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Isolation of antifungal bacteria from soil samples

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Abstract

Six strains of *Bacillus cereus* exhibiting antifungal activity were isolated from local environment and optimized for growth conditions. The isolates appeared neutrophiles, grew best at aeration and were represented by thermophiles and mesophiles. Growth inhibition zones of varying diameters were observed when the isolates were tested against *Saccharomyces cerevisiae* on nutrient agar. Largest inhibition zone measuring up to 16.25mm was observed for *B. cereus* Sn-21a after 48-72 hours of inoculation. Four of the isolates designated as Sn-18a, Sn-19a, Sn-20a and Sn-21a also showed antifungal activity against *Candida albicans*. For this test organism maximum growth inhibition zone (31mm) was noticed around filter paper disc loaded with 4X cell free cultural fluid of *Bacillus cereus* Sn-20a. The isolates Sn-20a and Sn-21a appeared thermophilic. Further work on these bacterial isolates is likely to define their antimycotic mode of actions and limitations regarding therapeutic applications of their antifungal exoproducts.

Key words: Antimycotic; *Bacillus cereus*; control of *Candida albicans*; bacterial exoproducts and fungal control.

Running Title: Antifungal *Bacillus* bacteria.

Introduction

Several species of bacteria and fungi live harmlessly on our skins and other areas representing normal microflora. However, invasion of certain pathogenic fungi or overgrowth of normally occurring harmless types can cause symptoms of fungal infections of the skin. The latter category of the organism is termed as opportunistic pathogens. Many types of infections and other skin conditions caused by fungi are quite common and persistent. Humans are usually affected by fungi that live on animals. Some types may be caught from farmyard and domestic animals. Some infections are not serious and usually not easily spread from person to person (BUPA's Health Information Team, 2004). Increased incidence of systemic fungal infections in the past two decades has been overwhelming. Before 1960s, only pathogenic dimorphic fungi were known to cause systemic infections. Later on opportunistic fungi had been reported to cause more number of infections, especially in immunocompromised hosts. Systemic fungal infections can be broadly classified into two groups: endemic mycosis caused by true pathogenic fungi and opportunistic fungal infections by a vast array of saprophytic fungi (Chakrabarti and Shivarakash, 2005).

Fungal rashes can sometimes be confused with other skin conditions, such as psoriasis and eczema. Cutaneous tissues can become infected when fungal organisms contaminate or colonize the epidermal surface or hair follicles. The skin can be portal of entry for fungal infections when the epithelial barrier is breached or it can be a site of disseminated systemic fungal disease (Catherine, 2006).

Most common fungal pathogens associated with invasive disease in humans are opportunistic yeast *Candida albicans* and filamentous fungi of *Aspergillus* spp. Numbers of fungi causing systemic disease as well as the number of systemic diseases caused by fungi are increasing. This global change in systemic fungal infections has emphasized the need to develop good diagnostic mycology laboratories as well as identification of proper antifungal drugs, to recognize this increasingly large group of potential pathogens and to find better and cheap remedies, respectively (Chakrabarti and Shivarakash, 2005). Major difficulty in treating fungal diseases is the availability of limited arsenal of antifungal compounds. The regulatory approval of three cell-wall active antifungal compounds encourages the search for additional clinical candidates that inhibit fungal cell wall formation (Richardson, 2003; George and Selitrennikof, 2006).

In Pakistan due to illiteracy related unhygienic practices and warm and humid climatic conditions prevailing most of the year, different skin fungal infections persist in the population. The present study was thus intended to isolate and

characterize antifungal producing bacteria from local environment. Six bacterial isolates being reported here appear potential candidates for production of antifungal antibiotics. Their antimycotic activities can be visualized to be more meaningful against the fungal infections prevailing in this area as compared to imported antifungal agents.

