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Antagonistic effect of *Pseudomonas aeruginosa* isolates from various ecological niches on *Vibrio* species pathogenic to crustaceans

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the antagonistic effects of *Pseudomonas* on the growth of *Vibrio*. The authors concluded that such organism (MCCB102, MCCB103) is effective for brackish water while MCCB117 and MCCB118 are effective for marine aquaculture.

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ABSTRACT

Objective: To abrogate pathogenic vibrios in aquaculture by testing the potential of *Pseudomonas* isolates from fresh water, brackish and marine environments as probiotics.

Methods: Purification and structural elucidation of antagonistic compound were carried out. Antagonistic activity of the compound against 7 *Vibrio* spp. was performed. Influence of salinity on the production of pyocyanin and the toxicity was done through the compound using brine shrimp lethality assay. Molecular characterization was performed to confirm that the isolates were *Pseudomonas aeruginosa*.

Results: Salinity was found to regulate the levels of pyocyanin production, with 5–10 g/L as the optimum. All *Pseudomonas* isolates grew at salinities ranging from 5 to 70 g/L. Isolates of marine origin produced detectable levels of pyocyanin up to 45 g/L salinity. Brackish and freshwater isolates ceased to produce pyocyanin at salinities above 30 g/L and 20 g/L, respectively. Culture supernatants of all 5 *Pseudomonas* isolates possessed the ability to restrict the growth of *Vibrio* spp. and maximum antagonistic effect on *Vibrio harveyi* was obtained when they were grown at salinities of 5 to 10 g/L. The marine isolate MCCB117, even when grown at a salinity of 45 g/L possessed the ability to inhibit *Vibrio* spp.

Conclusions: The present investigation showed that *Pseudomonas aeruginosa* MCCB119 would be ideal for application in freshwater, MCCB102 and MCCB103 in brackish water and MCCB117 and MCCB118 in marine aquaculture systems as putative probiotics in the management of vibrios.

KEYWORDS

Pseudomonas, Pyocyanin, *Vibrio*, Salinity, Antagonism

1. Introduction

Vibrios are ubiquitous bacterial pathogens in marine and brackish water aquaculture systems infecting almost all groups of cultured animals. To protect aquaculture stocks, antibiotics have been used over the years, at times indiscriminately due to which several pathogenic vibrios have acquired multiple drug resistance and become

untreatable, and the drug resistance has been transferred to pathogens of human concern^[1]. Due to these adverse effects, alternative measures of protecting aquaculture species against vibriosis are being explored including the enrichment of the culture systems with antagonistic probiotics. Among the probiotics, antagonistic *Pseudomonas* spp. have been gaining increasing attention as biological control agents against pathogenic fungi and bacteria

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in agriculture^[2–4], vibrios in aquaculture^[5,6] and also as bioaugmenters in bioremediation programs^[7–9]. Specifically, the versatile and ubiquitous bacterium, *Pseudomonas aeruginosa*^[10,11] (*P. aeruginosa*) has recently been recognized as an active antagonist of pathogenic *Vibrio harveyi* (*V. harveyi*) and thus as a candidate probiotic in aquaculture systems^[12–15]. The anti-vibrio compound produced by *P. aeruginosa* was proved to be pyocyanin^[16], a blue-green chloroform-soluble phenazine pigment, which possessed broad-spectrum antibacterial^[17], anti-fungal^[18,19] and antiprotozoan activities^[20].

Pyocyanin synthesis by *P. aeruginosa* is influenced by environmental factors including salinity^[21,22]. As the salinity requirement for aquaculture species vary widely, the effect of salinity on pyocyanin production by *P. aeruginosa* has been investigated here to identify specific isolates, from various ecological niches, with varying salinity tolerance and preference best suited for use in aquaculture systems. Assays have been conducted to assess the anti-vibrio effect of *P. aeruginosa* isolated from fresh water, brackish and marine environments, and to correlate this to pyocyanin production at different salinity levels. Antagonistic effects against several pathogenic vibrios as well as pyocyanin toxicity on brine shrimp [*Artemia salina* (*A. salina*)] nauplii have also been determined.

2. Materials and methods

2.1. Bacterial isolates

Five isolates of *Pseudomonas* (MCCB102, 103, 117, 118 and 119) were subjected for the study. The isolates MCCB102 and MCCB103 originated from brackish water environments off Chennai (Muthukkad estuary) and Cochin (foregut of shrimp collected from brackish water environment), India, respectively. Isolates MCCB117 and MCCB118 originated from marine sediments collected onboard FORV *Sagar Sampada* (Fishing and Oceanic Research Vessel, Government of India) cruise number 233 from Arabian Sea at depths of 500 m (7°00'19" N, 77°20'30" E) and 200 m (9°54'83" N, 75°55'00" E), respectively. The isolate MCCB119 was a freshwater one from the effluent discharge point of M/s Hindustan Organic Chemicals, Cochin. A reference strain of *P. aeruginosa*, MTCC741 (Microbial Type Culture Collection, Chandigarh, India), was also included in the study. Isolates from marine and brackish water environments were maintained in ZoBell's marine agar slants (2216E HiMedia, India) and the freshwater isolate and the type strain were maintained in nutrient agar slants. They were deposited in the microbial culture collection of National Centre for Aquatic Animal Health, Kerala, India. The marine and brackish water isolates of *Pseudomonas* were grown in media containing 0.5% peptone, 0.1% yeast extract, prepared in 30 and 15

g/L salinity seawater respectively, and the freshwater isolate and the type strain in the media having the same composition prepared in distilled water supplemented with 0.5% NaCl and incubated at (28±1) °C for all the below mentioned assays.

