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Phenazine-1-carboxamide, an Extrolite Produced by *Pseudomonas aeruginosa* Strain (CGK-KS-1) Isolated from Ladakh and India, and its Evaluation Against Various *Xanthomonas* spp.

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In the enduring investigation of the bioactive microbes, *Pseudomonas aeruginosa* strain (referred to as CGK-KS-1 (ICTB-315)), isolated from Chumathang hot spring, Ladakh, and India, was identified to possess a major bioactive fraction with antimicrobial and anti-biofilm properties. This bioactive metabolite was purified through bioactivity-guided fractionation. The chemical structure of this major compound was elucidated as phenazine-1-carboxamide (PCN) based on ^{1}H and ^{13}C NMR, FT-IR, EI-HR-MS and 2D NMR spectroscopic techniques. In the current study, PCN exhibited antimicrobial activity with MIC values ranging between 1.9–3.9 μg/ml against various test human pathogens and *Xanthomonas* spp. PCN showed the anti-biofilm property with the IC_{50} values ranging from 17.04 to 60.7 μM against different test pathogens. The *in silico* docking studies showed PCN strongly interacted with various proteins of different *Xanthomonas* spp. with high binding energies. We report herein for the first time the anti-biofilm property and the docking studies of PCN. The extrolite from *P. aeruginosa* strain CGK-KS-1 showed promising bioactivities and may be considered as a potential candidate for application in various biocontrol strategies.

Keywords: **Phenazine-1-carboxamide, *Pseudomonas aeruginosa*, *Xanthomonas*, antimicrobial, anti-biofilm, extrolite**

Introduction

In the current scenario, there is an imperative global demand for novel and effective drugs that can control drug resistant microbes to treat human, plant and animal ailments. Although microbes act as a better source to fulfil the demand; nevertheless only a small fraction of the microbial bioactive potential has been explored till now. Further, the bioactive potential of the extremophilic microbes is not much studied, with the exception of few reports. In this category fall few interesting examples like *Delftia* sp. KCM-0006 from the Godavari valley coalfields and its application for the green synthesis of Miconazole conjugated-silver based antifungal formulations [1], *Gillisia* sp. CAL575 from an Antarctica sponge and its ability to produce volatile organic compounds that inhibit *Burkholderia* members [2], rhamnolipid producing *Pseudomonas* strain BTN1 that inhibit *Burkholderia* strains [3]. Thus there is a need and scope for further extensive exploration of bioactive potential of microbes from untapped niches.

Phytopathogens have gained resistance to most of the

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existing chemical pesticides, fertilizers and these chemicals have posed serious hazards to the environment and human health. Considering this fact, the biocontrol strategies have gained importance due to their environmentally-friendly and sustainable nature [4]. Although plant pathogens producing biofilms were less studied, the existing few reports highlight the role of biofilm microconsortium in establishing the virulence and antimicrobial tolerance by plant pathogens. For instance, in a study with *Pseudomonas syringae* pv. *thae,* the causative agent of brown spot disease of beans, the biofilm grown cells were more tolerant to the bioactive Kasugamycin as compared to planktonic cells [5]. Biofilm formation by *Xylella fastidiosa* is observed in Pierce's disease of grapevines and citrus variegated chlorosis [6]. Biofilm formation and its dispersal were established to be crucial for the full virulence of few *Xanthomonas campestris* members, the causative agents of most destructive diseases of crucifers [7]. Biofilm formation is reported to play a role in virulence of *Ralstonia solanacearum* that cause damaging wilt in more than 150 plant varieties [8]. In this context, it is appropriate to search for antimicrobial agents that can inhibit the biofilm forming plant pathogens. Also in the biomedical sector, pathogens like *C. albicans, K. planticola* and *E. coli* form biofilms in catheters, stents, fracture fixings, dental prosthetics posing serious contamination problems in post-surgical cases [9].

