World Journal of Microbiology & Biotechnology (2005) 21:393–398
DOI 10.1007/s11274-004-2237-x

Thailand habitats as sources of pullulan-producing strains of *Aureobasidium pullulans*

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Keywords: *Aureobasidium pullulans*, exopolysaccharide, pullulan

Summary

A variety of habitats were sampled for the presence of *Aureobasidium* black yeasts with the attempt to find pullulan-producing strains. Habitats included leaves of mango (*Mangifera indica* Linn.), tamarind (*Tamarindus indica* Linn.), asoka (*Saraca indica* Linn.) and latex-painted and bathroom cement-wall surfaces. Parameters for the identification of the isolates included morphology, nutritional parameters, exopolysaccharide (EPS) production, and rDNA internal transcribed spacer (ITS) sequencing. All isolates of black yeasts were polymorphic with blastospores, hyphae, and chlamydospores. ITS analyses showed strong correlation with the GenBank *A. pullulans* sequences, with alignment using BLAST yielding greater than 95% similarity. All five isolates tested produced pullulan as deduced from infrared spectra and sensitivity to pullulanase. None produced aubasidan as evidenced from their IR spectra. The current studies support the notion that the hot, humid environments facilitate the development of *A. pullulans* and its tropical variants in diverse phylloplane and walls habitats, and merit support for further isolation and characterization of these black yeasts as a source of unique pullulan-producing strains.

Introduction

Aureobasidium pullulans is a yeast-like fungus common in a wide variety of environments from plant leaves to damp indoor surfaces. It is an ascomycetous yeast in the Order Dothideales, Family Dothideaceae. This species comprises two varieties, var. *pullulans* and var. *aubasidani* which are distinguished by molecular characteristics, nutritional assimilation patterns, and exopolysaccharide (EPS) structure (Yurlova & De Hoog 1997). This fungus is useful in a range of applications including being a potential source of industrial enzymes (amylase, xylanase, and pectinase), single cell protein, and the polysaccharide gum, pullulan (Deshpande *et al.* 1992; Leathers 2003). Pullulan, an extracellular linear homopolysaccharide, is composed of repeating maltotriose subunits linked through α-1,6 glucosidic bonds. Pullulan is exploited in various industries including pharmaceutical, food, electronic, and cosmetic companies (Leathers 2003).

A. pullulans is well recorded in the temperate-zones; however, in the tropics (such as Thailand), reports are scarce. Tokomasu *et al.* (1997) found *A. pullulans* as part of the fungal communities of pine-needle leaf litter on the pine forest of northern Thailand. Punnapayak *et al.* (2003) isolated airborne *A. pullulans* from various locations in Thailand. These appear the only major published reports of this black yeast found in Thailand. Moreover, though a phylloplane colonizer, there are no previous reports on the isolation of *A. pullulans* from fresh plant leaves or building surface environs in Thailand.

In this investigation, this fungus was isolated from diverse phylloplane habitats in Thailand and identified using morphology, nuclear ribosomal DNA internal transcribed spacer (ITS) sequencing, nutritional physiology, and determination of their EPS.

Materials and methods

Isolation of fungi

Fresh plant leaves (*Mangifera indica* Linn., *Tamarindus indica* Linn., *Hibiscus rosa-sinensis* Linn., *Ochna kirkii* Oliv., *Bougainvillea spectabilis* Linn., *Saraca indica* Linn., *Cassia fistula* Linn., *Eugenia uniflora* Linn., *Annona squamosa* Linn. and *Artocarpus heterophyllus* Lam.) were collected and disks (0.6 mm) were aseptically cut and placed on selective media plates-Corn Meal Agar (CMA) and Malt Extract Agar (MEA)-half strength. Other fungal habitats sampled included

bathroom cement-walls and latex-painted surfaces. Sterile cotton swabs were used for collection and this inoculum was smeared onto selective media plates in triplicate. All cultures were incubated at room temperature (30 °C). The initial yeast colonies were purified by using cross-streaking on Potato Dextrose Agar (PDA) and repeated until colony pure cultures were obtained. CMA, MEA, and PDA were from Difco (Detroit, MI).