A set of *Vibrio* spp. obtained from Belgium Culture Collection (BCCM-LMG) which included *V. harveyi* (BCCM-LMG 4044), *Vibrio parahaemolyticus* (*V. parahaemolyticus*) (BCCM-LMG 2850), *Vibrio vulnificus* (*V. vulnificus*) (BCCM-LMG 13545), *V. alginolyticus* (BCCM-LMG 4409), *V. fluvialis* (BCCM-LMG 11654), *Vibrio mediterranei* (*V. mediterranei*) (BCCM-LMG 11258) and *Vibrio nereis* (*V. nereis*) (BCCM-LMG 3895) along with a wild isolate of *V. harveyi* (MCCB111) deposited in the microbial culture collection of the National Centre for Aquatic Animal Health were used for the study.

2.1.1. 16S rRNA gene sequence analysis and identification

A 1.5 Kb fragment of the 16S rRNA gene was amplified from genomic DNA and sequenced to determine the uniqueness of each *Pseudomonas* isolate. Total genomic DNA was extracted by following standard method^[23] with slight modification. Briefly, aliquots of 2 mL overnight cultures grown in media containing 0.5% peptone, 0.1% yeast extract were centrifuged and the resultant pellet re-suspended in 1 mL lysis buffer (0.05 mmol/L Tris-HCl, 0.05 mmol/L ethylene diamine tetraacetic acid, 0.1 mol/L NaCl, 2% sodium dodecyl sulfate, 0.2% polyvinylpyrrolidone, 0.1% β-mercaptoethanol) followed by the addition of 20 µg/mL proteinase K and incubated at 37 °C for 1 h and then at 55 °C for 2 h. DNA was extracted by standard phenol-chloroform method^[24]. A region of the 16S rRNA gene was amplified by PCR using the universal primers NP1F (GAG TTT GAT CCT GGC TCA) and NP1R (ACG GCT ACC TTC TTA CGA CTT)^[25] in a 25 µL reaction mixture containing 1X Thermopol buffer (New England Biolabs, USA), 200 µmol/L dNTPs, 2.5 mmol/L MgCl₂, 10 pmol each primer, 75 ng DNA template, 1 IU *Taq* polymerase. Thermal cycling (Eppendorf, Germany) included 35 cycles of 94 °C for 20 seconds, 58 °C for 30 seconds, and 72 °C for 2 min followed by a final extension at 72 °C for 10 min. The 1.5 Kb DNA product was gel purified using a Wizard SV PCR purification kit (Promega, USA) and was sequenced in ABI 3700 sequencer at M/s Microsynth AG, Switzerland. The sequences obtained were matched with the GenBank database using the BLAST algorithm^[26] and submitted under the accession numbers EF062514 (MCCB102), EF053508 (MCCB103), EF062511 (MCCB117), EF062512 (MCCB118), and EF062513 (MCCB119).

2.1.2. Antagonism to *Vibrio* spp.

The culture supernatant from all the five isolates of *P. aeruginosa* along with the reference strain were examined for their antagonistic effect against *Vibrio* spp. obtained from BCCM-LMG including the wild isolate, *V. harveyi* (MCCB111) by disc diffusion assay. Each of the *Vibrio* spp. were grown on ZoBell's marine agar slants (2216E HiMedia, India)

and incubated at $(28 \pm 1)^\circ\text{C}$ overnight. These cultures were harvested in sterile 15 g/L salinity seawater, absorbance (Abs_{600}) adjusted to 1.5 and swabbed 500 μL onto ZoBell's marine agar plates. The isolates of *Pseudomonas* were grown in the above mentioned media and incubated at $(28 \pm 1)^\circ\text{C}$. About 1 mL aliquot of each of these 24 h grown *Pseudomonas* cultures were removed, centrifuged at 12 000 r/min for 15 min and 20 μL supernatant was spotted onto sterile discs of 6 mm in diameter (prepared from a stack of 6 Whatman No.1 filter papers) and placed on the plates swabbed with *Vibrio* spp. The plates were incubated at $(28 \pm 1)^\circ\text{C}$ for 18 h and the diameter of the zone of inhibition recorded using HI antibiotic zone scale (Himedia, Mumbai, India). The assays were conducted in triplicate.

