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ANTIFUNGAL POTENTIAL OF BACTERIAL METABOLITES: ISOLATION, SCREENING AND CHARACTERIZATION

Mahrukh Zaidi[1], Sehar Afshan Naz*[1], Nusrat Jabeen[1], Maryam Shafique[1] and Syed Abdus Subhan[2]

[1]*Department of Microbiology, Federal Urdu University of Arts, Science and Technology, Gulshan-e-Iqbal, Karachi, Pakistan.*
[2]*Department of Microbiology, University of Karachi, Karachi-75270, Pakistan.*
*****corresponding author email: **seharafshan@fuuast.edu.pk**

ABSTRACT

The increased use of antimicrobial agents resulted in the development of resistance against most of the commonly used antimicrobial drugs. Therefore the need for safe and effective antimicrobial agent have increased with time. This study was designed in an attempt to search for such natural antimicrobial agents against fungi. A total of fifty five indigenous bacterial strains were collected from different clinical specimens. These strains were identified by conventional methods as *Staphylococcus aureus, S. epidermidis, Bacillus subtilis, Pseudomonas aeruginosa, Proteus mirabilis and P. vulgaris.* The cell free culture supernatant (CFS) of these strains were used in antifungal screening by using agar well diffusion assay against different fungal strains. Of all the bacterial strains screened, 36.4% (20/55) showed antifungal potential. However, *S. aureus, B. subtilis and P. mirabilis* demonstrated bioactivity against some fungal strains but 71.4% (15/21) strains of *P.aeruginosa* demonstrated significant antifungal activity. The most potent antifungal strains *P. aeruginosa* strains MS 9 and MS 10 exhibited antifungal activity against *Rhizopus* sp., *Aspergillus* spp., *Penicillium* sp., *Microsporum* sp., *Trichophyton* spp. and *Saccharomyces* species. However, no activity was recorded against *Candida* spp. The physico-chemical characterization of these two strains MS 9 and MS 10 revealed retention of bioactivity of these metabolites both at low temperature (0^0C and 4^0C) and at high temperature up till 70^0C. Similarly exposure to organic solvents such as ethanol, methanol and formaldehyde also had no effect on their antifungal potential. The findings suggest further purification and possible application of these metabolites as antifungal agent in future.

Keywords: Antifungal, *Ps. aeruginosa*, phytopathogenic fungi, dermatophytes.

INTRODUCTION

There is a dramatic rise in the incidence of life threatening systemic fungal infections in the last few decades. This increase might be due to emergence of HIV infection, development of cancer chemotherapy, use of strong immunosuppressive drugs for organ transplantation and discriminate use of broad spectrum antibacterial therapy (Barrett, 2002). Antifungal therapy is still a most neglected field as it has been observed that Amphotericin B discovered in 1956 is still used as a Gold standard for antifungal therapy along with all its side effects on human health. Other antifungals also have many drawbacks in terms of their efficacy and toxicity. The frequent use of these drugs leads to emergence of drug resistance and other complications. There is a need to search for some other natural chemotherapeutic alternatives having selective toxicity with fewer side effects on human health (Abad *et al*., 2007).

Antimicrobial peptides (AMPs) have been gaining escalating attention nowadays because of their bioactivity against a wide range of microorganisms including bacteria, fungi, protozoa and metazoan parasites. These antimicrobial peptides are produced by most of the living organisms including bacteria, plants and animals including humans (Murray and Lieu, 2008). More interestingly; these AMPs have shown limited resistance compared to the bacteria tested in the clinical phase I and II trials. The AMPs produced by bacteria include toxins, bacteriolytic enzymes, by product of primary metabolic pathways, bacteriophage like structures, antibiotics and the bacteriocins (Cascales *et al.*, 2007, Jabeen *et al*., 2014).

Fungal inhibition due to bacterial metabolites is a well-recognized phenomenon. The role of *Pseudomonas* spp. in fungal inhibition is well documented in literature, with particular reference to *Ps. aeruginosa* of clinical origin that has been found to inhibit a variety of fungal strains. As observed, *Ps. aeruginosa* and *Ps. cepacia*, isolated from cystic fibrosis patients exhibited significant antifungal activity compared to other clinical isolates like *Staph. aureus, E. coli*, and *H. influenzae.* Both bacterial strains could inhibit *Aspergillus fumigatus, Saccharomyces cerevisiae* and *Candida* spp. (Kerr *et al*., 1999). Moreover, *Ps. fluorescens* and other *Pseudomonas* spp. also showed antagonistic activity against phytopathogenic fungi including *Botrytis cinerea, Fusarium* spp. and

Neurospora crassa (Annabel *et al.*, 2005; Shalini and Srivastava, 2008). Besides, *Lactobacillus* spp. *Bacillus* spp. and *Staphylococcus* spp. were also reported in literature as producers of antifungal agents (Galvez *et al.*, 1993; Yang and Clausen, 2004).