Fungal identification

Morphological observation

Slide cultures were made using PDA, which were stained with lactophenol-cotton blue and observed by wet mounting using bright field microscopy. The colony characteristics were observed daily. The *Aureobasidium* strains were compared with the standard strains, *A. pullulans* ATCC 42023 and NRRL 6992, and the descriptions of *Aureobasidium* by Barnett & Barry (1998), Domsch *et al.* (1993), and Hermanides-Nijhof (1977).

Nuclear ribosomal DNA internal transcribed spacer (ITS) Sequencing

Fresh cells from the *A. pullulans* cultures were ground in liquid nitrogen and genomic DNA extracted using the Dneasy Plant Protocol (Quiagen, Inc., Valencia, CA). The 5.8S rDNA and flanking ITS regions (ITS1&2) were amplified from 2 μl of undiluted genomic DNA in a 100 μl reaction using the primers ITS5 and ITS4 (White *et al.* 1990). Each reaction contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 12.5 pmol each dNTP, 50 pmol each primer, and 2 U Taq polymerase (Desai & Pfaffle 1995). PCR (25 cycles) was carried out using a GeneAmp 9600 thermocycler (Perkin-Elmer Corporation, Foster City, CA) set to 95 °C for 10 s, 56 °C for 30 s, and 72 °C for 1 min. Initial denaturation was conducted at 95 °C for 1 min with a final extension for 10 min at 72 °C. Successful PCR products were cleaned of primers and salts, using the QIAquick PCR Purification Kit (Quiagen, Inc., Valencia, CA). ABI PRISM® BigDye Terminators v3.0 Cycle Sequencing reactions (Applied Biosystems, Foster City, CA) were prepared according to the manufacturer's protocol, using primers ITS5 and ITS4 and the PCR product as template (White *et al.* 1990). Reactions were analysed on an ABI PRISM® 3100 Automated DNA Sequencer (Applied Biosystems, FosterCity, CA).

Nutritional physiology tests

Carbon and nitrogen assimilation were investigated according to Barnett *et al.* (1990). Inocula were cultivated in the Yeast Malt Broth (YMB) (Difco, Detroit, MI). The carbon (0.5 M, 0.5 ml) or nitrogen source (0.5 M, 0.5 ml) was added to 10× yeast nitrogen base (Difco, Detroit, MI) (0.5 ml) (Difco yeast carbon base for nitrogen assimilation) plus 4 ml of sterile distilled water. An inoculum (100 μl) of yeast culture (2.5×10^7 cell/ml) was added. Cultures were incubated at 25 °C. Distilled water was used as a control. Growth was assessed by cell turbidity of the dispersed mycelium.

EPS production and analysis

EPS was prepared by growing cultures in a production medium (PM) in shake flasks (100 ml/ 250-ml flask, 150 rev/min, room temperature). PM contained glucose (5%); $(NH_4)_2SO_4$ (0.06%); K_2HPO_4 (0.5%); $MgSO_4.7H_2O$ (0.04%); NaCl (0.1%); and yeast extract (0.04%), with the pH adjusted to 6.5. EPS was recovered after 5 days by removing the yeast mycelium by centrifugation ($10{,}000 \times g$, 15 min), and precipitating the EPS from the culture supernatant with 95% ethanol (2:1, ethanol:supernatant). EPS was dried at 60 °C.

The pullulan content was estimated by sensitivity to pullulanase (EC 3.2.1.41) from *Klebsiella pneumoniae* (Sigma, St. Louis, MO) according to Leathers *et al.* (1988). The IR spectra were determined using the potassium bromide (KBr) technique on an FTIR spectrometer (Perkin-Elmer, Norwalk, CT). Pullulan (Sigma) was used as the control standard.

Results and discussion

Aureobasidium spp. were isolated from different habitats around Thailand including a bathroom cement-wall (isolate BK4), a latex-painted surface (isolate BK6), and leaves of mango (*Mangifera indica* Linn.) (isolate NRM2), asoka (*Saraca indica* Linn.) (isolate LB3), and tamarind (*Tamarindus indica* Linn.) (isolate SK3). The isolates were generally recovered using MEA half strength. Isolate NRM2 were isolated using CMA half strength.