2.2. Purification of the antagonistic compound and its functional assessment

Among the 5 *Pseudomonas* isolates examined, MCCB117 was selected for extraction and characterization of the active compound based on its potential to exhibit higher inhibitory activity even at higher salinity (40 g/L). One litre of 24 h old MCCB117 culture was centrifuged at 12 000 r/min for 15 min at 4°C . The supernatant was filter sterilized (0.2 μm) and extracted using chloroform at a 1:1.5 (supernatant:chloroform) ratio. The chloroform fraction, blue in colour due to the presence of blue coloured pyocyanin, was run through 3 cm diameter, 60 cm long column, packed with 100–200 mesh size silica gel having methanol–chloroform mixture (ratio: 1:1) as the mobile phase. The blue coloured column fraction was collected and concentrated in a rotary evaporator under vacuum at 42°C . The concentrated pyocyanin was taken in a pre-weighed amber coloured bottle and passed a jet of nitrogen gas to remove solvents and to dry the product. Weight of the dried pyocyanin was determined gravimetrically. This was then dissolved in 500 μL dimethyl sulfoxide (DMSO) and used for all assays.

2.2.1. Antagonism of the purified compound to *Vibrio* spp.

The anti-microbial activity of the purified pyocyanin was determined by standard disc diffusion method, by using different concentrations of purified pyocyanin such as 5, 10, 20 and 30 mg/L obtained by adding Milli-Q water. An aliquot of 20 μL of these concentrations each was tested against *Vibrio* spp. obtained from BCCM-LMG and the wild isolate, *V. harveyi* (MCCB111) in triplicates. Discs containing 20 μL DMSO alone (100%) was used as control.

2.2.2. Identification of the purified compound

The purified compound along with an authentic standard of pyocyanin obtained from M/S Color Your Enzyme, Ontario, Canada, was analysed by high pressure liquid chromatography (HPLC)[27]. HPLC analysis was performed on a Dionex, model ultimate 3 000 (Germany) gradient elution

system attached to a C_{18} column (250.0 \times 4.6 mm) and a detector monitoring at 280 nm controlled by the software programme Chromeleon (version 6.80). A gradient method was used for eluting samples employing solvent systems A and B. Solvent A was water–trifluoroacetic acid (100:0.04, v/v) and solvent B acetonitrile–water–trifluoroacetic acid (90:10:0.04, v/v/v).

The structure of the antagonistic compound was further confirmed by nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy analysis was carried out in BRUCKER AVANCE IIIITM, FT-NMR spectrometer.

2.3. Influence of salinity on growth and antagonistic compound production

To determine the extent of salinity tolerance and preference of all the isolates of *P. aeruginosa* and the influence of salinity on their growth and antagonistic compound production, the isolates were individually inoculated into 100 mL media containing 0.5% peptone, 0.1% yeast extract (pH 7.5) at varying salinities of 5, 10, 15, 20, 30, 40, 50, 60, and 70 g/L obtained by adding sea water. For obtaining salinity above 30 g/L NaCl was added to seawater. All the experiments were done in triplicate. For assessing the growth and antagonistic compound production at zero salinity, the cultures were inoculated into medium having the same composition prepared in double distilled water. Cell suspensions of marine and brackish water isolates for inoculations were prepared from overnight grown slant cultures on ZoBell's marine agar prepared in 30 and 15 g/L salinity respectively. The cell suspensions of freshwater isolate and the type strain were prepared from nutrient agar slant grown cultures. The cells grown on the slants prepared with seawater having 15 and 30 g/L salinities were harvested in sterile seawater having the same salinity and freshwater isolate and the type strain in 0.5% saline. Absorbance of the suspension was adjusted to 1.0 at 600 nm (Abs_{600}) in a UV-vis spectrophotometer (Shimadzu, Japan) and the cells were seeded into the respective media (100 mL) to give a uniform initial absorbance of 0.01 at Abs_{600} . The cultures were incubated in shaker incubator (Orbitek, Scigenics Biotech, India) at $(28 \pm 1)^\circ\text{C}$ at 120 r/min.

Samples (6 mL) were withdrawn aseptically from each culture flask after 24 h incubation to quantify growth as well as antagonistic compound production and activity. Absorbance at 600 nm (Abs_{600}) of 1 mL culture was used to measure the growth of *P. aeruginosa*. The antagonistic compound production was quantified by extracting 5 mL culture supernatant with 3 mL chloroform and subsequently by adding 1 mL of 0.2 mol/L HCl to the chloroform extract which generated a red-coloured solution. The absorbance at Abs_{520} of the solution multiplied by a factor 17.072 was used to quantify the antagonistic compound ($\mu\text{g/mL}$)[28].

Antagonistic activity of the culture supernatants of all

Pseudomonas isolates, at all salinities was assayed against the wild isolate of *V. harveyi* (MCCB111) by way of disc diffusion, since *V. harveyi* has been reported as the most important aquaculture pathogen with multiple antibiotic resistance, causing mass mortalities in shrimp/prawn hatcheries[29].

2.4. Effect of NaCl on growth and antagonistic compound production

To investigate the impact of NaCl alone in the basal medium on growth and antagonistic compound production, a medium was prepared in de-ionized water containing 0.5% peptone, 0.1% yeast extract, supplemented with 5 g/L NaCl in triplicate. The specific NaCl concentration was selected since the antagonistic compound production of all the isolates were determined to be the highest at a salinity range of 5–10 g/L. Medium containing 0.5% peptone, 0.1% yeast extract prepared with seawater having 5 g/L salinity served as the control. The growth, antagonistic compound production and antagonism of *P. aeruginosa* were determined as described earlier.

