Designation of rice cake starters for fermented rice products with desired characteristics and fast fermentation

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Abstract This study aimed to control characteristics of fermented rice products by using functional fungi and yeasts isolated from traditional rice cake starters in Thailand. Amylolytic fungi, amylolytic yeasts, alcoholic yeasts and aromatic yeasts were isolated from rice cake starters through different isolation protocols. Among the protocols tested, the enrichment in rice cake fermentation prior to isolation was the most suitable protocol for isolation of amylolytic fungi from all rice cake starters. While the enrichment in submerged fermentation prior to isolation could increase the numbers of yeast isolates. The selected amylolytic fungus and amylolytic yeast were identified as *Rhizopus oryzae* F63S and *Saccharomycopsis fibuligera* Y71R, respectively. The yeast with high production of ethanol and aromatic ester was identified as *Pichia anomala* Y11E. Fermented rice cakes with different characteristics were prepared using various combinations of fungi and yeast. The combination of *R. oryzae* F63S with *S. fibuligera* Y71R exhibited strong amylolytic activity and produced an extra sweet fermented rice cake. While the combination of *R. oryzae* F63S with *P. anomala* Y11E showed higher alcoholic and aromatic flavors. Moreover, the pure yeast *P. anomala* Y11E added with commercial amylase has been proven as an innovative starter for fast fermentation. This concept may contribute greatly to the

further development of fermented food with desired properties at industrial level.

Keywords Alcoholic · Amylolytic · Fungi · Rice cake starter · Yeast

Introduction

Traditional rice cake starter or “Look-Pang” (in Thai) is a microbial starter containing a co-inoculum of fungi and yeasts in rice flour (Manosroi et al. 2011). During rice fermentation, the fungi produce enzymes that hydrolyze starch into sugars which are partially fermented to alcohols by the yeasts. Organic compounds such as esters and organic acids are also produced during fermentation (Chuenchomrat et al. 2008). The fermented products from rice cake starters are i.e. fermented rice cake, rice wine and rice vinegar. The traditional fermented rice cake in Thailand named “Khao-Maak” is made of glutinous rice fermented with traditional rice cake starter for 2–3 days. This fermented rice cake gives soft texture and sweet taste with a little alcoholic and aromatic ester flavor. It has been considered as a dietary supplement and could promote the growth development of malnourished children (Pitiporn 2008). Some of them have been reported that they contain live microorganisms which can promote health benefit of the host and can be called as probiotics (Tinrat et al. 2018).

It is well known that the quality of the fermented products depends on the variations of fungal and yeast species among rice cake starters. It was then expected that if the type and amount of functional species in starter could be designated, the fermented products with desired characteristics could be developed. Therefore, the isolation and selection of functional fungi and yeasts from traditional

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rice cake starters were attempted. To obtain as many fungal and yeast isolates as possible, isolation were carried out using 3 protocols; (1) direct isolation from starter, (2) submerged fermentation enrichment prior to isolation and (3) rice cake fermentation (solid state fermentation) enrichment prior to isolation. For application in rice cake fermentation, the suitable proportion of functional fungi and yeasts were manipulated in order to control the quality of the fermented products. The fermented rice cake with desired characteristics such as high alcoholic taste, high aromatic flavor and sweetened taste, were developed and their sensory evaluation were done. In addition, to develop pure rice cake starter and fasten fermentation, the commercial amylase was added instead of fungi into the pure yeast starter. The fermentation performance by this pure yeast starter was compared with the traditional rice cake starter.

Materials and methods

Isolation and screening of amylolytic fungi and yeasts from rice cake starter

Various traditional rice cake starters were collected from different local parts in Thailand. They were stored at 4 °C until use. To obtain as many fungal and yeast isolates, isolation were carried out using 3 protocols as follows:

1. Direct isolation from starter: One gram of rice cake starter was ground and diluted in 9 mL of 0.85% normal saline. The suspension was then tenfold-serial diluted. Each concentration of suspension was inoculated and spread on starch agar (0.3% meat extract, 0.5% peptone, 0.2% starch soluble, 1.5% agar). The inoculated agar plates were incubated at 30 °C for 48 h and pure colonies of fungi and yeasts were picked and collected. The culture plate was then flooded with Lugol's iodine solution (2 g iodine, 2 g ammonium sulfate and 300 mL deionized water) for 1 min. The amylolytic strains that exhibited clear zone around their colonies were selected (Saelim et al. 2008). The clear zone ratio was calculated as follows: clear zone ratio = clear zone size (mm)/colony size (mm).
2. Submerged fermentation enrichment prior to isolation: One gram of ground rice cake starter was inoculated in 50 mL of yeast extract peptone cassava broth (YPC) (0.05% yeast extract, 0.05% peptone and 3.0% cassava starch) and incubated at 30 °C with shaking speed of 150 rpm for 48 h. The filamentous fungi were partly removed by filtration. The culture suspension was then tenfold-serial diluted in 0.85% normal saline. The diluted solution were spread on starch agar and

incubated at 30 °C for 48 h. Pure colonies of fungi and yeast were picked and collected. The culture plate was flooded with Lugol's iodine solution for 1 min. The amylolytic strains that exhibited clear zone around their colonies were selected.

3. Rice cake fermentation enrichment prior to isolation: Ground rice cake starter was 0.2% inoculated in cooked glutinous rice and incubated at 30 °C for 48 h. The fermented rice cake was homogenized and tenfold-serial diluted and spread on starch agar plate. Pure colonies of yeast and fungi were picked and collected after incubation at 30 °C for 48 h. The culture plate was flooded with Lugol's iodine solution for 1 min. The amylolytic strains that exhibited clear zone around their colonies were selected.

Quantification of amylolytic activity of isolated fungi and yeasts

The seed cultures were prepared by incubating fungi and yeasts in YPC broth at 30 °C and 150 rpm for 7 days and 2 days, respectively. The fungal conidia were suspended and homogenized in aqueous solution containing 0.05% (w/v) Tween 80. The spore concentrations were determined using the Thoma cell counting chamber and viewing under microscope. The suspensions were diluted to adjust the final concentration of log 7 spores/mL for fungi and log 7 cells/mL for yeasts. One mL of spore suspension and yeast seed culture was inoculated to 100 mL of YPC broth. The inoculated broth was incubated at 30 °C and 150 rpm for 72 h. Amylolytic activity was determined using the modified dinitrosalicylic (DNS) method (Keharom et al. 2016). The enzyme sample (0.2 mL) was mixed with 1 mL of 1% soluble starch (in 0.02 M sodium phosphate buffer pH 6.9 containing 0.01 M NaCl) and incubated at 30 °C for 30 min. DNS was added to the reaction mixture and boiled for 10 min. The absorbance was measured at 540 nm and compared with glucose standard curve. One unit (U) of amylolytic activity was defined as the amount of enzyme that liberates 1 µg of reducing sugar in 1 min under assay conditions.

Isolation and screening of alcoholic yeasts and aromatic yeasts

To isolate alcoholic yeasts and aromatic yeasts, one gram of ground rice cake starter was inoculated in 50 mL of yeast extract peptone dextrose broth containing 18% w/v glucose (YPD₁₈) and incubated at 30 °C and 120 rpm for 24 h. The culture suspension was then tenfold-serial diluted in 0.85% normal saline. The solution in the dilution range of 10²–10⁶ folds was spread on YPD agar and

incubated at 30 °C for 48 h. Pure colonies of yeast were picked and collected (Lee et al. 2011).

The isolated strains were then screened for their ability to produce either ethanol or esters. The seed cultures were prepared in YPD broth and incubated at 30 °C and 150 rpm for 24 h. Ten percent of seed culture with the optical density of 2.0 was inoculated in YPD₁₈ and incubated at 30 °C and 150 rpm for 72 h. The growth was measured using spectrophotometer at 660 nm (LIBRA-S22, Biochrom, England). Alcohol (ethanol) and aroma ester (ethyl ethanoate) concentration were examined by gas chromatography (Shimadzu GC-2014) equipped with a flame ionization detector (FID) and Stabilwax column (dimensions 30 m × 320 µm × 0.25 µm). The temperature of the injection port and detector were 240 °C and 280 °C, respectively. The oven was performed using the following program: (1) 70 °C for 2.5 min, (2) 70–100 °C with a ramping of 40 °C/min and hold at 100 °C for 0.2 min, (3) 100–200 °C with ramping of 10 °C/min and hold at 200 °C for 2 min and helium was used for carrier gas at a flow rate of 1.8 mL/min. The isolated fungi and yeasts were identified by PCR amplification and sequencing of 26S ribosomal RNA (rRNA) and Internal Transcribed Spacer 1 and 2 regions (ITS1 and ITS2).