Examination of the cell morphology of the isolates by bright field light microscopy showed the classic *A. pullulans* polymorphology with blastospores, hyphae, and chlamydospores. The colonies grew rapidly, were smooth, slimy, pale pink or cream and became black with time (Figure 1). Isolates NRM2 and SK3 produced a pink and a yellow pigment, respectively, characteristic of so-called 'colour-variant' strains (Wickerham & Kurtzman 1975). The colony sizes ranged between 2.86 and 4.75 cm on the MEA after 7 days. Both morphological and colony characteristics corresponded well with the *A. pullulans* descriptions by Barnett & Barry (1998), Domsch *et al.* (1993), and Hermanides-Nijhof (1977) and to features of standard strains, ATCC 42023 and NRRL 6992.

Sequences for isolates BK4, BK6, NRM2, and LB3 were identical to each other and identical to other *A. pullulans* sequences in GenBank, including the following: AF121284 (ATCC 42457), AY 139395 (CBS 110373), AY 139393 (CBS 110376), AY 139392 (CBS 110375), AJ244236 (CBS 101160), AY 139391 (CBS 110377),

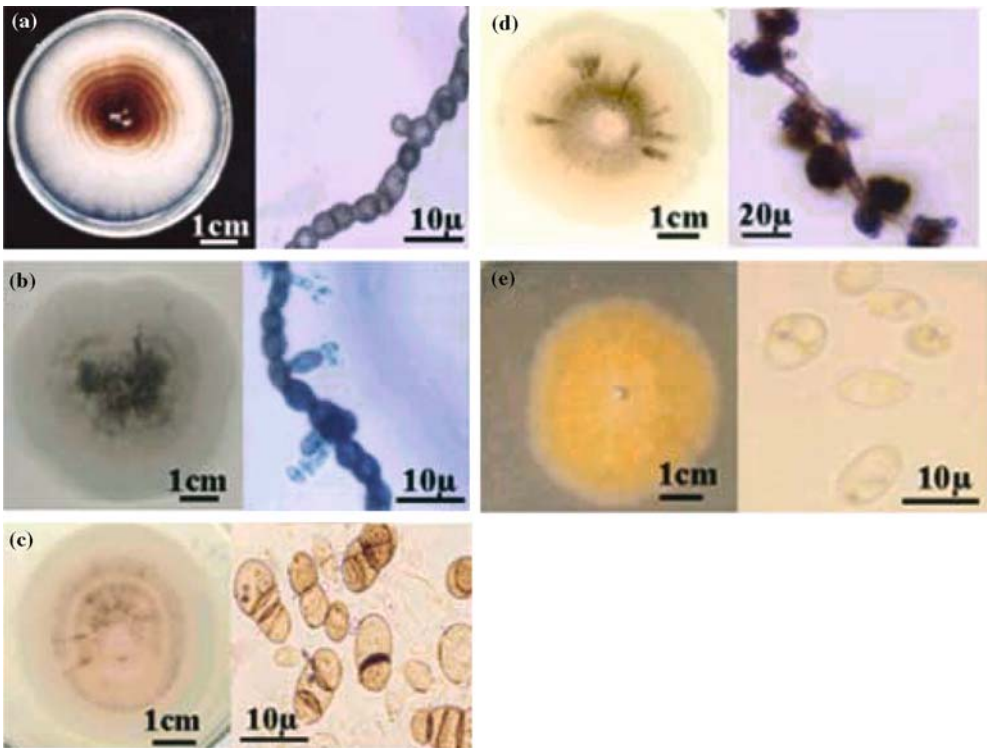

Figure 1. Colony and morphology of *Aureobasidium* isolates. (A) colony and hypha of isolate BK 4, (B) colony and conidial apparatus of isolate BK6, (C) colony and chlamydospores of isolate NRM2, (D) colony and hypha with conidia of isolate LB3, (E) colony and blastospores of isolate SK3.

