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Detection and identification of naturally-occurring yeasts in homemade fermented rice water

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ABSTRACT

Homemade fermented rice rinse water is a nutrient-rich due to certain yeasts produced during the process of fermentation. Nutrient-rich liquid from yeasts such as *Galactomyces,* isolated from Japanese sake wine, have gained commercial interest, and the ferment filtrate of *Galactomyces* is already used in some commercial cosmetic products. Unlike such familiar ferment filtrates, the particular yeast species present in homemade fermented rice water (FRW) and their possible benefits are yet to be identified. As an initial step to find out potential uses of yeasts in FRW, this study uses a combination of phenotypic and molecular methods to identify the yeast species present in homemade fermented rice water. FRWs were made from twenty samples of organic rice purchased from commercial shops. 42 samples were selected out of the total unidentified yeast isolates from FRW in order to investigate their biochemical characteristics using API ID 32C. PCR was then performed to amplify both the D1/D2 domain of the large subunit rDNA gene as well as the ITS1/ITS2 regions from the yeast genomic DNA. A total of nine yeast species belonging to seven genera were identified in the fermented rice water, including *Candida intermedia*, *C. parapsilosis*, *C. metapsilosis*, *Trichosporon asahii*, *Papiliotrema aspenensis, Meyerozyma caribbica, Hyphopichia burtonii, Cystobasidium calyptogenae* and *Rhodotorula toruloides.* This study is believed to be the first to identify the yeast species in homemade FRW. The findings expand knowledge of yeasts occurred in FRW and hold potential for developing useful applications from homemade FRW.

Key words: *Yeasts, Fermented rice water, Thailand*

Introduction

Water used for rinsing rice before cooking has been used in Asia for many centuries for improving the beauty of skin and hair. If rice rinse water is left out to sit for a period, natural fermentation will cause the rice water to sour. There is a report that Japanese women in Heian period use rice rinse water (Yu-Su-Ru) for hair washing as well as women in Huang Lu Village in southern China, and it resulted in long, flowing and shiny black hair (Inamasu *et al*., 2010, Lin, 2012). This Yu-Su-Ru extracted has properties to reduce friction and increase the flexibility of the hair (Inamasu *et al*., 2010). For the use of rice fermented water for skin care is beginning to be well known by the Procter & Gamble (P&G), the manufacturer of SK-II in Japan, which has products that are liquid-based skincare, called Pitera or Galactomyces ferment filtrate (GFF) made from yeast *Galactomyces* that isolated from rice fermentation process for sake production. GFF contains minerals, vitamins, amino acids, enzymes and various organic acids (SK-II, 2019), this has been used as a cosmetic ingredient for facial moisturizer. However,

due to the high price of GFF skincare products, there are online articles from various health and beauty websites offering alternatives as rice water-based fermentation recipes that can be made at home or D.I.Y. fermented rice water (FRW) to allow the rice rinse water to ferment for 1-2 days to achieve the fermented water at pH suitable for hair washing. In addition, the fermentation process may change substances in the rice water into beneficial substances similar to GFF (Hills, 2015, Daily Health Post, 2015, Amanpreet, 2016). Fermentation of rice water will occur from naturally-occurring yeast. There is no selection of appropriate yeast strains as a starter cultures for the homemade rice water fermentation to obtain the desired substances. The yeast species grows in the FRW will vary according to the raw materials used. Unfortunately, no previous studies on the microbiota involved in homemade FRW. The identification of yeast strains in rice water fermentation may lead to further research and development to produce quality FRW. In this study is therefore interested to investigate the yeast communities in the fermentation of rice water from various organic rice cultivation in Thailand using phenotypical methods combined with rDNA gene sequencing analysis.

Materials and Methods

Organic rice samples

Twenty samples of organic rice purchased from different commercial shops in Thailand were assigned code number 1 to 20 and kept at room temperature (Table 1).

Fermented rice water

Homemade FRWs were prepared from twenty organic rice samples. 100 g of each rice sample was soaked in 400 mL water for 30 mins with occasionally agitation. Rice rinse water was poured into a new bottle, capped and incubated at room temperature for 2 days.

