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Research Article

Evaluating the Antifungal property of *Pseudomonas aeruginosa DSGPM4* species on some food spoilage fungus

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ABSTRACT

There is an urgent need to survey for new novel antimicrobial agents due to the development of increased resistance power of large number of fungal and bacterial pathogens to common antimicrobial agents. In the present study the *Pseudomonas DSGPM4* sp capable of producing antimicrobial agent was isolated from waste water sample of Bangaluru, Karnataka, India. The metabolites of the isolated bacteria were screened for antifungal activity against six different fungal species. Screening was done by using paper disc containing 20µl of culture supernatant placed on nutrient agar plate seeded with test organisms. The activity was determined by measuring the inhibition zone diameter (in mm) both for crude and dilution with 0.1 (M) phosphate buffer (pH-7.0) in the ratio of 1:1 (Crude extract:Buffer) . The *Pseudomonas DSGPM4 sp.* showed inhibition zone diameter of 6.3 mm against *Beauveria sp.* with the application of crude metabolite and dilution of crude metabolite with 0.1 (M) phosphate buffer (pH-7.0) in the ratio of 1:1 (Crude metabolite: Buffer) showed inhibition zone diameter of 5.9 mm. Most stable and active pH was found for antifungal metabolites was 2.0. From this work it might be concluded that Pseudomonas DSGPM4 *sp.* can be used for the production of novel antifungal agent. Further study will be needed for characterization of the metabolites.

Key words: Bacteria, Fungi, Antifungal bacteria. Antifungal agents, Bacterial metabolites.

INTRODUCTION

There are many chemical preservatives in market, which can arrest the growth of food born pathogenic fungi and those have been approved now a days for preserving foods.From several survey it become cleared that consumers are looking and demanding those kind of food products which have long shelf life without adding any chemical preservatives. Food spoilage fungi can able to grow very fast and its growth have been a global concern because not only for economic loss, but they can cause many health hazard by producing several mycotoxins [1]. There is an urgent need to survey for new a novel antimicrobial compound against large number of fungal and bacterial pathogens. These pathogens are extremely difficult to control due to its complex epidemiology and virus pathogen of aerial infection [2]. Isolation of bacterial strains directly from waste water sample could be a good alternative for the selection of bio control agent,

since these cultures from nature are already well developed to survived in the field in contrast to laboratory strains [3] .With a view to isolate novel bacterial culture, having antifungal characters, waste water sample collected from water purifying plant and then screened their antimicrobial activities against several fungal species.

MATERIALS AND METHODS:

Sampling site and Isolation of microorganisms: Water samples were collected from a drainage system of Bangaluru, Karnataka, India. A quantity of 1ml of water from the collected sample was dissolved in 9ml sterile distilled waterand serial dilutions were made. Each dilution was plated on nutrient agar plate without antibiotics and incubated at 37^{o}C. After the growth of different microorganisms on the plate, each bacterial colony on the basis of its morphological characteristics was picked up and further purified by repeated streaking on nutrient agar plate . Each bacterial culture was then inoculated in nutrient broth, incubated and glycerol stocks were made and frozen at -70^{o}C [4].

Strain characterization:

Gram's staining and 16s rRNA sequencing: Gram's staining was performed to confirm a Gram positive/ Gram negative bacterial strain .16s rRNA sequencing from genomic DNA with universal primers was performed . To know the identity of organism, obtained sequences were compared with nucleotide databases like GenBank , EMBL and the Ribosomal Database Project (RDP) through BLAST programme [5,6].

Nucleotide sequence accession: The sequence was submitted to Gen Bank and accession numbers obtained.

Screening and identification of antifungal bacterial isolates : Dual culture method was performed to screen the antifungal activity of bacterial strain. Briefly, One 10-mm disk of a pure culture of test fungi was placed at the center of a Petri dish containing PDA. A circular line, made with a 6 cm diameter Petri dish dipped in a suspension of bioantagonistic bacteria (5 x 10^{9}-cfu mL^{-1}), was placed surrounding the fungal inoculum. Plates were cultured for 27±3°C for 72 h and growth diameter of the pathogen (fungal growth) was measured and compared to control growth where the bacterial suspension was replaced by sterile distilled water. Identification of bacterial isolates showing antifungal properties were based on cultural, microscopic, and biochemical characteristics with reference to Holt et al. (1994) [7].