Ladakh is a 45 million-year-old, 9000 to 25170 feet high-altitude Trans-Himalayan cold desert in the Indian subcontinent with temperatures ranging from +30 to -35℃ [10]. Microbes in this biosphere have a unique surviving endurance to tolerate and adapt hard climatic conditions including minimal nutrients, ultra low temperatures around the year (chilling stress), short-time intensive heating during the summers with UV irradiation [11]. However, there are only few reports on microbial bioactives from hostile niches like Ladakh including alkalitolerant *Bacillus lehensis* strain MLB_2 producing alkaline protease [12], dye degrading *Aspergillus* sp. strain CB-TKL-1 [13] and *Pseudomonas* sp. ICTB-745 producing antimicrobial metabolites like 1-hydroxyphenazine, phenazine-1-carboxylic acid, rhamnolipid-1, rhamnolipid-2 [14]. These facts have triggered the importance to search for novel bioactive metabolites from microbes isolated from Ladakh, India that can inhibit phytopathogens in specific.

The present study describes the purification and characterization of PCN from *P. aeruginosa* strain (referred to as CGK-KS-1) and evaluation of its antimicrobial property, minimum bactericidal/fungicidal concentration (MBC/MFC), anti-biofilm property, time-kill curves and *in silico* docking. Many earlier reports showed PCN to inhibit pathogens like *Rhizoctonia solani*, *Xanthomonas oryzae* pv. *oryzae* [15] MRSA strains [16] and *C. albicans* [17]. Anti-biofilm property of PCN against phytopathogens of *Xanthomonas* genus and *in silico* binding studies of PCN to various target proteins of *Xanthomonas* sp. has been reported for the first time.

Materials and Methods

Bacterial growth conditions

The strain CGK-KS-1 was isolated from a soil sample collected from Chumathang hot spring, Ladakh, India (Latitude: 33°21'33.39", Longitude: 78°19'25.95"). The pure culture has been maintained in the culture collection of our laboratory with the accession number ICTB-315 and was identified as *Pseudomonas aeruginosa* (GenBank Accession No. KY203649). The isolate ICTB-315 was cultured aerobically in the Luria-Bertani medium of pH 7.0 ± 0.2 at 30℃ with agitation at 150 rpm for 2 days in an orbital shaker (New Brunswick Scientific, USA). The supernatant was collected after centrifugation at 8,500 ×*g* for 20 min at room temperature.

Purification and analysis of bioactive metabolite

The major bioactive compound was extracted from the cell-free supernatant by ethyl acetate extraction followed by concentration and drying under reduced pressure on a rotary vacuum evaporator (Rotavapor R-205; Switzerland). The dried crude extract was loaded on a TLC silica gel 60 F_{254} plate (Merck, Germany) and profiled using a methanol-chloroform solvent system (10:90, v/v). The major compound was collected under UV Transilluminator (Camag, Switzerland), dissolved in methanol and filtered through 0.2 μm filters (Millipore, USA). The purity of the eluted compound was analyzed with HPLC (Shimadzu, Japan) interfaced with XBridge C_{18} (50 × 2.1 mm, Waters, Ireland) using the solvent system of 0.1% acetic acid buffered with Milli Q-water and acetonitrile (98:2, v/v).

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Structural characterization

The structure of the purified metabolite was elucidated employing spectroscopic studies. The UV spectrum was recorded at 30℃ on a UV-visible double-beam spectrophotometer (Lambda 25, Perkin-Elmer, USA). Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a Bruker New Avance 500 MHz NMR spectrometer (Bruker, Switzerland). The chemical shifts were shown in ppm where tetramethylsilane was used as the internal standard. Fourier transform infrared (IR) spectrum of the purified metabolite was recorded at a resolution of 4/cm in the wavenumber region of 400–4,000/cm using the Thermo Nicolet Nexus 670 FT-IR spectrophotometer (Thermofisher Scientific Inc., USA). The HR-MS spectrum (m/z) was recorded on AUTOSPEC-M (Micromass, UK) spectrometer. Elemental analysis in terms of carbon, hydrogen, nitrogen and sulfur content present in the purified metabolite were analyzed in helium-argon mixture using Vario Micro Cube elemental analyzer (Elementar Analysensysteme GmbH, Germany).