Isolation and maintenance of yeasts

FRWs were spread onto YM agar (glucose 1.0% w/v, peptone 0.5% w/v, yeast extract 0.3% w/v, malt extract 0.3% w/v, agar 2.0% w/v) containing 50 mg streptomycin and chloramphenicol to inhibit bacterial growth and incubated at 30 °C for 2 days. After total colony counts were made, a random number of each yeast colony type observed on YM plates were selected and streaked onto YM agar for further identification.

Yeasts phenotypic study

All yeast isolates were evaluated based on their morphology and the ability to assimilate and ferment different chemical sources using API ID 32C yeast identification kit (Biomerieux, France) and searched through its database to identify species. Extracellular enzyme activity for protease (casein), lipase (tributyrin) and amylase (starch) were tested following established protocols (Larsen *et al*., 1998; Freire, 1996; Schwan *et al*., 2007).

Molecular identification of yeasts

Yeast genomic DNA was extracted from single colony of each isolate using lysis solution (0.2 M Lithium acetate, 1%SDS) (Looke *et al*., 2011) and subsequently used as template for PCR. NL-1 (52-GCATATCAATAAGCGGAGGAAAAG-32) and NL-4 (52-GGTCCGTGTTTCAAGACGG-32) primers (Kurtzman and Robnett, 1998), and ITS1 (52-TCCGTAGGTGAACCTGCGG-32) and ITS4 (52-TCCTCCGCTTATTGATATGC-32) (White *et al*.,

Table 1. The lists of organic rice and their codes used in this study.

Rice	Code	Rice	Code
Chompoothip rice	1	Jasmine brown rice 2	11
Tubtimchumphae rice	2	Jasmine brown rice 3	12
Rice berry 1	3	Jasmine rice 1	13
Rice berry 2	4	Jasmine rice 2	14
Rice berry 3	5	Jasmin coarse rice 1	15
Rice berry 4	6	Jasmin coarse rice 2	16
Three-color brown rice 1	7	Red hawm rice	17
Three-color brown rice 2	8	Aromatic black rice	18
Three-color brown rice 3	9	Red brown hawm rice	19
Jasmine brown rice 1	10	Germinated brown rice	20

1990) were used for PCR amplification of the D1/D2 domain and ITS1/ITS2 regions, respectively. The PCR reactions were performed using OnePCR (GeneDirex, Taiwan) and following conditions: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, final extension at 72 °C for 10 min, holding at 4 °C. PCR amplified products were purified by the Pure Direx PCR Clean-Up and Gel Extraction Kit (Bio-Helix, Taiwan) and sent to Macrogen (Korea) for nucleotide sequencing. The sequences were then analyzed and compared with the sequences available in the GenBank using BLAST algorithm.

Results

The yeast counted from eighteen FRWs ranged from 6 x 10^1 cfu/mL to 3.1 x 10^4 cfu/mL, but no yeast colony observed from two FRWs (Table 2). A total of 42 yeast isolates were selected, grown on YM agar at temperatures of 30°C and identified using biochemical and molecular tests. All isolates were not be able to produce extracellular enzyme to degrade casein, but 3 isolates from fermented germinated brown rice water (code 20) were able to hydrolyze starch (R28) and tributyrin (R68 and R69) (Table 2 and Fig. 1).

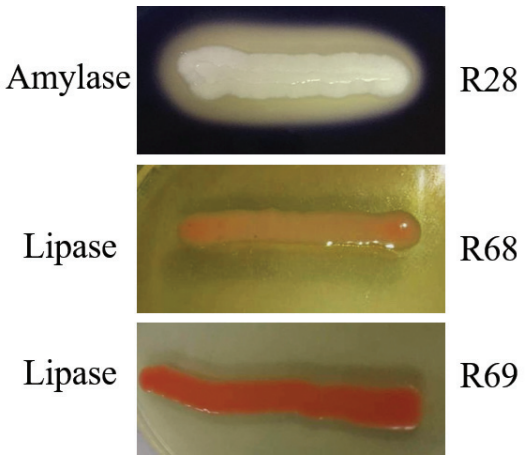

Fig. 1. Three yeast isolates: R28, R68 and R69 from fermented geminated brown rice water showing activity of amylase and lipase.