Separation of antifungal metabolites: Cell free supernatant of these cultures was collected, by growing them on nutrient broth (NB) for 48 h on a rotary shaker at 200rpm at 35±2^{0}C, centrifuged at 10,000rpm for 20 min. at 4^{0}C. Antibacterial compound was recovered from the filtrate by solvent extraction method following the process described by Westley *et al*, 1979 [4] . Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1 h for complete extraction. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath at 60°-70°C and the residue obtained were taken for the further studies.

Detection of antifungal activity: The antifungal activity was determined by agar well method [6.7,8]. The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1 ml 0.2 M phosphate buffer (pH 7.0). Then 100µl of it was loaded into well bored and test organism swabbed Potato Dextrose agar plates. The plates were incubated at 27±3°C for 72 h and examined. The diameter of the zones of complete inhibition was measured in milimeter.

Effect of pH: Overlay assay described above was used. The pH values of malt extract agar medium was changed at 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0

Detection of volatile substance: The detection of volatile antifungal compounds was evaluated by sealed Plates method[8] . *Pseudomonas aeruginosa DSGPM4* were inoculated on nutrient agar agar plates sealed with parafilm and allowed to grow at 30^{o} C for 24h, in parallel agar disc of mould was inoculated on other Petri dish and allowed to grow at 30^{o}C for 24h, and then the two plates were sealed together with parafilm and incubated at 30^{o}C for 72h. The growth of a fungus was determined by the measurement of colony diameter in mm compared to control to evaluate the inhibition by volatile substance.

RESULTS AND DISCUSSION:

The isolated bacterial strain was subjected to Gram's staining. Based on the result of Gram's staining, it was found Gram negative rod shape bacteria (Table 1). The isolate bacterial strain was used for the further biochemical characterization The bacterial strain was subjected for initial screening using test fungal isolates of *Aspergillus niger* and *Trichoderma sp.* These fungal strains were selected for initial screening, as it is the most common soil fungi and *Trichoderma* is used as biocontrol agent against a variety of plant pathogens [6,9,10]. The bacterial strain showing antogonostic activity was selected based on the inhibitory activity towards the test fungus.The spectrum of action of the metabolite against different fungal cultures is recorded

in Table 2 and Fig 1. *Pseudomonas aeruginosa DSGPM4 sp* showed inhibitory activity towards all the fungal pathogens. The cell free extracts of *Pseudomonas aeruginosa DSGPM4 sp*. inhibited the growth of *Trichoderma* sp. and *A. niger* sp. with an inhibition zone of 5.4 mm and 4.9 mm respectively.

Table 1
Morphological and biochemical characteristics of *Pseudomonas aeruginosa* DSGPM4

Morphological, Cultural, Physiological and Biochemical characteristics	***Results***
Cell shape	Rod
Size	5.25 µm
Gram's reaction	Gram negative
Capsule	Present
Spore	spore forming
Growth	Aerobic
Motility	Motile
Growth in NaCl (20%)	+
Growth temperature (°C)	35-37
Catalase	+
Oxidase	-
Glucose	Acid
Sucrose	Acid/Gas
Lactose	Acid
Fructose	Acid
Arabinose	Acid/Gas
Galactose	Acid/Gas
Mannitol	Acid
Raffinose	Acid/Gas
Xylose	Acid/Gas
Methyl red	-
VP-test	+
Indole	-
Citrate	-
Nitrate	+
Urease	+
Casein Hydrolysis	+
Starch	+
Gelatin	+
Identification	**Above characteristics indicates that the isolate belongs to the genus *Pseudomonas aeruginisa(GenBank Accn NoJN245880)***

Table 2
Antifungal effect of the cell free supernatant on fungal cultures.

