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Characterization and amylolytic activity of yeast and mold strains from Thai sweet rice

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ABSTRACT

Aims: The aim of this study was to isolate and identify the yeast and mold strains from the starter and to investigate their amylolytic activity.
Methodology and results: Thirty-two yeasts were isolated from ten samples of *look-paeng khao-mak*, a traditional starter culture in the production of *khao-mak* (sweet rice) in several places in Thailand. All isolates were identified based on their morphological and biochemical characteristics including the sequencing of D1/D2 domain of large subunit (26S) ribosomal DNA analyses. They were identified as *Saccharomycopsis fibuligera* (9 isolates), *Candida rugosa* (2 isolates), *C. tropicalis* (1 isolate), *Clavispora lusitaniae* (1 isolate), *Wickerhamomyces anomalus* (15 isolates) and *Meyerozyma guilliermondii* (4 isolates). All isolates of *S. fibuligera* showed high amylolytic activity. One-hundred isolates of mold were isolated from twenty-one samples of *look-paeng khao-mak.* They were belonged to *Amylomyces* sp. (42 isolates), *Rhizopus* sp. (30 isolates), *Mucor* sp. (12 isolates) and *Penicillium* sp. (16 isolates) based on their morphological characteristics. Four isolates, LK4-1, LK8-2, LK12-5 and LK17-1 showed amylase activity ranged from 32.24 to 39.74 unit/mL by dinitrosalicylic acid (DNSA) method. The isolates LK4-1, LK8-2 and LK12-5 were identified as *Amylomyces rouxii* and LK17-1 was *Rhizopus microsporus* based on their morphological characteristics and the ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer (ITS) sequences.
Conclusion, significance and impact study: The characterization and evaluation of yeast and mold species based on their phenotypic and genetic characteristics including their amylase activity will be useful for the diversity and sweet rice production.

Keywords: Look-paeng khao-mak, amylase, *Amylomyces*, ITS gene sequence, D1/D2 domain of 26S rRNA gene

INTRODUCTION

Look-paeng khao-mak, a traditional starter is produced in several places in Thailand. This starter mainly contained raw starch with mixed cultures of molds, yeasts and bacteria on rice or other cereals and was used in the production of various starch-based fermented foods for example syrup, *khao-mak* and vinegar. The fermentation starter has been used in many Asian countries with various local names, such as banh men in Vietnam, bubod in the Philippines, chu in China, koji in Japan, murcha in India, nuruk in Korea, ragi in Indonesia and Malaysia (Lee and Fujio, 1999; Limtong *et al*., 2005). The mold species that found in *look-paeng khao-mak* were *Rhizopus* spp., *Amylomyces* spp., *Actinomucor* spp., *Aspergillus* spp., *Mucor* spp., *Monascus* spp., and *Penicillium* (Limtong *et al*., 2005). *Rhizopus oryzae*, *Mucor indicus*, *Mucor circinilloides* and *Amylomyces rouxii* were obtained from banh men (Lee and Fujio, 1999; Dung *et al*., 2006). In ragi, *Amylomyces* spp., *Mucor* spp. and *Rhizopus* spp. were isolated (Hesseltine *et al.*, 1988). Molds are the major producer of amylase that degrade starch into sugar. Some mold species are reported to exhibit high amylase activity (Lee and Fujio, 1999; Shrestha *et al*., 2002; Limtong *et al*., 2005). The yeast species, *Saccharomycopsis fibuligera*, *Wickerhamomyces anomalus*, *Issatchenkia orientalis*, *Hyphopichia burtonii*, *Pichia fabianii*, *Candida rhagii*, *C. glabrata*, *Torulaspora globosa*, *Yamadazyma mexicana*, *P. heimii*, *Rhodotorula philyla*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Trichosporon asahii* commonly were found in *look-paeng khao-mak* (Kurtzman and Robnett, 1998). *Saccharomycopsis fibuligera*, *S. cerevisiae*, *Issatchenkia* sp., *P. anomala*, *C. tropicalis*, *P. ranonggensis* and *Clavispora lusitaniae* were obtained from banh men (Lemmel *et al*., 1980; Lee and Fujio, 1999). The yeast *S. fibuligera* which one of the common yeasts present in traditional starter was reported to have

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amylolytic activity (Lee and Fujio, 1999; Limtong *et al*., 2002; Thanh *et al*., 2008) and had been applied in food industry for producing sugar syrup (Sandhu *et al*., 1987), single cell protein (Lemmel *et al*., 1980), and ethanol (Verma *et al*., 2000). However, the yeast and mold species reported previously were identified based on their morphological and biochemical characteristics. This study deals with the isolation of the yeast and mold strains from *look-paeng khao-mak* and identify them based on phenotypic and genetic characteristics including the screening for their amylase activity.

MATERIALS AND METHODS

Source of samples and isolation of yeasts and molds

Twenty-one samples of *look-paeng khao-mak* were collected from Nakhon Pathom, Trad, Chumphon (4 samples), Songkhla (4 samples), Nakhon Si Thammarat (6 samples), Lopburi, Nakhon Ratchasima, Phatthalung, Krabi and Chachoengsao provinces. They were placed in clean plastic bags and stored at 4 °C prior to isolation. One gram of sample was suspended in 50 mL of Yeast and malt extract broth (YM Broth, Difco™ BD, USA) in 250 mL Erlenmeyer flask and incubated at 30 °C on rotary shaker at 180 rpm for 72 h. The culture broth was streaked onto Potato Dextrose Agar (PDA) plate and incubated at 30 °C for 72 h. The cultures were selected from colonies for the purification and then subcultured on PDA slant and stored at 4 °C. The yeast strains were isolated by suspending 1 g of sample into 50 mL of YM Broth in 250 mL Erlenmeyer flask and incubated at 30 °C on rotary shaker at 180 rpm for 72 h. The culture broth was streaked onto YM agar (Difco™ BD, USA) plate and incubated at 30 °C for 72 h. The cultures were selected from colonies for the purification and then subcultured on YM slant and stored at 4 °C.