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

PCN was tested for its in vitro antimicrobial activity employing agar well diffusion method [18]. The test human pathogens were *Micrococcus luteus* MTCC 2470, *Candida albicans* MTCC 3017, *Staphylococcus aureus* MTCC 96, *S. aureus* MLS-16 MTCC 2940, *Bacillus subtilis* MTCC 121, *Escherichia coli* MTCC 739 and *Klebsiella planticola* MTCC 530 which were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. Phytopathogens tested were *Xanthomonas oryzae* pv. *oryzae* strain BXO43, *X. oryzae* pv. *oryzicola* strain Y2, *X. campestris* pv. *campestris* TNAU, *X. campestris* pv. *citri*, *X. campestris* pv. *vesicatoria* strain 1, *X. campestris* pv. *vesicatoria* strain 65-1, *X. campestris* pv. *vesicatoria* strain 85-10 and *X. campestris* pv. *vesicatoria* strain 8004. *Xanthomonas* cultures were grown in sucrose (1%)-peptone (1%) broth at 30℃ for 24 h. In Mueller-Hinton broth, the human bacterial pathogens were grown at 37℃. In potato dextrose broth *C. albicans* MTCC 3017 was grown at 30℃. The human pathogenic and *Xanthomonas* strains with inoculum concentration of 10^6 cfu/ml (0.5 McFarland Standard) were seeded on Muller-Hinton agar and sucrose peptone agar plates respectively. Wells were prepared in the pathogen-seeded plates with a cork borer and the purified PCN at different concentrations ranging from 0 to 150 μg/ml was loaded into the wells. Appropriate positive (Ciprofloxacin and copper oxychloride standards for bacterial and *Xanthomonas* strains, respectively, while Miconazole standard for *C. albicans*) and negative (DMSO) controls were run in parallel at different concentrations ranging from 0 to 150 μg/ml. The plates were incubated for 24 h at 30℃ and the well containing the least concentration showing the inhibition zone is considered as the minimum inhibitory concentration. All the experiments were carried out in triplicates and mean values are represented.

Minimum Bactericidal/Fungicidal Concentration (MBC/MFC) assay [19] was performed in sterile 2.0 ml micro centrifuge tubes against a panel of human pathogens and *Xanthomonas* strains as listed above which were cultured overnight in Mueller-Hinton broth and sucrose peptone broth, respectively. Different concentrations of PCN were prepared from to 0.9 to 150 μg/ml. Overnight culture of different test strains were added to each dilution of PCN to achieve a final concentration equivalent to 0.5 McFarland standard and then incubated at 37℃/24 h for human pathogens and at 30℃/24 h for plant pathogens respectively. After 24 h of incubation, 10 μl of sample from each tube was seeded onto the Mueller-Hinton agar plates or sucrose peptone agar plates to examine the growth at each concentration of PCN. The least concentration of PCN required to kill the individual test pathogen is considered as Minimum Bactericidal/Fungicidal concentration. Mean values are presented from all the experiments performed in triplicates.

Anti-biofilm assay

The in vitro anti-biofilm assay [20] was performed using microtiter plates against the bacterial test strains and *C. albicans* which showed susceptibility to purified PCN. The human bacterial pathogens, *Xanthomonas* strains and *C. albicans* were cultured in Mueller-Hinton broth, sucrose-peptone broth and potato dextrose broth, respectively. The test strains were incubated in their respective culture media in a 96 well plate (in three replicates) at 37℃ for overnight without shaking. After incubation, the pre-formed biofilm cultures were treated

with PCN at various concentrations and incubated at 37℃ for 24 h. The biofilm was visualized by staining the cultures with 0.1% crystal violet for 30 min followed by elution with ethanol. The absorbance was measured at 540 nm using the Infinite M200Pro microtitre plate reader (Tecan Group Ltd., Switzerland) to determine the IC_{50} (μM) of PCN. Ciprofloxacin was used as standard for bacterial test pathogens. For *Xanthomonas* strains, copper oxychloride was used as a standard and Miconazole was the standard used for *C. albicans*.

