3.1. Introduction

Vibrios are important and common bacterial pathogens in marine and brackish water aquaculture systems that can infect and cause losses of almost all species of cultured crustaceans. *Vibrio* spp. are considered to be opportunistic or facultative pathogens with clinical vibriosis caused usually due to the consequences of suboptimal environmental factors and management procedures (Lightner, 1988). To help protect aquaculture stocks, antibiotics have been used over the years, sometimes indiscriminately due to which several pathogenic vibrios have acquired multiple drug resistance and become untreatable and the drug resistance being transferred to pathogens of human concern (Cabello, 2006). Due to these adverse effects, alternative measures of protecting aquacultured species against vibriosis are being explored including the enrichment of the culture systems with antagonistic probiotics.

Among the probiotics, antagonistic pseudomonads have been gaining increasing attention as biological control agents against pathogenic fungi and bacteria in agriculture (Anjaiah et al., 2003; Bano and Musarrat 2003; Rangarajan et al., 2003; Kumar et al., 2005), vibrios in aquaculture (Rattanachuay et al., 2011;
Saline dependent production of pyocyanin in *Pseudomonas aeruginosa* originated from different ecological niches and their selective application in aquaculture

Chaythanya et al., 2002) and also as augmenters in bioremediation programs (De Meyer et al., 1999; Chaerun et al., 2004; Hasanuzzaman et al., 2004; Tang et al., 2007). Pseudomonads constitute a large part of the microflora of gills, skin and intestinal tract of live fish and are often antagonistic against fish pathogenic bacteria and fish pathogenic fungi. Specifically, the versatile and ubiquitous bacterium *Pseudomonas aeruginosa* has recently been recognized as an active antagonist of pathogenic *Vibrio harveyi* and thus as a candidate probiotic in aquaculture systems (Pai et al., 2010; Hai et al., 2009a, b; Vijayan et al., 2006). Even though the clinical isolates of *Pseudomonas aeruginosa* have been identified as pathogens to humans (Gloyne et al., 2011; Muller et al., 2009) the environmental isolates have been accepted as a probiotic in aquaculture systems (Hai et al. 2009a, b; Pai et al., 2010; Preetha et al., 2010). The antagonistic compound produced by *P. aeruginosa* is pyocyanin, a blue-green chloroform-soluble phenazine pigment, which possesses broad-spectrum antibacterial (Preetha et al., 2010; Arunkumar et al., 1997), antifungal (Costa and Cusmana, 1975; Kerr et al., 1999) and antiprotozoan activities (Dive, 1973).

In nature, pyocyanin synthesis by *Pseudomonas aeruginosa* is influenced by environmental factors including salinity, as illustrated by Mavrodi, et al. (2006) and van Rij et al. (2004). On considering their application in aquaculture, salinity regime has been identified as the most prominent decisive environmental factor since salinities used for aquaculture species varies widely from place to place. This realization paved the way for a study on the effect of salinity on pyocyanin production by *Pseudomonas aeruginosa* for its meaningful application in aquaculture. To accomplish this target isolates of *Pseudomonas* from various ecological niches with varying salinity tolerance/preference were required. Accordingly, the study was undertaken to screen isolates of antagonistic *Pseudomonas aeruginosa* from freshwater, brackish and marine environments for growth and pyocyanin production under different salinity regimes so that their selective application could be achieved. The antagonism of pyocyanin produced by *P. aeruginosa* isolates at different salinities were examined against pathogenic *V. harveyi*. 
3.2. Materials and methods

3.2.1. Bacterial isolates

Five isolates of pyocyanin producing *P. aeruginosa* numbered MCCB102, 103, 117, 118 and 119 were subjected for the study. The isolates MCCB102 and 103 were from brackish water, off Chennai (east coast) and Kochi (west coast), India respectively. Isolates MCCB117 and MCCB118 were from marine sediment collected onboard FORV Sagar Sampada (Fisheries and Oceanographic Research Vessel, Govt. of India) cruise number 233 from Arabian Sea at depths of 500m (7°00'19″N, 77°20'30″E) and 200m (9°54'83″N, 75°55'00″E), respectively. MCCB119 was a freshwater isolate from the effluent discharge point of M/s Hindustan Organic Chemicals, Kochi. A reference strain, MTCC741 (Microbial Type Culture Collection, Chandigarh, India), was also included in this study. Isolates from marine and brackish water environments were maintained in ZoBell’s marine agar slants (2216E, HiMedia Laboratories, India), freshwater isolates and the type strain were maintained in nutrient agar slants. All the isolates were phenotypically and genotypically characterized as mentioned under chapter 2, and stored at -80°C and deposited in the microbial culture collection at the National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology, Kerala, India.