Screening and determination of amylase activity of yeast and mold isolates

Isolated yeast cultures were screened in 100 mL starch medium (3% soluble starch, 3 g yeast extract, 3 g peptone, 5 g glucose and 1000 mL of distilled water) in 500 mL Erlenmeyer flask and incubated at 30 °C for 96 h in a rotary shaker (150 rpm). The amylase activity was determined at 24, 48, 72 and 96 h of incubation by dinitrosalicylic acid method (Miller, 1959) using starch as the substrate. The reaction mixture containing 1 mL of 3% soluble starch in 0.25 M sodium acetate buffer pH 5.0 and 10% of culture broth (0.5 MacFarland) was incubated at 60 °C for 1 h. After that the reaction mixture was added with 1 mL of DNS solution and placed in boiling water bath for 10 min and then cooled. The amount of glucose produced was calculated by referring to the standard plot using glucose as the reducing sugar and absorbance was determined at 540 nm.

Isolated mold cultures were screened in 50 mL starch medium in 250 mL Erlenmeyer flask and incubated at 30 °C for 72 h in a rotary shaker (180 rpm). The amylase activity was determined by dinitrosalicylic acid method (Miller, 1959) using starch as the substrate. The reaction mixture containing 1 mL of 3% soluble starch in 0.25 M sodium acetate buffer pH 5.0 and 0.5 mL of culture broth (1×10^6 spores/mL using hemacytometer) was incubated at 60 °C for 1 h. After that the reaction mixture was added with 1 mL of DNS solution and placed in boiling water bath for 10 min and then cooled. The amount of glucose produced was calculated by referring to the standard plot using glucose as the reducing sugar and absorbance was determined at 540 nm.

Identification of yeast isolates

Phenotypic characterization

Cells and colony appearance of yeasts were determined on cells and colonies grown in YM broth and on YM agar, respectively after incubation at 30 °C for 72 h. Biochemical characterization of the isolates were determined by investigating the assimilation reactions of sugars using the ID 32C test (Bio-Mérieux, France) and followed to taxonomic keys as written in The Yeasts, A Taxonomic Study (Kurtzman and Fell, 1998) and Summary of specific characteristics. Yeast: Characteristics and identification (Barnett *et al*., 2000).

Sequencing of D1/D2 domain of 26S rRNA gene and phylogenetic analysis

DNA extraction was carried out by boiling of cells with lysis buffer (100 mM Tris (pH 8.0), 30 mM EDTA (pH 8.0), 0.5% SDS). A loopful of yeast cells was transferred to 1.5 mL Eppendorf tube. The 100 µL of lysis buffer was added. Cell suspensions were boiled in water bath or metal block bath for 15 min. After boiling, 100 µL of 2.5 M potassium acetate (pH 7.5) was added and placed on ice for 1 h, and centrifuged at 14,000 rpm for 5 min. Supernatant was extracted twice with 100 µL of chloroform:isoamyl alcohol (24:1 v/v). DNA was precipitated with isopropanol, placed at 20 °C for 10 min and centrifuged at 15,000 rpm for 15 min. DNA pellet was rinsed with 70% and 90% ethanol and then dried up (15-30 min at room temperature) The dried DNA was dissolved in 30 µL milli Q water.

Polymerase Chain Reaction (PCR) for D1/D2 domain of 26S rDNA by using the primers F63 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and LR3 (5'-GGTCCGTGTTTCAAGACGG-3'). Amplification was carried out in 100 µL reaction mixture conditioning 100 ng of genomic DNA, 2.5 U of *Taq* polymerase, 20 mM of each dNTP, 40 mM of each primer, 10 mM Tris-HCl and 1.5 mM $MgCl_2$. The reaction was pre-denatured at 94 °C for 5 min, then repeated for 30 PCR cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2.5 min and then followed by the final extension at 72 °C for 10 min. The amplified DNA was purified with QIAquick PCR Purification Kit according to the manufacturer's instruction. Visualization of the purified amplified DNA was performed by electrophoresis using 0.8% agarose gel in 1× TAE buffer

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and stained with ethidium bromide (8×10^{-5} µg/mL) and observed under UV illuminator.

After gene amplification, the PCR products were sent to the Macrogen, Korea to analyze the nucleotide sequence of the PCR products. The sequences of D1/D2 domain of 26S rDNA were compared by BLASTn similarity Search (http://www.ncbi.nlm.nih.gov/blast). Generated sequences were aligned with related species by using the CLUSTAL X ver. 1.8 computer programs (Thompson *et al*., 1997). The phylogenetic trees were constructed from the evolutionary distance data according to Kimura (1980) by the neighbor-joining method (Saitou and Nei, 1987). Bootstrap analysis (Felsenstein, 1985; 1988) is performed from 1,000 random re-samplings.

Identification of mold isolates

The mold isolates were identified based on the morphological characteristics by using slide culture techniques followed to taxonomic keys as written in Introduction to food and airborne fungi (Samson *et al*., 2002), Textbook of Fungi (Sharma, 1989), Introduction to Fungi (Webster and Weber, 2007). The potent isolates were selected for analysis of the ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer (ITS) gene sequences by using the primers ITS1 (5'-TCCGTAGGTGAACTGCGG-3' and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') (White *et al*., 1990).

