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Chinese Rice Pudding Fermentation: Fungal Flora of Starter Cultures and Biochemical Changes during Fermentation

DING-LING WEI and SHUNG-CHANG JONG*

Department of Biochemistry, National Yang-Ming Medical College Taipei, Taiwan, ROC;
American Type Culture Collection, 12301 Parklawn Drive,
Rockville, Maryland 20852, USA

Nineteen molds and fifteen yeasts were isolated from four commercial starter cultures. Four mold isolates (*Rhizopus arrhizus* ATCC 46429, *Rhizopus arrhizus* ATCC 46430, *Rhizopus formosaensis* ATCC 46431, and *Rhizopus javanicus* ATCC 46432) and four yeast isolates (*Torulopsis* sp. ATCC 44944, *Hansenula* sp. ATCC 44945, *Hansenula anomala* ATCC 46058, and *Torulopsis* sp. ATCC 46433) were selected for combination tests to study their abilities to convert steamed glutinous rice into good quality Chinese rice pudding. The progress of the fermentation was determined by the degree of change in the reducing sugars content and ethanol production. The results indicated that a combination of each mold culture studied and a yeast isolate, *Torulopsis* sp. ATCC 46433 or ATCC 44944, changed the sticky rice into a soft and juicy product with a sweet taste and a mild alcoholic flavor in 4 days. The mixture of a mold culture, *Rhizopus arrhizus* ATCC 46430 and a yeast culture, *Hansenula* sp. ATCC 44945 or *H. anomala* ATCC 46058, made a product with a sour taste and a strong alcoholic flavor.

Chinese rice pudding (*Tien-chiu-niang* in Mandarin and *Lao-chao* in Cantonese) is a popular Chinese fermented food with a sweet taste and a mild alcoholic flavor with a fruity aroma. It is served as a dessert and is also a traditional diet for new mothers who believe that it helps them regain their strength.

Preparation of Chinese rice pudding is quite similar to Indonesian *tapé ketan* fermentation[1,2] and can be done in any household. It is made by fermenting steamed glutinous rice with a small amount of commercial starter (*Chiu-Yüeh*) which contains a mold culture for saccharifying carbohydrates and a yeast culture for converting sugars into alcohol. Wang and Hesseltine[3] identified various organisms in commercial starters for the fermentation. Good results were obtained when they employed a mixture of pure cultures of *Rhizopus chinensis* and a species of *Endomycopsis*. The finished product was described as being soft, juicy, and sweet with a mildly alcoholic flavor (1–2% ethanol, and 20–30% reducing sugar measured as glucose).

In the present paper, fungal flora of four commercial starters and the biochemical changes that occur in the substrate during fermentation were studied.

Materials and Methods

Isolation and identification of microorganisms

Starters Four commercial starter cultures were used. Samples SC I and SC II were obtained from mainland China, SC III and SC IV were from Taiwan, ROC.

Media and cultivated methods One gram of each starter sample was crushed aseptically, suspended in 10 ml of distilled water, and rehydrated for one hour. The suspension was diluted 10^{-1} to 10^{-4} with distilled water. One ml of the diluted samples was mixed with 15 ml of molten potato dextrose agar (PDA) and yeast malt agar (YM) for isolation of molds and yeasts, respectively. The well-mixed agar media were then poured into petri dishes and incubated at 30°C for 24 to 48 h.

Taxonomic methods For diagnosis and taxonomical identification, the systems described in "Mucorales" by Zycha, Siepmann and Linnemann;[4] in "Certain species of *Mucor* with a key to all accepted species" by

Schipper;[5] in "Taxonomical studies on Genus *Rhizopus*" by Inui, Takeda and Iizuka;[6] in "The yeasts, a taxonomic study" by Lodder;[7] and in "A new key to the yeasts" by Barnett and Pankhurst[8] were followed.

Fermentation tests

Preparation of substrates Each 100 g portion of glutinous rice was washed and soaked in 150 ml of water overnight. After it was steamed in a rice steamer for 15 min, 60 g of the steamed rice was put into a 300 ml glass jar, covered and autoclaved for 15 min at 120°C.

Preparation of inocula Yeast inocula were prepared by introducing 5 ml of sterile water onto 48-h-old slant cultures on YM agar. The suspension of mold cultures was made by adding 5 ml of sterile water to each 4-day-old slant culture on PDA, and mycelia and sporangia were scraped off the agar with an inoculating wire.