3.2.2. Test of halophilism: saline-dependent production of pyocyanin and bacterial biomass

To determine the extent of salinity tolerance and preference and the influence of salinity on growth and pyocyanin production, in *P. aeruginosa*, all the isolates were inoculated into modified ZoBell’s broth (0.5% peptone, 0.1% yeast extract) prepared in seawater at varying salinities of 5, 10, 15, 20, 30, 40, 50, 60, and 70g l⁻¹. For zero salinity, the medium was prepared in double distilled water. Cell suspensions for inoculations were prepared from overnight cultures of isolates grown on respective agar slants. The cells grown on the slants were harvested using sterile saline (0.85% NaCl) and for zero salinity the cells were harvested using double distilled water. Absorbance of the suspension was adjusted to 1.0 at 600nm (Ab₆₀₀)
using sterile saline/double distilled water (for 0g l\(^{-1}\)) in a UV-Vis spectrophotometer (Shimadzu, Japan). The cells were seeded into flasks to give a uniform initial absorbance of 0.01 at Ab\(_{600nm}\). The flasks were incubated in shaker incubator (Orbitek, Scigenics Biotech, India) at 30±1°C at 120rpm. Samples (6ml) were withdrawn aseptically from each flask at 24 hour intervals to quantify growth as well as antagonistic compound production and activity.

3.2.2.1. Growth

Growth was measured in terms of absorbance at Ab\(_{600nm}\) and the specific cell count was determined from a standard curve generated based on absorbance vs cell count for *Pseudomonas aeruginosa*.

3.2.2.2. Quantification of pyocyanin

Antagonistic compound production was assayed by extracting 5ml culture supernatant with 3ml chloroform. This was then re-extracted in 1ml 0.2N HCl which gave a red-coloured solution due to the basic property of one of the N atom present in the pyocyanin structure (Friedheim and Michaelis 1931). Absorbance of this solution was measured at Ab\(_{520nm}\), and the concentration in micrograms of the compound produced per millilitre of culture supernatant was determined by multiplying the absorbance at Ab\(_{520nm}\) by the factor 17.072 following Essar et al. (1990).

3.2.2.3. Antagonistic activity

Antagonistic activity in the supernatants of the cultures at all salinity ranges were assayed against the shrimp pathogen *Vibrio harveyi*, MCCB111. Briefly, overnight cultures of *V. harveyi* grown on ZoBell’s marine agar slants were harvested in saline and the OD adjusted to 1.5 at Ab\(_{600nm}\) and 500µl was swabbed on to ZoBell’s marine agar plates. An aliquot of 20µl of the supernatant of 1ml culture of *P. aeruginosa* from each flask, was spotted on to sterile discs (prepared from a stack of six Whatman No.1 filter papers) placed on the previously swabbed plates. The plates were incubated at 28 ±1°C for 18 hours and zones of inhibition recorded using HI antibiotic scale (HiMedia Laboratories, India).
3.2.3. Effect of NaCl as substitute for seawater

To investigate the impact of NaCl as an alternative to seawater 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\(^{-1}\) NaCl. This NaCl concentration was selected since the antagonistic compound production of all the isolates were determined to be the highest at a salinity 5-10 g l\(^{-1}\). Modified ZoBell’s marine broth (0.5% peptone, 0.1% yeast extract) prepared with 5 g l\(^{-1}\) salinity seawater served as the control. The growth, pyocyanin production and antagonism of \textit{P. aeruginosa} were assayed as described earlier.

3.2.4. Statistical analysis

Data were analyzed employing analysis of variation (ANOVA) and regression analysis as applicable and significant differences were recorded based on \(p\)-value <0.05.

3.3. Results

3.3.1. Test of halophilism: saline-dependent production of pyocyanin and bacterial biomass

With all the 5 \textit{Pseudomonas aeruginosa} isolates, growth and pyocyanin production were influenced significantly by salinity (\(p <0.001\)) (Figs. 1, 2) even though they originated from various ecological niches. All isolates exhibited relatively uniform growth up to 70 g l\(^{-1}\) salinity (Fig. 1) but pyocyanin production varied distinctly among the isolates (Fig. 2). While the isolates of marine origin (MCCB117, MCCB118) produced detectable levels of pyocyanin in the medium prepared with salinities up to 40 g l\(^{-1}\), the brackish water isolates (MCCB102, MCCB103) ceased to produce pyocyanin with salinities above 30 g l\(^{-1}\). The freshwater isolate (MCCB119) did not produce pyocyanin with salinities above 20 g l\(^{-1}\). Maximum pyocyanin production of marine and brackish water isolates occurred at a salinity of 10 g l\(^{-1}\), however, with the freshwater isolate and reference strain at 5 g l\(^{-1}\). Pyocyanin production varied only marginally between the isolates from the same ecological niches. However, among the marine isolates, pyocyanin production was
significantly \((p<0.01)\) higher by MCCB117 compared to MCCB118 at all salinities tested (Fig. 2). Pyocyanin production of the brackish water isolate MCCB102 was slightly but not significantly \((p>0.05)\) higher than that of its counterpart MCCB103. Of all the isolates, the brackish water isolate MCCB102 produced the maximum concentration \((25.3\text{mg l}^{-1})\) of pyocyanin followed by the marine isolate MCCB117 \((21.8\text{mg l}^{-1})\).