Materials and Methods

1- Isolation of bacteria

Soil and water samples were obtained from different locations in the Quaid-e-Azam campus, University of the Punjab, Lahore. Soils collected from bank of canal, botanical garden and a sample from sewage holding pond were processed for isolation of antifungal bacteria. One gram of a given soil sample and one ml of the water sample were mixed with 10 and 9 ml of sterilized distilled waters in test tubes, respectively. The tubes were kept on orbital shaker at 130 rpm overnight. Contents of the tubes were then allowed to stand for 2 hours at room temperature. The soil extracts 200µl from each tube were spread on nutrient agar plates and incubated for 24 hrs at 37 °C. Following growth, bacterial colonies were purified and preserved on nutrient agar slants for further study.

2- Antagonistic effect against *S.cerevisiae*

A strain of the yeast, *Saccharomyces cerevisiae* was revived in nutrient broth. Then 200 µl of *S. cerevisiae* culture was spread on nutrient agar plates. By means of inoculating loop, a loop full from pure bacterial culture was placed on the surface of the yeast inoculated nutrient agar plate and incubated at 37 °C for 72 hrs. Six of the bacterial isolates designated as Sn-4a, Sn-17a, Sn-18a, Sn-19a, Sn-20a, and Sn-21a were selected on the basis of their yeast inhibition potential and then processed for optimization of their growth conditions, characterization and identification.

3- Optimization of growth conditions and characterization

Freshly inoculated bacterial cultures were incubated at 30ºC, 37ºC and 50ºC. For optimization of pH, the bacteria were grown in nutrient broths having pH 5±0.2, 7±0.2 and 9±0.2, at their respective optimum growth temperatures. The isolates were then incubated on orbital shaker at 100 rpm and without aeration at their corresponding optimum pH and temperatures. Nutrient broth tubes were inoculated with 1%, 5% and 10% inocula and incubated at their corresponding predetermined optimum growth conditions. In all the optimization experiments, the isolates were incubated in triplicates for 18 hours and the growth was determined by taking O.D. of the cultures at 600nm on Spectronic 20D spectrophotometer. The bacterial isolates were characterized physiobiochemically following the methods described by Benson (1994) and identified according to Konem *et al.* (1997) and Holt *et al*. (2000).

4- Antimycotic activity against *C. albicans*

From the six *Bacillus cereus* isolates, the four (Sn-18a, Sn-19a, Sn-20a, and Sn-21a) which showed more antifungal activity against *Saccharomyces cerevisiae* were selected for evaluation of antimycotic activity against *Candida albicans*. For this purpose, six test tubes containing 5 ml of nutrient broth were inoculated with a given bacterial strain and allowed to grow at their respective optimum conditions. The tubes were divided in two sets, one incubated for 24 hrs, and the other for 96 hrs. Optical density was taken at the end of each period. After centrifugation supernatant was kept in refrigerator. Culture of *C. albicans* was revived on Sabouraud 2% dextrose broth (Merck, 1996/97). The cell free culture fluids of bacterial strains were passed through Millipore filters (0.20 µm). Sterilized filter paper discs of 0.8 mm diameter were then loaded with 20 µl of the filter sterilized bacterial culture fluids which were placed on agar plates inoculated with *C. albicans* as described earlier. After incubation at 37 °C zones of growth inhibition were measured. For another experiment, 2 ml of cell free culture fluids were completely evaporated at 40 °C and then dissolved in 0.5ml of sterilized distilled water. The four times concentrated culture fluids were loaded on filter paper discs which were subsequently evaluated for their antifungal potential against *C. albicans* as described above.

Results and Discussion

Three (Sn-4a, Sn-17a and Sn-19a) of the six antifungal bacterial isolates from sewage and canal samples were found to be mesophiles as they showed maximum growth at 37 °C. While the isolates Sn-18a, Sn-20a and Sn-21 appeared thermophiles and aerobic. All the six bacterial isolates were found to be neutrophiles. From these isolates, four (Sn-4a, Sn-18a, Sn-19a and Sn-20a) showed best growth when the cultures were initiated with 5 % inoculum while the remaining two grew best with 1% and 10% of inocula. (Table 1). On the basis of physiobiochemical

characterization all the isolates were identified as *Bacillus cereus* (Table 2).

