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# Exploring Antagonistic Metabolites of Established Biocontrol Agent of Marine Origin

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**Abstract** Biocontrol ability of *Pseudomonas aeruginosa* ID 4365, a biocontrol agent of groundnut phytopathogens from marine origin, was previously attributed to the production of pyoverdin type of siderophores. However, pyoverdin-rich supernatants of this organism showed better antifungal activity compared to equivalent amount of purified pyoverdin indicating presence of undetected metabolite(s) in pyoverdin rich supernatants. On the basis of observation that antagonistic activity was iron-dependent and iron-independent, an attempt was made to detect the presence of additional metabolites. In addition to pyoverdin, strain produced additional siderophores, viz. pyochelin and salicylic acid. Two broad spectrum antifungal compounds, viz. pyocyanin and phenazine-1-carboxylic acid, were detected, characterized, and activity against phytopathogens was demonstrated. Iron- and phosphate-dependent co-production of siderophores and phenazines was confirmed. Strain showed additional features like production of hydrogen cyanide, indol-3-acetic acid, and phosphate solubilization.

**Keywords** Biocontrol agent · *Pseudomonas* · PGPR · Phenazines · Siderophores

## Introduction

Plant-growth-promoting rhizobacteria (PGPR), competitive root colonizers, not only stimulate plant growth but also get rid of unsolicited population on root surface through various means. They are being utilized in prophylactic manner in agriculture to avoid or minimize the use of chemical pesticides. Fluorescent pseudomonads have received status of "most eligible microbial biological control agents (MBCA)" due to its 'vibrant features' like competitive root colonization, small generation time, amenability to genetic

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manipulation, and 'strong metabolic arsenal', viz. production of plant growth regulators [1], siderophores [2], and antibiotics [3].

It is discouraging that after three decades of extensive research with vibrant tools of molecular biology, popularization and practicability of MBCA remains questionable. Most biocontrol agents studied to date were isolated from rhizosphere; moreover, its application has also remained region and plant/crop specific. During a screening program of our laboratory, Manwar et al. [4] studied *Pseudomonas aeruginosa* ID 4365 of marine origin and its biocontrol potential against groundnut phytopathogens [4]. This screening was based on quantitative estimation of siderophores produced by various strains of genus *Pseudomonas* as siderophores are known to exert biological control through competition for iron [5]. During further research, pyoverdin produced by this strain was identified and characterized. Pyoverdin-rich supernatants showed antagonistic activity against numerous soilborne phytopathogens [6]. Groundnut seed bacterization by *P. aeruginosa* ID 4365 proved to be an effective method for suppression of phytopathogens and plant growth promotion. This strain proved to be an efficient peanut root colonizer and conducive in improving nitrogen-fixing microbe–plant interactions and iron acquisition. In plate and pot assays and field trials, the influence of this organism was observed on plant growth, which was evident from improved number of root nodules, root- and shoot-length, chlorophyll content of leaves, and total biomass generated. In addition, nutritional value addition was evident from improved oil and carbohydrate content in nuts. Formulation of *P. aeruginosa* ID 4365 has better perspectives in plant growth promotion and nutritional value addition to plant products [7]. The use of marine strain as MBCA was a distinct feature of this study, where we focus additional metabolites of this strain in the context of its use as MBCA. On this basis, strain deserves strong recommendation for its prophylactic use in agriculture to control various soilborne root diseases of plants.

## Materials and Methods

### Materials

All media components were purchased from Hi-media Laboratories Pvt. Ltd, India. Unless otherwise specified in the text, throughout the experimentation, analytical/ guaranteed reagent (AR/GR) grade chemicals from S. D. Fine chemicals. Pvt. Ltd, India and double glass-distilled water was used. Authentic standard of pyocyanin (PYC) was chemically synthesized as per Knight et al. [8] in our laboratory. Authentic standard of phenazine-1-carboxylic acid (PCA) was kindly provided as gift by Dr. Patricia Slininger and Dr. Linda Thomashow (USDA), USA.