Time-kill curves

Xanthomonas strains exhibiting anti-biofilm susceptibility to PCN were studied to understand the quantitative effect of PCN by plotting the time-response growth curves [16]. In brief, each of the *Xanthomonas* strains (10^6 cfu/ml) were cultured in sucrose-peptone broth. Each strain was divided into the control group with only solvent (DMSO) and two treatment groups (32 and 62 μg/ml of PCN). PCN (20 μl) of each concentration was added to the treatment groups. A sample of 20 μl was withdrawn from each group at various time intervals and plated on sucrose-peptone agar. The plates were incubated at 30℃ for 24–28 h and were then quantified for the colony forming units.

Molecular docking

Molecular docking studies of PCN were performed with different protein targets including hydrolase of *X. oryzae* pv. *oryzae* (PDB ID: 5CP0), proline iminopeptidase of *X. campestris* pv. *citri* (PDB ID: 1AZW), c-di-GMP receptor essential for biofilm formation and virulence in *X. campestris* pv. *campestris* (PDB ID: 5B7W), Type-3-effector/polynucleotide kinase domain of *X. oryzae* pv. *oryzicola* (PDB ID: 4Z8V). The ligand was sketched in Sybyl 6.7. The molecular energy was minimized by adding Gasteiger-Huckel charges to attain a stable conformation. AUTODOCK 4.2 was used to predict the affinity, activity, binding orientation of the ligand with the target protein and to analyze the best conformations. Initially, the molecule was loaded; torsions were set and saved in PDBQT format. For docking purpose, the co-crystallized X-ray structure of the protein was loaded with the ligand binding site using Autodock 4.2 docking program. In the present investigation, we focused mainly on the binding energy, hydrogen bonds, and distance between the protein and ligand. All calculations for protein-ligand flexible docking were performed using the Lamarckian Genetic Algorithm (LGA) method. A grid box with the dimensions of X, Y, Z coordinates with a default grid spacing of 0.375 Å was used [21].

Results and Discussion

CGK-KS-1 produced 2.5 g/l of crude extract after fermentation for 48 h. The crude extract from strain CGK-KS-1 showed three spots on TLC developed in a combination of methanol: chloroform (10:90) (Fig. S1). The major compound 1 (Spot-1) was eluted from the TLC plate and solubilized in methanol. Further, the purity of the major compound-1 was assessed and the HPLC chromatogram showed a single peak with retention time of 21.717 min (Fig. S2). This compound was found to be UV-active and visualized as a dark green band under UV light at 254 nm, developed by spraying with iodine. The purified major compound was a greenish, non-fluorescent solid with no odour. It was soluble in different organic solvents such as methanol, ethanol, ethyl acetate, dimethyl sulfoxide, chloroform and sparingly soluble in water. The elemental compositional analysis of compound 1 is shown in Table S1. The productivity of compound-1 was 20 mg/l with a melting point of 242℃. The UV absorbance recorded in acetonitrile was 248 nm. ^{1}H NMR spectrum (Fig. 1) showed a broad singlet at δ = 11.0 ppm indicating the presence of amide protons. Multiplet of aromatic protons was displayed at δ = 7.71–8.55 ppm and 8.02–8.55 ppm. Two doublets were indicated by δ = 8.18–9.0 ppm (^{1}H, J = 5.6 Hz) and 8.37 ppm (^{1}H, J = 7.9 Hz). Two protons were displayed as multiplets

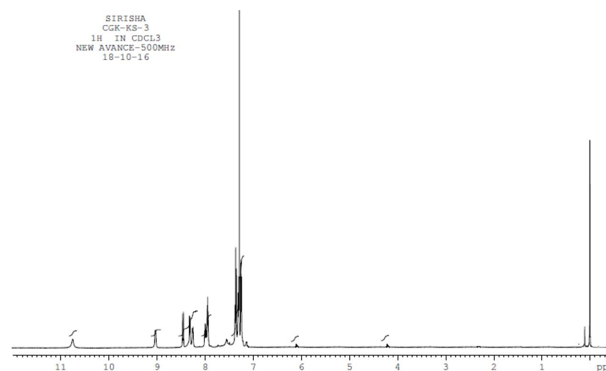

Fig. 1. ^{1}H NMR spectrum of phenazine-1-carboxamide produced by *P. aeruginosa* strain (CGK-KS-1).