AJ244269 (VKPM F-371), AJ276062 (MZ58) and AJ276061 (MZ65). The sequence for SK3 differed slightly from the other four by a single T to A transversion in the ITS1 and a single deletion (T) in the ITS2. Strain SK3 was more similar to sequences for isolates BK4, BK6, NRM2 and LB3 than to any other sequence in GenBank. The sequences were submitted to GenBank with the following accession numbers AY225163, AY225164, AY225165, AY225166, AY225167, respectively for the isolates BK4, BK6, NRM2, SK3, and LB3.

The carbon and nitrogen assimilation patterns of the isolates correlated with the assimilation patterns of the control strains (Tables 1 and 2). A diverse range of carbon sources was utilized including cellobiose, dulcitol, fructose, galactose, glucose, glycerol, methyl-α-D-glucoside, raffinose, sucrose, xylitol, and xylose, while cellulose, chitin, *p*-coumaric acid, sodium succinate, and sodium salicylate were not assimilated. Intra-specific variation of *Aureobasidium* isolates and standard strains was found in assimilation of dulcitol, glucosamine, sodium citrate (Table 1). Okagbue *et al.* (2001) reported that Zimbabwean isolates of *A. pullulans* (de Bary) Arnaud utilized a broad range of substrates including cellobiose, glucose, glycerol, sucrose, xylan, and xylose. Other workers reported *A. pullulans* to utilize cellobiose but not cellulose (Dennis & Buhagiar 1973; De Hoog & Yurlova 1994). Federici (1982) also noted a lack of cellulase activity. Chitinase activity was not detected from this fungus (Federici 1982; De Hoog & Yurlova 1994). The results are in agreement with previous reports in which *A. pullulans* was distinguished from *A. prunorum* and *Trichosporon pullulans* by its ability to utilize glycerol and galactose (Dennis & Buhagiar 1973). De Hoog & Yurlova (1994) noted that *A. pullulans* could utilize methyl-α-D-glucoside while *Hormonema* sp. could not. All isolates also utilized lactose and methyl-α-D-glucoside, in agreement with the data of *A. pullulans* var. *pullulans* (Yurlova & De Hoog 1997).

Nitrogen sources that were utilized included L-arginine, creatinine HCl, L-isoleucine, L-lysine, L-serine, sodium nitrate, sodium nitrite, and L-tryptophane but not creatine monohydrate, and L-threonine. Cooke & Matsuura (1963) reported that while *A. pullulans* P-66 assimilated a range of nitrogen sources including amino acids, it could not assimilate L-lysine. In contrast, Cernakova *et al.* (1980) and De Hoog & Yurlova (1994) stated that many tested strains of *A. pullulans* were able to utilize L-lysine. General utilization of amino acids is clear (Table 2), though the inability of specific strains to use asparagine, alanine, glutamine, proline, leucine, phenylalanine, and glycine is evident.

The EPS of all isolates showed sensitivities to pullulanase between 56 and 97% (Table 3). An apparent correlation between greater pullulan production by the lesser pigmented isolates was observed. This possibility was found by the previous reports (Leathers *et al.* 1988; West & Reed-Hamer 1993; Punnapayak *et al.* 2003).

Table 1. Carbon assimilation pattern of *Aureobasidium* isolates from Thailand.