2.5. Brine shrimp toxicity assay

Toxicity of the antagonistic compound was tested using *A. salina* nauplii (brine shrimp) as model animal representing crustaceans. Dried *Artemia* cysts were hatched (1 g cyst per liter) in filtered seawater at 27–30 °C with profuse aeration. Approximately 12 h after hatching, the nauplii were collected with a pipette and transferred a group of 20 nauplii to each of the wells of a 24 well plate filled with 1 mL sea water. Each test consisted of exposing the nauplii to different concentrations (50, 100, 150, 200, 250, 300, 350 and 400 mg/L) of the antagonistic compound dissolved in DMSO, added to the seawater in the 24 well plate in triplicate. One of the controls was nauplii in seawater without the antagonistic compound and the other was seawater added with DMSO in the same concentration as that in the test. The DMSO concentrations were 1% for 50 mg/L, 2% for 100 mg/L, 3% for 150 mg/L and so on till 8% for 400 mg/L pyocyanin. Toxicity was determined after 12 h and 24 h exposure by counting the number of survivors and calculating the percentage mortality. Larvae were considered dead if they did not exhibit any movement of appendages.

2.6. Statistical analysis

Data were subjected to analysis of variance (ANOVA) as applicable and significant differences were recorded based on *P*-value <0.05. Data from *Artemia* assay were analyzed statistically by probit analysis using SPSS software (SPSS 17.0, SPSS INC., Chicago, USA) to determine LC₅₀ values with 95% confidence.

3. Results

3.1. Identification of the bacterial isolates

Nucleotide sequences of the five isolates determined for a 1.5 Kb region of the 16S rRNA gene were 99% identical to *P. aeruginosa* in BLAST searches of GenBank.

Filter-sterilized cell-free supernatants of all isolates of *P. aeruginosa* inhibited growth of *Vibrio* spp. including *V. harveyi* (both BCCM-LMG 4044 and MCCB111), *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. mediterrani* and *V. nereis* with the inhibitory zones ranging from 13.5 to 31.0 mm in disc diffusion tests (Table 1). *P. aeruginosa* MCCB102 and MCCB117 generated the largest inhibitory zones against all *Vibrio* spp. examined.

Table 1

Antagonistic activity of culture supernatants of isolates of *P. aeruginosa* on *Vibrio* spp. measured in terms of diameter of the inhibition zone (mean±SD).

<i>Vibrio</i> spp.	Diameter of Zone (in mm) on <i>Vibrio</i> spp. by the isolates of <i>P. aeruginosa</i>					
	MCCB102	MCCB103	MCCB117	MCCB118	MCCB119	MTCC741
BCCM-LMG 4044	16.00±1.41	15.00±1.41	16.00±0.71	13.50±0.71	14.50±0.71	13.00±1.41
BCCM-LMG 11654	18.50±2.12	17.50±2.12	17.50±0.71	15.50±0.71	15.00±0.00	16.50±0.71
BCCM-LMG 11258	18.00±0.00	14.50±0.71	16.00±0.71	14.00±1.41	14.50±0.71	13.50±0.71
BCCM-LMG 3895	15.50±0.71	15.00±1.41	15.50±0.71	14.00±0.00	13.50±0.71	13.00±1.41
BCCM-LMG 2850	15.50±0.71	14.00±0.00	15.50±0.71	13.50±0.71	13.50±2.12	13.50±0.71
BCCM-LMG 13545	31.00±1.41	29.50±0.71	31.00±1.41	28.50±0.71	29.50±0.71	28.50±0.71
BCCM-LMG 4044	17.50±0.71	14.50±0.71	17.00±0.00	13.50±0.71	14.50±0.71	13.50±0.71
MCCB111	16.50±1.41	15.00±1.41	16.00±0.71	13.00±0.71	14.50±0.71	13.00±1.41

3.2. Identification of the antagonistic compound

The HPLC retention time (RT value) of the purified compound was 15 min, identical to that of the pyocyanin standard (Figure 1). In the ¹H-NMR spectrum, the peaks observed in the aromatic region (δ 7–9) corresponded with those determined for pure pyocyanin (Figure 2).

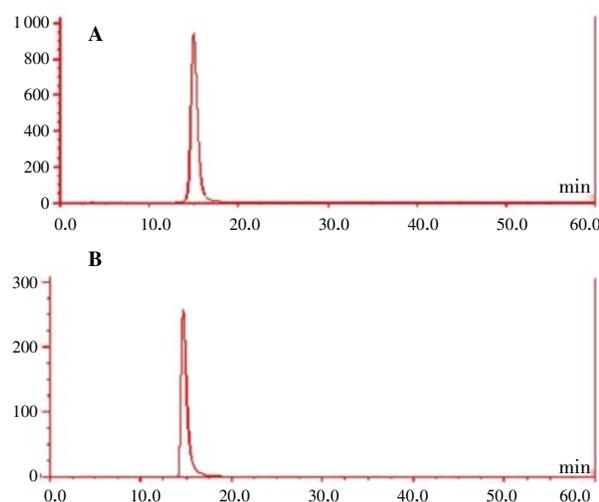


Figure 1. HPLC of pyocyanin.

