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Full Length Research Paper

Isolation of *Aureobasidium pullulans* from bathroom surfaces and their antifungal activity against some Aspergilli

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Ten *Aureobasidium* isolates were collected from bathroom surfaces in Thailand. They were identified as *Aureobasidium pullulans*. Cell extracts from all isolates were tested for antifungal activities against four selected *Aspergillus* species using a paper disc diffusion and conidial germination inhibition assay. BM1, KT1, HKW1 and HKW2 extracts inhibited *Aspergillus terreus*, whereas KT1 and BM1 extracts also inhibited *Aspergillus fumigatus*. BM1 extract alone inhibited *Aspergillus flavus*. From TLC analysis, an antifungal compound with an identical Rf to that of aureobasidin A was found in all extracts. Antifungal tests of TLC-separated compounds supported the paper disc diffusion and conidial germination inhibition assays.

Key words: *Aureobasidium pullulans*, antifungal activity, aureobasidin.

INTRODUCTION

Aureobasidium pullulans is a yeast-like Ascomycete (Order Dothideales, Family Dothideaceae) and is comprised of two known varieties, *A. pullulans* var. *pullulans* and *A. pullulans* var. *aubasidani* (Yurlova and De Hoog, 1997). This fungus is polymorphic in its life cycle, exhibiting hyphae, blastospores, conidiospores and chlamydospores (Punnapayak et al., 2003). *A. pullulans* is commonly found in a variety of habitats including plant leaves, painted wall and bathroom surfaces. The occurrence of this fungus has mostly been reported in temperate climates while that in tropical area was very scarce (Punnapayak et al., 2003). However, recently a number of *A. pullulans* strains were isolated from Thailand which displayed a wide range of variations in morphological and physiological characteristics. Most of those strains were isolated from leaves of tropical plants while only a few came from non-living surfaces (Prasongsuk et al., 2005). *A. pullulans* is of interest due to its abilities to produce the biopolymer pullulan and a number of industrial enzymes (amylases, xylanases and pectinases). In addition, Takesako et al. (1991 and 1993) reported the production of a cyclic decipeptide antibiotic named aureobasidin by a single specific strain of *A. pullulans* (R106, also referred to as BP-1938). This antibiotic exhibited antifungal activities against some fungal species including *Candida albicans*, *Saccharomyces cereviseae* and some *Aspergillus* spp. (Endo et al., 1997; Kurome et al., 1996; Takesako et al., 1993; Zhong et al., 2000). More recently, the gene *aba1* encoding an enzyme complex in aureobasidin A biosynthesis was cloned from *A. pullulans* R106 (Slightom et al., 2009). Up to present, the production of aureobasidin or any other antifungal agent(s) has never been reported in any of the tropical *A. pullulans* strains.

In this study, *A. pullulans* was isolated from different bathroom surfaces in Bangkok and vicinities, Thailand, and the obtained fungi were grown and subjected to assay for production of antifungal agents against four common *Aspergillus* species. Culture extracts that appeared positive for antifungal compounds were partially resolved by one-dimension thin layer chromatography and the resolved compounds were also tested by an *in situ* bioas-

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say for inhibitory effects against three selected species of *Aspergillus*.

MATERIALS AND METHODS

Fungal isolation

Fungal samples were collected from bathroom surface environs at different areas in Bangkok and vicinity using sterile cotton swabs. The swabs were aseptically smeared onto corn meal agar (CMA) and half- strength malt extract agar (MEA) plates. All *Aureobasidium*- like colonies were selected and subcultured on potato dextrose agar (PDA) and yeast malt agar (YMA) plates to pure cultures. The yeasts were then identified based on morphological observation (De Hoog and Yurlova, 1994; Domsch et al., 1993; Hermanides-Nijhof, 1977) and nutritional physiology test (Barnett et al., 2000). Identification of selected isolate was further confirmed by DNA sequencing of the ITS region. All yeast cultures were submitted into Fungal Section of the Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University (BCU), Bangkok, Thailand.