Realizing the significance of bacteria in production of natural antifungal chemotherapeutic agents, the present study targeted towards the analysis of antifungal spectrum of indigenous bacterial strains and their physic-chemical characterization.

MATERIALS AND METHODS

Collection of indigenous bacterial strains:

About Fifty five different bacterial strains were obtained from different pathological laboratories in Karachi. These strains were isolated from clinical specimens such as pus, urine, blood etc.

Identification of the bacterial strains:

The bacterial strains were identified up to species level using conventional standard diagnostic criteria (Holt *et al*., 1994; Forbes *et al*., 2002). The strains were identified as, *Staphylococcus epidermidis, S. aureus, Bacillus subtilis Proteus mirabilis, P. vulgaris,* and *Pseudomonas aeruginosa.*

Preparation of Cell free supernatant (CFS) for antimicrobial activity:

For the detection of antimicrobial potential, the cell free supernatant of these bacterial strains was prepared. The bacterial culture was grown in nutrient agar (Oxoid), incubated overnight at 37^0C. After incubation, the culture was centrifuged at 6000 rpm for 30 min. The cell free supernatant was collected carefully and stored in refrigerator until use (Zaidi *et al*., 2013; Khan *et al*., 2014).

Detection of Antifungal activity:

In order to detect antifungal activity of indigenous bacterial strains, agar well diffusion technique was used. The activity was checked against six indicator fungal strains (*Rhizopus* sp., *Aspergillus flavus*, *Penicillium* sp. *Candidia albicans, Microsporum canis and Trichophyton rubrum)* obtained from Department of Microbiology, Federal Urdu University. The indicator fungal strains were grown in Sabouraud`s dextrose agar (Oxoid).The indicator fungal inoculum was streaked on the sterile SDA plate with the help of sterile cotton swab to make a uniform lawn. 100 µl of CFS (producer bacterial strain) was introduced in the 7mm diameter wells made in the SDA plates. The plates were incubated at room temperature for 24 hours. Inhibition zones were recorded in mm. (Magnusson and Schnurer, 2001). The strains with good antifungal potentials were further screened for more clinically significant strains of fungi by employing agar well diffusion technique.

Characterization of potent producer strains:

The effect of different physical and chemical factors on the bioactivity of producer strains was observed. The CFS of potent producer was exposed to different ranges of temperatures such as 60^oC, 70^oC, 80^oC, 100^oC and 121^oC (at 15 psi for 15min.) and different organic solvents (ethanol, formaldehyde and chloroform) and the effect of these exposures was recorded by using *Aspergillus flavus* as indicator strains (Naz and Rasool, 2013).

RESULTS

This study was conducted to evaluate the antifungal potential of bacterial strains mainly isolated from clinical specimens. Fifty five different bacterial strains were collected for this research study. They were identified by conventional methods as *S. epidermidis* (n=06), *S. aureus* (n=09), *B. subtilis* (n=05), *P. mirabilis* (n=09), *P. vulgaris* (05), and *P. aeruginosa* (n = 21).

The antagonistic effect of these bacterial strains on fungi was checked by using their cell free supernatant in agar well diffusion method. The results revealed that 36.4% (20/55) of all the strains tested showed antagonistic activity against indicator fungal strains. *P. aeruginosa* was observed as the most predominant organism showing bioactivity against most of the fungal strains tested. Particularly significant antagonism was observed against *Rhizopus* sp., *A. flavus* and *Penicillium*. However few strains also demonstrated bioactivity again dermatophytes (*M. canis* and *T. rubrum*) too but no activity was recorded against *C. albicans.* Besides, some strains of *S. aureus* also showed a moderate antagonistic activity against some fungi such as *Rhizopus, A. flavus* and *M. canis*. Other bacterial strains tested in this study showed very little or no activity against any fungi (Table 1).

On the basis of good antifungal potential two strains of *P. aeruginosa* were selected for further study. These strains, *P. aeruginosa* MS 9 and *P. aeruginosa* MS 10, were further tested for their antifungal potential against several strains of clinically significant fungi. The results also revealed significant antifungal activity was produced by these strains against most of the fungal strains tested. However, there was no inhibition of *C. albicans* and *C. tropicalis* by any of the strain tested (Table 2).

Table 1. Antimicrobial spectrum of indigenously isolated bacterial strains.