A) Pyocyanin from the marine isolate *P. aeruginosa* MCCB117; B) pyocyanin authentic standard (Color Your Enzymes, Kingston, ON, Canada).

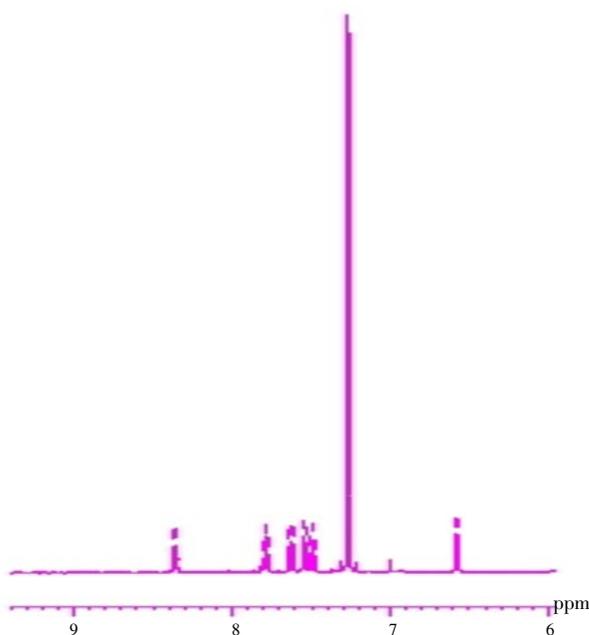


Figure 2. ^1H NMR spectra of pyocyanin extracted from marine isolate *P. aeruginosa* MCCB117.

The purified pyocyanin inhibited the growth of all *Vibrio* spp. above a concentration of 5 mg/L. At 5 mg/L, no zone of inhibition was observed. At 10 mg/L, the zones were less than 12 mm. Maximum zone of inhibition was observed at a concentration of 30 mg/L pyocyanin (Table 2). The control discs containing DMSO did not show any inhibitory activity.

Table 2

Antagonistic activity of purified pyocyanin (20 μL containing 30 mg/L pyocyanin) against different *Vibrio* spp. measured as the diameter of the inhibition zone (mean \pm SD).

<i>Vibrio</i> spp.	Zone Diameter (in mm)
<i>V. harveyi</i> BCCM–LMG 4044	27.50 \pm 0.71
<i>V. fluvialis</i> BCCM–LMG 11654	26.50 \pm 0.71
<i>V. mediterranei</i> BCCM–LMG 11258	26.00 \pm 1.41
<i>V. nereis</i> BCCM–LMG 3895	27.50 \pm 0.71
<i>V. parahaemolyticus</i> BCCM–LMG 2850	17.50 \pm 0.71
<i>V. vulnificus</i> BCCM–LMG 13545	38.00 \pm 1.41
<i>V. alginolyticus</i> BCCM–LMG 4044	28.50 \pm 0.71
<i>V. harveyi</i> MCCB111	27.50 \pm 0.71

3.3. Influence of salinity on growth and pyocyanin production

Growth and pyocyanin production of all isolates of *P. aeruginosa* were influenced, significantly by salinity ($P<0.001$) (Figures 3 and 4). All the isolates exhibited growth up to 70 g/L salinity (Figure 3), but the extent of pyocyanin production with respect to salinity varied distinctly among the isolates (Figure 4). While the isolates of marine origin (MCCB117, MCCB118) produced detectable levels of pyocyanin in the media having salinities up to 45 g/L, the brackish water isolates (MCCB102, MCCB103) ceased to produce

pyocyanin in the media with salinities above 30 g/L. The freshwater isolate (MCCB119) did not produce pyocyanin in the medium having salinities above 20 g/L. The reference strain MTCC741 could produce pyocyanin up to salinity 30 g/L, but only in very small quantity. Maximum pyocyanin production of marine and brackish water isolates occurred at a salinity of 10 g/L, but it was at 5 g/L with the freshwater isolate and reference strain. Among the marine isolates, pyocyanin production was significantly ($P<0.01$) higher in MCCB117 compared to MCCB118. Similarly, pyocyanin production of the brackish water isolate MCCB102 was significantly ($P<0.05$) higher than MCCB103, when the production at all salinities were considered together. Of all the isolates, the brackish water isolate MCCB102 produced the highest concentration (25.3 mg/L) of pyocyanin followed by the marine isolate MCCB117 (21.8 mg/L).