Designation of fungi and yeast ratio

To evaluate the appropriate ratio between amylolytic fungi and yeast for the product with various properties, the fungi with the highest amylase activity was inoculated at log 6 spores/g-starch with the specific yeasts at various inoculum sizes of log 6–log 8 cells/g-starch in YPC broth medium and incubated at 30 °C and 150 rpm for 72 h. The sample was collected every 24 h and analyzed for amylase activity, ethanol and aroma ester as described above. The suitable fungi and yeast ratio for application in rice cake fermentation were determined.

The fermented rice cake with different characteristics including (a) high alcoholic taste, (b) high aromatic flavor and (c) sweetened taste, were produced by inoculating the suitable fungi with the suitable yeast at appropriate ratio. The inoculated glutinous rice was incubated at 30 °C for 72 h and the sample was collected every 24 h to measure ethanol and ester concentration by gas chromatography and measure reducing sugar by DNS method. The sensory evaluation of fermented products was performed with 30 trained panelists (21 females and 9 males) aged between 23 and 60 years from Faculty of Agro-Industry at Prince of Songkla University, Thailand. The panelists were served with 3 g of each sample together with a bottle of water in random order in three different sessions. Panelists evaluated the intensity of alcohol and ester flavor using 10-point scale (Phat et al. 2016).

Process development for fast fermentation of rice cake

To develop pure rice cake starter and shorten fermentation time, commercial amylase (iKnowZyme MTAA EC Code 3.2.1.1, Thailand) was added instead of fungi with the selected functional yeast. The inoculated glutinous rice was incubated at 30 °C for 48 h and the samples were collected for the measurement of sugar, ethanol and aromatic ester. The performance was compared with the tradition rice cake starter.

The experiments were performed at least in triplicates. The statistical significances were evaluated using one-way analysis of variance (ANOVA) and Duncan's multiple range tests ($P < 0.05$).

Results and discussion

Isolation and screening of amylolytic fungi and yeasts from rice cake starters

Amylolytic fungi (name started with F) and yeasts (name started with Y) were isolated from local rice cake starters using three protocols including direct isolation from starter (name ended with D), submerged fermentation enrichment prior to isolation (name ended with S) and rice cake fermentation enrichment prior to isolation (name ended with R). The direct isolation from all rice cake starters could detect fungal colonies ranging from 10^4 - 10^5 CFU/g-starter. Five fungal isolates showing obvious clear zone of amylolytic activity with different morphologies were selected. Among them, F21D showed the largest clear zone ratio of 1.8 while fungal isolate F22D and F51D presented larger colonies but had smaller clear zone ratio (1.03 and 1.1) than F21D. For yeast isolation, there were only some rice cake starters, in which the yeast colonies were detected in the range of 10^4 – 10^5 CFU/g-starter. Among the yeast colonies detected, yeast isolates Y61D and Y71D exhibited obvious clear zone ratio (4.3 and 5.0, respectively) around their colonies indicating the amylolytic activity.

From submerged fermentation enrichment prior to isolation, 11 fungi and 6 yeasts with different morphologies were selected. Isolate F31S, F63S and F71S showed measurable clear zone (clear zone ratio ranged from 1.1 to 1.4). The largest clear zone ratio was observed from the isolate F63S. Among 6 yeast isolates, only 2 isolates (Y71S and Y81S) exhibited clear zone around their colonies. Rice cake fermentation prior to isolation was performed in order to imitate natural habitat of fungi. The concentrations of reducing sugar detected in rice cake samples were in the range of 154–181 mg/g-rice cake. The ethanol and ester in rice cake products ranged from 4.0 to 26.5 mg/g-rice cake.

Compared with the above two isolation protocols, the rice cake fermentation prior to isolation was the most suitable technique to isolate the amylolytic fungi from all rice cake starters. With this protocol, 17 fungal isolates with different morphologies were found on starch agar. Among them, there were 8 isolates showing obvious clear zone on starch agar (clear zone ratio ranged between 1.3 and 1.6). Among 5 yeast isolates found, 2 isolates (Y61R and Y71R) exhibited large clear zone (clear zone ratio of 3.5–4.8) around their colonies. Limtong et al. (2002, 2005) who isolated yeasts by rice cake fermentation enrichment prior to isolation methods, also found that the clear zone ratio of isolated yeasts were in the range of 1.93–3.25.

