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Original Article

Antimicrobial Activity of Indigenous Strains of *Aureobasidium* Isolated From *Santalum Album* Leaves

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Abstract

Although more than 3000 antibiotics have been reported from Actinomycetes/ higher fungi; biotechnological potential of yeasts and yeast-like fungi with respect to production of antimicrobial compounds has not been sufficiently investigated. We examined the antimicrobial activity of 11 strains of *Aureobasidium pullulans* (two new isolates and nine standard strains). All the strains of *Aureobasidium pullulans* inhibited *Ps. fluorescen s,* but none of these strains could inhibit *Candida albicans* and *S. cerevisiae*. Interestingly, the yeast *Pichia angusta* was inhibited by six of the *A. pullulans* strains used in the present investigation. Two indigenous isolates of *Aureobasidium* Natural Isolate 1 (NI. 1) and Natural Isolate 2 (NI. 2) showed antibacterial activity against the Gram-negative cultures; most of which were resistant to Gentamicin. This study provides evidence that *A. pullulans* is a promising producer of antimicrobial agents for better chemotherapeutic agents, possibly against Pseudomonals infections.

Keywords: *Aureobasidium*; Antimicrobial; Pseudomonas.

Introduction

Aureobasidium pullulans (de Bary) Arn. is a cosmopolitan yeast- like fungus that occurs in diverse habitats, including the phyllosphere of many plants and also on various tropical fruits (1, 2). *Aureobasidium pullulans* is industrially important because of its' capacity to produce the polysaccharide "pullulan" (3-6). In addition, it produces various potentially useful products such as xylanase (7-8), β-D- fructofuranoside fructohydrolase (4, 9), sucrase (10), esterase (11) and β-galactosidase (4). Moreover, World Health Organization has placed *A. pullulans* within risk "Group I" (12), where there is no possibility of infection to either society or laboratory workers. There are relatively few reports on antimicrobial compounds obtained from various yeasts and yeast-like fungi (13), and very little work has been carried out on antibacterial activity of *A. pullulans*.

McCormack *et al*. (14) reported for the first time the inhibition of *Ps. aeruginosa* and *Staphylococcus aureus* by compounds obtained from *A. pullulans*. Takesako *et al*, (15) reported a group of antifungal antibiotics, named aureobasidins, from *A. pullulans. Aureobasidium* appears to be a promising organism for development of newer antimicrobial agents, both for chemotherapy as well as non-medical applications.

This article reports on the isolation of two natural isolates of *Aureobasidium pullulans*

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strains from the leaf surface of *Santalum album*. Studies on antimicrobial activity of these two isolates and nine standard cultures of *Aureobasidium pullulans* were carried out.

Experimental

Organism and their cultivation:

Target Organisms: *Pseudomonas aeruginosa, Pseudomonas Putida, Pseudomonas fluorescens, Klebsiella pneumoniae, Acinetobacter calcoaceticus,*

Escherichia coli, Salmonella typhi, Staphylococcus aureus, and Bacillus spp were maintained on nutrient agar slants containing 20% glycerol and stored at 4-10ºC. *Candida albicans, Saccharomyces cerevisiae, Pichia angusta* and *A. Pullulans* strains were maintained on SDA medium containing 20% glycerol and stored at 4-10ºC (Table 1).

Isolation of Aureobasidium Pullulans from aerial surfaces of plants:

Aureobasidium pullulans strains were isolated from Santalum album leaves, essentially based on a procedure described by Pollock *et.al.* (6). Briefly, Leaves of *Santalum album* were washed gently with water and cut into small pieces, 0.5 g of which was soaked in 10 ml of sterile distilled water and then incubated for three days at 28º C on a shaker set at 120 rpm. An aliquot of 0. 1 ml was transferred to a 10 ml medium, which included the following (per liter of deionised water) ingredients: 2.0g of yeast extract, 0.5 g of $(NH_4)_2Hpo_4$, 1.0 g of NaCl, 0.2 g of $MgSO_4$. $7H_2O$, 3.0g of K_2HPO_4, 0.01 g each of $FeSO_4$, $MnSO_4$ and $ZnSO_4$ pH7, 20.0g Sucrose and 10 mg ml^{-1} of chloramphenicol. After two days of shaking at 28º C, the turbid culture was allowed to stand stationary to settle down filaments and aggregates. bout 100 µl of the diluted sample was spread on the same medium, and looked upon for isolated colonies after 2- 6 days. The cultures were observed periodically, under a phase contrast microscope, to examine their morphology.