### Culture Maintenance and Inoculum Development

Culture of *P. aeruginosa* ID4365 was obtained from repository of School of Life Sciences, North Maharashtra University, Jalgaon. It was maintained on solid King's B medium (g $l^{-1}$ peptone, 20; $K_2HPO_4$ 1.5; $MgSO_4 \cdot 7H_2O$, 1.5; glycerol, 15 ml; pH 7.0) and preserved at 4 °C. All phytopathogen strains (*Aspergillus niger*, *Colletotrichum falcatum*, *Colletotrichum capsicum*, *Fusarium oxysporum*, and *Sclerotium rolfsii*) were maintained on potato dextrose agar (PDA) slants and preserved at 4 °C. The inoculum of *Pseudomonas* strain under study was raised in King's B medium on rotary shaking incubator at 180 rpm and 28 °C for 18–20 h.

### Antifungal Activity Against Various Phytopathogens and PGPR Traits of Strain

Strain was grown on King's B Medium agar plate for 72 h at 28 °C. This growth was then exposed to chloroform for 30 min to kill bacteria by inverting the bottom of Petri dish over chloroform containing upper lid. After removal of this chloroform by evaporation at room temperature, the mycelia plug (8 mm) of actively growing phytopathogen was placed in center and incubated at 28 °C [9]. Control plate was treated in same manner except inoculation of bacterial strain. Each experiment was performed in triplicate.

### Detection of Siderophores

For preliminary detection of siderophores, synthetic succinate medium [10] was inoculated with 18-h-old culture (1% *v*/*v*) and incubated on rotary shaker at 120 rpm at 28 °C for 40 h. Siderophore production was detected by mixing 0.5 ml cell-free supernatant with 0.5 ml of chrom-azurol sulfonate (CAS) reagent and observed for color change from blue to orange red [11]. Production of pyochelin and salicylic acid was established in modified casamino acid (CAA) medium. Briefly, 0.5% casein enzyme hydrolysate (pH 7.5 with 1 M KOH) was de-ferreted by adding 20 g of $MgCO_3$ per liter and was removed by sedimentation after 2 h shaking, followed by centrifugation. A 0.4-mM magnesium chloride was supplemented, and the medium was sterilized by passing it through membrane filters of 0.45-μ pore size. Inoculum (1%) was added in medium and incubated on a rotary shaker (Steelmet, Pune) at 28 °C and 120 rpm for 24 h. For the detection of pyochelin and salicylic acid, the method described by Cox and Graham [12] was employed. Briefly, biomass was removed by centrifugation (7,500 rpm for 15 min at 30 °C), and supernatant was brought to pH 2.5 with HCl. This supernatant was then extracted with ethyl acetate, and organic layers were concentrated by evaporation at reduced pressure. Residue obtained was dissolved in minimal methanol. The methanol solutions were applied to Whatman paper No. 1 by micro-pipette, and the chromatograms were developed by ascending chromatography in water–acetic acid (90:1), with no saturation. After development and drying, the paper chromatogram was viewed under UV light, and the fluorescent bands were marked. A test strip of the chromatogram was sprayed with an iron reagent (0.1 M FeCl3 in 0.1 N HCl), and another test strip was treated with a phenolate spray reagent (1 volume of iron spray reagent added to 1 volume of 0.1 M potassium ferricyanide).

### Detection of Phenazines

Pigment production medium D (PPMD) [23] inoculated with 18-h-old culture (1% *v*/*v*) and incubated on rotary shaker at 120 rpm at 28 °C for 72 h was explored for detection of phenazine compounds. For PYC detection, chloroform extract of cell-free supernatant was analyzed on thin layer chromatography (TLC) with authentic sample wherein chloroform/methanol 1:1 was used as a mobile phase. Whereas for the detection of PCA, chloroform extract of acidified cell-free supernatant was analyzed on TLC with authentic sample. Benzene/acetic acid 19:1 was used as mobile phase.

### Detection of Hydrogen Cyanide

Production of hydrogen cyanide (HCN) was established under microaerophilic conditions. Organism was heavily inoculated on King's B agar supplemented with 50 mM glycine and 0.01 mM $FeCl_3$. A small filter paper impregnated with the hydrogen cyanide detection

solution [13] was taped inside the upper lid of Petri plate. Plates were sealed with Para film and incubated at 28 °C for 96 h.