Detection of antifungal activity

Antifungal production was based on the method of Takesako et al. (1991) with slight modifications. Each selected *A. pullulans* isolate was grown in seed culture medium containing 0.67 % (w/v) yeast-nitrogen base and 2% (w/v) glucose for two days at room temperature (28 ± 2°C) with agitation before being transferred to production I medium containing (w/v) 2% glucose, 0.5% $(NH_4)_2SO_4$, 0.15% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2.2H_2O$, 0.01% NaCl, 0.5 ppm $FeCl_3.6H_2O$ and 0.5 ppm $ZnSO_4.7H_2O$ at one tenth ratio (v/v). After growing for 56 h, production II medium containing (w/v) 10% glucose, 5% Bacto-peptone, 2.5% $(NH_4)_2SO_4$, 0.75% KH_2PO_4, 0.25%$MgSO_4.7H_2O$, 0.05% $CaCl_2.2H_2O$, 0.05% NaCl, 2.5 ppm $FeCl_3.6H_2O$, and 2.5 ppm $ZnSO_4.7H_2O$ was added at one fifth ratio (v/v) and the culture was incubated for another 78 h. Cells were harvested by centrifugation (3824 x g, 15 min) prior to grinding and 95% (v/v) ethanol extraction. Cell debris was removed by filtration through Whatman paper and the crude extract was concentrated by evaporation.

The ethanol-dissolved compounds were then extracted by ethyl acetate three times. After final evaporation, the dried extracted was dissolved in a small volume of dimethylsulfoxide (DMSO, Sigma, USA) and stored at 4°C for further experiments.

The DMSO-dissolved extracts were tested for antifungal activity against the four *Aspergillus* species, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus terreus*, using a paper disc diffusion method (Tachibana et al., 2005) and a conidial germination inhibition assay (Lavermicocca et al., 2000).

For the paper disc diffusion test, each *Aspergillus* species spore suspension comprising 1 x 10^5 cells/ml in 1% (v/v) Tween 80 was evenly spread onto each PDA plate. Four paper discs (6 mm) either blank (negative filter toxicity control) or separately soaked with one of DMSO (solvent negative control), DMSO-dissolved extracts or aureobasidin A (Sigma, USA; 0.1 mg/ml in DMSO; reference standard and positive control) were placed on each *Aspergillus*-spread plate. All plates were incubated at room temperature for four days. Clear zones were detected and the ratios between the diameter of each clear zone of DMSO-dissolved extract and that of aureobasidin A were calculated. For the conidial germination assay, each DMSO-dissolved extract was added to *Aspergillus* spore suspensions comprising 1 x 10^3 cells/ml in 1% (v/v) Tween 80 at a one tenth ratio and the suspension mixtures were incubated at room temperature (28 ± 2°C) for 30 min with agitation. Spore suspensions mixed with DMSO were carried out as negative control. After incubation, the suspension mixtures were spread on PDA plates (0.1 ml per plate) and incubated at room temperature for three days. The number of growing colonies was counted and the percent germination was calculated (100% germination in negative control). All experiments were conducted in triplicate.

Analyses of compounds

DMSO-dissolved extracts of selected *A. pullulans* isolates and aureobasidin A (0.1 mg/ml DMSO) were spotted on a TLC plate (Silica gel 60. Merck, Germany). Ethyl acetate was used as the solvent and the separated compounds were detected by iodine fumigation. The retention factor (Rf) value of each separated compound was calculated. To test the antifungal activity of separated compounds, the resolved TLC plates were subjected to an *in situ* bioassay by spraying with the appropriate *Aspergillus* species spore suspension (1 x 10^6 cells/ml in tomato juice) and incubating for three days at room temperature. Thereafter, fungal hypha growing on the plates were stained with lactophenol blue for 5 min and destained with 5% (v/v) acetic acid for 10 min. Clear zones were observed and Rf values were calculated. All experiments were carried out in triplicate.