Further, assimilation tests (carbohydrates, organic acids, and amino acids), susceptibility test (cycloheximide) and colorimetric test (esculin) were carried out using API ID 32C kit. The result of API test (Table 3) showed 21, 12, 3 and 2 isolates as *Cryptococcus humicola*, *Candida parapsilosis*, *Trichosporon asahii* and *Cryptococcus laurentii*, respectively. Other 5 isolates were identified as *Candida famata*, *C. guilliermondii*, *C. sake*, *Rhodotorula minuta* and *R. glutinis*.

Table 2. Total yeast number isolated from FRWs and some phenotypical characteristics.

Rice code	Total yeast count cfu/mL	Number of isolates selected	Hydrolysis of		
			Casein	Tributyrin	Starch
1	3.0×10^3	1			
2	4.4×10^2	3			
3	3.0×10^3	2			
4	3.0×10^3	2			
5	1.5×10^2	2			
6	3.1×10^4	4			
7	4.4×10^2	3			
8	2.0×10^2	2			
9	-				
10	3.0×10^3	2			
11	1.4×10^2	1			
12	1.3×10^2	1			
13	2.1×10^3	1			
14	2.3×10^3	3			
15	3.0×10^3	2			
16	2.8×10^2	2			
17	-				
18	1.1×10^2	3			
19	6.0×10^1	3			
20	3.0×10^3	5		2	1
	Total	42	0	2	1

However, differences in the assimilation of chemical sources were found within the isolates identified species, *C. humicola*, *C. parapsilosis*, *T. asahii* and C. *laurentii* (Table 3). For example, the isolates identified as *C. humicola* found differences in the ability to assimilate D-saccharose and N-acetyl-glucosamine, and susceptible to cycloheximide. The isolates identified as *C. parapsilosis* also showed variable assimilation patterns of L-arabinose, lactic acid, D-xylose, glycerol, potassium gluconate, levulinic acid and L-sorbose. All 42 yeast isolates identified according to biochemical characteristics were confirmed by molecular techniques. Both the D1/D2 domain of the large subunit (LSU) ribosomal DNA gene and the ITS1-5.8S-ITS2 regions of all isolates were amplified by PCR. The PCR products showed differences in size by gel electrophoresis depending on yeast species (data not shown). The D1/D2 and ITS1/ITS2 sequences were compared with the sequences in GenBank using BLAST search and the

yeast identification was analyzed. The closest sequence identity to GenBank was used to identify the 42 isolates into 7 genera and 9 species as shown in Table 4. All sequenced isolates homology with sequences in the GenBank were 99-100%, except for the ITS1/ITS2 sequence of isolate R28 that showed the lowest identity to the GenBank sequence at 92%. Both analysis of D1/D2 domain and ITS1/ ITS2 regions sequences were identified each isolate of 40 isolates as the same species. The D1/D2 sequences analysis of 2 isolates (R35 and R38) were able to identify at species level, whereas the ITS1/ITS2 sequences analysis were able to identify only at genera level. The 21 isolates (50%) were identified as *C. intermedia*, 11 isolates (26%) as *C. parapsilosis*, 3 isolates (7%) as *T. asahii*, 2 isolates (4%) as *Papiliotrema aspenensis* (*Cryptococcus aspenensis*) or *Cryptococcus* sp., 1 isolate (2%) as *Meyerozyma caribbica*, 1 isolate (2%) as *Hyphopichia* (*Pichia*) *burtonii*, 1 isolate (2%) as *Cystobasidium calyptogenae* (*Rhodotolura calyptogenae*) and 1 isolate (2%) as *Rhodotolura* (*Rhodosporidium*) *toruloides* (Table 4 and Fig. 2).

Discussion

After fermentation of rice waters for 2 days, the total solid (TS) content and pH of each FRW were decreased (data not shown). This could be due to the

Table 3. Chemicals assimilation and growth of yeasts isolated from FRWs using API ID32 kit.