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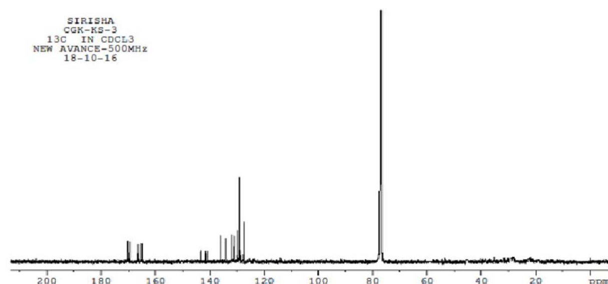

Fig. 2. ^{13}C NMR spectrum of phenazine-1-carboxamide produced by *P. aeruginosa* strain (CGK-KS-1).

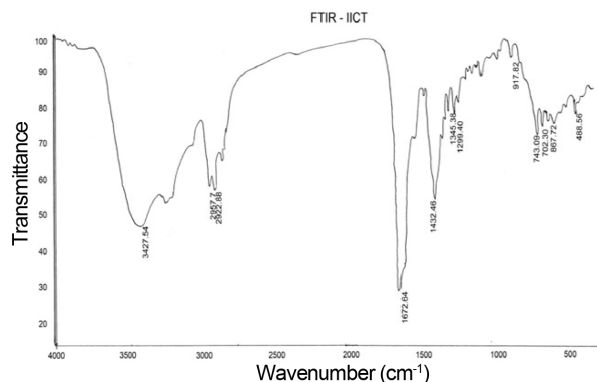

Fig. 3. FT-IR spectrum of phenazine-1-carboxamide produced by *P. aeruginosa* strain (CGK-KS-1).

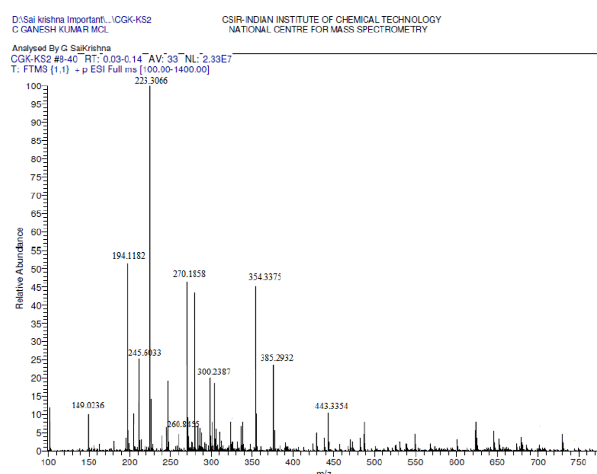

Fig. 4. EI-HR-MS spectrum of phenazine-1-carboxamide produced by *P. aeruginosa* strain (CGK-KS-1).

between δ = 8.18 and 8.27 ppm. ^{13}C NMR spectrum (Fig. 2) gave signals at δ = 170.36, 169.24, 166.37, 165.09, 142.65, 143.44, 140.67, 136.49, 134.07, 131.98, 130.75, 128.88, 127.66 ppm. A signal at δ = 166.37 ppm indicated the presence of carbonyl carbon of the amide group. The remaining signal peaks from δ = 127.66 to 170.36 ppm corresponded to aromatic carbons. The protons of the PCN were determined from the TOCSY, COSY, HMBC and HSQC spectra (Fig. S3–S6). The ^{1}H NMR spectrum of compound-1 (Fig. 1) indicated an ABCD pattern in the aromatic ring, which is a typical feature observed among phenazine derivatives [22], while the ^{13}C NMR spectrum (Fig. 2) in $CDCl_3$ displayed 13 well-resolved signals, suggesting a symmetrical structure for the molecule. IR (KBr) (ν_{max}) showed characteristic peaks at 3427 cm^{-1} which corresponded to amide NH group, ν = 2957 and 2922 cm^{-1} attributed to aromatic CH stretching vibrations and peak at ν = 1672 cm^{-1} showed the presence of amide C=O stretch (Fig. 3); HR-MS (ESI) *m/z*: calculated mass for $C_{13}H_9N_3O$, $[M+H]^+$ is 223.3, found: 223.306 (Fig. 4). Based on ^{1}H and ^{13}C NMR, 2D NMR, FT-IR and HR-MS spectral analysis (Table S2), the purified compound-1 was identified as phenazine-1-carboxamide (PCN).