RESULTS

Ten isolates of *A. pullulans* collected from various bathroom surfaces in the Bangkok vicinity, Thailand were submitted into BCU including BM1, KT1, HKW1, HKW2, HKW3, HKW4, JB1, PH1, TB1 and VM1 (voucher material: Denarn #001 to 010 respectively). They were identified based on morphological characteristics and nutritional physiology profiles. More than one type of cells such as pseudohypha, clamydospores, blastospores, budding cells and swollen cells which were typical of *A. pullulans* were observed in all isolates (data not shown). Variation in colony color was also detected when the fungi were cultivated on MEA for seven days. The nutritional physiology profiles of all isolates corresponded well to those of standard strains, *A. pullulans* NRRL Y-2311-1 and *A. pullulans* NRRL Y-7469 (Table 1). They were able to utilize various carbon sources such as D-glucose, D-galactose, D-fructose, D-cellobiose, D-mannose, D-xylose, D-sucrose, D-salicin, mannitol, methyl-α-D-glucose, β-lactose, and starch while α-cellulose, ethanol, and methanol were not assimilated. A range of nitrogen sources were utilized by all isolates including ammonium acetate, am-monium chloride, ammonium nitrate, ammonium sulfate, glycine, asparagine, lysine, sodium nitrate, and urea. The ITS region of one isolate, BM1, was amplified and sequenced (GenBank accession number EU719542), and the nucleotide sequence comparison using Blast al-gorithm indicated that this isolate was *A. pullulans* (data not shown).

Out of the ten isolates, extracts of four isolates (BM1, HKW1, HKW2, and KT1) exhibited a significant inhibitory effect against at least one of the tested four *Aspergillus* species. Extracts from all four *A. pullulans* isolates could inhibit *A. terreus* with clear zone ratios of 1.00, 0.87, 1.00 and 0.53 for BM1, KT1, HKW1 and HKW2, respectively, whilst failed to affect *A. niger*. Furthermore, the KT1 extract could inhibit *A. fumigatus* (clear zone ratio of 1.00)

Table 1. Carbon and nitrogen utilization profile of selected *Aureobasidium pullulan* isolates showing antifungal activity in this study.

Carbon and nitrogen source	Isolate					
	KT 1	BM 1	HKW 1	HKW 2	NRRL Y -2311-1[a]	NRRL Y-7469[a]
D-arabinose	+	w	+	w	nd	nd
L-arabinose	+	+	+	+	nd	nd
D-cellobiose	+	+	+	+	+	+
α-Cellulose	-	-	-	-	-	-
Ethanol	-	-	-	-	nd	nd
D-fructose	+	+	+	+	+	+
D-galactose	w	w	+	w	+	+
β-D-glucose	+	+	+	+	+	+
Glycerol	+	w	w	+	w	w
β-lactose	+	+	+	+	+	+
D-maltose	+	+	+	+	+	+
Maltrotriose	+	+	+	w	nd	nd
Mannitol	+	+	+	+	+	+
D-mannose	+	+	+	+	nd	nd
D-melezitose.2H_2O	+	+	+	+	nd	nd
Methanol	-	-	-	-	nd	nd
Methyl-α-D-glucose	+	+	+	+	+	+
D-salicin	+	+	+	+	+	+
Sorbitol	+	w	+	+	+	+
L-sorbose	w	w	+	+	nd	nd
Starch (soluble)	+	+	+	+	+	+
D-sucrose	+	+	+	+	+	+
D-trehalose.2H_2O	+	+	+	+	+	+
D-xylose	+	+	+	+	+	+
Ammonium acetate	w	w	w	+	nd	nd
Ammonium chloride	+	+	+	+	nd	nd
Ammonium nitrate	+	+	+	+	nd	nd
Ammonium sulfate	+	+	+	+	nd	nd
L-glycine	+	+	+	+	+	+
L-asparagine. H_2O	+	+	+	+	+	+
L-Lysine	+	+	+	+	+	+
Sodium nitrate	+	+	+	+	+	+
Urea	+	+	+	+	nd	nd

Growth was determined by OD_{600} measurement after 7-day cultivation.
+: growth OD >0.05) - : no growth OD <0.02) : weak growth 0.05>OD >0.02) nd = not determined
[a] standard isolates, . *pullulans* NRRL Y-2311-1 *A. pullulans* NRRL Y-7469 Prasongsuk et al. 2005).

whilst that of BM1 was found to be very effective against both *A. fumigatus* and *A. flavus* with clear zone ratios of 1.00 and 0.70 respectively.