### 3.3.2. Antagonism to *Vibrio harveyi* at different salinities

Filter-sterilized cell-free culture supernatant of the isolates of *Pseudomonas aeruginosa* inhibited growth of *Vibrio harveyi* with the inhibitory zones (Fig. 3) ranging from 10.5 to 18mm in disc diffusion tests (Table 1). Differences in inhibition zone diameters related to pyocyanin quantities produced by each isolate grown at different salinities. For all 5 isolates, maximum inhibition zones were obtained when they were grown in media at 5-10g l\(^{-1}\) salinity and the marine isolate MCCB117 could inhibit vibrios even when grown at a salinity of 40g l\(^{-1}\).

### 3.3.3. Effect of NaCl substituted for sea water

When seawater was replaced with NaCl at 5g l\(^{-1}\), there was no significant reduction in bacterial growth or pyocyanin production \((p>0.05)\) (Figs. 4, 5).

### 3.4. Discussion

Salinity has significant influence on the metabolism, diversity and functions of microbial communities, particularly when they occupy various ecological niches (Abed et al., 2007). Production of phenazine compounds by fluorescent pseudomonads is profoundly influenced by environmental factors (Mavrodi et al., 2006). Though microbial metabolite production depends on several environmental factors, salinity fluctuations are more significant than any other physical factors in aquaculture systems. Even though the use of *Pseudomonas aeruginosa* in aquaculture as a putative probiotic is well established (Hai et al., 2009a,b; Chythanya et al., 2002; Vijayan et al., 2006; Pai et al., 2010) and a commercial product has also been made available (PS-1\(^{TM}\), NCAAH, India, www.ncaah.org), the isolates from
various ecological niches and their saline dependent production of pyocyanin has not yet been studied. Pyocyanin has been identified as the key molecule produced by *Pseudomonas aeruginosa* that inhibits growth of vibrios in aquaculture systems (Preetha et al., 2010), and culture fermentation conditions have been optimized to maximize its production (Preetha et al., 2007). It has also been demonstrated that *Pseudomonas* can control vibrios and improve larval survival in shrimp hatchery systems (Pai et al., 2010). However, the salinity tolerance and preference of *Pseudomonas* isolates to maximize their probiotic efficacy have not been investigated in aquaculture systems that operate under salinity ranges from zero to as high as 40g l⁻¹. In this context, appropriate isolates of *Pseudomonas aeruginosa* selected judiciously were examined for their growth and pyocyanin production, salinity preferences and for their antagonist effects against pathogenic *V. harveyi*.

Production of pyocyanin, by the 5 isolates of *Pseudomonas aeruginosa* examined were salinity-dependent even though biomass increased over time and were relatively stable for all isolates at all salinities. Though 16S rRNA gene sequence of all bacterial isolates possessed 99% nucleotide sequence identity to *P. aeruginosa*, they varied significantly in salinity-dependent pyocyanin production. Pyocyanin was produced in highest amounts when the bacteria were grown in media with salinities ranging from 5 to 10g l⁻¹, having its cessation above 40g l⁻¹. Replacement of seawater by NaCl in the growth medium did not significantly change pyocyanin production even though marginal declines were evident. This feature was common to all 5 isolates, irrespective of them being sourced from freshwater, brackish and marine environments.