Antifungal activity of the bacterial isolates against *Saccharomyces cerevisiae*, was recorded. Growth inhibition zone (GIZ) around the growth of Sn-4a after 24 hrs was observed to be 4mm which increased to 6.25 and 11.5mm after 48 and 72 hours of incubations, respectively. In case of other isolates, no growth inhibition zone was observed after 24hrs but the zones were noticed as 13, 11.5, 18 and 13mm after 48hrs and 18, 13, 19 and 14 mm after 72 hrs of incubation around the growths of Sn-17a, Sn-18a, Sn-19a and Sn-20a, respectively (Table 3, Fig. 1). Only four isolates showed antifungal activity against *C. albicans*. When filter paper discs loaded with 20µl of 24 hrs incubated cell free cultural fluids were tested against *C. albicans,* the zones of inhibition around the discs were recorded to be 3.5, 4, 4.25 and 0.00mm. For 96 hrs incubated bacterial cultures, the ZGI measured 4.25, 4.5, 4 and 4 mm for Sn-18a, Sn-19a, Sn-20a and Sn-21a, respectively. When four times concentrated cultural fluids were employed, ZGI measured as 18, 2.85, 13.25 and 0.00 mm for 24hrs incubated cultures of Sn-18a, Sn-19a, Sn-20a and Sn-21a, respectively. Corresponding figures of GIZ for the 96 hrs incubated cultures of sn-18a to sn-21a appeared as to be 20, 31, 18 and 21.5mm, respectively (Table 4, Fig 1).

In recent years, there has been an increasing trend towards identification of more efficient antifungal drugs owing to the increased incidence of fungal infections (Andriole, 2000; Odd, 2003; Mathew and Nath, 2009). The present study indicated that the bacterial isolates have considerable antifungal activity against *Saccharomyces cerevisiae.* Increases of diameters of the yeast growth inhibition zones around the bacterial cell mass with increase in incubational periods suffest that antifungal bacterial exoproducts had continued to be produced in later phases of the bacterial growth. When *Candida sp.* was exposed to four times concentrated cell free cultural fluid on Sabouraud agar surface, growth of the yeast was inhibited following more or less the same pattern in terms of bacterial incubation periods as discussed for *S. cerevisiae*.

Over the last decade, the incidence of fungal infections has increased dramatically and increased incidence of cryptococcosis is reported from all centers with the emergence of AIDS (Chakrabarti & Shivarakash, 2005). Infection due to *Candida* and *Cryptococcus* are the most common. These situations necessitate search for novel and more potent antifungal drugs. Although nature of the antifungal compounds produced by the bacteria reported here as well as their mode of actions have not been worked out. Further work on these compounds may throw light on their therapeutic usefulness. It is pertinent here to stress that these antifungal bacteria have been isolated from local environment and their therapeutic or other antimycotic potential may prove more useful as compared to the drugs/ antibiotics sourced from other parts of the world.

Table 1: Optimization of different growth conditions of the bacterial isolates grown in nutrient broths.

Isolate #	**Experimental Conditions**										
	Temperature			pH			Oxygen supply		Inoculum size		
	RT (30 °C)	**37 °C**	**50 °C**	**5**	**7**	**9**	**Aeration**	**Non-aeration**	**1%**	**5%**	**10%**
Sn-4a	0.203 ± 0.04	0.166 ± 0.002	0.096 ± 0.009	0.043 ± 0.02	0.335 ± 0.05*	0.106 ± 0.02 ♠	0.209 ± 0.008	0.114 ± 0.006*	0.346 ± 0.02	0.418 ± 0.006	0.358 ± 0.02
Sn-17a	0.109 ± 0.019	0.193 ± 0.01*	0.156 ± 0.01	0.024 ± 0.01	0.309 ± 0.05 ♠	0.105 ± 0.008 ♠	0.224 ± 0.01	0.147 ± 0.02*	0.423± 0.03	0.42 ± 0.01	0.37 ± 0.02
Sn-18a	0.07 ± 0.004	0.145 ± 0.01*	0.169 ± 0.001*	0.012 ± 0.007	0.034 ± 0.009	0.026 ± 0.009	0.209 ± 0.037	0.115 ± 0.003*	0.112 ± 0.007	0.137 ± 0.011	0114 ± 0.038
Sn-19a	0.081 ±	0.186 ±	0.149 ±	0.029	0.302 ±	0.102 ±	0.218 ±	0.130 ±	0.362	0.376	0.368