Biochemical characterization of natural isolates of A. pullulans:

Our natural isolates were compared to standard strains of *A. pullulans,* using the auxanogram method, in order to determine the utilization of Carbon and Nitrogen sources (16). Based on these, tests similarity coefficients of natural isolates with the standard *A. pullulans* ATCC 16625 were calculated as S=a / a+b+c, where a= number of positive characteristics shared by the two strains; b= number of positivecharacteristics in the first strain when the second strain was negative; c= number of positivecharacteristics in the second strain when first strain was negative. Cultural characteristics of all the *A. pullulans* strains were studied on potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) after incubation for 2-6 days.

Table 1. Organisms used in this study.

Sr.No.	Organism	Source
1	*Aureobasidium pullulans* 1049	NCIM*
2	*Aureobasidium pullulans* 1048	NCIM
3	*Aureobasidium pullulans* 976	NCIM
4	*Aureobasidium pullulans* 1226	NCIM
5	*Aureobasidium pullulans* 153	MTCC**
6	*Aureobasidium pullulans* 1991	MTCC
7	*Aureobasidium pullulans* 2013	MTCC
8	*Aureobasidium pullulans* 2160	MTCC
9	*Aureobasidium pullulans* 16625	ATCC
10	*Aureobasidium pullulans* NI. 1	Our isolate***
11	*Aureobasidium pullulans* NI.2	Our isolate
12	*Escherichia coli* 2810	NCIM
13	*Acinetobacter calcoaceticus* 127	NCIM
14	*Pseudomonas aeruginosa* 2036	NCIM
15	*Pseudomonas putida* 2102	NCIM
16	*Pseudomonas fluorescens*	Lab culture collection
17	*Staphylococcus aureus*	Lab culture collection
18	*Klebsiella pneumoniae* 2719	NCIM
19	*Bacillus spp*	Lab culture collection
20	*Salmonella typhi* 2501	NCIM
21	*Candida albicans* 3100	NCIM
22	*Pichia angusta* 26012	ATCC
23	*Saccharomyces cerevisiae*	Lab culture collection

A .pullulans strains and yeasts cultures were maintained on Sabouraud dextrose agar (SDA) medium containing 20% glycerol and all the bacterial cultures maintained on nutrient agar medium and stored at 4-10^{0}C.

*NCIM= National Collection of Industrial Micro-organisms, National Chemical Laboratory, Pune, India.

**MTCC= Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

***Lab Culture Collection= Department of Microbiology, University of Pune, India.

Growth conditions and fermentation:

A loopful of cells (10^8 cells / ml) taken from the slant culture of *A. pullulans* were was suspended in three ml of sterile distilled water. From this suspension one ml was transferred into 10 ml of Sabouraud Dextrose Broth (SDB), incubated for 48 h on a shaker setat 120 rpm, in order to prepare inoculum for all the experiments. One ml of the inoculum was added to 100 ml of malt extract broth and incubated at 28ºC on a shaker set at 120 rpm for three days. The entire content of the flask was centrifuged at 5000 rpm for 20 min and the supernatant was extracted three times with 10 ml of ethyl acetate, concentrated up to 2 ml under a stream of nitrogen gas and then used for testing the antimicrobial activity.

Testing the antimicrobial activity:

Antimicrobial activity of the above extract was determined by the disc assay procedure (17). The concentrated ethyl acetate extract (100 µl) was added to an ampoule containing 10 sterile (Whatman paper number 1; 5 mm dia.) disks and kept in a refrigerator for 24 h. Control disks were prepared using an ethyl acetate extract of the un-inoculated medium.

Results and Discussion

Identification of natural isolates:

On the basis of morphological characteristics and assimilation of different carbon and nitrogen sources, natural isolates were identified as *A. pullulans*. Further details on the growth behavior

Table 2. Nutritional physiological pattern of *Aureobasidium pullulans* strains:

Test	*Aureobasidium pullulans* strains.										
	NI.1	NI.2	1049	1048	976	1226	153	2160	2013	1991	16625
Glucose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	W	W	+	+	+	+	+	+	+
Maltose	+	+	W	W	+	+	+	+	+	-	+
Lactose	+	+	W	+	W	+	+	+	W	+	+
Galactose	W	+	+	+	+	+	+	+	+	W	+
Trehalose	+	+	+	+	W	+	+	+	W	W	+
Raffinose	+	+	W	+	+	W	+	+	+	-	+
Rhamnose	+	+	W	W	+	+	+	+	W	+	+
Mannose	+	+	+	+	+	+	+	+	W	+	+
Adonitol	-	+	+	+	+	+	+	+	+	-	+
Mesoinositol	W	W	W	+	+	+	W	+	W	W	+
Methanol	W	W	W	-	+	+	+	+	+	+	W
Melibiose	+	+	W	+	+	+	+	W	W	-	+
Cellobiose	W	+	W	+	W	+	+	+	W	-	+
Fructose	W	W	W	+	W	W	+	+	+	W	+
D-Ribose	W	+	+	+	W	+	W	+	W	W	+
Starch	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	-	+	+	+	+	+	W	-	+
Peptone	+	+	+	+	+	+	+	+	+	+	+
Yeast Extract	+	+	+	+	+	+	+	+	+	+	+
L-Aspargine	+	+	+	W	+	+	+	+	+	+	+
Ammonium nitrate	-	W	W	W	W	+	+	+	+	+	W
Ammonium sulphate	-	+	W	W	W	+	+	+	+	+	+
Na. nitrite	-	-	-	-	-	-	-	-	-	-	W
Arginine	W	+	W	W	W	+	+	+	-	W	W
Alanine	W	W	W	W	W	+	+	+	W	-	+

Table 2. (Continued)

		NI.1	NI.2	1049	1048	976	1226	153	2160	2013	1991	
Growth in presence of †	0% NaCl	+	+	+	+	+	+	+	+	+	+	+
	2.5% NaCl	+	+	+	+	+	+	+	+	+	+	+
	5% NaCl	+	+	+	+	+	+	+	+	+	+	+
	7.5 NaCl	+	+	+	+	+	+	+	+	+	+	+
	10% NaCl	+	+	+	+	+	+	+	+	+	+	+
	Cycloheximide (100µg/ml)	S	S	S	S	S	S	S	S	S	S	S
	Cu ‡	-	-	-	-	-	-	-	-	-	-	-
	Fe	-	-	-	-	-	-	-	-	-	-	-
	Zn	-	-	-	-	-	-	-	-	-	-	-
Enzymes tested §	A Amylase	+	+	+	+	+	+	+	+	+	+	+
	Gelatinase	-	-	-	-	-	-	-	-	-	-	-
	Protease	-	+	+	-	-	+	+	+	+	+	+
	Cellulase	-	-	-	-	-	-	-	-	-	-	-
	Urease	+	+	+	+	+	+	+	+	+	+	+
	Xylanase	-	+	-	-	-	-	-	+	-	+	-

*Assimilation tests were carried out in YNBA medium with different carbon (0.2 % *w/v*) and nitrogen (0.1 % *w/v*) sources.
†A loopful of inoculums was spot inoculated on YNBA containing different concentrations of salts.
‡5 µg of metal solution was added into wells in SDA plates seeded with *A. pullulans* (10^6 - 10^7 cells ml^{-1}).
§A loopful of inoculum of *A. pullulans* was spot inoculated on YNBA containing appropriate substrate and screened for enzyme activity.
W= weak, S= Sensitive, + = Positive, - = Negative

of the two isolates and ATCC 16625 are given in Table 2. In the early stage of their growth, oval budding cells were predominant and at a later stage elongated cells with blastospores present on swollen assimilation of adonitol, tips and chlamydospores could be seen. Similarity coefficients of NI.1 and NI.2 with *A. pullulans* (ATCC 16625) were estimated to be 87 and 92% respectively. Isolates NI.1 and NI.2 isolates belonged to the same species, as could be seen from the similarity coefficient with the ATCC strain of *A. pullulans* 16625. These isolates were taken to be different strains, as they showed variations from the ATCC 16625 with respect to assimilation of adonitol, ammonium sulphate and protease activity (NI.1); assimilation of adonitol and xylanase activity (NI.2). At the beginning, both the strains produced creamy white colonies, which turned black during the latter stages of growth. Strain NI.1 formed mucoid colonies (Table 3).