Chemical Source	API ID32 identified yeast species (number of isolates)								
	C. humicola (21)	*C. parapsilosis* (12)	*C. famata* (1)	*C. guilliermondii* (1)	*T. asahii* (3)	*C. sake* (1)	*C. laurentii* (2)	*R. minuta* (1)	*R. glutinis* (1)
D-galactose	+	+	+	+	+	+	+	+	+
cycloheximide	+ (17)*	+ (1)	-	+	+	-	+ (1)	-	+
D-saccharose (sucrose)	+ (19)	+	+	+	+	+	+ (1)	+	+
N-acetyl-glucosamine	+ (19)	+	+	+	+	+	+ (1)	-	-
lactic acid	+	+ (7)	-	-	+	-	+	-	-
L-arabinose	+	+ (11)	-	+	+	-	+	+	+
D-cellobiose	+	-	+	+	+	-	+	+	-
D-raffinose	+	-	+	+	-	-	+	-	+
D-maltose	+	+	+	+	+	+	+	-	-
D-trehalose	+	+	+	+	+ (2)	+	+	+	+
potassium 2-ketogluconate	+	+	+	+	+	+	+	+	-
methyl-áD-glucopyranoside	+	+	+	+	+	-	-	-	-
D-mannitol	+	+	+	+	+ (1)	+	-	+	+
D-lactose	+	-	+	-	+	-	-	+	-
inositol	-	-	-	-	-	-	-	-	-
D-sorbitol	+	+	+	+	+ (2)	+	+	-	-
D-xylose	+	+ (11)	+	+	+	-	+	+	+
D-ribose	+	-	-	+	+	-	+	+	+
glycerol	+	+ (8)	+	+	-	+	-	-	+
L-rhamnose	+	-	+	+	-	-	+	+	+
palatinosE	+	+	+	+	+	+	+	-	-
erythritol	+	-	+	-	+	+	-	-	-
D-melibiose	+	-	+	-	-	-	+ (1)	-	-
sodium glucuronate	+	-	+	-	+	-	+	-	-
D-melezitose	+	+	+	+	-	-	+	+	-
potassium gluconate	+	+ (11)	-	+	+	-	+	+	+
levulinic acid	+	+ (10)	-	+	-	-	-	+	-
D-glucose	+	+	+	+	+	+	+	-	-
L-sorbose	+	+ (9)	+	+	+ (1)	-	+ (1)	+	+
glucosamine	+	+	+	+	+	+	+(1)	+	-
esculin ferric citrate	+	+	+	+	+	+	+	-	-

+, Positive; -, negative and (17) *, among 21 isolates only 17 isolates showed positive results.

Table 4. Identification of yeast isolates by sequencing of the D1/D2 rDNA and ITS1/ITS2 regions and comparing with the sequences in GenBank.