### Detection of Indol-3-Acetic Acid

Strain was inoculated in nutrient medium supplemented with 500 μg/ml of tryptophan, a precursor of indol-3-acetic acid (IAA). Culture was incubated on rotary shaker at 28 °C for 7 days. IAA was detected by a method described previously [14]. Briefly, bacterial cells were removed from the culture medium by centrifugation (10,000 rpm, 15 min). A 2-ml aliquot of the supernatant was acidified with two drops of ortho-phosphoric acid and mixed with 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid+1 ml 0.5% $FeCl_3$) and allowed to stand at room temperature for 20 min. Development of pink color would indicate presence of IAA.

### Detection of Phosphate Solubilization Activity

Pikovskaya's agar [15] was spot inoculated with growing culture of strain to study it ability to dissolve water-insoluble phosphate salts. Zone of clearance around colony would indicate ability to dissolve insoluble phosphates,

### Production of Phenazines in Various Media and on Various Carbon Sources

Organism was grown in various media, viz. synthetic medium (g/l $MgCl_2{\cdot}6H_2O$, 4.1; $Na_2SO_4$, 7.1; $FeSO_4{\cdot}7H_2O$, 0.005; $K_2HPO_4$, 0.1; urea, 2.0; glycerol 20, pH 7.0) [16]; alanine medium (g/l $MgCl_2{\cdot}6H_2O$, 4.06; $Na_2SO_4$, 14.2; ferric citrate, 0.1; $K_2HPO_4$, 1.39; DL-alanine, 10; glycerol 20, pH 7.0) [17]; iron-free succinate medium [g/l $K_2HPO_4$, 6.0; $KH_2PO_4$, 3.0; $(NH_4)_2$ $SO_4$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; succinic acid, 4.0, pH 7.0] [10]; and PPMD (g/l NaCl. 5.0; $KNO_3$, 1.0; peptone 20, glycerol 20, pH 7.0) [18] for 72 h at 120 rpm and 28 °C. Influence of different carbon sources on the production of phenazines was studied wherein each carbon source was added to synthetic medium (excluding original carbon source) in concentrations corresponding with 180 mM carbon atoms [19]. The following concentrations were used: citric acid (30 mM), fumaric acid (45 mM), lactic acid (60 mM), L-pyroglutamic acid (36 mM), malic acid (45 mM), propionic acid (60 mM), pyruvic acid (60 mM), succinic (45 mM), fructose (30 mM), glucose (30 mM), arabinose (36 mM), ribose (36 mM), xylose (36 mM), lactose (15 mM), maltose (15 mM), sucrose (15 mM), and glycerol (60 mM).

### Factors Determining Co-production of Siderophores and Phenazines

PPMD media with varying phosphate concentrations (in the form of $K_2HPO_4$ in the range 0–2%) and mineral media [16] of varying iron concentrations (0–50 μM) were inoculated with active culture 1% (*v*/*v*) and incubated at 120 rpm, 28 °C for 48–72 h. Detection of metabolites was done variously, i.e., either by TLC, universal CAS assay for siderophores and UV-spectro-photo-metrically at specific wavelength.

### Purification of Active compounds

Small-scale production of phenazines was carried out by culturing the strain in 7 l PPMD medium in a 12-l capacity reactor (NAPRO, Pune, India). After 72 h of incubation