Among three isolation protocols, the direct isolation from starter showed least number of isolates. This could be because the original cell concentration in the rice cake starters might be low and/or some fungal and yeast strains might not be active enough to grow on agar plate. It could be suggested that the enrichment in submerged fermentation could increase the numbers of isolates, especially the yeasts which are highly active in submerged environment. While solid state fermentation is more suitable for fungal growth. Mrudula and Murugammal (2011) have also reported the advantages of solid state fermentation over submerged fermentation for fungal growth and enzyme production. They found that the productivities of fungal enzymes using solid state fermentation were higher than those using submerged fermentation.

Quantification of amylolytic activity of isolated fungi and yeasts

Figure 1 shows the quantitative amylase activity of fungi (a) and yeasts (b) isolated from rice cake starters during 24–72 h of cultivation in yeast extract-peptone-cassava starch broth (YPC broth). The activities of isolated fungi were in the range of 38.6–416.2 U/mL. Among the fungal isolates compared, F63S and F91R exhibited high quantitative amylolytic activity of 387.3 and 351.3 U/mL at 48 h, respectively. While F31R and F71R showed higher final amylolytic activity of 346.0 and 416.2 U/mL at 72 h, respectively (Fig. 1a). These values were higher than those of other types of fungi such as *Aspergillus niger* which produced α -amylase at the levels of 81.9 U/mL (Aliyah et al. 2017) and 5.9–10.5 U/mL (Sakthi et al. 2012) and *Rhizopus oryzae* which produced amylase at the levels of 3.1–3.8 U/mL (Freitas et al. 2014). Compared to the amylolytic fungi, the amylolytic yeasts produced lower amylolytic activity of 39.8–160.2 U/mL (Fig. 1b) but these values were higher than those previously reported. For example, the amylolytic yeasts *Saccharomycopsis fibuligera* and *Geotrichum* sp. showed relatively low amylase activity of 0.15–0.7 U and 130 U/mL, respectively, during

Fig. 1 Amylase activity of fungi **a** and yeast **b** isolated from rice cake during 24–72 h of cultivation in yeast extract-peptone-cassava starch broth (YPC)

24–72 h of cultivation (Yalçın and Çorbacı 2013; Gen et al. 2014).

Screening of alcoholic yeasts and aromatic yeasts

To isolate the suitable yeasts for production of ethanol and aroma flavor in fermented rice cake, YPD₁₈ was used as an isolation medium (isolate name ended with E). Both alcoholic and aromatic yeasts were detected in all rice cake starters. The number of isolated yeasts was varied from 1 to 10⁷ CFU/g-starter. These members were close to those reported by Limtong et al. (2002) and Luangkhlapho et al. (2014). The ethanol and ester production by (1) amylolytic yeasts (2) alcoholic yeasts and (3) aromatic yeasts were compared (Table 1). During 48–72 h of cultivation, the isolates Y11E, Y21E and Y41E exhibited high ethanol production ranged from 32.8 to 62.1 g/L in YPD₁₈ medium. Limtong et al. (2002) and Shittu et al. (2016) reported that the yeasts isolated from traditional rice cake starters included *Saccharomycopsis fibuligera*, *Rhodotorula phylla*, *Trichosporon asahii*, *Pichia anomala*, *Issatchenkia orientalis*, *Torulaspora globosa*, *Rhodotorula minuta*,

Table 1 Ethanol and ethyl ethanoate production by isolated yeasts from rice cake starters in YPD₁₈ medium determined using gas chromatography

Yeast	Ethanol (g/L)		Ethyl ethanoate (g/L)	
	48 h	72 h	48 h	72 h
Y11E	39.3 ± 2.3 ^b	45.8 ± 4.6 ^b	2.0 ± 0.25 ^a	2.5 ± 0.16 ^a
Y21E	32.8 ± 2.7 ^{bc}	39.2 ± 4.1 ^{bc}	1.3 ± 0.11 ^b	1.5 ± 0.12 ^b
Y31E	—*	—	1.2 ± 0.11 ^b	1.3 ± 0.02 ^b
Y41E	52.4 ± 4.6 ^a	62.1 ± 3.6 ^a	—	—