PCN showed promising antimicrobial activity against various human pathogens and *Xanthomonas* strains. The results of the antimicrobial activity, MBC and/or MFC are shown in Table 1, which showed that the PCN possess a promising antibacterial activity against all the Gram-positive bacteria, Gram-negative bacteria, *C. albicans* and *Xanthomonas* strains except for *X. axonopodis* pv. *malvacearum*. In the current study, PCN showed MBC value of 3.9 μg/ml against *S. aureus* MTCC 96, *B. subtilis* MTCC 121, *S. aureus* MLS16 MTCC 2940, *M. luteus* MTCC 2470 and *K. planticola* MTCC 530. MBC of PCN against *E. coli* MTCC 739 and *C. albicans* was 7.8 μg/ml. Among the phytopathogens, PCN showed MBC of 3.9 μg/ml against the rice pathogens such as *X. oryzae* pv. *oryzae* strain BXO43, 7.8 μg/ml against *X. oyrzae* pv. *oryzicola* strain Y2, *X. campestris* pv. *vesicatoria* strain 8004, *X. campestris* pv. *citri*, *X. campestris* pv. *campestris* strain TNAU, *X. campestris* pv. *vesicatoria* strain 1 and strain 65-1. Earlier reports indicated that PCN inhibited phytopathogens like *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *X. oryzae* pv. *oryzae* [15, 23] and human pathogens like *C. albicans* [17], which corroborates our findings.

A biofilm is a surface attaching sessile microbial life form comprising of secreted polysaccharides which are helpful for adhesion, enzymes capable of degrading anti-

Table 1. Antimicrobial activity of the purified phenzine-1-carboxamide (PCN) from *P. aeruginosa* strain (CGK-KS-1).

Test pathogen	PCN		Ciprofloxacin		Miconazole		Copper oxychloride	
	MIC[a] (μg/ml)	MBC[b] (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MFC[b] (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
S. aureus MTCC 96	3.9	3.9	0.9	0.9	-[c]	-[c]	-	-
S. aureus MLS16 MTCC 2940	3.9	3.9	0.9	0.9	-	-	-	-
B. subtilis MTCC 121	1.9	3.9	0.9	0.9	-	-	-	-
M. luteus MTCC 2470	1.9	3.9	0.9	0.9	-	-	-	-
E. coli MTCC 739	3.9	7.8	0.9	0.9	-	-	-	-
K. planticola MTCC 530	3.9	3.9	0.9	0.9	-	-	-	-
C. albicans MTCC 3017	3.9	7.8	-	-	7.8	7.8		
X. oryzae pv. *oryzae* BXO43	3.9	3.9	-	-	-	-	62.5	125
X. oryzae pv. *oryzicola* strain Y2	3.9	7.8	-	-	-	-	62.5	125
X. campestris pv. *vesicatoria* strain 1	3.9	7.8	-	-	-	-	62.5	125
X. campestris pv. *vesicatoria* strain 65-1	3.9	7.8	-	-	-	-	62.5	125
X. campestris pv. *vesicatoria* strain 85-10	3.9	15.6	-	-	-	-	125	250
X. campestris pv. *vesicatoria* strain 8004	7.8	7.8	-	-	-	-	125	250
X. campestris pv. *campestris* strain TNAU	3.9	7.8	-	-	-	-	125	250
X. campestris pv. *citri*	3.9	7.8	-	-	-	-	125	250