RESULTS AND DISCUSSION

Isolation and amylase activity of yeast and mold isolates

Thirty-two yeast strains were isolated from ten samples of *look-paeng khao-mak* in several places in Thailand (Table 1). Amylase activity was determined for all yeast strains by dinitrosalicylic acid method using 3% starch as the substrate. The strains LY7-1, LY7-2, LY16-1 LY19-1, LY19-2, LY19-3, LY21-1, LY21-2 and LY21-3 showed amylase activity ranged from 70.8 to 171.2 unit/mL and these strains produced highest amylase activity after 96 h of incubation, whereas the strain LY7-2 and LY21-2 produced highest amylase activity after 72 h of incubation (Figure 1). One-hundred mold strains were isolated from twenty-one samples of *look-paeng khao-mak* in several places in Thailand (Table 1). All the mold isolates were primary screened and the strains LK1-2, LK1-3, LK1-4, LK4-1, LK6-5, LK7-3, LK7-8, LK12-5, LK15-5, LK16-5, LK17-1 and LK19-1 showed high amylase activity were selected (Data not shown). All 13 isolates were determined for amylase activity using the inoculum of 1×10^6 spores/mL. One unit (U) was defined as the amount of enzyme liberating 1 µmol of reducing sugar per minute under the standard assay conditions. Four isolates, LK4-1, LK8-2, LK12-5 and LK17-1 showed strong amylase activity ranged from 32.24 to 39.74 unit/mL respectively (Figure 2). These strains were highly capable of starch hydrolysis as reported by Limtong *et al.* (2005).

Identification of yeast isolates

Thirty-two yeast isolates were separated into six groups based on their phenotypic characteristics as described below and as shown in Tables 1 and 2. The analysis of D1/D2 domain of 26S rRNA gene sequences of 17 representative isolates of six groups were also carried out and the phylogenetic tree indicated their taxonomic position was shown in Figure 3.

Group 1 contained 9 isolates, LY7-1, LY7-2, LY16-1, LY19-1, LY19-2, LY19-3, LY21-1, LY21-2 and LY21-3. Four isolates (LY7-1, LY19-1, LY19-2 and LY21-2) were selected as the representative isolates for their phenotypic characterization. They assimilated cyclohexamide, D-sucrose, D-cellobiose, D-raffinose, D-maltose, methyl-α-D-glucopyranoside, glycerol, D-glucose and esculin ferric citrate. They produced hat-shape ascospores and pseudohyphae (Kurtzman and Fell, 1998; Barnett *et al*., 2000). The representative six isolates had identical sequences (100% similarity) in the nucleotide sequence of the D1/D2 domain of the 26S rRNA gene and were located within the cluster of *Saccharomycopsis fibuligera* as in Figure 3. Therefore, they were identified as *S. fibuligera* (Kurtzman and Robnett, 1998).

All nine isolates in Group 1 showed high amylase activity (Figure 2). These isolates identified as *S. fibuligera* had ability to hydrolyse starch and produced high amylase activity agreed with the hydrolysis of starch to sugar by the yeast isolates in *look-paeng* that has been reported by many authors (Lee and Fujio, 1999; Limtong *et al*., 2002; Tsuyoshi *et al*., 2005; Aidoo *et al*., 2006; Thanh *et al*., 2008). Therefore, the isolates involved and played important role in the fermentation of sweet rice.

Group 2 contained 2 isolates, LY8-1 and LY8-2. They assimilated D-galactose, N-acetyl-glucosamine, lactic acid, D-mannitol, D-sorbitol, D-xylose and glucosamine. They produced pseudohyphae (Kurtzman and Fell, 1998; Barnett *et al*., 2000). The representative LY8-1 had identical sequence (100% similarity) in the nucleotide sequence of the D1/D2 domain of the 26S rRNA gene and was located within the cluster of *Candida rugosa* as in Figure 3. Therefore, they were identified as *C. rugosa* (Lachance *et al.,* 2011).

Group 3 contained 1 isolate, LY16-4. It assimilated D-galactose, D-sucrose, N-acetyl-glucosamine, D-maltose, D-trehalose, potassium 2-keto gluconate, methyl-α-D-glucopyranoside, D-mannitol, D-sorbitol, D-xylose, palatinose, D-melezitose, D-glucose, L-sorbose and glucosamine. They produced pseudohyphae (Kurtzman and Fell, 1998; Barnett *et al*., 2000). The representative LY16-4 had identical sequence (100% similarity) in the nucleotide sequence of the D1/D2 domain of the 26S rRNA gene and was located within the cluster of *Candida tropicalis* as in Figure 3. Therefore, it was identified as *C. tropicalis* (Lachance *et al*., 2011).

