5.1. Introduction

_Pseudomonas aeruginosa_ produces a variety of extra-cellular pigments of which phenazines comprise the most significant portion. The phenazine compounds have a variety of biotechnological applications especially as redox agents and antibiotics. The non-aqueous electrochemistry of phenazine and substituted phenazines is of interest because of their use as dye stuffs, the biological importance of some derivatives and the structural relationship to isoalloxazine and to flavoproteins, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). Pyocyanin has been identified as a good model for flavin systems (Morrison et al., 1978) and it has been used as electron transfer agent and catalyst in the studies on photosynthetic systems of bacteria and green plants (Vernon et al., 1963; Zaugg, 1964; Ohfuji et al., 2004). It has been postulated that pyocyanin can be used as electron shuttle in microbial fuel cells by enabling bacterial electron transfer towards the microbial fuel cells anode (Pham et al, 2008). There is a very early report that pyocyanin behaved as a reversible dye of the quinoid type with a redox potential similar to that of menaquinone (Friedheim and Michaelis, 1931).

Pyocyanin is a nitric oxide antagonist in various pharmacological preparations and has various pharmacological effects on eukaryotic and prokaryotic cells (Ohfuji
The phenazine-based pyocyanin is of particular interest for its capability of producing reactive oxygen species (Baron and Rowe, 1981; Mavrodi et al., 2001) besides the significant influence it exert on the respiration of its producer (Hernandez et al., 2004). Pyocyanin also has got application in biosensors as a redox compound for carrying out electron transfer between enzyme molecules and the electrode material (Ohfuji et al., 2004). An amperometric biosensor system using pyocyanin as a mediator was developed by Ohfuji et al. (2004) for a more accurate determination of glucose. Therefore, the biosensors using pyocyanin was also expected to apply to some fields such as medicine, food and environment.

Pyocyanin produced by \textit{P. aeruginosa} has been recognized as a major factor in controlling pathogenic vibrios in aquaculture. The bioprocess and downstream processing of pyocyanin for aquaculture application have not been reported much. Preetha et al. (2007) optimized the carbon and nitrogen sources and growth factors of \textit{Pseudomonas MCCB103} using the statistical design, Response Surface Methodology (RSM) and reported increased biomass and pyocyanin production. However, since, it was required to further enhance the pyocyanin production, overexpression of the genes encoding pyocyanin biosynthesis in the marine isolate \textit{P. aeruginosa MCCB117} was hypothesized and accomplished as genetically modified strain named PA-pUCP-Phz++ with increased yield.

Despite the various applications of pyocyanin, it remains a costly compound in the market. However, for application such as in aquaculture, we require a commercially viable product. Traditionally, the pyocyanin is isolated from active cultures of \textit{Pseudomonas} by solvent extraction followed by purification. In the present study, the pyocyanin was processed from the genetically modified PA-pUCP-Phz++ strain and the purity of the product was confirmed by structural elucidation in comparison with the authentic pyocyanin standard. The functional analysis of the purified product was carried out by anti-vibrio activity screening, dose-dependent activity, and luminescence assay. The shelf life determination and the economic feasibility of the purified pyocyanin were studied to develop it as a commercial product for application in aquaculture industry.
5.2. Materials and Methods

5.2.1. Development of a downstream process for large scale production of pyocyanin

5.2.1.1. Bacterial strain and culture condition

The genetically modified *P. aeruginosa* PA-pUCP-Phz\(^{++}\) strain which contains increased copy number of pyocyanin biosynthetic genes was used for the downstream process to maximize pyocyanin yield and thus the large scale production. The PA-pUCP-Phz\(^{++}\) strain was developed from the wild strain *Pseudomonas aeruginosa* MCCB117 obtained from marine sediment collected at 500 m depth (7°00'19"N, 77°20'30"E) and the details of genetic modification were explained in the previous chapter (Chapter 4). The PA-pUCP-Phz\(^{++}\) strain was maintained by strict bioethics norms under safe custody at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kerala, India.

The PA-pUCP-Phz\(^{++}\) strain was inoculated into 100ml ZoBell’s broth (0.5% peptone, 0.1% yeast extract) prepared in sea water with a salinity 10g l\(^{-1}\) containing 200µg ml\(^{-1}\) gentamycin and incubated at 30°C in a shaking incubator at 150rpm to produce pyocyanin. Pyocyanin from the untransformed *P. aeruginosa* strain MCCB117 was also extracted and purified as a control for comparison.

5.2.1.2. Collection of supernatant and extraction using chloroform

A 24 hour old culture broth was transferred into a 50ml Oakridge tube and centrifuged at 10,000g for 20 minutes at 4°C. The supernatant was transferred into a 1000ml separating funnel (Magnum, India) and mixed with chloroform at 1:1.5 ratio (supernatant : chloroform). This mixture was shaken well and kept undisturbed for 5-10 minutes for the pyocyanin fraction in the broth to get extracted to the chloroform layer.