[a]Minimum inhibitory concentration (MIC) is defined as the lowest concentration of the compound that inhibited 90% of the growth when compared with that of the control growth. Values are means of triplicate ± SD.
[b]Minimum bactericidal concentration (MBC) or Minimum fungicidal concentration (MFC) which corresponds to the minimum concentration of the bioactive compound to reduce the number of CFU for 0.1% of the initial inoculum.
[c]No activity.

microbials, charged moieties like DNA. Bacterial and fungal biofilms make the inhabiting bacteria and fungi resistant to antimicrobial agents and the host immune network [24, 25]. In the biomedical sector, pathogens like *C. albicans, K. planticola* and *E. coli* form biofilms in catheters, stents, fracture fixings, dental prosthetics posing serious contamination problems in post-surgical cases [9]. Biofilm formation has been reported in phytopathogens like *X. oryzae* pv. *oryzae, X. oryzae* pv. *oryzicola, X. campestris* pv. *citri*, *X. campestris* pv. *campestris* [26–29]. In *X. axonopodis* pv. *citri*, sessile biofilm formation is an important factor for infection and the inability to form a biofilm disables the organism to cause the disease [29, 30]. In plant and human pathogens transformation into biofilm is involved in the upsurge of antimicrobial resistance [31]. In the present study, we showed for the first time that PCN exhibited biofilm inhibition activity against phytopathogenic *Xanthomonas* strains. The mode of action of phenazine-1-carboxamide might be its intercalation with grooves of DNA, enzymes like topoisomerases, and charge-transferring moieties [32]. Further, PCN inhibited the biofilm formation of some of the Gram-positive, Gram-negative bacteria, *C. albicans* MTCC 3017 and some of the *Xanthomonas* strains and the results to this regard are shown in Table 2. In this context, the IC_{50} (μM) values of PCN against *M. luteus* MTCC 2470, *K. planticola* MTCC 530 and *E. coli* MTCC 739 were 17.25, 17.46 and 34.82 μM, respectively. The IC_{50} value of PCN for anti-biofilm activity against *C. albicans* MTCC 3017 was 34.82 μM. The anti-biofilm activity values of PCN against *X. oryzae* pv. *oryzae* strain BXO43 and *X. oryzae* pv. *oryzicola* strain Y2 were 17.04 and 34.82 μM, respectively. While the anti-biofilm activity values of PCN against *X. campestris* pv. *campestris* strain TNAU, *X. campestris* pv. *vesicatoria* strain 1, *X. campestris* pv. *vesicatoria* strain 65-1, *X. campestris* pv. *citri* and *X. campestris* pv. *vesicatoria* strain 85–10 ranged between 29.01 to 60.7 μM. From the growth-kill curve analysis, the cfu/ml decreased by almost 10 fold on treatment with PCN (62 μg/ml) for 24 h (Fig. S5). (Fig. S7).

The molecular docking studies were performed to pre-

Table 2. Anti-biofilm activity of the purified PCN from *P. aeruginosa* strain (CGK-KS-1).

Test pathogen	IC_{50} (μM)			
	PCN	Ciprofloxacin	Miconazole	Copper oxychloride
M. luteus MTCC 2470	17.25 ± 0.09	3.01 ± 0.08	-	-
K. planticola MTCC 530	17.46 ± 0.24	3.04 ± 0.11	-	-
E. coli MTCC 739	34.82 ± 0.2	3.02 ± 0.09	-	-
C. albicans MTCC 3017	34.82 ± 0.1	-	18 ± 0.09	-
X. oryzae pv. *oryzae* BXO43	17.04 ± 0.14	-	-	585 ± 0.17
X. oryzae pv. *oryzicola* strain Y2	34.82 ± 0.07	-	-	550 ± 0.26
X. campestris pv. *vesicatoria* strain 1	32 ± 0.14	-	-	592 ± 0.23
X. campestris pv. *vesicatoria* strain 85-10	60.7 ± 0.14	-	-	650 ± 0.32
X. campestris pv. *vesicatoria* strain 65-1	29.01 ± 0.14	-	-	650 ± 0.23
X. campestris pv. *campestris* strain TNAU	29 ± 0.14	-	-	-
X. campestris pv. *citri*	33.01 ± 0.13	-	-	750 ± 0.27

