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Research Note

Diversity and killer activity of yeasts in Malaysian fermented food samples

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Abstract. The biodiversity and the killer activity of yeasts isolated from various types of fermented food in Malaysia were investigated in this study. Of 252 yeasts isolated from 48 fermented food samples in this study, 19 yeast species were identified based on sequence analysis of the ITS1-5.8S-ITS2 partial fragments of the yeasts. A total of 29 (11.5%) of the yeast isolates demonstrated killer activity to at least one *Candida* species tested in this study; including 22 isolates of *Trichosporon asahii*, 4 isolates of *Pichia anomala*, and one isolate each of *Pichia norvegensis*, *Pichia fermentans* and *Issatchenkia orientalis*, respectively. The presence of killer yeasts reflects antagonism that occurs during microbial interaction in the fermented food, whereby certain yeasts produce killer toxins and possibly other toxic substances in competition for limited nutrients and space. The anti-*Candida* activity demonstrated by killer yeasts in this study should be further explored for development of alternative therapy against candidiasis.

Food fermentations are usually driven by a complex microbial population, in which bacteria, yeasts and moulds interact in the same environment striving for nutrients and spaces. The production of organic acids, hydrolytic enzymes and precursors for aroma by microorganisms is essential for the improvement of the physiochemical, nutritional and safety characteristics of fermented food. Killer yeasts are widespread in natural population of fruits and decaying vegetables and may have important effects on the development and composition of other flora sharing the same habitat. The killer characteristics of the yeasts have been applied to combat undesirable organisms during the production of beer and wine, and for food preservation (Druvefors *et al.*, 2005). Yeast killer toxins, analogous to bacteriocins, are either fungicidal or fungistatic to sensitive strains of their own species or other species. These yeasts including *Debaryomyces*, *Pichia*, *Torulopsis* and *Williopsis* species have been reported to have agronomic, environmental and industrial applications (Buzzini *et al.*, 2007). In addition, the killer toxins of *Pichia anomala* (Sawant *et al.*, 1989) and *Williopsis mrakii* (Hodgson *et al.*, 1995) have been proposed as antimycotic agents.

In recent years, the frequency of *Candida* infections has increased especially among severely immunosuppressed patients. *Candida albicans* is the most frequently encountered yeast in the clinical specimens and the most pathogenic *Candida* species, however; increasing prevalence of infections caused by nonalbicans *Candida* species such as *Candida parapsilosis*, *Candida tropicalis* and *Candida glabrata* has been reported

(Pfaller & Diakema, 2007). Search for effective antimycotic therapy is needed in view of the emergence of antifungal drug resistance in *Candida* species.

In our effort to search for potential antimycotic agents from natural sources, this study was conducted to determine the yeast biodiversity and the occurrence of killer yeasts from fermented food samples in our region.

Eight various types of fermented food samples obtained from markets in Klang valley, Malaysia were investigated in this study. These included tapai (fermented glutinous rice or tapioca, n=14), fermented fruit and vegetables (n=13), soy sauce (n=6), tempeh (fermented soy beans, n=5), rice wine (n=2), yogurt (n=2), fermented beans and miso (n=5), vinegar (n=1). Briefly, 0.1 gram of the food samples was homogenized in sterile 1% peptone water. The homogenate was then serially diluted from 10^2 to 10^8 and inoculated onto potato dextrose agar (PDA). Yeast colonies were selected from each agar plate and stored in brain heart infusion broth with 20% glycerol at -80°C for long term preservation.

The yeast was identified by amplification and sequence analysis of ITS1-5.8S-ITS2 gene fragment, as described by White *et al.* (1990). Briefly, a single colony of yeast cells was suspended in 10µl of sterile distilled water. The yeast suspension (2 µl) was then added into a PCR (polymerase chain reaction) mixture containing 0.25 µl of *Taq* polymerase (5 U/µl) (MBI Fermentas, Vilnius, Lithuania), 0.5 µl of deoxyribonucleotide triphosphate mix (10mM of each nucleotide), 2.5 µl of 10X PCR reaction buffer, 1.5µl of $MgCl_2$ and 0.125 µl of 10mM each of primer ITS 1 (5' TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC-3'). The amplification reaction was carried out in BIORAD Mycycler with an initial denaturation step at 95°C for 5 min, followed by 36 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and a final step at 72°C for 10 min. The amplification products of ITS1-5.8S-ITS2 fragment were separated by electrophoresis on 1% (w/v) agarose gel at 90V for 45 minutes. The amplicons were then purified using GENEALL™ PCR SV kit (General Biosystem, Seoul, Korea). Sequencing was performed using Big Dye® Terminator Cycle Sequencing Kit (Applied Biosystems, CA) on an ABI-3730 Genetic Analyzer (Applied Biosystems, CA), using ITS1 and ITS4 as primers. The sequences were assembled and analyzed with Nucleotide-nucleotide BLAST (BLASTN) program (http://blast.ncbi.nlm.nih.gov/Blast).