5.2.1.3. Phase separation and concentration

The blue colored chloroform layer along with pyocyanin formed below the aqueous layer in the separating funnel was collected into a covered conical flask to protect from light to prevent oxidation. This chloroform fraction was then transferred
5.2.1.4. Silica gel column purification

The vacuum concentrated pyocyanin fraction was purified by silica gel column having 3 cm diameter and 60 cm length (Magnum, India). The column was packed with silica having a mesh size 100-200 and equilibrated using chloroform-methanol solvent system in the ratio 1:1 and the concentrated pyocyanin fraction was loaded into the column. Chloroform-methanol solvent system in the ratio 1:1 was used as the mobile phase to separate pyocyanin. The blue coloured pyocyanin fraction was then collected into a 250ml conical flask, protected from light.

5.2.1.5. Vacuum evaporation of solvent system to concentrate the purified pyocyanin

The column purified pyocyanin fraction in chloroform-methanol solvent was further concentrated in vacuum rotary evaporator at 40°C (BÜCHI, Switzerland). The concentrated and purified pyocyanin was collected in amber coloured bottle and stored at -20°C till use.

5.2.1.6. Quantification and preparation of purified pyocyanin for application

The concentrated pyocyanin was taken in a pre-weighed amber coloured bottle and passed through a jet of nitrogen gas to remove solvents and completely dried. The weight of the dried pyocyanin was taken and subtracted from the weight of empty bottle to get the dry weight of pyocyanin in milligrams. This was then dissolved in appropriate amount of Dimethyl sulfoxide (DMSO) for applications.

5.2.2. Structural elucidation and confirmation of purity of the compound

5.2.2.1. HPLC analysis

The purified pyocyanin along with an authentic sample of pyocyanin standard obtained from M/s Color Your Enzyme, Ontario, Canada were analysed by High pressure liquid chromatography (HPLC) according to a method described by Fernandez and Pizarro (1997). HPLC analysis was performed on a Dionex, model ultimate 3000 (Germany) gradient elution system attached to a C_{18} column (250 X
4.6mm) and a detector monitoring at 280nm 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 was acetonitrile-water-trifluoroacetic acid (90:10:0.04, v/v/v). Elution was carried out as follows: Solvent A was maintained for 15 minutes and then changed to 90% A and 10% B. This mixture was used for 10 minutes. Subsequently a linear gradient with a ratio of 70% A and 30% B was applied for another 15 minutes. These conditions were maintained for further 5 minutes (until 45 minutes from onset). Finally, solvent composition was changed with a ratio 64% A and 36% B and maintained until the end.

5.2.2.2. UV – Visible spectra of purified pyocyanin in different solvents

The purified pyocyanin and pyocyanin standard were subjected to spectroscopic analysis. Ultraviolet and visible absorption spectra of purified pyocyanin and pyocyanin standard, dissolved in solvents such as chloroform and 0.1N HCl were recorded on Shimadzu UV 102 spectrophotometer.

5.2.2.3. Mass spectrometry

Mass spectrometric analysis of pyocyanin and pyocyanin standard was done on a quadrupole mass spectrometer at the Department of Applied Chemistry, Cochin University of Science and Technology, India.

5.2.2.4. ¹H NMR spectral analysis

¹H NMR spectra of the purified pyocyanin were recorded on a Bruker Avance III FT NMR spectrometer operating at 400MHz at the Sophisticated Test and Instrumentation Centre (STIC), Cochin University of Science and Technology, India. CdCl₃ was used as the solvent and tetramethylsilane (TMS) was used as the internal standard.

5.2.3. Functional analysis of the purified pyocyanin

Followed by the structural elucidation of the purified pyocyanin obtained through downstream process, functional analysis of the compound was performed to confirm its activity. Antagonistic assay against *Vibrio* spp. along with the
determination of minimum inhibitory concentration (MIC) and inhibition on luminescence of luminescent *V. harveyi* of the purified compound were studied. Moreover, the stability testing and shelf life determination of purified pyocyanin were experimented for a period of two years.

### 5.2.3.1. Antagonistic effect of purified pyocyanin against *Vibrio* spp.

Antagonism of purified pyocyanin against pathogenic *Vibrio* spp. was examined. *Vibrio* spp. obtained from Belgium Culture Collection (BCCM-LMG) such as *V. harveyi* (BCCM-LMG 4044), *V. parahaemolyticus* (BCCM-LMG 2850), *V. vulnificus* (BCCM-LMG 13545), *V. alginolyticus* (BCCM-LMG 4409), *V. fluvialis* (BCCM-LMG 11654), *V. mediterranei* (BCCM-LMG 11258), *V. proteolyticus* (BCCM-LMG 3772), and *V. nereis* (BCCM-LMG 3895) were used and the antimicrobial effect was screened by spot inoculation test. Briefly, overnight cultures of *Vibrio* spp. grown on ZoBell’s marine agar slants were harvested in saline, absorbance was adjusted to 1.5 at A$_{600}$, and swabbed (500µl) on to ZoBell’s marine agar plates. An aliquot of 2µl of the purified pyocyanin was spotted on the swabbed plates. The plates were incubated at 28 ± 1°C for 18 hours and the zones of inhibition recorded using HI antibiotic scale (Himedia, Mumbai, India). The experiments were conducted in triplicates.