(Temp: RT, aeration 1 vvm, agitation 500 rpm, antifoam: groundnut oil added manually when required), cell-free supernatants were obtained by centrifugation (10,000×*g*, 4 °C for 10 min). For concentration of phenazines, NPA-1 resin (Indion, India) column was prepared by treating with 0.2% sodium nitrate solution followed by washing by 500 ml distilled water. Cell-free supernatant was allowed to pass through the column. The column was washed with 1 l distilled water, and the adsorbed phenazines were eluted with methanol. For extraction of PCA, elute was acidified and extracted with benzene. Benzene layer separated after addition of small amount of water was washed with slightly acidic water and extracted in 5% $Na_2CO_3$ solution for active compound. This bicarbonate layer was again extensively washed with benzene and PCA was re-extracted in benzene after acidification of bicarbonate layer with con HCl. This extract was concentrated using rotary vacuum evaporator (Buchi R-124, Switzerland) and applied on silica gel column equilibrated with benzene. Elution was performed by benzene/acetic acid (19:1), and PCA containing fractions (confirmed by TLC) were pooled together, concentrated, and subjected to crystallization. Crystals separated by decanting were washed twice with chilled chloroform. Purity of crystals was assessed with high-performance liquid chromatography (HPLC). Isocratic HPLC was carried out on Knauer, Germany (model LC 6600) system equipped with detector (190–600 mm, Model LC 6600) and integrated CSW32 software, using Eurospher C18 column (250×4.6 mm, 5 μM integrated pre-column). Mobile phase was a mixture of MeOH–5 mmol/l phosphate buffer (pH 5.0; 60:40 volume ratios). The flow rate was 1.0 ml/min, and chromatograms were monitored at 248 nm. Purity was reconfirmed using gradient solvent system. Solvent A (ACN/$H_2O$/THF—30:70:0.1) was gradually replaced with solvent B (ACN/$H_2O$/THF—90:10:0.1) from 0–100% in 30 min, at the flow rate of 0.7 ml/min on YMC PaCK ODS-A (250×4.6 mm) column. Chromatograms were monitored at 254 nm. For extraction and purification of PYC, same elute (leftover after extraction of PCA) was neutralized and extracted with chloroform. For further purification, chloroform solution was first washed two to three times with 5% $Na_2CO_3$ and two three times with neutral distilled water. This chloroform fraction was then partitioned with 0.1 N HCl (2:1 ratio). Red colored PYC in 0.1 N HCl solution was then washed two to three times with equal volumes of chloroform. After neutralization, PYC was extracted in equal volume of chloroform. The process was repeated ten times. Finally, blue chloroform fraction was extracted in least amount of dilute HCl. For crystallization, PYC solution in dilute HCl was neutralized with least amount of sodium hydroxide, and pH was adjusted to 7.5. This suspension was stored at 4 °C to obtain crystals. Purified crystals of PYC and PCA obtained as above where subjected to chemical characterization with TLC, MP, UV spectrum, Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR).

## Determination of Antifungal Activity of Purified Compounds

Evaluation of inhibitory property of both compounds was undertaken. PCA/PYC crystals were dissolved in dimethyl sulfoxide (DMSO) to yield a 10,000 μg/ml solution. Appropriate amount of this solution was introduced in 100 ml PDA flasks separately to obtain agars with effective concentration of PCA in the range of 5–50 μg/ml, whereas for PYC with effective concentration was in the range of 50–500 μg/ml. Plates prepared using these agars were center inoculated with agar plugs with actively growing phytopathogens. Control plates with added DMSO were also prepared. All plates were incubated at 28 °C. The study was performed in triplicate and repeated once. Measurement of fungal growth was taken when fungal mycelium in the control treatment reached/approached to edge of

plate. However, in some cases, observations were limited on seventh day of incubation. The study was performed at pH 5.0 and 7.0. Similarly inhibitory activity of 1:1 mixture of PCA and PYC was also accessed in the range of 0–65 μg/ml. Inhibitory activity was also evaluated in broth in same range.

## Results and Discussion

Manwar [6] evaluated the biocontrol ability of the *P. aeruginosa* ID 4365 by artificially infecting the soil with groundnut pathogens, namely, *S. rolfsii* and *F. oxysporum*, and found it excellent, and this potential was attributed to pyoverdin produced by the strain. During the present study, it was observed that growth of phytopathogens, including *S. rolfsii*, *C. falcatum*, *A. niger*, *F. oxysporum*, and *C. capsicum* was hampered in plates treated with *P. aeruginosa* ID 4365. For each phytopathogen studied, more than 95% inhibition was observed which re-established the potential of strain. Many researchers have shown that siderophore-producing pseudomonads are potent biocontrol agents [20]. However, production of pyoverdin is not a sole mechanism and present exploration lead to detection of additional metabolites in arsenal of *P. aeruginosa* ID 4365. It is further demonstrated that strain has tendency to co-produce various other metabolites which is important for better establishment of biological control.