Although pyocyanin production was obtained even in nutrient medium prepared in deionized water without salt, it was lesser compared to that obtained in the presence of salts. This suggests that while presence of salts may not be an absolute requirement for pyocyanin production, it can enhance it, and the level of
salinity tolerance vis-à-vis pyocyanin production is strain dependent. Though pyocyanin production had declined at salinity above 40g l\(^{-1}\), there was substantial cell biomass built up to salinity 70g l\(^{-1}\). Studies by Khan et al. (2007) showed that there was little difference among the marine, river and clinical isolates in their response to high sodium chloride concentration indicating high tolerance of \(P.\ aeruginosa\) to high salt condition. The genome of \(Pseudomonas\ aeruginosa\) PAO1 (Stover et al., 2000) contains a \(nqr\) operon encoding a \(\text{Na}^+\)-translocating NADH-quinone oxidoreductase, the primary sodium pump mainly found among marine bacteria (Kogure, 1998; Hase et al., 2001) and which probably allows them to survive in high saline environments. It has been postulated that \(P.\ aeruginosa\) strains of marine environments have a freshwater origin (Kimata et al., 2004; Yoshpe-purer and Golderman, 1987). Studies that have been made on the different aspects of \(P.\ aeruginosa\) with respect to their habitat or geographical origin could not reveal any distinguishing features between the different isolates both phenotypically and genotypically (Pirnay et al., 2002). However studies so far have not compared the pyocyanin production of such isolates and our results indicate that it is significantly higher in marine/brackish water isolates compared to their freshwater counterparts. Also these isolates were more halotolerant in terms of pyocyanin production than the freshwater ones. 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 aquaculture species. Pyocyanin production and halotolerance were not statistically significant (\(p>0.05\)) between brackish water and marine isolates. The 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 occupy specific ecological niches.

The data reported here indicate that salinity influences pyocyanin levels produced by \(Pseudomonas\ aeruginosa\) types and that isolates originating from marine, brackish or fresh water can be selected for putative probiotic applications.
based on their ability to grow well and produce pyocyanin at varying salinities used for various aquaculture species. Even though all 5 isolates examined grew relatively uniformly in fresh water to water with a salinity of 70g l$^{-1}$, pyocyanin production levels dropped markedly at salinity tested above 5-10g l$^{-1}$, which proved optimal for all isolates. However, marine isolates of \textit{P. aeruginosa} were able to produce pyocyanin when grown in water with salinity levels up to 40g l$^{-1}$, and thus some flexibility existed in selection of what isolates might prove most advantageous as probiotics for various aquaculture species. Accordingly, the data suggest that \textit{Pseudomonas aeruginosa} isolate MCCB119 would be the most suitable organism for application in fresh water aquaculture systems, isolates MCCB102 and NCCB103 in brackish water aquaculture systems and isolates MCCB117 and MCCB118 in marine aquaculture systems. Moreover this is the first report on the application of \textit{P. aeruginosa} isolated from marine environment of 200m (MCCB118) 500m (MCCB117) depth.

\textbf{Table 1} Antagonistic activity of individual isolates of \textit{Pseudomonas aeruginosa} against \textit{Vibrio harveyi} at different salinities (g l$^{-1}$).

<table>
<thead>
<tr>
<th>Salinity (g l$^{-1}$)</th>
<th>MCCB102</th>
<th>MCCB103</th>
<th>MCCB117</th>
<th>MCCB118</th>
<th>MCCB119</th>
<th>MTCC741</th>
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<tr>
<td>0</td>
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<td>14±0.00</td>
<td>15.5±0.70</td>
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<td>10±0.0</td>
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<td>16±0.70</td>
<td>17±0.00</td>
<td>14±1.41</td>
<td>12.5±0.70</td>
<td>12±1.41</td>
</tr>
<tr>
<td>10</td>
<td>18±1.41</td>
<td>17±0.00</td>
<td>17±0.00</td>
<td>13.5±0.70</td>
<td>11.5±2.12</td>
<td>11±1.41</td>
</tr>
<tr>
<td>15</td>
<td>17±1.41</td>
<td>16±0.70</td>
<td>16.5±0.70</td>
<td>13.5±0.70</td>
<td>10.5±0.70</td>
<td>11.5±0.70</td>
</tr>
<tr>
<td>20</td>
<td>16.5±2.12</td>
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<td>16.5±0.70</td>
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<td>-</td>
<td>10.5±0.70</td>
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<td>25</td>
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<td>12±0.7</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>15±1.41</td>
<td>11±0.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>-</td>
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<td>10.5±0.70</td>
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<tr>
<td>45</td>
<td>-</td>
<td>-</td>
<td>10.5±0.70</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Chapter 3

Saline dependent production of pyocyanin in *Pseudomonas aeruginosa* originated from different ecological niches and their selective application in aquaculture

![Fig. 1 Growth of *Pseudomonas* isolates at different salinities.](image1)

![Fig. 2 Pyocyanin production by *Pseudomonas* isolates at different salinities.](image2)
Saline dependent production of pyocyanin in *Pseudomonas aeruginosa* originated from different ecological niches and their selective application in aquaculture

**Fig. 3** Demonstration of antagonistic activity of *Pseudomonas aeruginosa* against *V. harveyi* (MCCB111) 1) MCCB102 2) MCCB 103 3) MCCB117 4) MCCB118 5) MCCB119 6) MTCC741.

**Fig. 4** Growth of *Pseudomonas* isolates in seawater based/NaCl supplemented growth media.
Fig. 5 Pyocyanin production by *Pseudomonas* isolates in seawater based/NaCl supplemented growth media.