For yeast killing activity determination, yeast extract potato dextrose agar supplemented with methylene blue (YEPD-MB) agar was used as described by Fuentefria *et al.* (2008). The sensitive yeast strains used in this study were *C. albicans* ATCC90028, *C. parapsilosis* ATCC22019, *Candida krusei* ATCC6258, and clinical isolates of *Candida dubliniensis*, *C. tropicalis*, *C. glabrata*, *Candida guilliermondii* and *Candida rugosa*. Overnight cultures of sensitive strain were suspended in sterile distilled water to obtain about 10^5 cells/ml (80% transmittance at 530 nm followed by 10X dilution) and spread using sterile swab on a YEPD-MB agar plate. Yeasts isolated from food samples were then point-inoculated in duplicate on the agar plate and incubated at 30°C for 72 h. The plate was inspected daily for the appearance of inhibition zone surrounding the inoculated area as an indication of killer yeast activity.

Sequence determination and Genbank database search for yeast species with matching sequence provide accurate identification of yeasts up to the species level. However, as the sequence determination method was costly; only 73 isolates from various samples were identified using this approach. A total of 19 yeast species were identified (Table 1), with *Saccharomyces cerevisiae* and *Pichia* species being more frequently isolated from the samples. Several *Candida* species including *Candida orthopsilosis*, *C. glabrata*, *C. tropicalis* and *C. guillermondii* identified in this study, have been described as human normal flora and opportunistic pathogens (Pfaller & Diakema, 2007). The isolation of *Candida* yeasts suggests human involvement in the preparation and handling of the fermented food.

Twenty-nine (11.5%) of 252 yeasts isolated from this study demonstrated killer activity to at least one *Candida* species (Table 2). The killer yeasts were identified as *Trichosporon asahii* (22 isolates), *Pichia anomala* (4 isolates), *Pichia norvegensis* (1 isolate), *Pichia fermentans* (1 isolate), and *Issatchenkia orientalis* (1 isolate).

Although *S. cerevisiae* is the most dominant type of yeasts in tapai, other yeasts such as *C. orthopsilosis*, *C. glabrata*, *Issatchenkia orientalis* (teleomorph of *C. krusei*), *Kluyveromyces marxianus*, *P. anomala* and *Pichia caribbica* were also isolated (Table 1). In another study, microorganisms such as *Saccharomycopsis fibuligera*, *Amylomyces rouxii*, *Mucor circinelloides*, *Mucor javanicus*, *Rhizopus oryzae* and *Rhizopus chinensis* have also been identified from tapai (Ko, 1972).

Pichia anomala, the only killer yeasts isolated from three of fourteen tapai samples in this study (Table 1) demonstrated killing activity against *C. rugosa* and *C. guillermondii*. The killer yeast been reported previously to have a wide range of activity against important opportunistic fungal pathogens such as *C. albicans*, dimorphic fungi and a variety of moulds (Druvefors *et al*., 2005; Passoth & Schnürer, 2003). In this study, no killer activity of *P. anomala* was detected against *C. albicans*, however; the killer yeast displayed two killing patterns which were distinguishable by the ability to inhibit *C. dubliniensis* and *C. tropicalis* (data not shown).

Tempeh is produced from the fermentation of dehulled and cooked soybeans with moulds of the genus *Rhizopus* (mainly *Rhizopus oligosporus*). Many yeast

Table 1. Yeast isolates identified from fermented food samples in this study

Fermented food samples	Yeast strains (no. isolates identified by molecular method)
Tapai (n=14)	*Candida orthopsilosis* (1)
	Candida glabrata (5)
	Issatchenkia orientalis (3)
	Kluyveromyces marxianus (1)
	*Pichia anomala** (6)
	Pichia caribbica (1)
	Saccharomyces cerevisiae (9)
Fermented fruits and vegetables (n=13)	*Zygosaccharomyces bailii* (1)
	*Issatchenkia orientalis** (2)
	Candida rugosa (1)
	Yarrowia lipolytica (1)
Tempeh (n=5)	*Pichia guilliermondii* (7)
	Candida tropicalis (1)
	*Pichia norvegensis** (1)
	Sporopachydermia lactativora (1)
	*Trichosporon asahii** (22)
Miso and other fermented beans (n=5)	*Zygosaccharomyces rouxii* (1)
	Candida glabrata (1)
	Candida parapsilosis (2)
Yogurt (n=2)	*Clavispora lusitaniae* (3)
	Saccharomyces cerevisiae (2)
	*Pichia fermentans** (1)
Soy sauce (n=6)	No yeast isolated
Rice wine (n=2)	No yeast isolated
Vinegar (n=1)	No yeast isolated