### Detection of Additional Metabolites

Production of pyoverdin was evident by characteristic yellow green fluorescence in medium [6]. Additional siderophores, pyochelin, and salicylate were detected by paper chromatography in ethyl acetate extracts of modified CAA medium. Pyochelin appeared as a yellow-green fluorescent spot when viewed under UV light. A fluorescent blue spot in sample co-migrated with standard of salicylic acid, which also showed same fluorescence. Yellow-green fluorescent spot as viewed under UV light turned red when sprayed with 0.1 M $FeCl_3$ in 0.1 N HCl, whereas on separate strip, this spot turned deep blue with the phenolate spray reagent, which confirmed the presence of pyochelin [12]. Pyochelin contributes in biological control by iron-independent methods, as its complex with copper and zinc are more stable and thus deprive phytopathogens in rhizosphere [20]. Biocontrol mechanism of siderophores may not always be through iron chelation. Salicylic acid, the third siderophore detected in present study, which is structurally most simple siderophore, has ability to induce systemic resistance (ISR). Ongena et al. [21] showed that, under certain conditions, ISR play predominant role over siderophores and antibiosis.

Presence of spots co-migrating with authentic standards revealed detection of PYC and PCA. $R_f$ of PYC in solvent system chloroform/methanol (1:1) was found to be 0.62, whereas that of PCA in solvent system chloroform/methanol (9:1) was found to be 0.59. These values matched with previous reports [22, 23]. Hydrogen cyanide, the volatile biocide, was detected as yellow color of Castric and Castric reagent-soaked paper taped inside lid of Petri plate turned deep brown. Modified King's B agar in the plate turned to deep green blue indicated that HCN and phenazines were co-produced. The role of each antibiotic mentioned above in biological control has been studied and established at various stages, viz. identification of genes involved in biosynthesis, detection of metabolite in rhizosphere, study of antibiotic negative mutants, monitoring expression of genes in vivo by employing reporter genes, analysis of contribution of metabolite in ecological fitness of

producer organism, and demonstration of activity in vitro [24]. Moreover, some antibiotics, viz. PCA, are being studied to control foliar phytopathogens [25], and hence, fermentative mass production is also being optimized [26].

Additional PGPR traits were detected in the form of production of IAA in tryptophan-enriched nutrient broth and solubilization of phosphates in tri-calcium-phosphate-enriched media. Role of IAA in plant growth promotion has been well studied and reviewed [27]. However, Sharaf and Farrag [14] for the first time reported that disease suppression in tomato plants, exerted by application of IAA, was achieved through increasing plant growth and exerting a direct harmful effect on target pathogen and/or inducing resistance in host. These workers have observed reduced spore germination, mycelia dry weight, and protein content in tomato wilt pathogen *F. oxysporum* lycopersici.

## Production of Phenazines in Various Media and on Various Carbon Sources

Among various media tested for phenazine production, as shown in Fig. 1, alanine medium [17] and synthetic medium [16] supported maximum production of PCA and PYC. These two media are remarkably similar except the content of sodium sulfate, which is almost twice in alanine medium and urea is replaced with DL-alanine. PPMD media that contain higher amounts of peptone, however, do not support production of phenazines to the extent of defined media. Iron-deficient succinate medium, most suitable for production of pyoverdin, supported production of phenazines especially when special care was not taken to avoid iron contamination.

Various carbon compounds were utilized by organism, however, with varied efficiency, and this may significantly affect secondary metabolite profile of organism. Effect of two types of carbon sources, viz. organic acids and sugars, was studied (Fig. 2).

Among various organic acids, only malic acid failed to support growth and production of phenazines. Citrate and pyruvate severely repressed the formation of PYC but not PCA. Among sugars, pentose sugars and disaccharides failed to support growth and production of phenazines. Fructose, glucose, and glycerol proved to be the best carbon sources for growth and production of metabolites perhaps being readily available.

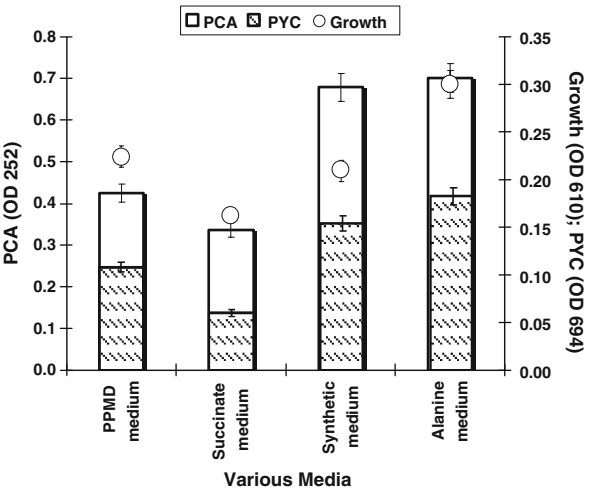


**Fig. 1** Secretion of phenazines in various media. Production of PCA and PYC are comparable in synthetic and alanine media