Similar results were obtained from the conidial germination assay where extracts of these four isolates inhibitted conidial germination in at least one *Aspergillus* species. The percent inhibition ranged from 39 to 63 % depending on both the *A. pullulans* isolate and *Aspergillus* species tested. *A. terreus* was inhibited by extracts from all four isolates with 63, 49, 42, 39 % inhibition for BM1, KT1, HKW2 and HKW1 extracts, respectively. The growth of *A. fumigatus* could be inhibited by extracts of BM1 (47 %) and KT1 (43 %), whilst that of *A. flavus* could only be inhibited by the extract of BM1 (63 %).

The extracts of all four *A. pullulans* isolates (BM1, KT1, HKW1 and HKW2) were subjected to TLC. Three compounds (Rf values of 0.40, 0.55 and 0.675) were detected in extracts of BM1, HKW1 and HKW2 whereas only two compounds (Rf values of 0.40 and 0.675) were detected in the KT1 extract (Figure 1) . An analytical grade aureobasidin A was also included as a reference standard and positive control in this experiment, yielding an Rf of 4.0.

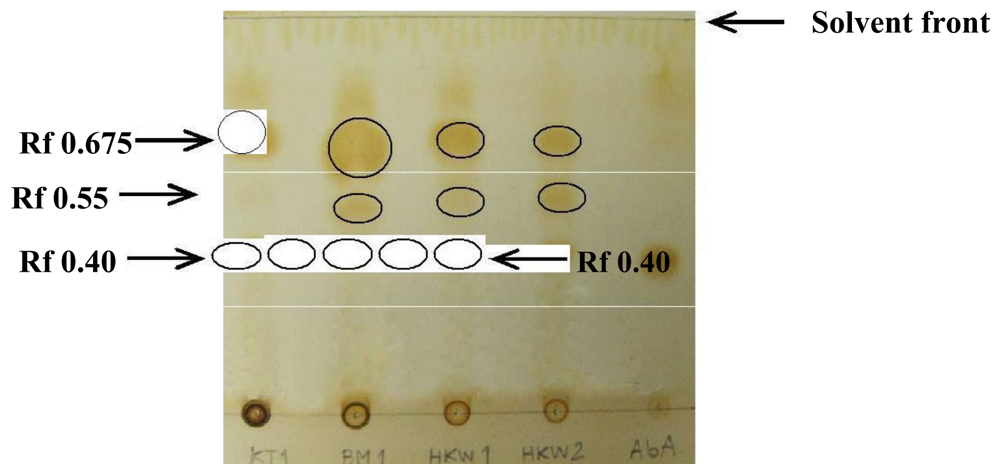

Figure 1. Thin layer chromatogram resolution of extracts of *A. pullulans* isolates KT1 (lane 1), BM1 (lane 2), HKW1 (lane 3) and HKW2 (lane 4), and the reference standard aureobasidin A (lane 5). The TLC solvent was ethyl acetate and the chromatogram was developed by iodine fumigation.