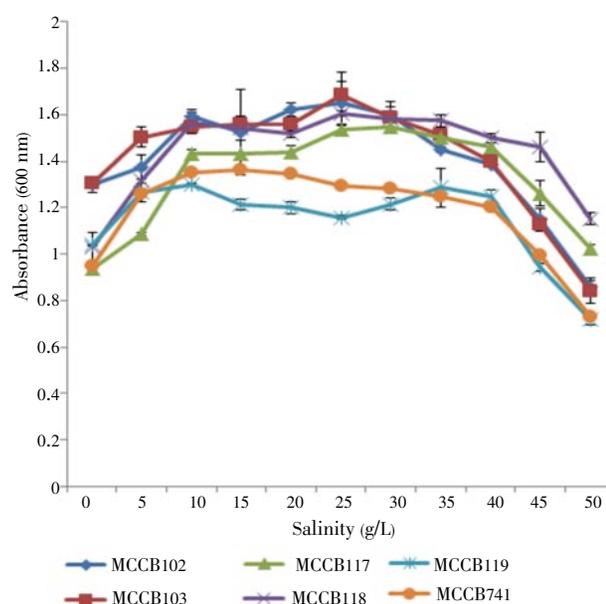


Figure 3. Growth of *P. aeruginosa* isolates cultured at different salinities. $P<0.001$.

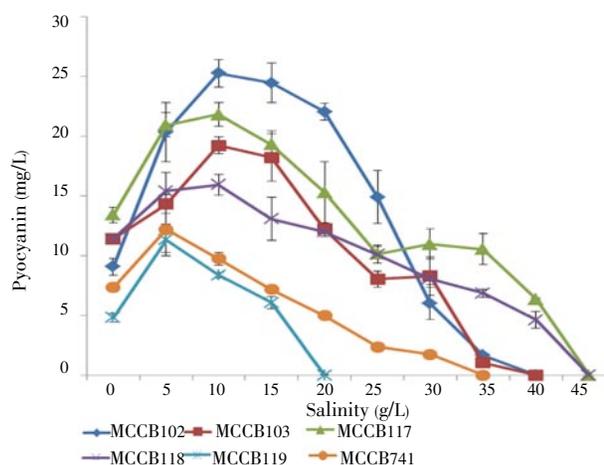


Figure 4. Pyocyanin production by *P. aeruginosa* isolates cultured at different salinities. $P<0.001$.

3.4. Antagonism to *V. harveyi* at different salinities

Filter-sterilized cell-free culture supernatant of all the isolates of *P. aeruginosa* grown at different salinities inhibited the growth of *V. harveyi* (MCCB111) with the inhibitory zones ranging from 10.5 to 18.0 mm in disc diffusion assay (Table 3). Differences in the diameter of the inhibition zones were related to the quantity of pyocyanin produced by each isolate grown at different salinities. For all the isolates, maximum inhibition zones were obtained when they were grown in media at 5–10 g/L salinity and the marine isolate MCCB117 could inhibit vibrios even when grown at a salinity of 40 g/L.

Table 3

Antagonistic activity of culture supernatants of *P. aeruginosa* on *V. harveyi* (MCCB111) at different salinities (g/L) measured in terms of diameter of the inhibition zone (mean±SD).

Salinity (g/L)	Diameter of Zone (in mm)					
	MCCB102	MCCB103	MCCB117	MCCB118	MCCB119	MTCC741
0	13.50±0.70	14.00±0.00	15.50±0.70	12.00±1.41	10.00±0.00	11.00±1.41
5	16.00±0.70	16.00±0.70	17.00±0.00	14.00±1.41	12.50±0.70	12.00±1.41
10	18.00±1.41	17.00±0.00	17.00±0.00	13.50±0.70	11.50±2.12	11.00±1.41
15	17.00±1.41	16.00±0.70	16.50±0.70	13.50±0.70	10.50±0.70	11.50±0.70
20	16.50±2.12	15.00±0.00	16.50±0.70	13.00±0.00	0	10.50±0.70
25	15.50±0.70	13.00±0.70	15.50±0.70	12.50±0.70	0	0
30	12.50±0.70	12.00±0.70	15.00±1.41	12.00±0.00	0	0
35	0	0	15.00±1.41	11.00±0.00	0	0
40	0	0	12.50±0.70	10.50±0.70	0	0
45	0	0	10.50±0.70	0	0	0

3.5. Effect of NaCl on growth and antagonistic compound production

When seawater was replaced with NaCl at 5 g/L, there was no significant reduction ($P>0.05$) in growth (Figure 5). The pyocyanin production of all the isolates were generally higher in seawater compared to NaCl, especially in the brackish water isolates MCCB102 and MCCB103 (Figure 6).

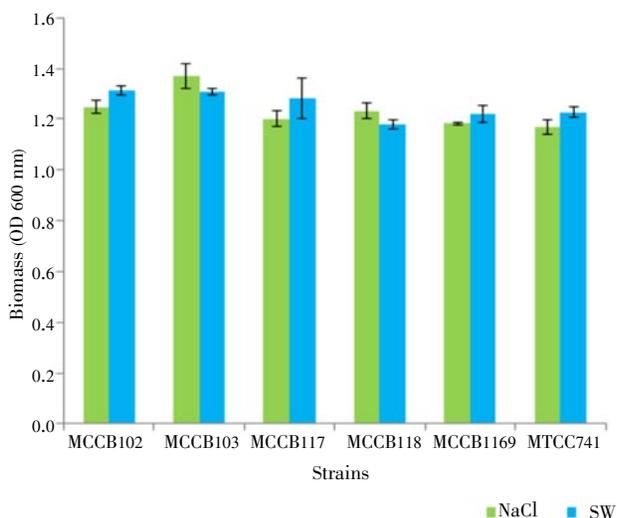


Figure 5. Growth of *P. aeruginosa* isolates in seawater based/NaCl supplemented growth media. $P>0.05$. OD: Outside diameter; SW: Seawater.