One ml of yeast suspension and two ml of mold suspension were used for the inoculation of 60 g of sterilized glutinous rice, mixing as well as possible. The rice was then covered and incubated at 30°C for 9 days.

Ability of molds and yeasts to digest glutinous rice The mold and yeast isolates were tested for their ability to convert the steamed glutinous rice into a soft and juicy product on which mold growth is not noticeable. Two ml of each isolate was used for inoculation.

Progress of fermentation The progress of fermentation was determined by the changes of pH, reducing sugars content, and alcohol production.

Reducing sugars were measured by a modification Bernfeld's method.[9] Ten gram samples of fermenting rice were taken after 2, 4, 6, 7, and 9 days incubation and blended with 90 ml of distilled water for 2 min. A 10 ml aliquot was centrifuged for 30 min at 2,500 rpm. The supernatant solution was filtered through Whatman no. 1 filter paper. One ml of dinitrosalicylic acid reagent[9] was added to 1 ml of filtrate, heated in a boiling water bath for 5 min, and then cooled in running tap water. The optical density of the solution or its dilution was determined at 540 nm with a Baush and Lomb Spectronic 20 spectrophotometer. A calibration curve established with maltose (0.1 to 1.0 mg/ml) was used to convert the spectrophotometer readings into milligrams of maltose.

Ethanol was separated and determined quantitatively by gas chromatography. A Hewlett Packard Model 5830A gas chromatograph was used. A coiled stainless steel column, 6 feet by 1/8 inch with a wall thickness of 2.1 mm (ID) was fitted into the injection port to allow on column injection. The column packing was 10% SP 1000/1% H_3PO_4 on 100/120 chromosorb WAW. The column oven was maintained at 130–150°C with the detector oven and the injector port maintained at 250°C and 200°C, respectively. The flow rate of the carrier gas (helium) was 30 ml/min. Ethanol was detected at a sensitivity of 0.05 AUFS. A Hewlett Packard GC Terminal Integrator model 18850A was connected in series to the gas chromatographic instrument to determine the peak areas.

Standard aqueous solution containing 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% (v/v) ethanol in diethyl ether were prepared. A linear curve was obtained when different concentrations (0.5–3.0%, v/v) of standard ethanol solutions were injected into the column. Retention times (Tr) were 0.26 and 0.49 for ether and ethanol, respectively.

One ml of homogenized fermented product was transferred to a centrifuge tube, and 0.4 g NaCl and 1.0 ml ethyl ether (Labindustries Co.) were added. The tube was stoppered, and the contents were mixed well and centrifuged for 5 min at 1,500–2,000 rpm. The top (ether) layer was removed to a separate tube, and a small amount of $CaCl_2$ was added to remove any H_2O. Three μl of the sample solution was injected into the column with a no. 701 Hamilton syringe.

The changes in the pH of all fermentations were determined, using a Corning model 7 pH meter.

Results and Discussion

Nineteen molds and fifteen yeasts, as shown in Table 1, were isolated from the four commercial starter cultures. Colonies of each kind of microorganism were selected on the basis of differences in features between them, although many isolates subsequently proved to be identical. According to the morphological characteristics described by Zycha *et al.*,[4] Schipper[5] and Inui *et al.*,[6] all the nineteen mold isolates were considered to belong to the family Mucoraceae. Eleven of them were classified into the genus *Rhizopus*. Of the remainding eight mold isolates, two from sample SC I were identified as *Mucor racemosus*,[10] the other six isolates from SC I, SC II, and SC IV were considered to belong to *Amylomyces rouxii* (=*Chlamydomucor oryzae*) as described by Ellis *et al.*[11] These six strains showed the following characteristics: colonies growing rapidly within 7 days at room temperature on PDA, near grayish brown in color; sporagiophores hyaline to light brown with yellowish contents; sporangia abortive, globose, 17–55 μm in diameter, hyaline to

Table 1. Microflora of starter cultures.