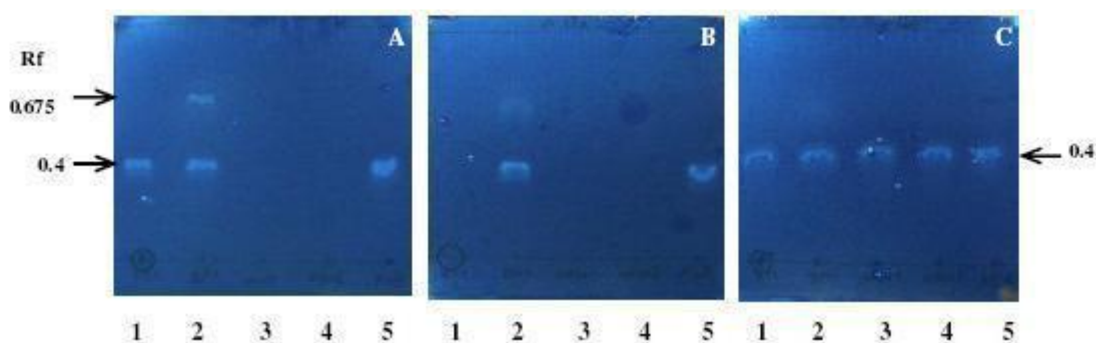

Figure 2. Thin layer chromatogram showing inhibition zones against *A. fumigatus* (A); *A. flavus* (B); and *A. terreus* (C). Lane 1: extract of KT1; lane 2: extract of BM1; lane 3: extract of HKW1; lane 4: extract of HKW2; and lane 5: aureobasidin A.

When the resolved TLC plate was sprayed with an *A. terreus* spore suspension and incubated at room temperature for three days, compound(s) with an Rf value of 0.40 in all extracts inhibited spore germination and the hyphal growth of the fungus (Figure 2). Additionally, antifungal activity against *A. fumigatus* was also detected in compound(s) with an Rf value of 0.4 for KT1 and with Rf values of 0.4 and 0.675 for BM1 extracts. In contrast, antifungal activity against *A. flavus* was only detected in compounds with Rf values of 0.40 and 0.675 in extracts of BM1.

DISCUSSION

Major reports of aureobasidin appeared to have come from *A. pullulans* strain R106 (Kurome et al., 1996; Takesako et al., 1991 and 1993; Slightom et al., 2009). None has been reported before among the tropical isolates. Moreover, previous studies on tropical isolates emphasized more on isolates from phylloplane habitats rather than other types of ecological niche (Prasongsuk et al., 2005). In the attempt to search for *A. pullulans* in an abiotic environment, we found that the occurrence of the fungus on bathroom surfaces around Bangkok was not uncommon. *A. pullulans* isolates were identified based on morphological characteristics and nutritional physiology profiles.

Different types of cells and variation in colony color were observed which were not uncommon for the fungus (Prasongsuk et al., 2005). The nutritional utilization profiles corresponded well to those of several *A. pullulans* strains previously isolated from Thailand (Prasongsuk et al., 2005) . Moreover, the inability to utilize cellulose by *A. pullulans* has been reported (Prasongsuk et al., 2005; De

Hoog and Yurlova, 1994).

Results from paper disc diffusion and conidial germination inhibition tests showed that four *A. pullulans* isolates had antifungal activity against selected *Aspergillus* spp.

The extract of BM1 in particular was found to be very effective in both terms of the number of species inhibited and the percentage of inhibition. The results also suggested that the antifungal agents produced by *A. pullulans* in this study could inhibit both hyphal growth and spore germination. This finding corresponded with earlier reports that aureobasidin A could inhibit *A. niger* , *A. flavus* , *A fumigatus*, and *A. terreus* via the inhibition of inositol phosphoryceramide synthase, the key enzyme in fungal sphingolipid biosynthesis (Takesako et al., 1993; Zhong et al., 2000).

Antifugal agents produced by all *A. pullulans* isolates were further investigated by TLC. The close similarity (no detectable difference by one-dimensional resolution) of the Rf value (0.40) between the apparent antifungal agents produced by all *A. pullulans* isolates obtained in this study and aureobasidin A suggested a possible common identity. However, the inhibition of different *Aspergillus* species was not an exact match making it possible that these antifungal agents might be aureobasidin with different structural variations or different forms of antifungal antibiotics as detected from their TLC profiles. Relevantly, eighteen types of aureobasidin, each different in functional groups, have so far been reported (Ikai et al., 1991).

Considering the fact that most studies of aureobasidin A appeared to concentrate only on a single strain, R106, it was rather significant to find this type of inhibitory compound(s) existed quite commonly (40%) among the tropical *A. pullulans* isolated from the bathroom surfaces. Further investigation including the purification and analysis to positively identify the compounds which could perhaps lead to the discovery of new forms of antibiotics with different activities or specificities remains to be elucidated.

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