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Table 1: Source, isolate number and identification of yeast and mold isolates from *look-paeng khao-mak.*

Sample location (province)	Isolate no.			
	Yeasts	100% Similarity/ Group	Molds	Identification
Nakhon Pathom	-	-	LK1-1, LK1-2, LK1-3, LK1-4	*Amylomyces* sp.
			LK1-5	*Rhizopus* sp.
Trad	-	-	LK2-1, LK2-2	*Penicillium* sp.
			LK2-3, LK2-4	*Amylomyces* sp.
Mueang, Chumphon	-	-	LK3-1	*Amylomyces* sp.
			LK3-2, LK3-3	*Penicillium* sp.
Sathing Phra, Songkhla (I)	LY4-1, LY4-2, LY4-3, LY4-4	*Wickerhamomyces anomalus*/ Group 5	LK4-1, LK4-2	*Amylomyces* sp.
			LK4-3, LK4-4, LK4-5	*Rhizopus* sp.
Tha Sae, Chumphon	-	-	LK5-1	*Penicillium* sp.
Krabi	-	-	LK6-1, LK6-2, LK6-3	*Penicillium* sp.
			LK6-4, LK6-5	*Amylomyces* sp.
Lang Suan, Chumphon	LY7-1, LY7-2	*Saccharomycopsis fibuligera*/ Group 1	LK7-1, LK7-2, LK7-3, LK7-4	*Rhizopus* sp.
			LK7-5, LK7-6	*Mucor* sp.
			LK7-7, LK7-8	*Amylomyces* sp.
Bang Khan, Nakhon Si Thammarat	LY8-1, LY8-2	*Candida rugosa*/ Group 2	LK8-1, LK8-2	*Amylomyces* sp.
			LK8-3, LK8-4	*Rhizopus* sp.
Nakhon Ratchasima	-	-	LK9-1, LK9-2, LK9-3	*Amylomyces* sp.
Mueang, Nakhon Si Thammarat	LY10-1, LY10-2	*Wickerhamomyces anomalus*/ Group 5	LK10-1, LK10-2	*Amylomyces* sp.
			LK10-3, LK10-4	*Rhizopus* sp.
Tha Sala, Nakhon Si Thammarat	-	-	LK11-1, LK11-2	*Rhizopus* sp.
			LK11-3	*Amylomyces* sp.
Lan Saka, Nakhon Si Thammarat	-	-	LK12-1, LK12-4	*Mucor* sp.
			LK12-3	*Rhizopus* sp.
			LK12-2, LK12-5, LK12-6	*Amylomyces* sp.
Phatthalung	-	-	LK13-1, LK13-2	*Amylomyces* sp.
			LK13-3, LK13-4, LK13-5, LK13-6	*Rhizopus* sp.
Chumphon	-	-	LK14-1, LK14-2	*Rhizopus* sp.
			LK14-3, LK14-4	*Amylomyces* sp.
Songkhla	LY15-1, LY15-2, LY15-3 LY15-4	*Meyerozyma guilliermondii*/ Group 6	LK15-1, LK15-2	*Rhizopus* sp.
			LK15-3, LK15-4, LK15-5	*Amylomyces* sp.
			LK15-6, LK15-7	*Mucor* sp.
Bang Nam Priao, Chachoengsao	LY16-1	*Saccharomycopsis fibuligera*/ Group 1	LK16-1, LK16-2	*Amylomyces* sp.
			LK16-3, LK16-4	*Mucor* sp.
	LY16-2 , LK16-3	*Wickerhamomyces anomalus*/ Group 5	LK16-5, LK16-6, LK16-7, LK16-8	*Penicillium* sp.
	LY16-4	*Candida tropicalis*/ Group 3		
	LY16-5	*Clavispora lusitaniae*/ Group 4		
Sathing Phra, Songkhla (II)	LY17-1, LY17-2, LY17-3, LY17-4, LY17-5	*Wickerhamomyces anomalus*/ Group 5	LK17-1, LK17-3, LK17-4	*Rhizopus* sp.
			LK17-2	*Penicillium* sp.
Hat Yai, Songkhla	-	-	LK18-1, LK18-2	*Rhizopus* sp.
			LK18-3	*Penicillium* sp.
Lopburi	LY19-1, LY19-2, LY19-3	*Saccharomycopsis fibuligera*/ Group 1	LK19-1, LK19-2, LK19-5, LK19-6	*Amylomyces* sp.
			LK19-3	*Rhizopus* sp.
			LK19-4	*Penicillium* sp.
Ron Phibun, Nakhon Si Thammarat	LY20-1, LY20-2	*Wickerhamomyces anomalus*/ Group 5	LK20-1	*Rhizopus* sp.
			LK20-2, LK20-3	*Mucor* spp.
			LK20-4, LK20-5	*Amylomyces* sp.
			LK20-6	*Penicillium* sp.
Chian Yai, Nakhon Si Thammarat	LY21-1, LY21-2, LY21-3	*Saccharomycopsis fibuligera*/ Group 1	LK21-1, LK21-2, LK21-3	*Amylomyces* sp.
			LK21-4, LK21-5	*Mucor* sp.

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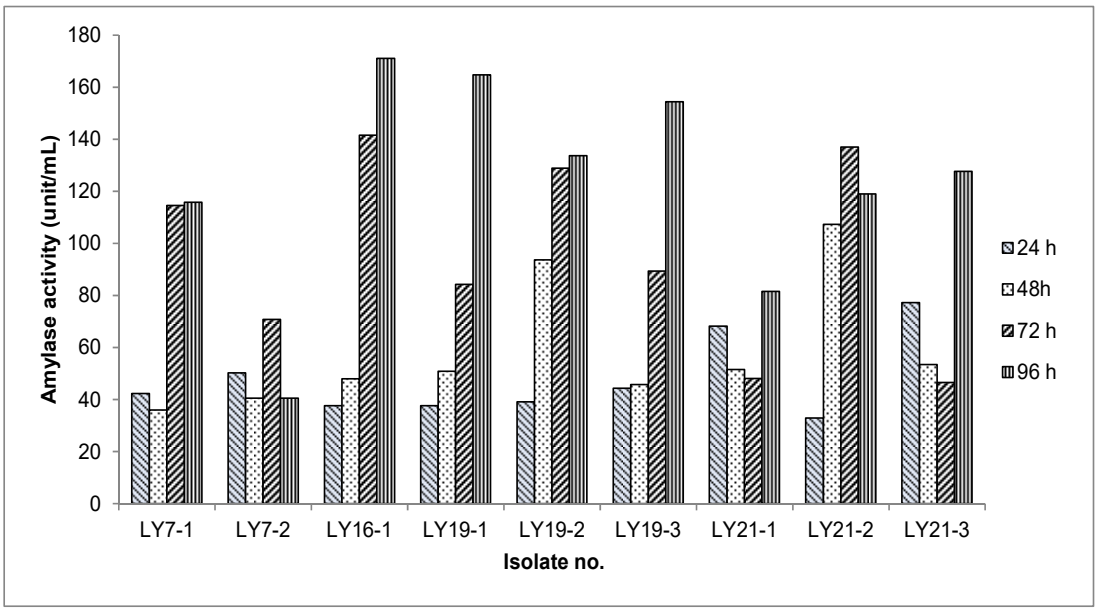