	0.002	0.02*	0.003*	± 0.019	0.01	0.009	0.009	0.01*	± 0.017	± 0.01	± 0.01
Sn-20a	0.090 ± 0.006	0.158 ± 0.003*	0.181 ± 0.005* ,♠	0.015 ± 0.002	0.024 ± 0.004	0.021 ± 0.01	0.239 ± 0.01	0.135 ± 0.01*	0.172 ± 0.009	0.192 ± 0.03	0.174 ± 0.001*
Sn-21a	0.101 ± 0.014	0.145 ± 0.013	0.169 ± 0.003*	0.019 ± 0.008	0.059 ± 0.008*	0.025 ± 0.001 ♠	0.21 ± 0.007	0.12 ± 0.003*	0.139 ± 0.03	0.158 ± 0.002	0.161 ± 0.020

Values represent optical density (600nm) of 18 hrs incubated cultures and are means of triplicates ± S.E.M.
Values with asterisk are significantly different from those in the first row of the respective experiment. Significant difference between the values of second and the third row is indicated by ♠. *, ♠ = $p \leq 0.05$, single-factor analysis of variance.

Table 2: Physiobiochemical characterization and identification of the bacterial isolates.

Bacterial Isolate	*Characteristic*								
	Gram Reaction (Size in µm)	Endos-pore	Moti-lity	Oxid-ase	Catal-ase	Nitrate Reduction	Sulfide Form-ation	Lecthin-ase	Iden-tified as
Sn-4a	+ ve Diplobacilli (2.5 X 1)	+ ve Round	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	*Bacillus cereus*
Sn-17a	+ ve Diplobacilli (4 X 1)	+ ve Round	+ ve .	+ ve	+ ve	+ ve	+ ve	+ ve	*Bacillus cereus*
Sn-18a	+ ve Diplobacilli (3.8 X 1)	+ ve Round	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	*Bacillus cereus*
Sn-19a	+ ve Diplobacilli (3 X 1)	+ ve Round	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	*Bacillus cereus*
Sn-20a	+ ve Diplobacilli (3.2 X 1.2)	+ ve Round	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	*Bacillus cereus*
Sn-21a	+ ve Diplobacilli (3.8X 1)	+ ve Round	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	*Bacillus cereus*

Table 3: Zones of growth inhibition (mm) of *Saccharomyces cerevisiae* around growths of different *Bacillus cereus* isolates.

Bacterial isolate	**Incubation period**		
	24 hrs	**48hrs**	**72hrs**
Sn-4a	4 [a]	6.25	11.5
Sn-17a	No zone	13	18
Sn-18a	No zone	11.5	13
Sn-19a	No zone	18	19
Sn-20a	No zone	13	14
Sn-21a	No zone	16.25	16.25

[a] : Diameter (mm) of the yeast growth inhibition zone around the bacterial colonies.

Table 4: Zones of growth inhibition (mm) of *Candida albicans* against *Bacillus cereus* isolates, cultures fluids.

Bacterial isolate	Incubation period	
	24 hrs	**96 hrs**
Sn-18a	18[a] (3.5)	20 (4.5)
Sn-19a	28.5 (4)	31 (4.5)
Sn-20a	13.25 (4.2.5)	18 (4)
Sn-21a	No zone	21.5 (4)

[a]: Diameter (mm) of the yeast growth inhibition zone around discs loaded with 4x of cell free culture fluid. Values in parentheses indicate the growth inhibition zones around discs loaded with respective non-concentrated cell free culture fluids.

