.2 | 0.0254 |
| 6986.5 | 0.0318 |
| 7325.1 | 0.0199 |
| 7385.7 | 0.0481 |
| 7635.3 | 0.0195 |
| 8460.4 | 0.0079 |
| 8788.4 | 0.0275 |
| 9655.4 | 0.0238 |
| 9697.4 | 0.0476 |
| 9932.0 | 0.0079 |
| 11604.8 | 0.0027 |
### 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                                     |
| Gentiobose Assimilation                   | + Escluin 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                 |                                                                  |