Bacterial strains N=55	**No of antifungal Producer**	**Indicator fungal strains**					
		Rhizopus sp.	***Aspergillus flavus***	***Penicillium sp.***	***Microsporum canis***	***Trichophyton rubrum***	***Candida albicans***
S. epidermidis n=06)	*0*	0/6	0/6	0/6	0/6	0/6	0/6
S. aureus (n=09)	*3*	3/9	3/9	0/9	2/9	0/9	0/9
B. subtilis (n=05)	*1*	1/5	1/5	0/5	0/5	0/5	0/5
P. aeruginosa (n=21)	*15*	11/21	15/21	13/21	6/21	4/21	0/21
P. mirabilis (n=09)	*01*	0/9	1/9	1/9	0/9	0/9	0/9
P. vulgaris (n=05)	*0*	0/5	0/5	0/5	0/5	0/5	0/5

*Producer strains giving zone of inhibition more than 10mm were considered bioactive

The effect of different physical and chemical stimulus on the bioactivity of these potential antifungal strains *P. aeruginosa* MS9 and *P. aeruginosa* MS10 were also determined. Cell free supernatant of both the strains retained their bioactivity at low temperature (4^0C and 0^0C) up till one month. *P. aeroginosa* MS9 retain the activity up to 70^0 C, however, loss of activity was noted above this temperature. Other strain *P. aeruginosa* MS10 was more heat stable and retained its bioactivity up till 80^0C. Retention of activity after exposure to different organic solvents was also carried out. All the organic solvents tested had no effect on bioactivity of both the strains but exposure to chloroform led to the loss of antagonistic activity against fungi (Table 3).

DISCUSSION

Antagonism against fungi is relatively a common characteristic of bacteria which confers them to survive in mixed microbial population. There are many mechanisms which explain the inhibition of one organism by another such as competitive inhibition due to nutrients depletion, production of siderophores, antibiotics, bacteriocins, enzymes, volatile substances etc. The bacterial species which significantly produce these antifungal substances mainly belongs to Actinomycetes, *Bacillus*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, *Enterococcus* etc. ((Kerr, 1999 ; Naz and Rasool, 2013). The present study focused on the production of antifungal substances by indigenous bacterial strains isolated from clinical specimens. Of these isolates, 36.4% were found to be producer of such antifungal substances and were identified as *S. aureus*, *P. mirabilis*, *B. subtilis* and *P. aeruginosa*. This phenomenon of fungal growth inhibition was detected in both pathogenic and non-pathogenic bacteria by using different techniques in earlier studies too (Kerr, 1999).

Staphylococcus species are Gram positive cocci and are commonly found in our environment. *S. aureus* is the well-recognized species responsible for causing many infections in human (Leshem *et al*., 2013). When this specie was screened for its antifungal potential, 33% strains were found to inhibit growth of Rhizopus sp., *A. flavus* and *M.*

canis. Whereas *S. epidermidis* didn't give any antifungal activity against tested fungi. The role of S*taphylococcus* spp. in fungal inhibition is not so much recognized but has been reported (Saeed, *et al*., 2006).

Similarly *Bacillus subtilis* which is a Gram positive bacilli also showed inhibitory effect against *Rhizopus* and *A. flavus.* Although only 20% of this species showed antifungal activity but it has been observed in literature that the role of Bacillus species are well recognized in inhibition of fungi particularly phyto-pathogen and human pathogenic strains such as *Candida*, *Aspergillus* species and *Saccharomyces cerevisiae* (Klich *et al*.,1994: Eshita *et al* ., 1995; Kajimura *et al*., 1995). Other species of bacillus such as *B. cereus* and *B. licheniformis* revealed activity against *C. albicans, S. cerevisiae, M. canis, Mucor* spp., *T. mentagrophyte, Sporothrix schenkii* and *Aspergillus* spp. (Aoki *et al*., 1994; Lebbadi *et al* ., 1994).

Table 2. Inhibition pattern of inhibitory compound of MS9 and MS10 producer strain against different indicator fungi.

Indicator fungal strains	***P. aeruginosa* MS9**	***P. aeruginosa* MS10**
Rhizopus spp. (MS10)	++	++
Rhizopus spp. (MS11)	++	++
Rhizopus spp. (MS12)	++	-
Rhizopus spp. (MS13)	++	++
Mucor spp. (MS14)	+++	+++
Mucor spp. (MS15)	+++	+++
Aspergillus niger (MS1)	++++	+++
A. niger (MS2)	+++	+++
A. niger (MS3)	+++	+++
A. flavus (MS4)	+++	+++
A. flavus (MS5)	+++	-
A. terreus (MS6)	+++	++++
A. terreus (MS7)	+++	+++
Penicillium sp. (MS17)	++++	+++
Saccharomyces sp. (MS16)	-	-
Trichophyton tonsurans (MS18)	++	+
T. mentagrophyte (MS19)	++	+++
Microsporum canis (MS20)	++	+++
Candida albicans (MS8)	-	-
C. tropicalis (MS9)	-	-

Key: +++ = Inibition zone > 30mm, ++ = Inibition zone of 20-29mm, + = Inhibition zone 10-19mm, - = No inhibition zone or <9mm.