Figure 1: Amylase activity of nine yeast isolates at 24, 48, 72 and 96 h of incubation.

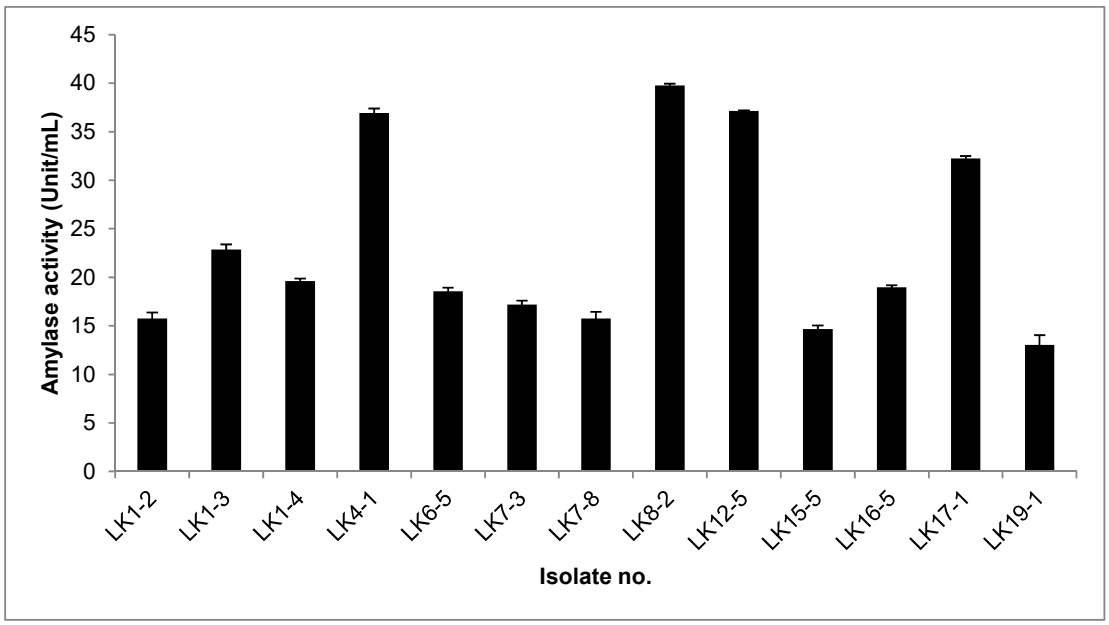

Figure 2: Amylase activity of selected mold isolates.

Group 4 contained 1 isolate, LY16-5. It assimilated D-galactose, D-sucrose, D-maltose, D-trehalose, potassium 2-keto gluconate, methyl-α-D-glucopyranoside, D-mannitol, D-sorbitol, D-ribose, glycerol, D-rhamnose, palatinose, D-melezitose, potassium gluconate, D-glucose, glucosamine and esculin ferric citrate. They produced one or two clavate ascospores and pseudohyphae (Kurtzman and Fell, 1998; Barnett *et al.*, 2000). The representative LY16-5 had identical sequence (100% similarity) in the nucleotide sequence of the D1/D2 domain of the 26S rRNA gene and was located within the cluster of *Clavispora lusitaniae* as in Figure 3. Therefore, it was identified as *Cl. lusitaniae* (Kurtzman and Robnett, 1997).

Group 5 contained 15 isolates, LY4-1, LY4-2, LY4-3, LY4-4, LY10-1, LY10-2, LY16-2, LY16-3, LY17-1, LY17-2, LY17-3, LT17-4, LY17-5, LY20-1 and LY20-2. Four isolates (LY4-1, LY4-3, LY16-2 and LY20-1) were selected as the representative isolates for their phenotypic characterization. They assimilated D-sucrose, lactic acid, D-raffinose, D-maltose, D-trehalose, methyl-α-D-glucopyranoside, D-mannitol, D-sorbitol, D-xylose,

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Table 2: Morphological characteristics and assimilation reactions of yeast isolates.