*Not detected. Different superscript letters indicate significant differences between treatments

Rhodotorula mucilagnosa, *Candida krusei*, *Kodamara ohmeri* and *Pichia burtonii*. They all produced ethanol in the range of 0–47.6 g/L. However, these yeast strains produced relatively lower ethanol than brewer's yeast, *Saccharomyces cerevisiae* which could produce ethanol as high as 71–145 g/L (Vu and Kim 2009; Khamkeaw and Phisalaphong 2016). Ethyl ethanoate as aromatic flavor was produced by Y11E, Y21E and Y31E at the levels of 1.2–2.5 g/L during 48–72 h of cultivation (Table 1).

Identification of selected fungi and yeasts

The isolated fungus F63S which exhibited the highest amylolytic activity during 48 h, was identified as *Rhizopus oryzae* with the similarity of 100% (Supplementary Fig. S1). This specie has long been used for production of various enzymes especially amylase and glucoamylase and organic acids. It has also been applied in various kinds of fermented food (Yamane and Tanaka 2013; Freitas et al. 2014; Ibarruri and Hernández 2018). The isolated yeast Y11E which produced the highest aroma ester (ethyl ethanoate) was identified as *Pichia anomala* with the similarity of 99% (Supplementary Fig. S2). This specie is also known as *Wickerhamomyces anomalus* and *Hansenula anomala*. It is a non-*Saccharomyces* yeast in alcoholic beverages, being applied to enhance the sensory quality by producing particular volatile compounds. It is also recognized as naturally occurring biocontrol agent which can prevent mold spoilage and enhance preservation of moist grain (Kurita 2008; Kim et al. 2013). The isolated alcoholic yeast Y41E was identified as *Clavispora lusitaniae* with the similarity of 99%. It is also known as *Candida lusitaniae*. As *P. anomala* has not been reported as pathogen and its ethanol production was close to that of *C. lusitaniae*, *P. anomala* Y11E was then selected for further study. The yeast Y71E exhibiting high amylase activity was identified as *Saccharomycopsis fibuligera* (Supplementary Fig. 2S). This specie is a food-borne and representative producer of

amylolytic enzymes among ascomycetous yeasts, being widely used as a microbial starter in various fermented foods (Saelim et al. 2008; Choi et al. 2014; Carroll et al. 2017). In this study, the isolated fungus F63S (*Rhizopus oryzae*), yeast Y11E (*Pichia anomala*) and yeast Y71E (*Saccharomycopsis fibuligera*) were used for further studies. Limtong et al. (2002, 2005), Saelim et al. (2008) and Khamkeaw and Phisalaphong (2016) also reported that among the yeasts isolated from rice cake starters most of them were identified as *Saccharomycopsis fibuligera*. The other yeast species were *Pichia anomala*, *P. burtonii*, *P. fabianii*, *P. maxicana*, *P. heimii*, *Rhodotorula philyla*, *Candida rhagii*, *C. glabrata*, *Torulaspora globosa*, *S. cerevisiae*, *T. delbrueckii* and *Trichosporon asahii*. Most fungi isolated from rice cake starters belonged to the genus of *Amylomyces* and *Rhizopus* and the remaining fungi belonged to the genus of *Actinomucor*, *Aspergillus*, *Mucor*, *Monascus* and *Penicillium*. Among them, the genus *Amylomyces* and *Rhizopus* possess relatively strong amylolytic activity.