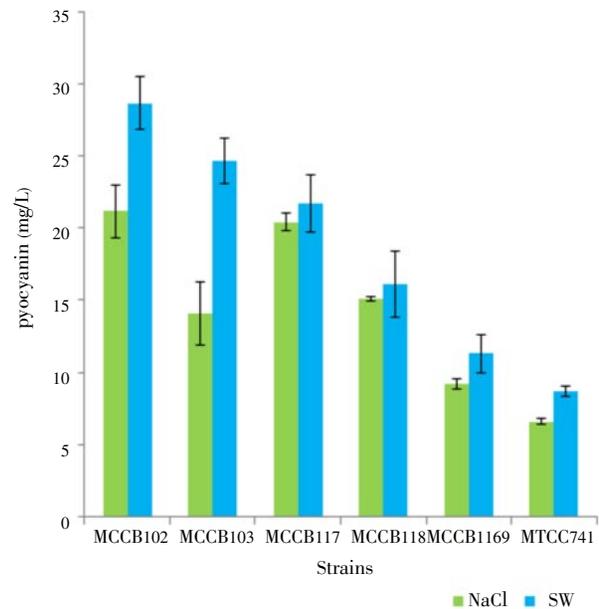


Figure 6. Pyocyanin production by *P. aeruginosa* isolates in seawater based/NaCl supplemented growth media. $P>0.05$. SW: Seawater.

3.6. Brine shrimp toxicity assay

Percentage mortalities amongst groups of brine shrimp nauplii exposed to pyocyanin for a period of 12 and 24 h are shown in Figure 7. From probit analysis, LC_{50} values were found to be (296.65±18.15) mg/L and (215.312±35.660) mg/L during an exposure period of 12 h and 24 h, respectively. No toxicity was observed upto 50 mg/L pyocyanin for 12 h and 24 h and upto 100 mg/L pyocyanin for 12 h. DMSO added controls showed toxicity above 2%. There was no death of nauplii in seawater added control.

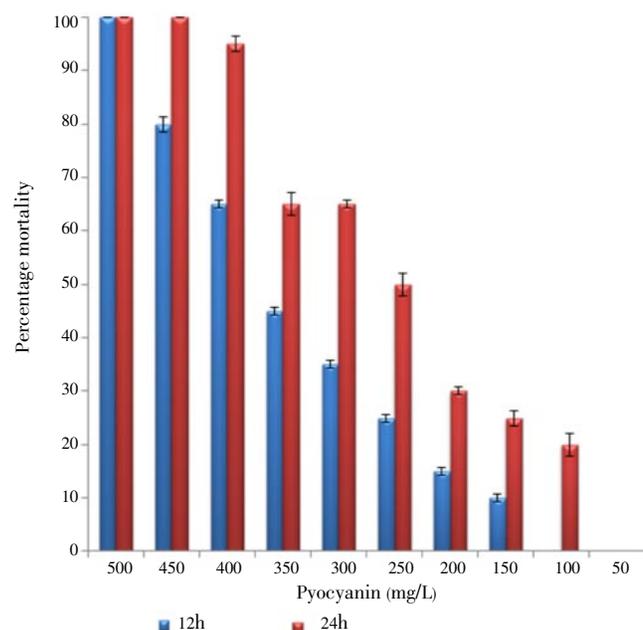


Figure 7. Brine shrimp lethality assay of pyocyanin produced from *P. aeruginosa*, marine isolate MCCB117.

4. Discussion

The aim of this study was to provide an insight to the antagonistic effects of pyocyanin produced by *P. aeruginosa* isolates at different salinity, for its selective application in aquaculture to control pathogenic vibrios. Pyocyanin produced from environmental isolate of *P. aeruginosa* has been identified as the key molecule that inhibits growth of vibrios in aquaculture systems^[16]. It has also been demonstrated that environmental isolate of *Pseudomonas* can control vibrios and improve larval survival in shrimp hatchery systems^[15]. In natural habitats salinity has a significant influence on the metabolism, diversity and functions of microbial communities, particularly when they occupy various ecological niches^[30]. Though microbial metabolite production depends on several environmental factors, salinity fluctuations are more significant than any other physical factor in aquaculture systems^[31,32]. Even though the use of *P. aeruginosa* in aquaculture as a putative probiotic is well established^[5,12–15] and a commercial product has also been made available (PS-1TM, NCAAH, India), the salinity tolerance and preference of *Pseudomonas* isolates to maximize their probiotic effect due to pyocyanin production have not been investigated in aquaculture systems, that operate under salinity ranges from zero to as high as 45 g/L. In this context, *P. aeruginosa* isolated from various ecological niches were judiciously examined for their growth and pyocyanin production, salinity preferences and for their antagonistic effects on *Vibrio* spp., especially to the luminous *V. harveyi*.