	Group 1	SF[a,b]	Group 2	CR [a,b]	Group 3	CT[a,b]	Group 4	CL[a,b]	Group 5	WA[a,b]	Group 6	MG[a,b]
No. of isolate	4		2		1		1		4		2	
Ascospore[c]	+[d]	+[d]	-	-	-	-	+[e]	+[e]	+[f]	+[f]	+[f]	+[f]
Pseudohyphae	+	+	+	+	+	+	+	+	-	v	+	+
Arabinose	-	-	-	-	-	d, -	-	v	-	-	+	v
D-Cellobiose	+ (d1)	+	-	-	d	v	d	+, d	- (+1)	v	d	v
Cycloheximide[g]	+	+, d	-	v	d	+, d	-	-	-	-	+	v
Erythritol	-	v	-	-	-	-	-	-	+	v	-	-
Esculin	+	nd	d	nd	d	nd	+	nd	+	nd	+	nd
D-Galactose	-	-	+	+, d	+	+	+	v	-	v	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Potassium gluconate	- (d2)	+, d	-	v	d	v	+	v, d	- (d1)	v	d	v
Sodium glucuronate	- (d2)	+	-	-	-	-	-	-	-	-	-	-
Glucosamine	-	-	+	v	+	d, -	+	v	-	-	+	+, d
N-acetyl-glucosamine	+ (-2)	-	+	nd	+	+	-	+	-	-	+	+
Potassium 2-keto gluconate	-	v	-	-	+	+, d	+	+, d	-	v	+	+
Methyl-α-D-glucopyranoside	+	+	-	-	+	v	+	v	+	v	+	v
Glycerol	+	+	-	v	d	v	+	+, d	+	+	d	+, d
Lactic acid	-	nd	+	nd	d	nd	-	nd	+	nd	-	nd
D-Lactose	-	-	-	-	d	-	-	-	-	-	-	-
Levulinic acid	-	nd	-	nd	d	nd	-	nd	-	nd	d	nd
Inositol	+ (-1)	v, d	-	-	-	-	-	-	-	-	-	-
D-Maltose	+	+	-	-	+	+	+	+	+	v	+	+, d
D-Mannitol	- (+1)	v	+	v, d	+	+	+	+	+	+	+	v
D-Melibiose	-	-	-	-	-	-	-	-	-	-	-	v
D-Melezitose	- (d1)	v, d	-	-	+	v	+	v	+	v	+	v
Palatinose	- (+2)	nd	-	nd	+	nd	+	nd	+	nd	+	nd
D-Raffinose	+ (d1)	v	-	-	d	-	-	-	+	v	+	+, d
D-Rhamnose	-	-	-	-	d	-	+	v	-	-	-	v
D-Ribose	-	v	-	-	d	d, -	+	v	-	v	-	+, d
D-Sorbitol	- (+1)	v	+	+, d	+	+	+	+, d	+ (d1)	+, d	+	v
L-Sorbose	-	-	-	v	+	v	-	v	-	-	d	v

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Table 2: (continued)

	Group 1	SF[a,b]	Group 2	CR [a,b]	Group 3	CT[a,b]	Group 4	CL[a,b]	Group 5	WA[a,b]	Group 6	MG[a,b]
D-Sucrose	+	v	-	-	+	v	+	+	+	+	+	+
D-Trehalose	- (d1)	v, d	-	-	+	+	+	+, d	+	+, d	+	+
D-Xylose	-	-	+	v	+	+	-	+, d	+ (d1)	v	+	+

Group 1, LY7-1, LY19-1, LY19-2, LY21-1; Group 2, LY8-1, LY8-2; Group 3, LY16-4; Group 4, LY16-5; Group 5, LY4-1, LY4-3, LY16-2, LY20-1.
SF, *Saccharomycopsis fibuligera*; CR, *Candida rugosa*; CT, *Candida tropicalis*; CL, *Clavispora lusitaniae*; WA, *Wickerhamomyces anomalus*; MG, *Meyerozyma guilliermondii*.
+, positive; d, delayed positive; v, variable; -, negative reaction; nd, not determined. Numbers in parentheses indicate the number of strains showing a positive, delayed positive or negative reaction.
[a]Kurtzman and Fell (1998)
[b]Barnett *et al*. (2000)
[c]For ascospore shape
[d]2-4 Hat-shaped ascospores
[e]one or two clavate ascospores
[f]1-4 Hat-shaped ascospores
[g]0.1 g/100 mL Cycloheximide for reference strains

