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Research Note

Anti-*Candida* activity and biofilm inhibitory effects of secreted products of tropical environmental yeasts

Tan, H.W. and Tay, S.T.*
Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
* Corresponding author email: tayst@um.edu.my
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Abstract. This study describes the killer phenotypes of tropical environmental yeasts and the inhibition effects of the culture filtrates on the biofilm of *Candida albicans*. A total of 26 (10.5%) of 258 yeast isolates obtained from an environmental sampling study demonstrated killer activity to *Candida* species. The killer yeasts were identified as species belonging to the genus *Aureobasidium*, *Pseudozyma*, *Ustilago* and *Candida* based on sequence analysis of the ITS1-5.8S-ITS2 region of the yeasts. *Pseudozyma* showed the broadest killing effects against sensitive strains of *Candida*. New species of *Ustilago* and *Pseudozyma* demonstrating killer phenotypes were identified in this study. Interestingly, more than 50% reduction in the metabolic activity of *Candida albicans* biofilm was noted after exposure to the culture filtrates of the nine killer yeasts. Purification and characterization of toxin and metabolites are essential for understanding the yeast killing effects.

Candida albicans is an opportunistic yeast pathogen that is capable of causing a variety of infections in susceptible patients. The occurrence of *Candida* infection among hospitalized patients has been associated with catheter colonization, due to the capability of the yeast to form biofilms on plastic intravascular devices (Ramage *et al.*, 2002). The emergence of drug resistance of this fungal pathogen has spurred the interest to look for alternative chemotherapies. Among the new antimicrobial molecules under investigation, yeast killer toxins, analogous to bacteriocins, represent promising candidates because of their wide spectra of activity (Magliani *et al.*, 1997; Polonelli *et al.*, 2000). In addition, quorum sensing molecules present in the secreted products of certain microorganisms are attractive therapeutic targets for treatment of biofilm-associated infection due to the role it plays in the global regulation of virulence factors of the microorganisms. Identification of these molecules is important as the molecules have potential to be used for effective treatment of biofilm infections. A quorum-sensing molecule produced by planktonic cultures of *C. albicans*, farnesol, has been shown to prevent the germination of yeast cells into mycelia, a phenomenon that is pertinent to *C. albicans* biofilm formation (Hornby *et al.*, 2001).

Yeast killer systems have been described in a number of yeasts such as, *Saccharomyces cerevisiae*, *Candida*, *Cryptococcus*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Torulopsis*, *Ustilago*, *Williopsis* and *Zygosaccharomyces* (Magliani *et al.*, 1997; Chen *et al.*, 2000; Guyard *et al.*, 2000). However, very few studies have been conducted over the past to reveal the

natural occurrence of killer yeasts in the tropical environment (Abranches *et al.*, 1997; Buzzini & Martini, 2000). In this study, we attempted to isolate killer yeasts from the environment and to determine the inhibitory effects of the yeasts against nine *Candida* species. The potential application of the secreted products (present in the culture filtrates) of the killer yeasts in reducing and eliminating biofilms of *C. albicans* is also investigated. It is hoped that the findings of this study may contribute to the discovery of effective compounds for treatment of candidiasis.

In this study, a total of 100 samples consisting of leaves, flowers, and soils collected from the campus of University of Malaya, Kuala Lumpur, Malaysia were cultured for yeasts. Approximately 0.5 g samples were mixed with 5 ml sterile distilled water in a MacCartney bottle and vortexed. One hundred microliters of the suspension was then spread onto Sabouraud's dextrose agar (SDA, Oxoid) supplemented with chloramphenicol (250 mg/l) and incubated at 30ºC for up to 10 days. Upon daily inspection, any colonies resembling yeasts (confirmed by gram staining) were subcultured onto Potato dextrose agar (PDA, Scharlau, Spain) and stored at 4ºC for further testing.

The isolates in this study were identified by sequence determination of the yeast internal transcribed spacer gene regions. DNA isolation was performed by a simple phenol-chloroform extraction method (Graham *et al.*, 1994). Briefly, a loopful of the yeast colony was suspended in 500 µl lysis buffer (0.1 M Tris, pH 8.0, 50 mM EDTA, 1% SDS) and vortexed briefly with an equal volume of acid-washed glass beads (approximately 0.05 g). The cell lysate was then centrifuged at 8000 rpm for 2 min. The supernatant was added with 400 µl of Tris-saturated phenol-chloroform, and centrifuged. Yeast DNA was precipitated using 95% ethanol followed by 70% ethanol and air-dried. The DNA was resuspended in 25 µl of sterile distilled water prior to use. Two microliters of DNA was used as template in a 25 µl reaction volume containing 0.25 µl of *Taq* polymerase (5 U/µl) (MBI Fermentas), 0.5 µl of deoxyribonucleoside triphosphate mix (10mM of each nucleotide), 2.5 µl of 10X PCR reaction buffer, 1.5 µl 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') (White *et al.*, 1990). The amplification reaction involved an initial cycle of 95ºC for 5 min, followed by 36 cycles of 95ºC for 1 min, 52ºC for 1 min, 72ºC for 1 min, and a final cycle step at 72ºC for 10 min. The amplification products were 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 analysed with Nucleotide-nucleotide BLAST (BLASTN) programme (http://blast.ncbi.nlm.nih.gov/Blast).