Rice code	Isolates	D1/D2			ITS1/ITS2		
		Sequence length (bp)	% Identity to Gen Bank sequence	Closet GenBank species	Sequence length (bp)	% Identity to GenBank sequence	Closet GenBank species
1	R25	492	100	*C. intermedia*	209	99	*C. intermedia*
2	R59	518	100	*C. parapsilosis*	383	100	*C. parapsilosis*
	R61	540	100	*T. asahii*	257	100	*T. asahii*
	R63	525	100	*T. asahii*	438	100	*T. asahii*
3	R45	461	100	*C. intermedia*	245	100	*C. intermedia*
	R49	461	100	*C. intermedia*	255	100	*C. intermedia*
4	R13	533	100	*C. parapsilosis*	361	100	*C. parapsilosis*
	R14	429	100	*C. intermedia*	271	100	*C. intermedia*
5	R16	460	100	*C. intermedia*	271	100	*C. intermedia*
	R18	471	100	*C. intermedia*	277	100	*C. intermedia*
6	R55	521	100	*C. parapsilosis*	369	100	*C. parapsilosis*
	R56	522	100	*C. parapsilosis*	424	100	*C. parapsilosis*
	R57	446	100	*C. intermedia*	271	100	*C. intermedia*
	R58	445	100	*C. intermedia*	271	100	*C. intermedia*
7	R29	489	100	*C. intermedia*	275	100	*C. intermedia*
	R30	517	100	*C. parapsilosis*	424	100	*C. parapsilosis*
	R41	468	100	*C. intermedia*	271	100	*C. intermedia*
8	R20	490	99	*C. parapsilosis*	374	100	*C. parapsilosis*
	R21	494	100	*C. intermedia*	271	100	*C. intermedia*
10	R9	460	100	*C. intermedia*	271	100	*C. intermedia*
	R11	522	100	*C. parapsilosis*	310	100	*C. parapsilosis*
11	R5	499	100	*C. parapsilosis*	424	100	*C. parapsilosis*
12	R6	468	100	*C. intermedia*	269	100	*C. intermedia*
13	R1	501	100	*C. intermedia*	271	100	*C. intermedia*
14	R22	486	100	*C. intermedia*	271	100	*C. intermedia*
	R65	522	100	*C. parapsilosis*	332	100	*C. parapsilosis*
	R67	464	100	*C. intermedia*	271	100	*C. intermedia*
15	R50	461	100	*C. intermedia*	231	100	*C. intermedia*
	R51	461	100	*C. intermedia*	277	100	*C. intermedia*
16	R53	532	100	*C. parapsilosis*	421	100	*C. parapsilosis*
	R54	517	100	*C. parapsilosis*	401	100	*C. parapsilosis*
18	R35	546	100	*P. aspenensis*/ *C. aspenensis*	454	99	*Cryptococcus* sp.
	R38	550	100	*P. aspenensis*/ *C. aspenensis*	454	99	*Cryptococcus* sp.
	R39	472	100	*C. intermedia*	247	100	*C. intermedia*
19	R31	461	100	*C. intermedia*	271	100	*C. intermedia*
	R32	560	100	*C. metapsilosis*	450	99	*C. metapsilosis*
	R43	461	100	*C. intermedia*	247	100	*C. intermedia*
20	R26	469	99	*M. caribbica* (anamorph)/ *C. fermentati*	502	100	*M. caribbica* (anamorph)/ *C. fermentati*
	R27	576	100	*T. asahii*	462	100	*T. asahii*
	R28	485	100	*H. burtonii*	400	92	*H. burtonii*
	R68	535	99	*C. calyptogenae*/ *R. calyptogenae*	505	100	*C. calyptogenae*/ *R. calyptogenae*
	R69	534	100	*R. toruloides*	395	100	*R. toruloides*

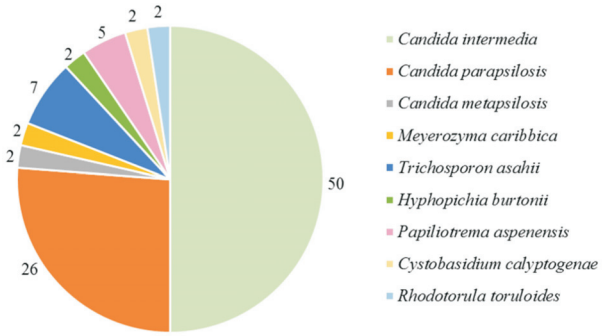

Fig. 2. The percentage of yeast species isolated from FRWs.