Test organisms	Concentrated Culture fluid of *Pseudomonas aeruginosa DSGPM4(Average Zone of inhibition in mm)*	Culture fluid of *Pseudomonas aeruginosa DSGPM4*was diluted with 0.1M phosphate buffer 1:1*(Average Zone of inhibition in mm)*
A. niger	4.9	4.6
Trichoderma	5.4	4.9
Mucor	2.3	2.0
Penicillum	3.5	3.2
Beauveria	6.3	5.9
A.ochraceous	3.0	2.8

Table 3
Antimicrobial effect of the cell free supernatant on fungal cultures after using different percentage of Ammonium sulphate

% Ammonium sulphate	0-50	50-60	60-70	70-80	Test organism
Bacterial cultures	Average Zone of inhibition in mm				
Pseudomonas aeruginosa DSGPM4	1.5	1.9	2.23	2.18	*Aspergillus niger*
Pseudomonas aeruginosa DSGPM4	2.23	2.20	2.00	2.33	*Trichoderma* sp.
Pseudomonas aeruginosa DSGPM4	2.6	2.4	1.7	1.5	*Beauveria sp*

Table 4
Antifungal properties of ethyl alcohol precipitated metabolite on test organisms.

Test organism	*Aspergillus niger*	*Trichoderma* sp.	*Beauveria sp*
Bacterial cultures	Average Zone of inhibition in mm		
Pseudomonas aeruginosa DSGPM4	2.6	1.9	3.2

Table 5
.Antifungal properties of ethyl alcohol precipitated metabolite on test organisms at pH different pH and 27^{0}C (unit mm).

Test organism	*Aspergillus niger*	*Trichoderma* sp.	*Beauveria sp*
Bacterial cultures	Average Zone of inhibition in mm		
Pseudomonas aeruginosa DSGPM4	5.6 (pH 2) 5.0(pH 3) 4.6(pH 4) 4.2(pH 5) 3.5(pH 6) 2.6(pH 7)	3.6 (pH 2) 3.0(pH 3) 2.6(pH 4) 2.2(pH 5) 1.5(pH 6) 0.6(pH 7)	4.6 (pH 2) 4.0(pH 3) 3.6(pH 4) 3.0(pH 5) 2.5(pH 6) 1.6(pH 7)

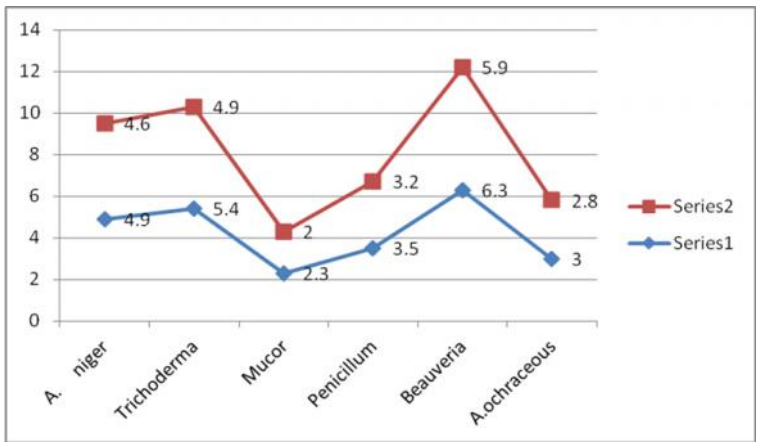

Fig 1

Antifungal effect of the cell free supernatant on fungal cultures with and without dilution (unit mm).