Starter culture	Mold		Yeast	
SC I	*Rhizopus arrhizus*	2/7*	*Torulopsis* sp.	5/8
	Mucor racemosus	2/7	*Hansenula* sp.	3/8
	Amylomyces rouxii	3/7		
SC II	*Rhizopus javanicus*	1/2	*Torulopsis* sp.	2/2
	Amylomyces rouxii	1/2		
SC III	*Rhizopus stolonifer*	1/4	*Hansenula* sp.	5/5
	Rhizopus formosaensis	2/4		
	Rhizopus delemar	1/4		
SC IV	*Rhizopus arrhizus*	3/6	0	
	Rhizopus oryzae	1/6		
	Amylomyces rouxii	2/6		

* Two of the seven isolates from the SC I sample were identified as *Rhizopus arrhizus*.

brown, with enlarged apophysis; sporangiospores globose, oval, ellipsoidal to irregular shaped, gray, smooth with thin walls, 5–7 × 3–7 μm; chlamydospores abundantly produced in the aerial and substrate hyphae, hyaline to light brown, thick walled, globose, oval, ellipsoidal with granular contents, 12–30 × 10–20 μm, single and in series.

Fifteen yeast colonies were isolated from samples SC I, SC II and SC III; none was found in SC IV. On the basis of morphological and physiological characteristics (Table 2), these yeast isolates can be separated into four groups, Y I (including SC I-a, b, c, g, h isolates), Y II (including SC I-d, e, f isolates), Y III (including SC II-a, b isolates), and Y IV (including SC III-a, b, c, d, e isolates). From taxonomical studies, group Y I and Y III were classified into the genus *Torulopsis*; group Y II and Y IV were identified as *Hansenula*.[7,8] As shown in Table 2, the *Hansenula* possessed strong abilities for nitrogen utilization, carbon assimilation, and fermentation. In contrast, the *Torulopsis* assimilated a few kinds of organic compounds and utilized only glucose for fermentation.

Each of the mold isolates was tested alone for its ability to convert steamed glutinous rice into a soft and juicy product on which mold growth is almost unnoticeable. Only four mold isolates, ATCC 46429, ATCC 46430, ATCC 46431, and ATCC 46432, met these requirements and were used in the fermentation tests (Table 3). None of the fifteen yeast isolates alone was able to convert the rice into a soft and juicy product.

Many reports[1–3] indicated that a good fermented rice product was obtained when a mixture of molds and yeasts was used as inoculum. Wang[12] discovered that if a pure culture of *Rhizopus* spp. isolated from commercial starter (Chiu-Yuen) was used as a single inoculum, the rice product showed an increase in sweetness but a decrease in alcoholic content, which is not considered satisfactory. Djien (1) studied the Indonesian *tape* fermentation and concluded that the cooperation of a mold *Chlamydomucor oryzae* and a yeast *Endomycopsis fibuligera* was necessary to convert steamed glutinous rice into a sweet-sour, slightly alcoholic paste. Although the precise nature of the interaction of yeast and mold in the fermentation is not clear, it is possible that during fermentation the molds secret some digestive enzymes such as amylase, lipase, and proteinase into the rice and initiate the fermentation by conversion of the rice starch into sugars, which are partially transformed into alcohols and acids by the yeasts; esters are also formed to give the product its delightful aroma. In this study, combinations of one amylolytic mold and one alcohol-producing yeast isolate (Table 3) were tested for ability to convert

Table 2. Characteristics of yeasts isolated from starter cultures.

Characteristics	Group of strains			
	Y I	Y II	Y III	Y IV
Colonies	White, slimy	White, smooth	White, smooth	White, smooth or wrinkled
True mycelia	−	−	−	−
Shape of cells	Round & elongated	Round	Round	Round & oval
Ascospores	−	2 to 4 per ascus spherical	−	2 to 4 per ascus, hat-shaped
Vitamin-free growth	+	+	+	+
Growth at temp.				
22–37°C	+	+	+	+
45°C	+	+	+	−
Nitrogen utilization				
NO_3	−	+	−	+
NO_2	−	+	−	+
Ethylamine	+	+	−	+
Splitting of arbutin	+	+	−	+
Carbon assimilation				
Glucose	+	+	+	+
Galactose	±	+	−	+
L-Sorbose	−	−	−	−
Maltose	±	+	−	+
Sucrose	+	+	−	+
Cellobiose	−	+	−	+
Trehalose	±	+	+	+
Lactose	−	±	−	−
Melibiose	−	±	−	−
Raffinose	±	+	−	+
Melezitose	−	+	−	+
Inulin	−	−	−	−
Soluble starch	−	+	−	+
D-xylose	−	+	−	±
L-arabinose	−	±	−	−
D-arabinose	−	−	−	−
D-ribose	−	+	−	±
L-rhamnose	−	−	−	−
D-glucosamine	−	+	−	−
Ethanol	+	+	+	+
Glycerol	+	+	−	+
i-Erythritol	−	−	+	+
Adonitol	−	−	−	±
Dulcitol	−	−	−	−
D-mannitol	−	+	−	+
D-sorbitol	−	+	−	+
α-methyl-D glucoside	±	+	−	+
Salicin	±	+	−	+
Inositol	±	−	−	−
Lactic acid	+	+	+	+
Citric acid	+	+	−	+
Succinic acid	+	+	−	+
Valine	−	−	−	−
Glycine	−	−	−	−
Proline	+	+	+	+
Arginine	−	−	−	±
Fermentation				
Glucose	+	+	+	+
Galactose	−	+	−	+
Maltose	−	+	−	+
Sucrose	−	+	−	+
Lactose	−	−	−	−
Raffinose	−	+	−	+
Melibiose	−	±	−	−
Inulin	−	−	−	+
Species name	*Torulopsis* sp.	*Hansenula* sp.	*Torulopsis* sp.	*Hansenula anomala*