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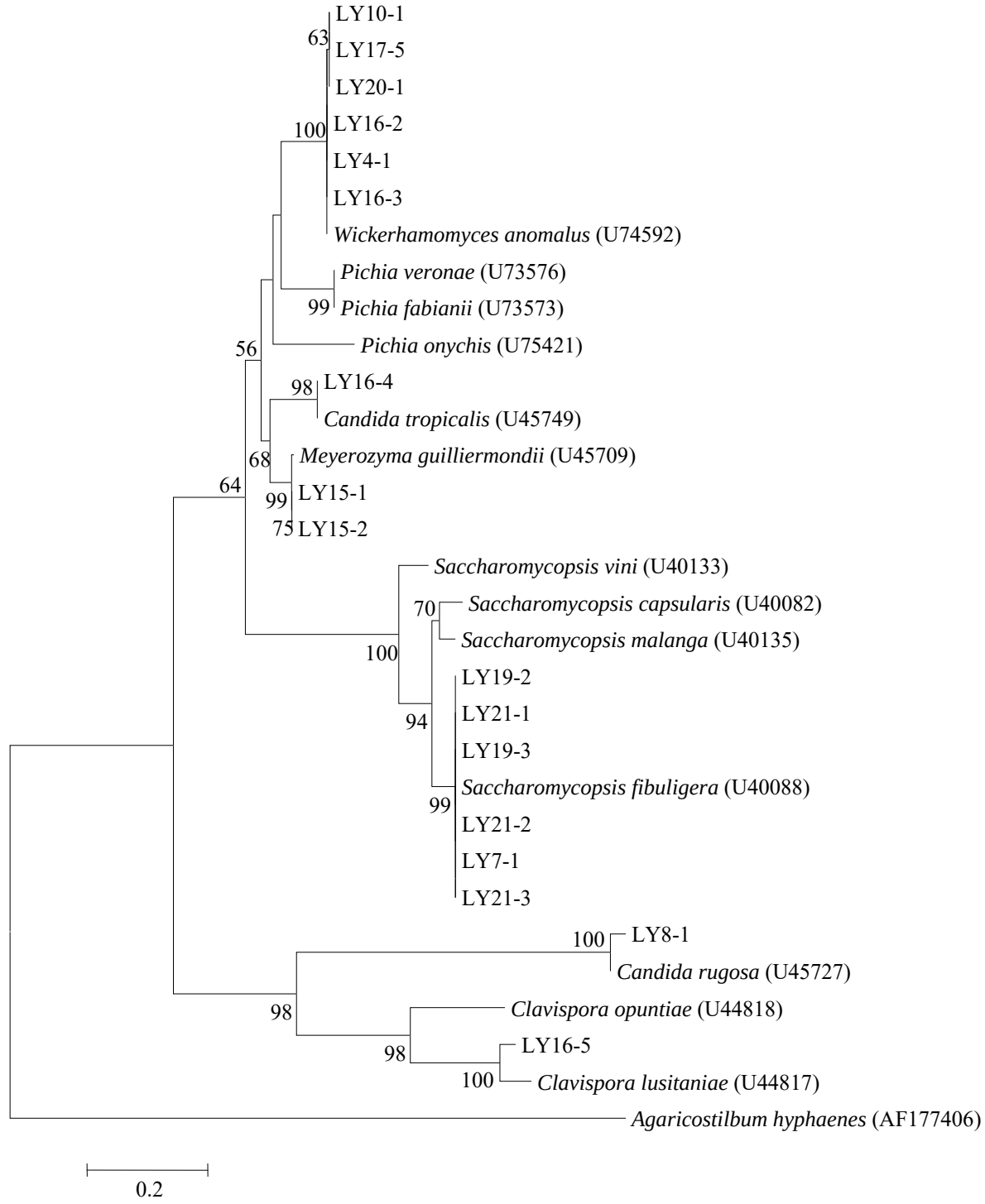

Figure 3: The phylogenetic tree of seventeen yeast isolates and related taxa based on the D1/D2 domain of 26S rRNA gene sequences.

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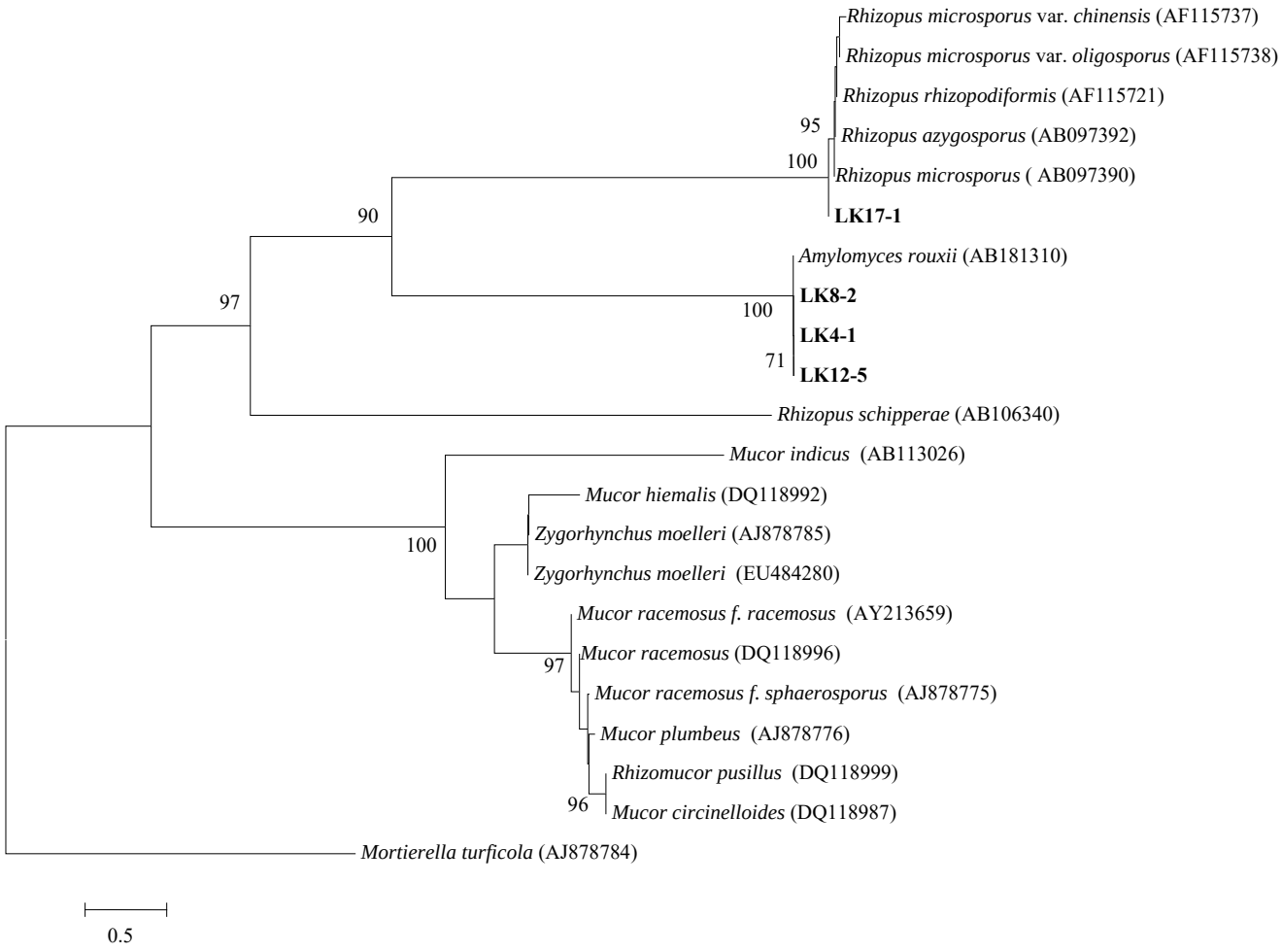