Carbon substrates/Strains	BK4	BK6	SK3	NRM2	LB3	NRRL Y-2311-1[a]	NRRL Y-7469[a]
1. Caffeic acid	–	–	w	–	–	–	-
2. D-Cellobiose	+	+	+	+	+	+	+
3. Cellulose powder (1% fibrous)	–	–	–	–	–	–	–
4. Chitin (colloidal)	–	–	–	–	–	–	–
5. *p*-Coumaric acid	–	–	–	–	–	–	–
6. D-Glucose	+	+	+	+	+	+	+
7. Dulcitol	+	+	+	+	+	–	+
8. Fructose	+	+	+	+	+	+	+
9. D-Galactose	+	+	+	+	+	+	+
10. D-(+)-Glucosamine	w	w	–	–	–	+	–
11. Glycerol	+	+	w	+	+	w	w
12. *Myo*-inositol	+	+	+	+	+	+	+
13. Lactose	+	+	+	+	+	+	+
14. Mannitol	+	+	+	+	+	+	+
15. Methyl-α- D-glucoside	+	+	+	+	+	+	+
16. Maltose	+	+	+	+	+	+	+
17. Quinic acid	+	+	+	+	+	+	+
18. Raffinose	+	+	+	+	+	+	+
19. Rhamnose	+	+	–	+	+	+	+
20. Ribose	+	+	+	+	+	+	+
21. Sodium citrate	–	–	–	–	+	+	+
22. D–Sorbitol	+	+	+	+	+	+	+
23. Sodium succinate	–	–	–	–	–	–	–
24. Sodium acetate	–	–	–	–	–	–	w
25. Sodium salicylate	–	–	–	–	–	–	–
26. Starch (soluble)	+	+	+	+	+	+	+
27. Sucrose	+	+	+	+	+	+	+
28. Salicin	+	+	+	+	+	+	+
29. Trehalose	+	+	+	+	+	+	+
30. D-Xylose	+	+	+	+	+	+	+
31. D-Xylitol	+	+	+	+	+	+	+

[a] Standard strains, *A. pullulans* NRRL Y-2311-1 and *A. pullulans* NRRL Y-7469.
+ = assimilation, – = non-assimilation, w = weak assimilation.

Table 2. Nitrogen assimilation pattern of *Aureobasidium* isolates from Thailand.

Nitrogen substrates/Strains	BK4	BK6	SK3	NRM2	LB3	NRRL Y-2311-1[a]	NRRL Y-7469[a]
1. L-Aspartic acid	+	w	+	+	+	+	+
2. L-Threonine	–	–	–	–	–	–	–
3. L-Asparagine	–	–	–	+	–	+	+
4. Lysine	+	+	+	+	+	+	+
5. L-Methionine	w	w	+	+	+	+	+
6. Creatine monohydrate	–	–	–	–	–	–	–
7. L-Valine	+	+	+	w	+	+	+
8. Sodium nitrite	+	+	+	+	+	+	+
9. Sodium nitrate	+	+	+	+	+	+	+
10. Creatinine	+	+	+	+	+	+	+
11. L-Alanine	w	+	+	–	+	+	+
12. L-Arginine	+	+	+	+	+	+	+
13. L-Serine	+	+	+	+	+	+	+
14. L-Tryptophan	+	+	+	+	+	+	+
15. L-Ornithine	+	w	+	+	+	+	+
16. L-Glutamic acid	+	+	+	+	+	+	+
17. L-Glutamine	+	w	+	–	+	+	+
18. L-Proline	+	+	+	–	–	+	+
19. L-Leucine	+	+	+	+	–	+	+
20. L-Isoleucine	+	+	+	+	+	+	+
21. L-Phenylalanine	+	+	+	–	–	+	+
22. Glycine	+	+	–	+	+	+	+
23. L-Histidine	w	+	+	+	+	+	+

[a] Standard strains, *A. pullulans* NRRL Y-2311-1 and *A. pullulans* NRRL Y-7469.
+ = assimilation, – = non assimilation, w = weak assimilation.

Table 3. Pullulan content and degree of pigmentation of the EPS.

Isolates	Pullulan content (%)[a]	Degree of pigmentation[b]
BK4	97	+
BK6	56	+ + +
NRM2	61	+ +
LB3	80	+
SK3	90	+

[a] % Pullulan content was calculated from the amount of reducing sugar (maltotriose equivalent) released from the reaction between the EPS and pullulanase enzyme.
[b] Degree of pigmentation was determined by visual observation.

Leathers *et al.* (1988) noted that melanin, which contaminated pullulan, could be inhibitory to pullulanase.

The IR spectra of EPS from all isolates were similar to that of the pullulan standard (Figure 2), with a pullulan-like peak at around $\lambda = 850\ \text{cm}^{-1}$ which indicates the ∝-configuration within the EPS (Yurlova & De Hoog, 1997). Madi *et al.* (1997) also reported a peak at $\lambda = 859.6\ \text{cm}^{-1}$of EPS from *A. pullulans* (de Bary) Arnaud (IMI145194) which they interpreted as an ∝-configuration.