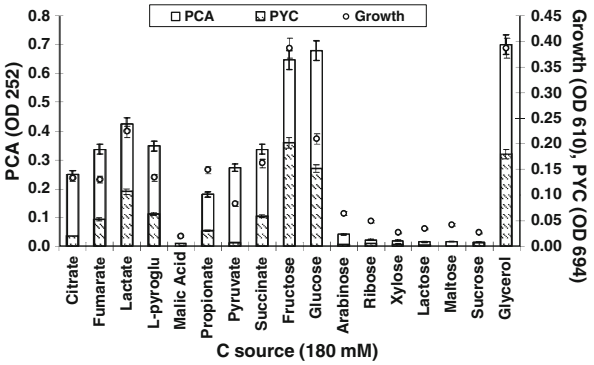


**Fig. 2** Effect of various carbon sources. Organic acids do not support production of metabolites to the extent supported by hexose sugars and glycerol. Pentose sugars and disaccharides are not metabolized by organism

## Factors Determining Co-production of Siderophores and Phenazines

Phosphorus is commonly deficient in the most natural soils due to its fixation as insoluble iron and aluminum phosphates in acidic soils or calcium phosphates in alkaline soils [28]. Previous study showed that an organism had the capability to solubilize insoluble phosphates and also to chelate iron through production of siderophores. Manwar [6] found that siderophores were produced in more quantities in media, which was comparatively low in phosphates, and concluded that phosphates inhibited synthesis of siderophores in iron-deficient media. In the present study, it was observed that increasing concentration of phosphates in PPMD medium lead to the inhibition of phenazines but induced production of siderophores. However, excess phosphates inhibited production of phenazines and siderophores. Thus, this study showed that co-production of metabolites was possible under certain set of conditions. The same conclusion can be reached from another set of experiment in which ferrous sulfate of mineral medium was excluded, and iron was supplemented in the form of $FeCl_3$. Iron has profound effect on growth of microorganisms. As observed in Fig. 3, with the addition of iron, growth increases gradually; however, the production of various metabolites, viz. pyoverdin, PYC, and PCA, vary in context of each other. Pyoverdin production increased up to 2 μM of iron and ceased to 20 μM; similarly, production of PCA increased up to 6 μM of iron and then decreased. In contrast to PCA, PYC synthesis began at 4 μM and continued to increase gradually thereafter. It was

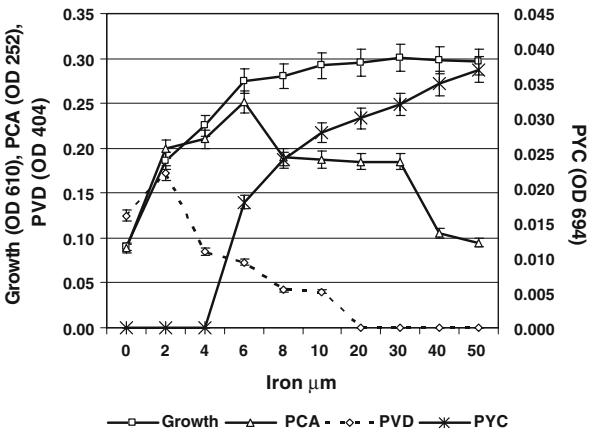


**Fig. 3** Effect of iron on metabolic profile of strain. Increasing concentration of iron discouraged production of PVD but improved production of PYC. PCA production is optimum at 6 μM of iron

observed that a decrease in biosynthesis of PCA was accompanied with an improved biosynthesis of PYC, indicating that the conversion of PCA to PYC was iron dependent, which indicated that the organism under study has the ability to produce siderophores (PVD, PCH, and salicylate), phenazines (PCA and PYC), volatile antibiotic (HCN), and plant hormone cum pathogen inhibitory IAA. Although, the organism might not produce all these metabolites in rhizosphere at once, the chance of expression of at least a few metabolites under most climatic conditions may ensure consistent plant protection.