Yeast extract potato dextrose agar supplemented with methylene blue (YEPD-MB agar) was used in the killer activity assay as described by Fuentefria *et al.* (2008). Briefly, an overnight culture of a sensitive strain was suspended in sterile distilled water to obtain about 10^5 cells/ml and lawn on YEPD-MB agar using a sterile cotton swab. An overnight culture of killer yeast was then inoculated on the agar and incubated aerobically for 72 hours at 30ºC. The plate was inspected daily for the appearance of inhibition zone surrounding the inoculated area. A total of nine sensitive yeast strains were included in this study: *C. albicans* ATCC90028, *C. parapsilosis* ATCC22019, ATCC90018, *Candida krusei* ATCC6258, and clinical isolates of *Candida dubliniensis*, *Candida tropicalis*, *Candida glabrata*, *Candida guilliermondii* and *Candida rugosa* in our culture collection.

The secreted products present in the culture filtrates of killer yeasts were assayed for inhibitory effects against *C. albicans* biofilm. Briefly, the killer yeast was cultured in brain heart infusion broth for 6 days at 30ºC with shaking (100 rpm).

The supernatant was collected by centrifugation at 5000 g for 30 min and filtered using a 0.20 µm filter membrane (Sartorius Stedim, Germany). The biofilm of *C. albicans* ATCC strain 90028 was prepared as described by Jin *et al.* (2003). The biofilm was washed twice with sterile phosphate buffered saline and added with 100 µl of a culture filtrate. Examination of biofilm morphology was carried out with an inverted light microscope (Olympus, USA) at 200x magnification. The metabolic activity of the biofilm after 48 h of exposure to a culture filtrate was measured by XTT reduction assays, as described by Jin *et al.* (2003). The experiments were performed in quadruplicate for each isolate. Inhibitory effects of the culture filtrate were determined based on the percentage reduction in the metabolic activity of the treated biofilm as compared with the metabolic activity of the biofilm without exposure to the culture filtrate.

A total of 258 environmental yeasts were isolated and screened for killer activity in this study. Of the 46 yeasts randomly subjected to molecular identification, *Aureobasidium pullulans* was the most abundant yeast species, constituting 50% of the isolates identified in this study. Other yeasts included members of the genus *Candida* (4.3%), *Rhodosporidium* (8.7%), *Sporidiobolus* (4.3%), *Pseudozyma* (15.2%) and *Ustilago* (17.4%). A total of 26 (10.1%) isolates were identified as killer yeasts (Table 1). A total of 23 killer sensitivity patterns were recognized in this study based on the specific killing effects of the yeasts on the nine sensitive strains (Table 1). Among *Candida* sensitive strains tested in this study, *C. rugosa* was the most vulnerable strain, being susceptible to 15 (57.7%) of the killer yeasts.

Aureobasidium pullulans is the most predominant killer yeast identified in this study (Table 1). The yeast is commonly found in diverse habitats including the phyllosphere of many plants and various tropical fruits, painted walls and bathroom surfaces (Lotrakul *et al.*, 2009). The organism produces aureobasidin A, an antifungal depsipeptide antibiotic, which has strong fungicidal activity *in vitro*

Table 1. Killer phenotypes of environmental yeasts investigated in this study

Killer yeast	No. isolates demonstrating killer phenotypes on YEPD-MB agar	No. killer sensitivity patterns on YEPD-MB agar*	No. culture filtrate causing ≥50% reduction in the metabolity activity of *C. albicans* biofilms
Aureobasidium pullulans	10	8	2
Candida species			
C. parapsilosis	1	1	0
C. tropicalis	1	1	1
Ustilago species			
U. sparsa	2	2	1
U. trichophora	2	2	0
U. tragana	4	3	3
Pseudozyma species			
P. hubeiensis	3	3	0
Pseudozyma spp.	3	3	2
Total	**26**	**23**	**9**

*killing sensitivity patterns were determined based on the specific killing effects of each isolate on nine sensitive yeast strains

against many pathogenic fungi, including *C. albicans*, *C. neoformans*, and *Aspergillus* spp. (Ikai *et al.*, 1991; Takesako *et al.*, 1993). *Ustilago maydis*, a fungal pathogen of maize, is the only *Ustilago* species that has been well reported so far to have a killer system. The killer system of the yeast is characterized by three different toxins (Koltin & Day, 1975). In this study, killer activity was identified in four isolates of *Ustilago tragana*, two isolates of *Ustilago sparsa* and two isolates of *Ustilago trichophora*. The killer activity of these *Ustilago* species has not been reported before.