In conclusion, *A. pullulans* was successfully isolated from distinct habitats in Thailand. This furthers our knowledge of the occurrence of this organism in tropical climates. The *A. pullulans* isolates were from very different habitats from leaves to painted surfaces. On the basis of morphology, nutritional physiology, ribosomal DNA ITS sequencing, and the types of EPS, all isolates were identified as *A. pullulans* var. *pullulans*. Isolates included typical black colonies and colour variants. Although *Aureobasidium* is ubiquitous, colour variant strains have thus far only been isolated from tropical or subtropical sites. Because of the polymorphism of this fungus, morphological criteria are not sufficient for identification; however, molecular techniques (ITS sequencing) were also unable to resolve the isolates. Differences in nutritional physiology and EPS characterization were useful to define the isolates. All isolates produced a pullulan EPS, a commercial biopolymer gum raising the concept that further *Aureobasidium* isolates from Thailand should be evaluated for potential commercial exploitation.

Acknowledgement

The authors wish to thank The Royal Golden Jubilee (RGJ) Ph.D. grant 4.S.CU/42/Q1 contract number PHD/0143/2542, The Thailand Research Fund and Eveleigh-Fenton fund for the financial support. We thank Cletus P. Kurtzman, Timothy D. Leathers (UDSA, Peoria, IL), James F. White Jr., Marshall Bergen (Plant Pathology/Biology, Rutgers University) for helpful discussion. This research was also supported by the establishment fund of the Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, and the Project for the Promotion of Efficiency and Capability of the National Competition by the Ministry of Education, Thailand.

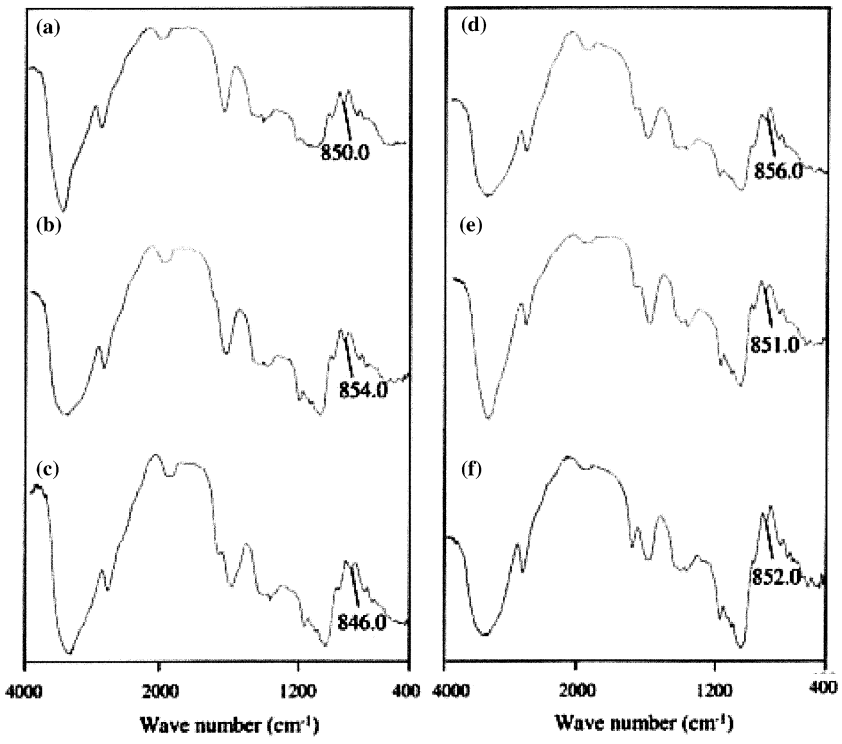

Figure 2. Infrared (IR) spectra. (a) Pullulan standard (Sigma), (b) EPS from *Aureobasidium* sp. BK4, (c) EPS from *Aureobasidium* sp. BK6, (d) EPS from *Aureobasidium* sp. NRM2, (e) EPS from *Aureobasidium* sp. LB3, (f) EPS from *Aureobasidium* sp. SK3.

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