Designation of fungi and yeast ratio

The amylolytic fungus *R. oryzae* F63S was inoculated with the alcoholic and aromatic yeast *P. anomala* Y11E and the amylolytic yeast *S. fibuligera* Y71E in YPC broth medium at various inoculum sizes in order to evaluate their appropriate ratio. Figure 2a shows amylase activity of the co-culture of *R. oryzae* F63S with *P. anomala* Y11E. In the co-culture with the same proportion of fungi and yeast at log 5 cells/mL, the amylase activity increased approximately from 200 to 400 U/mL during 24–72 h of cultivation. However, when the yeast inoculum size was increased up to log 6 and log 7 cells/mL the amylase activity dropped dramatically to the level less than 200 U/mL at 72 h. This might be because the high amount of yeast generated high amount of ethanol which might inhibit the fungal growth and suppress the amylase production by the fungi. Figure 2b shows the amylase activity of the co-culture of *R. oryzae* F63S with *S. fibuligera* Y71E. The amylase activity of this co-culture was higher than that of the co-culture of *R. oryzae* F63S with *P. anomala* Y11E (Fig. 2a) by 1.0–2.5 times. The co-culture of *R. oryzae* F63S and *S. fibuligera* Y71E promoted the highest amylolytic activity of 447.9 U/mL at 72 h when using inoculum ratio of fungi to yeast at log 5 to log 5 cells/mL. It should be noted that the amylolytic activity during 24 h of cultivation, increased with increasing yeast inoculum size.

Figure 3 shows the effect of inoculum sizes on ethanol, aromatic ester and sugar production. Ethanol and ester production were not detected in the co-culture of amylolytic fungus *R. oryzae* F63S and yeast *S. fibuligera* Y71E (Fig. 3a, b). It was obvious that yeast *P. anomala* Y11E

Fig. 2 Effect of yeast inoculum size on amylase activity by co-culture of fungi and yeast in YPC medium

played an important role in ethanol and ester production. The ester production was increased from 18.5 to 35.5 mg/L with an increased yeast inoculum size from log 5 to log 7 cells/mL while the ethanol production slightly decreased from 466.3 to 417.2 mg/L. Figure 3c shows reducing sugar detected in the co-culture. The reducing sugar slightly increased with an increase of *S. fibuligera* Y71E inoculum size because this yeast could also produce amylase together with the fungi and both effectively converted starch into sugars. Pereira et al. (2013) have reported that the ethanol production by brewer's yeast *Saccharomyces cerevisiae* increased with increasing inoculum size while aromatic ester, ethyl ethanoate, decreased. The synthesis of ethyl ethanoate needs ethanol as one of the substrates together with acetate by the reversed reaction of enzyme-catalyzed hydrolysis. It should be noted that the pathway for ethanol and ester synthesis differed between *S. cerevisiae* and *P. anomala* Y11E. *P. anomala* Y11E produced most of esters by the inverse esterase reaction using acetate and the corresponding alcohol (Fu et al. 2018) while *S. cerevisiae* primarily used alcohol acetyltransferase pathway for ethanol production and both pathways for ethyl acetate production (Nancolas et al. 2017).

Fig. 3 Effect of yeast inoculum size on ethanol, ethyl ethanoate and sugar production by co-culture of fungi and yeast in YPC medium at 72 h of fermentation

Application of selected fungi and yeasts for production of fermented rice cake

The fermented rice cake with desired characteristics including (a) alcoholic taste, (b) aromatic flavor and (c) sweetened taste, were produced using the suitable combination between fungi and yeasts from the previous section. The results are shown in Table 2. The co-inoculation of *R. oryzae* F63S with *P. anomala* Y11E could produce fermented rice cake with high alcohol and aromatic flavors. Aromatic ester in fermented rice cake increased from 4.0 ± 0.04 to 5.0 ± 0.04 mg/g-rice cake with increasing the inoculum size of yeast *P. anomala* Y11E from log 5 to log 7 cells, while ethanol concentration slightly decreased from 57.6 ± 5.3 to 46.1 ± 3.8 mg/g-rice cake (Table 2a, b). The results showed that the fermented rice cake with high amount of this yeast specie

Table 2 Quantitative analysis and sensory score of rice cake fermented with quality controlled starter for 72 h

Fermented rice products	Quantitative analysis			Sensory score		
	Reducing sugar (mg/g-rice cake)	Ethanol (mg/g-rice cake)	Ethyl ethanoate (mg/g-rice cake)	Sweetness	Alcoholic flavor	Aromatic flavor
(a) Alcoholic fermented rice cake	176 ± 1.7 ^b	57.6 ± 5.3 ^a	4.0 ± 0.04 ^b	6.0 ± 1.79 ^b	7.4 ± 2.36 ^a	5.1 ± 1.91 ^a
(b) Aromatic fermented rice cake	117 ± 3.8 ^c	46.1 ± 3.8 ^b	5.0 ± 0.04 ^a	6.0 ± 1.85 ^b	5.9 ± 1.86 ^{ab}	6.4 ± 1.74 ^a
(c) Sweetened fermented rice cake	196 ± 9.6 ^a	5.7 ± 1.5 ^c	0.96 ± 0.04 ^c	7.1 ± 1.48 ^a	4.4 ± 1.69 ^b	3.9 ± 1.33 ^a