activities of the fermenting microorganisms and yeasts which utilized solid content such as carbohydrates, proteins, and lipid washed from rice grains for growth, and produced some organic acids. Several yeast colonies were detected in eighteen of twenty FRWs (Table 2), indicating that rice water fermentation can occur by spontaneous yeasts. Moreover, it was found that no yeast growth from FRW made from two rice samples (code 9 and 17) that stored for a long time or stale rice. The biochemical characterization results of 42 yeast isolates using API ID 32C kit (Table 3) are not sufficiently reliable because the similar metabolism of related species and a limited API database of yeast species, may lead to incorrect identification results. Hasejima *et al*., (2011) reported that *C. intermedia* identified by molecular method has been misidentified as *C. humicola* using API ID32C which is consistent to the results found in this study. Since, molecular method by analyzing the nucleotide sequence of the D1/D2 domains of LSU rDNA gene and the ITS regions are useful and effectiveness in identification of yeast species in both the Ascomycota and Basidiomycota (Fell *et al*., 2000; Kurtzman and Robnett, 1998; Scorzetti *et al*., 2002). Furthermore, there is a comprehensive reference database available for yeast classification studies. Therefore, the identification of yeast species was performed using biochemical methods and molecular techniques in parallel. As it is shown in Table 4, the use of molecular method based on the D1/D2 and ITS1/ITS2 sequences has identified all 42 yeasts isolated from homemade FRW samples into 7 genera and 9 species, which had not been described previously. *C. intermedia* was the most common yeast species isolated, representing 50% of the total yeast isolated, followed by *C. parapsilosis* (26%), *T. asahii* (7%), *P.* (*Cryptococcus*) *aspenensis* (5%), *C. metapsilosis* (2%), *M. caribbica* (2%), *H. burtonii* (2%), *C. calyptogenae* (2%) and *R. toruloides* (2%) (Fig. 2). The sources of yeast found in homemade FRWs could be from environments, producers, rice sources and water (Olajuyigbe *et al*., 2017; Maneewatthana *et al*., 2000). *C. intermedia* is related with the rice-cassava fermentation, the Brazilian beverage (Schwan *et al*., 2007) and able to use as biocontrol agent against fungal plant pathogens (Huang *et al*., 2011; Rosa-Magri *et al*., 2011). *C. parapsilosis* and *T. asahii* are also found in rice-cassava fermentation (Schwan *et al*., 2007). *C. metapsilosis*, *M. caribbica*, *Cystobasidium* and *Rhodotorula* had been described as endophytic yeasts detected in rice leaf and *P. aspenensis* found in corn and sugarcane leaf in Thailand (Khunnamwong *et al*., 2018). Therefore, these yeast species may be transmitted from rice leaf tissue to rice seeds during harvesting and able to grow in rice water. Hydrolysis of tributyrin and starch observed in *C. calyptogenae* (R68), *R. toruloides* (R69) and *H. burtonii* (R28) isolated from FRW made from germinated brown rice (code 20), indicating that they can produce lipolytic and amylolytic enzymes (Table 2). Germination of rice produces bioactive compounds and bran oils, and breaks down the amylose into smaller units by action of amylolytic enzymes (Cho and Lim, 2016), which may be suitable for the growth of amylase/lipase-producing yeast. In addition, the growth of yeast that produces enzymes may generate some factors promoting further yeast development, resulting in more diverse yeast species found in fermented germinated brown rice water compared to others (Table 4). *H. burtonii* is one of yeasts isolated from traditional starter of murcha: a Nepali rice grain liquor, that might be involved in saccharification by hydrolysis of starch (Takeuchi *et al*., 2006). As observed in this study, *H. burtonii* can produce amylolytic enzyme or amylase to hydrolyze starch and may help yeast to utilize carbohydrate and grow in rice water. *R. toruloides* (also known as *Rhodotorula glutinis* or *Rhodotorula gracilis* or *Rhodosporidium toruloides*) is a pink yeast and accumulate lipids up to 70% of dry cell weight (Li *et al*., 2007). *Rhodotorula* yeast can be used in the industry as source of carotenoids and lipolytic enzymes such as lipase (Hatzinikolaou *et al*., 1999, Strehaiano *et al*., 2006). Interestingly, yeast isolated in this study that capable of producing enzymes can be a good candidate for further study. Overall, the results of

this study indicate that the source of yeast in rice water fermentation is primarily from rice microflora and surrounding environments. Thus, the chemical characteristics and presence of specific moleculesin the homemade fermentation of rice water may vary depending on the spontaneous yeast species, which is responsible for the production of substances in FRW. However, be consider that some of yeast species present in FRW have been reported as opportunistic pathogens such as *C. parapsilosis*, *C. intermedia* and *T. asahii* (Miceli *et al*., 2011; Taj-Aldeen *et al*., 2014; Fernández-Ruiz *et al*., 2017). Therefore, it might be necessary to understand the role of the yeasts involved in the homemade rice water fermentation in order to develop into a yeast starter cultures for consistent quality of FRW production and safe to use as facial skin and hair care.

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