*Killer yeast

species, e.g. *T. asahii* (formerly known as *Trichosporon beigelii)*, *Candida lusitaniae*, *Candida maltosa*, *Candida intermedia*, *Yarrowia lipolytica*, *Lodderomyces elongisporus*, *Rhodotorula mucilaginosa*, *Candida sake*, *Hansenula fabiani*, *C. tropicalis*, *C. parapsilosis*, *Pichia membranaefaciens*, *Rhizopus rubra*, *C. rugosa*, *C. curvata*, *Hansenula anomola*) were reported from tempeh (Samson *et al.*, 1987). In this study, we reported the identification of *Pichia guilliermondii*, *P. norvegensis* and *Sporopachydermia lactativora* in addition to those reported previously.

Trichosporon asahii, the most frequently isolated killer yeast species from tempeh in this study, has been reported as an incident contaminant that disturbs tempeh production and possibly poses a hazard for human health (Han *et al.*, 2000; Feng *et al.*, 2007). *Trichosporon* species is also known to cause mild superficial cutaneous infections and life-threatening disseminated infections characterized by resistance to amphotericin and echinocandins with poor prognosis (Miceli *et al.*, 2011).

In this study, *T. asahii* demonstrated a broad killing activity against various *Candida* species except for *C. albicans* (Table 2). The broad killer phenomenon has been previously reported for other *Trichosporon* species including *Trichosporon pullulans*, *Trichosporon asteroids* (Golubev, 2006) and *Trichosporon porosum* (Kulakovskaya *et al.*, 2010). The killing phenomenon exhibited by *T. asahii* is yet to be investigated.

The other killer yeast isolated from tempeh in this study was *P. norvegensis*, which demonstrated killing activity to three *Candida* species (*C. rugosa*, *C. glabrata* and *C. guillermondii*) (Table 2). The yeast has been reported to cause contamination in non-heat sterilizable food such as soft cheese (Bouakline *et al.*, 2000). *Pichia fermentans* and *I. orientalis* are the two killer yeasts isolated from yogurt and salted vegetable samples, respectively in this study. The killing phenomena of these yeasts have not been reported previously.

Very little is known on the mechanisms of killing activity of yeast. Although yeast killer toxins are antifungal proteins which have been reported to have glucanase and chitinase activities (Izgu *et al.*, 2006; Santos & Marquina, 2004); however, other metabolites for instance, cellobiose lipids produced by *T. porosum* are also fungicidal (Kulakovskaya *et al.*, 2010). It appears to us that non-albicans *Candida* species such as *C. glabrata*, *C. rugosa* and *C. parapsilosis* are more susceptible to the killer yeasts in our study as compared to *C. albicans* and its closely related species, *C. dubliniensis* (Table 2). Purification and characterization of the responsible metabolites of the yeasts merit further investigations.

Table 2. Anti-*Candida* activity of killer yeasts identified in this study

Killer yeast	No. of isolates demonstrating killing activity on the sensitive strain							
	Candida albicans ATCC90028	*Candida dubliniensis*	*Candida tropicalis*	*Candida parapsilosis* ATCC 22019	*Candida krusei* ATCC 6258	*Candida glabrata*	*Candida guilliermondii*	*Candida rugosa*
T. asahii (n=22)	0	0	2	22	14	22	9	18
P. anomala (n=4)	0	2	2	0	0	0	4	4
P. norvegensis (n=1)	0	0	0	0	0	1	1	1
I. orientalis (n=1)	0	0	0	0	1	1	0	1
P. fermentans (n=1)	0	0	0	0	0	1	0	0
Total	**0**	**2**	**4**	**22**	**15**	**25**	**14**	**24**

The killer activity of yeasts originating from fermented food samples has not been reported previously in Malaysia. The presence of killer yeasts in the fermented food samples reflects antagonism that occurs during microbial interaction, whereby certain yeasts produce killer toxins and possibly other toxic substances in competition for limited nutrients and space. The occurrence of killer yeasts has important effects on the development and composition of other flora in the fermented food. For instance, the introduction of known food-grade of yeasts has been reported to improve the nutritional quality and simultaneously reduce the risk of unwanted yeasts, moulds and bacteria in tempeh (Feng *et al.*, 2007). In addition, the anti-*Candida* activity as demonstrated by the killer yeasts should be further explored for development of alternative therapy against candidiasis, a persistent public health problem (Pfaller & Diakema, 2007).

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