Figure 4: The phylogenetic tree of *A. rouxii* LK4-1, LK8-2 and LK12-5, and *R. microsporus* LK17-1 and related taxa based on rDNA- ITS1/ITS4 sequence.

glycerol, palatinose, erythritol, D-melezitose, D-glucose and esculin ferric citrate. They produced 1-4 hat-shaped ascospores (Kurtzman and Fell, 1998; Barnett *et al*., 2000). The representative six isolates had identical sequences (100% similarity) in the nucleotide sequence of the D1/D2 domain of the 26S rRNA gene and were located within the cluster of *Wickerhamomyces anomalus* as in Figure 3. Therefore, they were identified as *W. anomalus* (Kurtzman, 2011).

Group 6 contained 4 isolates, LY15-1, LY15-2, LY15-3 and LY15-4. Two isolates (LY15-1 and LY15-4) were selected as the representative isolates for their phenotypic characterization. They assimilated D-galactose, cyclohexamide, D-sucrose, N-acetyl-glucosamine, arabinose, D-raffinose, D-maltose, D-trehalose, potassium 2-keto gluconate, methyl-α-D-glucopyranoside, D-mannitol, D-sorbitol, D-xylose, palatinose, D-melezitose, D-glucose, glucosamine and esculin ferric citrate. They produced 1-4 hat-shaped ascospores and pseudohyphae (Kurtzman and Fell, 1998; Barnett *et al*., 2000). The representative two isolates had identical sequences in the nucleotide sequence (100% similarity) of the D1/D2 domain of the 26S rRNA gene and were located within the cluster of *Meyerozyma guilliermondii* as in Figure 3. Therefore, they were identified as *M. guilliermondii* (Kurtzman and Robnett, 1997).

Identification of mold isolates

All mold strains were identified based on the morphological characteristics by using slide culture techniques. The result revealed that most isolates obtained from *look-paeng khao-mak* formed abortive sporangium and non septate hyphae (Webster and Weber, 2007), therefore, they were *Amylomyces rouxii* (42 isolates) (Table 1). The remaining isolates that formed rhizoids and non-septate hyphae were *Rhizopus* sp. (30 isolates) (Webster and Weber, 2007). The isolates that formed dark green color, granular powdery colonies, pale

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yellow in color of the back side of colonies and they also formed septate hyphae, simple and branched conidiophores, and conidia was round were *Penicillium* sp. (16 isolates) (Webster and Weber, 2007) whereas the isolates that formed white to grey colonies and formed sporangiospores and non-rhizoids were *Mucor* sp. (12 isolates) (Webster and Weber, 2007).

The highest amylase activity isolates LK4-1, LK8-2 and LK12-5 were identified based on the sequences of the ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer (ITS). These strains had identical ITS gene sequence with 100% similarity to *Amylomyces rouxii* and the phylogenetic tree was in Figure 4. Therefore, they were identified as *Amylomyces rouxii*. In addition, strain LK17-1 had identical ITS gene sequence with 99% similarity to *Rhizopus microsporus.* Therefore it was identified as *Rhizopus microsporus* and its phylogenetic tree was in Figure 4. Isolates of *A. rouxii* and *R. microsporus* showed high amylase activity, which indicated that these two mold strains could play an important role in the hydrolysis of starch to sugar in sweet rice fermentation.

In this study, a sweet rice starter (*look-paeng khao-mak*) contained yeast isolates identified as *S. fibuligera*, *C. rugosa*, *C. tropicalis*, *Cl. lusitaniae*, *W. anomalus* and *M. guilliermondii* based on their phenotypic and genetic characterization. The mold isolates distributed were *Amylomyces* sp., *Rhizopus* sp., *Mucor* sp. and *Penicillium* sp. based on their morphological characteristics. *A. rouxii* strains were dominant mold as previous reports (Lee and Fujio, 1999; Limtong *et al*., 2005) and the dominant yeast isolates that had ability to hydrolyse starch to sugar were *S. fibuligera* same as previous reports (Lee and Fujio, 1999; Limtong *et al*., 2002; Tsuyoshi *et al*., 2005). The yeast and mold isolates found are useful in sweet rice fermentation.

CONCLUSION

The yeast isolates distributed in *look-paeng khao-mak,* a traditional starter culture were identified as *S. fibuligera*, *C. rugosa*, *C. tropicalis*, *Cl. lusitania*e, *W. anomalus* and *M. guilliermondii* based on their phenotypic characteristics and the sequencing of D1/D2 domain of large subunit (26S) ribosomal DNA analyses. *S. fibuligera* isolates LY7-1, LY7-2, LY16-1 LY19-1, LY19-2, LY19-3, LY21-1, LY21-2 and LY21-3 showed amylase activity ranged from 70.8 to 171.2 unit/mL. The mold isolates LK4-1, LK8-2 and LK12-5 identified as *A. rouxii* and LK17-1 identified as *R. microsporus* based on their morphological characteristics and the rDNA ITS sequences showed amylase activity ranged from 32.24 to 39.74 unit/mL.

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