Table 3. Cultures used in the fermentation tests.

Organisms	ATCC no.	Source
Molds:		
Rhizopus arrhizus	46429	Starter culture I
Rhizopus javanicus	46432	Starter culture II
Rhizopus formosaensis	46431	Starter culture III
Rhizopus arrhizus	46430	Starter culture IV
Yeasts:		
Torulopsis sp.	44944	Starter culture I
Hansenula sp.	44945	Starter culture I
Torulopsis sp.	46433	Starter culture II
Hansenula anomala	46058	Starter culture III

steamed glutinous rice into juicy and alcoholic rice pudding. Tables 4 and 5 show the changes in pH, reducing sugars (RS) content, and ethanol production during fermentation with various combinations of mold and yeast isolates. The taste of the fermented rice after 48 h incubation was also reported.

The pH of rice fermented with different combinations of mold and yeast isolates as shown in Tables 4 and 5 decreased sharply from 6.7 to below 4.7 during the first 48 h indicating that the acidity of the fermenting rice was increasing. Beyond 48 h the pH fluctuated between 4.7 and 3.7 throughout the 216 h (9 days).

The changes in reducing sugars content during the fermentation with the mold isolate *Rhizopus arrhizus* ATCC 46430 plus each of four yeast isolates (*Torulopsis* sp. ATCC 44944, *Hansenula* sp. ATCC 44945, *Torulopsis* sp. ATCC 46433 and *Hansenula anomala* ATCC 46058) are shown in Table 4. For all combinations studied, the reducing sugars content continually increased during the first 96 h, and thereafter increased in combination with *Torulopsis* sp. ATCC 44944 and *Hansenula* sp. ATCC 44945 but decreased in combination with *Torulopsis* sp. ATCC 46433 and *Hansenula anomala* ATCC 46058. The highest level (12.4%) of reducing sugars was attained with a combination of *R. arrhizus* ATCC 46430 and *Torulopsis* sp. ATCC 46433 at 96 h; whereas the lowest concentration (3.4%) was obtained when the *R. arrhizus* ATCC 46430 was combined with *Hansenula* sp. ATCC 44945. These results indicate that different species of yeasts may play an important role in determining the reducing sugars content in the fermented rice.

By comparing the reducing sugars and ethanol level of the four yeast isolates in combination with *R. arrhizus* ATCC 46430, *Hansenula* sp. ATCC 44945 produced the highest concentration of ethanol but a lower level of reducing sugars at 96 and 168 h. As seen from Table 2, the *Hansenula* strains possess a strong tendency to ferment glucose,

Table 4. Changes in pH, reducing sugars (RS) content and ethanol production occurring in rice fermented with *R. arrhizus* ATCC 46430 in combination with various yeasts.

Fermentation with:	Time (h)	pH	RS (%)	Ethanol (%) (v/v)	Taste (during 48–96 h)
Unfermented rice	0	6.7	0	0	—
R. arrhizus ATCC 46430					
Plus *Torulopsis* sp. ATCC 44944	48	4.7	1.7	—	Sweet mild, alcoholic and fruit aroma
	96	3.9	7.3	3.08	
	168	3.9	10.0	2.84	
Plus *Hansenula* sp. ATCC 44945	48	4.7	1.0	—	Sweet-sour, strong alcoholic flavor
	96	3.8	3.4	5.13	
	168	3.8	7.8	7.10	
Plus *Torulopsis* sp. ATCC 46433	48	3.8	6.7	—	Sweet, pleasant fruit aroma & mild alcoholic flavor
	96	4.0	12.4	2.52	
	168	3.9	9.8	3.52	
Plus *Hansenula anomala* ATCC 46058	48	4.6	3.0	—	Sweet-sour, strong and unpleasant alcoholic flavor
	96	4.3	8.3	2.51	
	168	4.3	7.2	2.88	

Table 5. Changes in pH, reducing sugars (RS) content, and ethanol production occurring in rice fermented with *Torulopsis* sp. ATCC 46433 in combination with various molds.