All the 5 *P. aeruginosa* isolates investigated stand out as potent antagonists of *Vibrio* spp. such as *V. parahaemolyticus*, *V. nereis*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. mediterranei* and *V. harveyi*, confirming their potential application in aquaculture as antagonistic probiotics. Likewise, in recent studies, researchers identified six isolates of *P. aeruginosa* from estuarine habitats of Cochin, India for fish health management, and confirmed their antibacterial activity against the pathogenic species of *Vibrio* of fishes^[33]. Toxicity assessments in brine shrimp showed pyocyanin to be non-toxic at concentrations up to 100 mg/L, and the LC₅₀ values were (296.65±18.15) mg/L and (215.312±35.660) mg/L for the exposure times of 12 h and 24 h respectively. The cytotoxicity (IC₅₀) of pyocyanin from environmental isolates of *P. aeruginosa* in primary haemocyte cultures from *Penaeus monodon* was found to be (1.40±0.31) mg/L, and on *V. harveyi* at 0.5 mg/L, it showed

static effect and cidal at 1.0 mg/L^[16]. NMR spectroscopic study confirmed that the extracted compound, antagonistic to *Vibrio*, was pyocyanin.

Production levels of pyocyanin by all isolates of *P. aeruginosa* were salinity-dependent even though biomass increased over time upto 70 g/L salinity. While 16S rRNA gene sequence analysis showed that all the isolates possessed 99% nucleotide sequence identity to *P. aeruginosa*, they varied significantly between each other in salinity-dependent pyocyanin production. Pyocyanin was produced in highest amounts when the isolates were grown in media with salinities ranging from 5 to 10 g/L. In addition, irrespective of the pyocyanin production, all the isolates grew at salinities ranging from 5–70 g/L. However, recent findings indicated the growth of estuarine isolates of *P. aeruginosa* upto 50 g/L. Replacement of seawater by NaCl in the growth medium did not significantly inhibit growth and pyocyanin production^[33]. All these findings support the well-adapted lineage with distinct ecophysiological features due to the cladogenic processes observed in environmental isolates of *P. aeruginosa*^[34]. These findings are relevant to aquaculture as the selection of a bacterial isolate as a putative probiotic can be based on salinity requirements of the cultured species. Marine and brackish water isolates were more halotolerant than the freshwater isolate with respect to pyocyanin production and production levels were also significantly higher. All these differences in salinity tolerance/preference with respect to pyocyanin production among the different environmental isolates of *P. aeruginosa* appear to be due to their adaptation to suit to specific ecological niches^[34,35].

In conclusion, the study proposed five environmental isolates of *P. aeruginosa* for their selective application in aquaculture. The organism can either inhibit or kill *Vibrio* in aquaculture systems as antagonistic probiotics providing protective effect to the reared animals from vibriosis. The present scenario dictates that aquaculture industry is in dire necessity of either antagonistic compounds or antagonistic probiotics in lieu of antibiotics. Brine shrimp assay points to the fact that there is no toxicity up to 50 mg/L pyocyanin, and the quantity required for cidal effect is only 1.0 mg/L. In this context, the *Pseudomonas* species isolated and studied here has wider scope of application at field level. Based on the data generated, *P. aeruginosa* MCCB119 would be ideal for application in freshwater, MCCB102 and MCCB103 in brackish water and MCCB117 and MCCB118 in marine aquaculture systems as putative

probiotics in the management of vibrios.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Aquaculture is the industry of the century to supply the world by the fish. *Vibrio* spp. have a significant effect on such industry. The magnitude of the antagonistic effect of *Pseudomonas* depends to a large extent on the tolerance and preference of *Pseudomonas* to salinity. To identify *Pseudomonas* isolates potentially useful for abrogating pathogenic *Vibrio* in aquaculture, the effect of salinity on growth and pyocyanin production were evaluated.

Research frontiers

This research discusses the antagonistic effect of *P. aeruginosa* isolates, which is an antimicrobial metabolite that restricts the growth of *Vibrio* species.

Related reports

The culture from the isolates of *P. aeruginosa* along with the reference strain were examined for their antagonistic effect against *Vibrio* spp.

Toxicity of the antagonistic compound was tested using *A. salina* nauplii as model animal representing crustaceans.

Innovations and breakthroughs

Important research trying to solve a big problem in fish farms and the methods used with such organisms are considered new ways in such problems.

Applications

From the literature survey, it has been found that brine

shrimp is used to examine the toxicity of antagonistic compounds. This scientific study supports and suggests the use of this to confirm the previous results.

Peer review

This is a valuable research work in which authors have demonstrated the antagonistic effects of *Pseudomonas* on the growth of *Vibrio*. The authors concluded that such organism (MCCB102, MCCB103) is effective for brackish water while MCCB117 and MCCB118 are effective for marine aquaculture.

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