dict the interaction of PCN with different target proteins from different *Xanthomonas* species. Molecular docking studies exhibited a good correlation between the predicted binding energies of the PCN and the experimental binding affinities. The observed binding modes suggests that PCN binds at the active site of peptidyl deformylase (PDB ID: 5CP0) from *X. oryzae* pv. *oryzae* with a high binding energy of -7.91 kcal/mol by interacting with the Leu_{100} (Fig. 5a). It is presumed that PCN interacts with the active site of the enzyme which might possibly lead to inhibition of protein synthesis due to lack of removal of *N*-formyl group from nascent peptides. PCN interacts with Asn_{279} and Gly_{337} of type 3 effector/ polynucleotide kinase (PDB ID: 4Z8V) of *X. oryzae* pv. *oryzicola* with a binding energy of -6.02 kcal/mol (Fig. 5b). Further the docking of PCN with proline iminopeptidase (PDB ID: 1AZW) showed that PCN interacted with Gly_{42} of the protein with a binding energy of -5.69 kcal/mol (Fig. 5c). Docking of PCN with a receptor of c-di-GMP, involved in motility and biofilm formation in *X. campestris* pv. *campestris* (PDB ID: 5B7W) showed that PCN has high affinity with a binding energy of -6.7 kcal/ mol (Fig. 5d). Ligand interactions and their respective interacting residues are depicted in Table S3.

In conclusion, the PCN yielded from *P. aeruginosa* strain CGK-KS-1 showed promising antimicrobial activity against both human and against various *Xanthomonas* strains. We report for the first time the biofilm inhibition activity of PCN against Gram-positive, Gram-negative bacteria, *C. albicans* MTCC 3017 and various phyto-

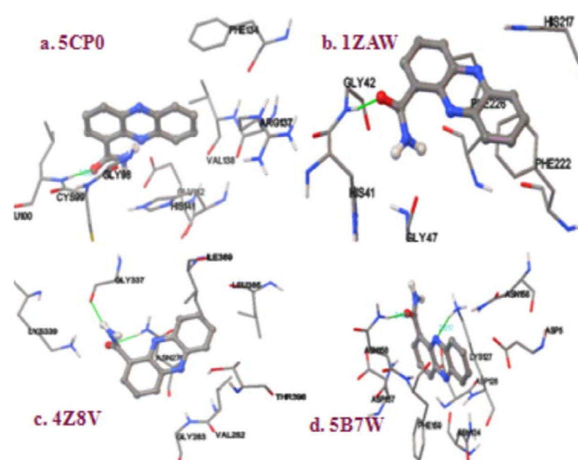

Fig. 5. Molecular docking of phenazine-1-carboxamide produced by *P. aeruginosa* strain (CGK-KS-1) with various target proteins of different *Xanthomonas* strains. (a) Interaction with peptide deformylase (PDB: 5CP0) of *X. oryzae* pv. *oryzae*, (b) Interaction with proline iminopeptidase (PDB: 1ZAW) of *X. campestris* pv. *citri*, (c) Interaction with 3 effector/ polynucleotide kinase (PDB: 4Z8V) of *X. oryzae* pv. *oryzicola*, (d) Interaction with c-di-GMP receptor (PDB: 5B7W) of *X. campestris* pv. *campestris*.

pathogenic *Xanthomonas* strains. The *in silico* docking studies suggest that PCN binds different enzymes of *Xanthomonas* species with high affinity. This anti-phytopathogenic property of PCN can be further explored as an alternative to the conventional practice of agrochemical usage for the biocontrol of bacterial blight diseases caused by *Xanthomonas* sp. For elucidating the biocontrol mechanism of phenazine-1-carboxamide further

research is mandatory. Greenhouse experiments that involve the treatment of *Xanthomonas* infected plants with PCN and observing its effect on disease progression and control could reveal the applicability of PCN as eco-friendly and safe microbial biocontrol agent.

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Conflict of Interest

The authors declare that there is no conflict of interest with any researcher or funding agency.

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