Fermentation with:	Time (h)	pH	RS %	Ethanol (%) (v/v)	Taste (during 48–96 h)
Unfermented rice	0	6.7	0	0	—
Torulopsis sp. ATCC 46433					
Plus *Rhizopus arrhizus* ATCC 46429	48	4.4	1.8	0.03	Sweet, mild alcoholic and pleasant fruit aroma
	96	3.9	6.7	1.00	
	144	4.0	9.3	1.96	
	216	3.9	8.3	3.63	
Plus *R. javanicus* ATCC 46432	48	4.2	3.8	0.07	〃
	96	3.7	7.5	1.11	
	144	3.8	7.9	1.70	
	216	3.8	8.3	3.44	
Plus *R. formosaensis* ATCC 46431	48	4.6	1.8	0.02	〃
	96	4.3	8.5	0.47	
	144	4.1	5.2	2.01	
	216	4.0	7.2	3.51	
Plus *R. arrhizus* ATCC 46430	48	4.2	5.6	0.13	〃
	96	4.3	9.6	0.95	
	144	4.1	6.4	1.94	
	216	4.0	5.6	3.92	

galactose, maltose, sucrose, and raffinose into ethanol, and therefore consumed large amounts of sugars to produce high levels of ethanol (5.13% at 96 h and 7.10% at 168 h) and retained low concentrations of sugar (3.4% at 96 h and 7.8% at 168 h) in the rice. Therefore, the final products gave a sweet-sour taste and strong alcoholic flavor (Table 4).

Hansenula anomala ATCC 46058 produced more reducing sugars and less ethanol (Table 4) when compared with *Hansenula* sp. ATCC 44945 at 96 h. The products appeared to have a sweet-sour taste and a strong but unpleasant alcoholic odor which could not be considered as tasty rice pudding. A similar result was also reported by Cronk *et al.*[2] on the studies of Indonesian *tape ketan* fermentation. They observed that when the rice was fermented in part by *Hansenula anomala* it developed a strong odor of ethyl acetate, which might decrease palatability to some consumers. The esterification of acetic acid with ethanol to form ethyl acetate accounts partially for the decreased concentration of ethanol.

As shown in Table 2, the fermenting ability of the *Torulopsis* sp. was quite weak. It converted only glucose into ethanol, therefore retaining a large quantity of sugars in the rice at 96 and 168 h (Table 4), and developing a sweet taste and a mild alcoholic flavor.

The fermentation of rice by various molds including *Rhizopus arrhizus* ATCC 46429, *R. javanicus* ATCC 46432, *R. formosensis* ATCC 46431, *R. arrhizus* ATCC 46430, each in combination with the same yeast isolate *Torulopsis* sp. ATCC 46433, resulted in similar changes in pH, reducing sugars and ethanol production (Table 5). The percentages of reducing sugars and ethanol at 96 h for all four combinations maintained a proper ratio to make the rice products with a sweet taste, mild alcoholic flavor, and pleasant fruit aroma; these are considered good qualities in *Tien-chiu-niang*.

Wang and Hesseltine[3] studied *Lao-chao* (*Tien-chiu-niang*) fermentation and found that a good fermented rice was made when a mold, *Rhizopus chinensis* NRRL 3671, and a yeast, *Endomycopsis* sp. NRRL Y7067, were used as inocula instead of a commercial starter. In the present work, when a similar mold, *R. chinensis* ATCC 22958, and *Endomycopsis*

fibuligera ATCC 24945 (=*Saccharomyces fibuligera*) were employed for rice fermentation, an unsatisfactory product with a sour taste and strong alcoholic flavor was obtained. This result confirmed the observations reported by Cronk *et al.*[2] that *E. fibuligera* showed higher alcohol producing ability than *Hansenula* sp., therefore consuming more sugars to produce high concentrations of alcohol and decreasing the sweetness of the product.

The results of the present studies demonstrate that to make a delicious rice pudding a combination of a selected strain of the yeast *Torulopsis* and one of the amylolytic molds may be used as a starter culture.

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