Antimicrobial activity:

Almost all the *A. pullulans* strains showed activity against Gram-negative bacteria, but none of them inhibited *Staphylococcus aureus* and *Bacillus spp*. Strikingly, *Pseudomonals* seem to be apparently much more sensitive

Table 3. Cultural characteristics of different strains of *A. pullulans* on different media

	PDA		SDA	
	2 days	6 days	2 days	6 days
NI.1	mucoid, shining creamy white	dark green, mycelium abundant	Black color, chlamydospores and mycelium	black color, chlamydospores and mycelium
NI.2	unicellular, creamy color	chlamydospores, scanty mycelium , black color	unicellular budding form, creamy white	dark color, mycelium
ATCC 16625	creamy white, unicellular	black color, thick chlamydospores and mycelium	conidia, sporulation at the margin	black color, mycelium abundant

Table 4. Antimicrobial activity of *A. pullulans* strains jugged as the diameter (mm) of the inhibition zone

Target organisms	*Aureobasidium pullulans* strains											
	Genta* 10µg disk^{-1}	NI.1	NI. 2	1049	1048	976	1226	2160	1991	2013	153	16625
E.coli	15 (S)	12	18	0	0	19	0	0	16	0	0	0
Ps. aeruginosa	11 (R)	7	11	7	7	0	9	8	10	0	7	0
Ps. putida	0 (R)	11	9	10	6	0	8	8	9	10	8	8
Ps. fluorescens	0 (R)	13	11	7	7	7	7	7	8	8	8	12
A.calcoaceticus	11 (R)	11	13	13	0	11	15	7	11	0	7	0
S. typhi	12 (R)	0	0	0	0	10	9	9	0	0	0	8
K. pneumonia	0 (R)	0	7	0	0	8	9	0	0	0	0	0
S. aureus	10 (R)	0	0	0	0	0	0	0	0	0	0	0
Bacillus spp	12 (R)	0	0	0	0	0	0	0	0	0	0	0
C. albicans	0	0	0	0	0	0	0	0	0	0	0	0
P. angusta	0	0	12	0	0	0	8	10	10	0	10	8
S. cerevisiae	0	0	0	0	0	0	0	0	0	0	0	0

100µL bacterial culture (OD_{660} = 0.7- 0.8) and yeasts culture (OD_{660} 1.3- 1.5) were spread on Muller Hinton agar or Sabouraud dextrose agar. Disks were placed on the medium and incubated for 1-2 days at 37 ^{0} C. Each disk contained 10 µL of ethyl acetate extract. S= Sensitive, R= Resistant * Genta. = Gentamycin, based on disk susceptibility, from Hi Media Company, India.

to *A. pullulans*. *Ps. fluorescens, Ps. aeruginosa* and *Ps. putida* were inhibited by 11, 10 and eight strains of *A. pullulans,* respectively. Similarly, out of the 11 strains of *A. pullulans,* eight strains showed inhibition of *A. calcoaceticus,* an emerging nosocomial pathogen. Three/ four of the *A. pullulans* strains showed inhibition of *E. coli, S. typhi* and *K. pneumoniae*. Though *P angusta* was inhibited by six strains of *A pullulans*, none could inhibit *C. albicans* and *S. cerevisiae* (Table 4). The natural isolates were compared with nine standard strains of *A. pullulans* and identified as *A. pullulans* on the basis of morphological, physiological and cultural characteristics. Production of antibiotic has been reported most often from the eubacteria-like *Bacillus*, Actinimycetes, e.g. *Streptomycete*s and lower fungi e.g. *Cephalosporium*. It should be pointed out that more than 3000 antibiotics have been isolated from actinomycetes and relativelyfew from fungi (18). Mac William (19) has found that yeasts and yeast-like fungi, as compared to other microorganisms, are not a promising source of novel antibiotics.

Because of the emergence of pathogens resistant to most currently available antimicrobial agents and a concomitant increase in the number of immunosuppressed patients, screening of antimicrobial compounds is becoming increasingly important. This study provides comprehensive evidence that *A. pullulans*, a safe non-pathogenic (12) and ubiquitous organism (1), could be a promising producer of antimicrobial compounds. In this study the ntimicrobial activity of *A. pullulans* strains was predominantly against Gram- negative bacteria and *Pseudomonas spp*.. In particular and unlike the study of McCormack *et al* (14), no activity was seen against *Staphylococcus aureus*. Studies in our laboratory indicate that there are intracellular compounds inhibiting *Staphylococcus aureus* (Data not shown).

It is worth noting that *Pseudomonas aeruginosa* has been a notorious organism for chemotherapy .It is highly resistant to β-lactam antibiotics and intractable to treatment with most potent antipseudomonal agents (20-21). It is also an opportunistic pathogen responsible for a wide range of infections.

Taken together, the results described here provide hope for obtaining better chemotherapeutic agents, possibly against *Pseudomonas* infections.

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