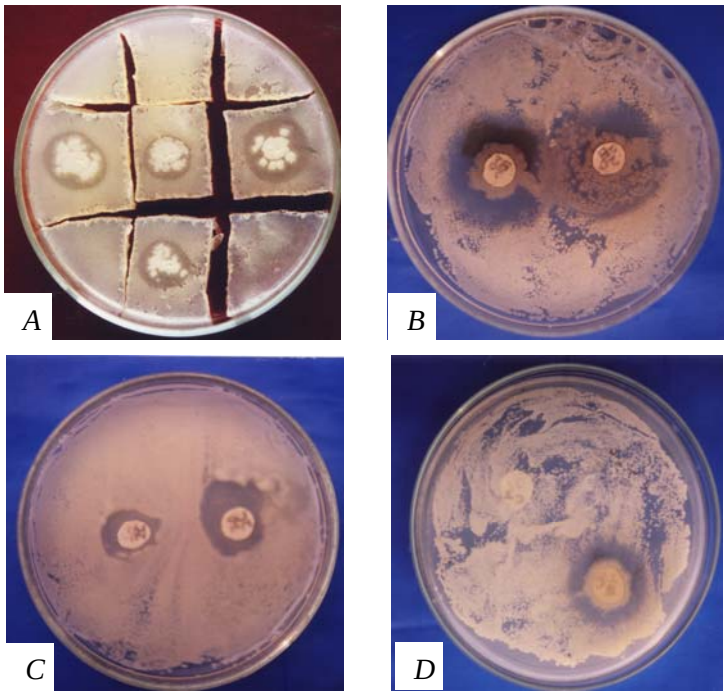

Fig 1: Growth inhibition zone (GIZ) against *Saccharomyces cerevisiae* of *B. cereus* isolates **[A]** after 72 hrs around growth of Sn-17a (left), Sn-18a (center), Sn-19a (right) and Sn-20a (bottom). The GIZ against *Candida albicans* around filter paper discs loaded with 20 µl of 4X cell free cultural fluid (CFCF) of the bacterial isolates, Sn-19a **[B]**, Sn-20a **[C]** and Sn-21a **[D]**. In the figures B, C and D discs loaded with 24and 96 hrs incubated CFCF are shown on left and right sides of the plates, respectively.

References

Andriole VT, 2000. Current and future antifungal therapy: new targets for antifungal therapy. *Int. J. Antimicrobial Agents*, **16**: 317-321.

Benson HJ, 1996. *Microbiological Applications, Laboratory Manual in General Microbiology*, W. M. C. Brown Publishers, Dubuque, U.S.A.

BUPA's Health information Team, 2004. *Fungal skin infections*. United Kingdom.

Catherine A, 2006. Mycologic disorders of the skin. *Health Science Journal*, **21**: 128-134.

Chakrabarti A, Shivarakash MR, 2005. Microbiology of systemic fungal infection. *J. Postgraduate Medicine*, **51**: 16-20.

George S, Selitrennikof CD, 2006. Identification of novel cell wall active antifungal compounds. *Int. J. Antimicrobial Agents*, **28**: 361-365.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Stanley TW, 2000. *Bergey's manual of determinative bacteriology*. 9th ed., Lippincott Williams and Wilkins, New York, USA.

Konem EW, Allen SD, Janda WM, Schreckenberger PC, Winn Jr WC, 1997. *Atlas and textbook of diagonistic microbiology*. 5th ed. Lippincott Williams &Wilkins, 530 Walnut street, Philadelphia, PA 19106-3621 USA.

Mathew BJ, Nath M, 2009. Recent approaches to antifungal therapy for invasive mycosis. *Chem. Med. Chem.,* **4**: 310-323.

Merck, 1996-1997. *Microbiology Manual*. Merck kgat, Darmstaut, Germany.

Odd FC, 2003. Antifungal agents: their diversity and increase sophistication. *Mycologist*, **17** : 51-55

Richardson MD, Warnock DW, 2003. *Fungal infection*: Diagnosis and management. 3rd ed., Blackwell Publishing Limited.