Table 3. Effect of different treatments on the bioactivity of *P.aeruginosa* MS 9 and *P.aeruginosa* MS 10.

Treatment	**Effect on Bioactivity**	
	P. aeruginosa **MS 9**	***P. aeruginosa*** **MS 10**
Control (CSF without any treatment)	**R**	**R**
Temperature		
0^0C (One month)	R	R
4^0C (one month)	R	R
60^0C (15 min)	R	R
70^0C (15 min)	R	R
80^0C (15min)	S	R
100^0C (15min)	S	S
121^0C at 15psi (15 min)	S	S
Organic solvents		
Ethanol 1% (15min)	R	R
Methanol 1% (15min)	R	R
Chloroform 1% (15min)	S	S
Formaldehyde 1% (15min)	R	R

Key: R= Resistant to treatment (No loss of bioactivity); **S**= Sensitive to treatment (loss of bioactivity)

Proteus species are Gram negative inhabitant of human gastrointestinal tract. Most of these species are human pathogen and responsible for causing urinary tract infections and septicemia. However, the role of *Proteus* spp. is not restricted to pathogenesis only. There are certain *Proteus* spp. which are inhibitory for *C. albicans* and many phytopathogenic fungi such as *Gelasinospora ceralis, Fusarium oxysporum, Trichoderma viride, Penicillium viridicatum* and *Zygorhychus vuilleminii* (Kerr, 1999). This fungal inhibition by *P. mirabilis* was also detected against *A. flavus* and *Penicillium* sp. in the present study while surprisingly *P. vulgaris* did not show any such activity.

Pseudomonas, a Gram negative bacillus, is another ubiquitous organism distributed widely in nature. Many species of this genus are well recognized as human, animal and plant pathogen. Particularly, *P. aeruginosa* is considered as a notorious opportunistic pathogen responsible for causing life threatening infections in burn patients and patients with existing cystic fibrosis (Kerr, 1999; Naz and Tariq, 2005). To establish its dominance in an environment, *P aeruginosa* produces many antifungal factors such as pyocins, Dihydro aeruginoic acid, Pyocyanin and I- hydrooxyphenazine. These factors have inhibitory effect on the growth of phytopathogenis fungi as well as human pathogens such as *C. albicans and A. fumigatus* (Kerr *et al*., 1999)

The fungal inhibition was demonstrated well in our study when 71.4% of the total *P. aeruginosa* revealed antifungal potential against most of the fungi tested. These strains were mainly active against *A. flavus, Penicillium* sp. *Rhizopus* sp. and dermatophytes. Among these strains, two strains *P. aeruginosa* MS 9 and MS 10 because of their good antifungal activity were further tested against more fungal strains. Interestingly, both strains exhibited excellent fungal inhibition of both saprophytic and dermatophytic fungi. Whereas, they were ineffective against *Candida* species. These findings are in consistent with previous studies where *P. aeruginosa* and *P. cepacia* of clinical origin showed significant antifungal activity against *A. fumigatus, S. cerevisiae* and *Candida* spp. as compared to other clinical isolates tested (Kerr, 1994; Kerr *et al.*, 1999).

Different physical and chemical factors may have significant impact on the antifungal bioactivity of bacterial strains therefore the effect of some of these factors was also determined. The effect of varying temperature on the bioactivity of both potent antifungal strains *(P. aeruginosa* MS 9 and MS 10) revealed their stability at low temperature as well as at high temperature up till 70^0C. The effect of different organic solvents on the bioactivity of

P. aeruginosa MS 9 and MS 10 also demonstrated the retention of bioactivity after treatment with most of organic solvents used. The determination of these impacts could have importance in the possible application of these metabolites as a chemotherapeutic agent.

CONCLUSION

Because of the side effects and developing resistance against currently available antifungal agents, inhibition of fungal growth by natural substances has great significance in the development of antifungal chemotherapeutic agents. The results revealed substantial potentials of antifungal agents in the metabolites produced by bacterial strains *P. aeruginosa* MS9 and *P. aeruginosa* MS10 and suggested its further purification and application as chemotherapeutic agent.

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