The antimycotic activity of another ustilaginaceous yeast, *Pseudozyma* species (*Pseudozyma fusiformata*, *Pseudozyma flocculosa*, *Pseudozyma prolific* and *Pseudozyma tsukubaensis*), against yeasts and filamentous fungi, including phytopathogenic and medically important species of the *Filobasidiella*, *Malassezia*, *Taphrina* and *Ustilago* has been demonstrated previously (Golubev *et al.*, 2006). The glycolipids and mycocin of the yeast has been associated with killing activity (Kulakovskaya e*t al.*, 2003; Golubev *et al.*, 2006). *Candida* (*Torulopsis*) *glabrata* was the first *Candida* species reported to have killer toxin being detected (Bussey & Skipper, 1975). In this study, two environmental isolates of *Candida*, namely C. *tropicalis* and *C. parapsilosis*, were identified as killer yeasts.

More than 50% reduction was noted in the metabolic activity of *C. albicans* biofilm after exposure to the culture filtrate of nine killer yeasts in this study (Table 1). The highest percentage (71.9%) of reduction in biofilm metabolic activity was shown by the culture filtrate of a *Pseudozyma* isolate (data not shown). Figure 1 shows the morphology of *C. albicans* biofilm after 48 hr of exposure to the culture filtrate. The clear areas seen in the microtiter well is an indication of the killing effect of the culture filtrate.

There are many factors that can influence biofilm formation of *C. albicans*. It is thought that the presence of alcoholic products such as ethanol, isoamyl alcohol, 2-phenylethanol, 1-dodecanol, E-nerolidol, glycolipid biosurfactant and signaling molecules found in the yeast culture filtrates may affect the growth of *C. albicans* biofilm (Jeniel *et al.*, 2008; Muthusamy *et al.*, 2008). In particular, farnesol has been identified as a quorum-sensing molecule that prevents pseudohyphae transition in *C. albicans* (Hornby *et al.*, 2001; Henriques *et al.*, 2007), and hence, inhibits the induction of

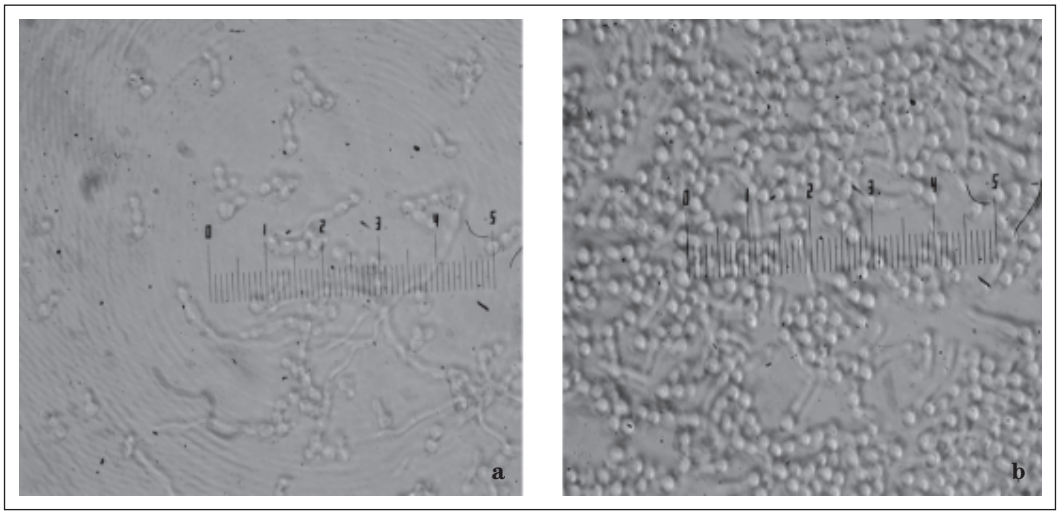

Figure 1. Appearance of *C. albicans* biofilms after 48 hours of exposure to (a) the culture filtrate of a *Pseudozyma* spp. (b) negative control (normal growth medium)

hyphal growth and biofilm formation in *C. albicans*.

The diversity and anti-*Candida* activity of tropical environmental yeasts is presented in this study. Further identification and characterisation of secreted products of the killer yeasts will be helpful in formulating strategies to limit biofilm formation of *C. albicans* on medical indwelling devices.

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