## Purification and Determination of Active Compounds

Purification of three runs of batches (21 l of broth) yielded 5.79 g PYC and 2.57 g PCA. Authentic sample, mixture of test and authentic sample, and crystalline PYC separately all melted sharply at 134 °C. This value matched with the characterization described previously [29]. In another test, spots of PYC, authentic sample, and a mixture of purified PYC and authentic sample migrated equal distance on TLC plates. In UV spectrum, three peaks were observed at wavelengths 242, 279, 388 for the sample. These observations were in agreement with the authentic sample and literature [29]. The IR spectrum of PYC sample (3,454, 3,396, 2,360, 2,073, 1,975, 1,730, 1,627, 1,556 and 1,411 $cm^{-1}$) was compared with that of authentic PYC sample and found to be super imposable.

In case of PCA, authentic sample, a mixture of test and authentic sample, and crystalline PCA separately all melted sharply at 244 °C [23]. In another test, spots of sample, authentic sample, and a mixture of sample and authentic sample migrated equal distance on TLC plates. Sample and standard PCA showed same retention time, i.e., 3.008 min in linear system and 15.68 min in gradient system. In UV spectrum, two peaks were observed: the major peak being at 251 nm and the minor peak at 370 nm, which were in agreement with the authentic sample and literature [23]. The IR spectrum of PCA sample showed absorption band at 3,600 (OH), 3,020, 2,950, 2,360 (broad), 1,733 (CO), 1,600, 1,523, and 1,460 $cm^{-1}$. The absorption at 1,733 and 3,600 $cm^{-1}$ confirmed the presence of carboxylic group. All other bands matched with IR of the authentic sample and to that of a previous study [30]. In the $^{1}H$ NMR recorded in $CDCl_3$, the singlet at δ15.55 indicated the presence of carboxylic acid group in the compound. The NMR exhibited ABCD pattern in the aromatic ring, which is a characteristic of phenazine derivatives. The chemical shifts and the splitting pattern were comparable with the reported $^{1}H$ NMR of PCA. Similarly, the $^{13}C$ NMR is in full agreement with the reported spectrum of PCA. It showed 13 well-resolved signals including the signal at 165.88 assigned for carbonyl group. Characterization of PCA has been recently reported by Liu et al. [9] also.

## Antifungal Activity of Purified Compounds

PYC showed weak inhibitory activity against any phytopathogens at pH 5; however, at pH 7.0, *F. oxysporum* NCIM 1008, *C. falcatum* and *S. rolfsii* NCIM 1084 were inhibited up to 46.35%, 61.67%, and 94.72%, respectively, at 500 μg/ml, indicating that antifungal activity of PYC is pH-dependent. PCA inhibited all fungal phyto-pathogens at 10 ppm. PCA at 50 μg/ml concentration inhibited growth of *C. falcatum* up to 96.57% and *A. niger* up to 95.57%, whereas 89.29% inhibition of *F. oxysporum* NCIM 1008 was observed at 40 μg/ml. Minimum inhibitory concentration (MIC) of PCA for *S. rolfsii* NCIM 1084 was found to be 29 μg/ml. MIC of PCA for *S. rolfsii* NCIM 1084 when studied in liquid medium was found to be 27 μg/ml. The MIC of PCA against *Cochliobolus sativus*, *Gaeumannomyces graminis* var. *tritici*, *Poria subincarnata*, *Pythium aristosporum*,

*Pythium heterothallicum*, *Pythium volutum*, *Rhizoctonia solani*, *Rigidoporus nigrescens*, *Trametes serialis*, and *Trametes variiformis* was between 1 and 5 mg $kg^{-1}$. Moreover, more than 90% fungi tested were inhibited by concentrations below 50 mg $kg^{-1}$ [31]. Our observations are in agreement with these reports. To find out if the two phenazines studied show some synergistic activity, we studied the inhibitory activity of mixture of PCA and PYC; however, no such activity was observed. However, these negative results do not rule out the possibility of such activity in nature as the study was made on purified metabolites and a third factor may be involved during activity in rhizosphere.

To conclude, *P. aeruginosa* ID 4365 is a well-studied biocontrol agent. Its abilities have been proven in field, and through the present investigation, influential traits of this strain have been identified. Significance of these traits is well established in the literature; moreover, the interaction and synergistic effect of metabolites has been appreciated [32]. *P. aeruginosa* ID 4365 is strongly recommendation for its use in agriculture to control various soilborne root diseases of crops.

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