Superscript letters indicate significant differences among the tested samples

The sensory score were derived from an average score (of 10 scores) of 30 panelists

(a) *R. oryzae* F63S and *P. anomala* Y11E at a ratio of log 5 spores/log 5 cells

(b) *R. oryzae* F63S and *P. anomala* Y11E at a ratio of log 5 spores/log 7 cells

(c) *R. oryzae* F63S and *S. fibuligera* Y71E at a ratio of log 5 spores/log 5 cells

could increase the concentration of aromatic flavor. The sugar concentrations in the liquid squeezed from the fermented rice cake were in the range of 0.6–0.9% w/v corresponding to 117–196 mg/g-rice cake. It should be noted that the fermented rice cake produced by co-inoculation of *R. oryzae* F63S with *S. fibuligera* Y71E contained higher amount of sugar than the fermented rice cake produced by co-inoculation of *R. oryzae* F63S with *P. anomala* Y11E.

The sensory evaluation of fermented rice cake was performed as shown in Table 2. The intensities of sweetness, alcoholic and aromatic flavors in fermented rice cake were evaluated by 30 panelists. The strongest intensities of sweetness, alcoholic and aromatic flavors were found in the sweetened fermented rice cake (c), alcoholic fermented rice cake (a) and aromatic fermented rice cake (b), respectively. In the fermentation of rice cake, after the fungi converted starch into sugar, the yeast then consumed sugar and produced alcohol. Alcohol was then converted to aromatic ester by the yeast. As the aromatic fermented rice cake (b) contained 100 times higher amount of yeast cells, its aromatic ester was then higher than that in the alcoholic fermented rice cake (a). Interestingly, the aromatic flavor score from sensory evaluation was also higher. In the sweetened fermented rice cake (c), both amylolytic fungi and yeast produced amylase that could convert starch into sugar. Therefore, the sugar concentration and the sweetness score of the fermented rice cake were highest. However, the evaluation score of alcoholic and ester flavors were lower than those of other two fermented rice cakes.

Process development for fast fermentation of rice cake

To fasten fermentation time of rice cake, commercial amylase was added with the alcoholic and aromatic yeast

P. anomala Y11E and the pure rice cake starter was formulated. The performance of the pure rice cake starter was compared with the traditional rice cake starter (Fig. 4). There was no sugar, ethanol and ester detected in the rice cake inoculated with the yeast only. This was because this yeast could not ferment rice directly. When the commercial amylase was added with the yeast, sugar concentration increased rapidly during 6 h of fermentation time and remained constant before slightly decreased after 24 h (Fig. 4a). The addition of higher amount of amylase led to higher amount of sugars present in the rice cake. The ethanol was produced rapidly after 6 h due to the activity of the yeast to ferment available sugars and produce ethanol. With higher sugar concentration, higher ethanol concentration was produced (Fig. 4b). The concentration of ester also increased when the amount of amylase increased (Fig. 4c). While the fermentation rate by traditional starter was much lower than that by pure yeast starter added with amylase. These results indicate the possible development of innovative starter for fast fermentation of rice cake and other starchy materials.

Conclusion

This study has shown that the rice cake fermentation prior to isolation was the most suitable technique to isolate the amylolytic fungi while the enrichment in submerged fermentation could isolate more numbers of the yeasts. Compared to the amylolytic fungi *R. oryzae* F63S, the amylolytic yeast *S. fibuligera* Y71E produced relatively lower amylolytic activity. The co-culture of amylolytic fungi and yeast exhibited stronger amylolytic activity than the pure culture of each strain. The co-inoculation of amylolytic fungi with alcoholic and aromatic yeast could

Fig. 4 Rice cake fermentation using pure yeast starter added with commercial amylase to fasten the fermentation compared with the use of traditional rice cake starter

produce rice cake with high levels of alcohol and aromatic flavors. In addition, this study has proven that the pure yeast starter with the addition of commercial amylase was effective for fastening the rice cake fermentation. These techniques may contribute greatly to the further development of fermented food with desired properties at industrial level.

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