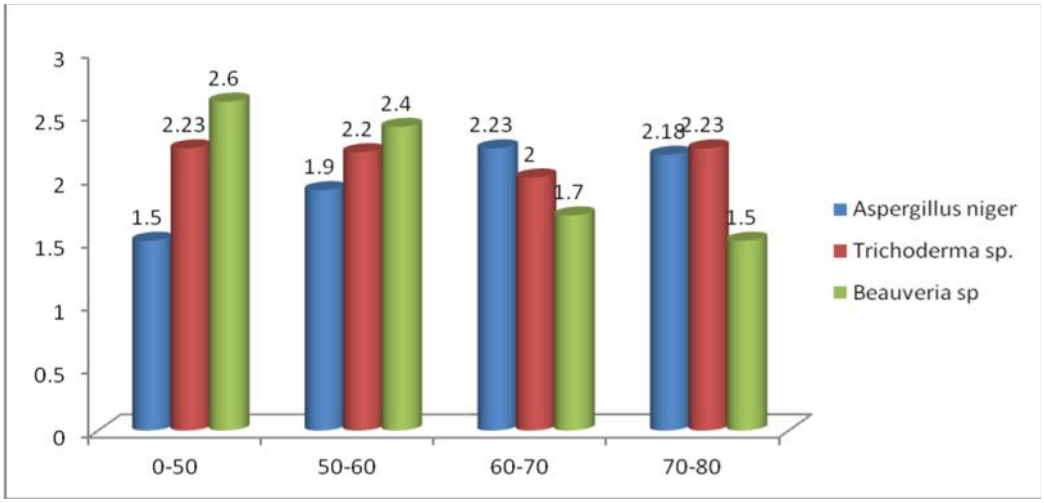

Fig 2

Antimicrobial effect of the cell free supernatant on fungal cultures after using different percentage of Ammonium sulphate (unit mm).

NOTE: X axis % of ammonium sulphate and Y axis zone of inhibition in mm.

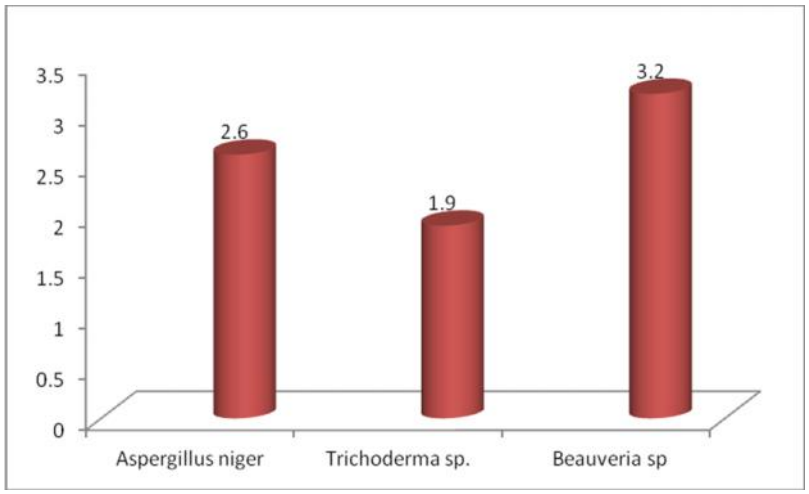

Fig 3

Antifungal properties of ethyl alcohol precipitated metabolite on test organisms (unit mm).

NOTE: Y axis zone of inhibition in mm

However, *Mucor,Penicillum and A.ochraceous* were inhibited by the bacterial extracts with a low degree of inhibition (2-3.5 mm). The diluted (1:1) extract of the *Pseudomonas aeruginosa DSGPM4 sp*. inhibited the growth of *Trichoderma sp*. however, the diluted extract inhibited the other pathogens with a low degree of inhibition (Table3,4 and Fig 2,3). The cell free extract of *Pseudomonas aeruginosa DSGPM4 sp* was highly active. However, the other fungal pathogens were inhibited at a moderate level at 1:1 dilution. However, this is the first report on antifungal activity of *Pseudomonas aeruginosa DSGPM4 sp*.The control mechanisms used by the bacteria may involve the secretion of fungal cell wall hydrolytic enzymes [11,12,13]. No inhibition of mould was observed on any of the dishes sealed with the test strains. A total inhibition growth of *Aspergillus* spp.by DSGPM4 at the pH 2.0 was observed. But the use of malt extract agar medium with pH values at 3.0, 4.0 and 5.0, decreased the antifungal activity of all strains, therefore most probably to the secretion of organic acids which depends strongly on the pH, since they are active in the undissociated form (Table 5). In this form, their lipophilic condition permits them to penetrate across the membrane. At a higher intracellular pH, the acid dissociates to release protons and conjugate bases, which disrupt the membrane proton motive force [3].Microbial action cannot be restricted to one niche; so it is necessary to test their adaptation to other sites especially when bacteria have expressed a good antagonistic activity against specific phytopathogenic fungi [10,13]. The data reported here indicate that the same antagonistic strains of bacteria that protect from plant pathogens. This strain can be also used as seed inoculants to prevent the seed borne pathogens and their infections.

CONCLUSION

From our results it might be concluded that *Pseudomonas DSGPM4 sp*. can be used for the production of novel antifungal agent in future.

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