### 5.2.3.2. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of purified pyocyanin was tested against *Vibrio harveyi* (MCCB111). The purified pyocyanin was added to 18 hours old *V. harveyi* culture ($10^5$ cells ml$^{-1}$) in a 96-well microplate to obtain final concentrations of 0, 1, 2.5, 5.0, 7.5 and 10.0mg l$^{-1}$. Triplicates were performed for each concentration. The growth of *Vibrio harveyi* was measured from absorbance at 600 nm in a microplate reader (Tecan InfiniteTm, Austria) for 0 to 24 hours at an interval of 6 hours. At 0 and 24 hours, 100µl of the culture from each concentration, in duplicate, was plated onto ZoBell’s agar (Himedia, Mumbai, India), incubated at 30°C for 48 hours and the total plate count was determined.
5.2.3.3. Luminescence inhibition assay

Luminescence inhibition assay of purified pyocyanin was tested against luminescent *Vibrio harveyi* (MCCB111). The purified pyocyanin was added to 18-hour-old *Vibrio harveyi* culture of 0.1 OD (Ab 600nm) in a 15ml micro centrifuge tube to obtain final concentrations of 0, 1.0, 2.0, 3.0, 4.0, 5.0, and 10mg l⁻¹. The bioluminescence was measured in a luminometer (Modulus, Turner Biosystems, USA) for 0 to 12 hours at an interval of 2 hours.

5.2.3.4. Stability testing and shelf life determination of purified pyocyanin

The purified and concentrated pyocyanin dissolved in chloroform/methanol mixture (1:1) were stored at -20°C for two years and tested for antagonistic activity against *V. harveyi* MCCB111 at regular intervals using the disc diffusion assay.

5.2.3.5. Economic feasibility of the process using genetically modified *P. aeruginosa* PA-pUCP-Phz⁺⁺ strain

Economic feasibility of the technology has been evaluated by comparing with the cost of commercially available pyocyanin (Sigma Life Sciences). The approximate production cost of the downstream process was calculated based on media components, solvents and column materials used for pyocyanin extraction and purification.

5.3. Results

5.3.1. Development of a downstream process for the large scale production of pyocyanin from PA-pUCP-Phz⁺⁺

The PA-pUCP-Phz⁺⁺ strain inoculated into ZoBell’s broth after 24 hours of incubation at 30°C with shaking at 150rpm developed a deep bluish green colour due to the release of the mixture of phenazine compounds including pyocyanin into the medium (Fig. 1). The extraction of pyocyanin from the broth using chloroform yielded a deep blue coloured chloroform-pyocyanin mixture in the separating funnel (Fig. 2). Subsequently, the chloroform-pyocyanin mixture was concentrated in vacuum rotary evaporator before column purification using silica gel column. After column purification using the solvent mixture chloroform - methanol (1:1) as mobile phase,
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Structural elucidation and functional analysis of the purified compound

Two coloured fractions; an upper blue coloured pyocyanin fraction and a lower yellow coloured fraction were observed (Fig. 3). The blue coloured pyocyanin fraction alone was collected and concentrated in a vacuum rotary evaporator and stored.

5.3.2. Structural elucidation and confirmation of purity of the compound

The results on purity of pyocyanin obtained from PA-pUCP-Phz++ through the downstream process was analysed by structural elucidation. In HPLC analysis, the retention time (RT value) of the purified pyocyanin was 15 minute which was identical to that of the pyocyanin standard (Fig. 4).

UV-Visible spectroscopic analysis of the purified pyocyanin showed four absorption maxima, at wavelengths such as 699nm, 529nm, 310nm and 254.5nm in the solvent chloroform, and five absorption maxima in 0.1N HCl at wavelengths such as 553nm, 390nm, 284nm, 246nm and 224nm. This was comparable to the absorption maxima obtained by the pyocyanin standard at wavelengths such as 691nm, 529nm, 306nm, and 255.5nm in chloroform and 555nm, 388nm, 284nm, 247nm and 225nm in 0.1N HCl (Table 1).

The structure of purified compound was confirmed by nuclear magnetic resonance (NMR) spectroscopy. In the ¹H NMR spectrum the peaks observed in the aromatic region (δ 7-9) corresponded with those reported for pure pyocyanin (Fig. 5).

Mass spectroscopic analysis of the purified compound and pyocyanin standard demonstrated a protonated molecular ion at m/z 211 further confirming the purity of the compound (Fig. 6).

5.3.3. Functional analysis of the purified compound

The purified pyocyanin inhibited the growth of *Vibrio* spp. such as *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *V. fluvialis*, *V. mediterranei*, *V. proteolyticus* and *V. nereis* with the diameter of the zone of inhibition ranging from 13.5 to 31mm when tested by disc diffusion method (Table 2).

Luminescence inhibition assay of purified pyocyanin showed inhibition of luminescence even at a concentration of 2mg l⁻¹ (Fig. 7).
The dose-dependent activity of the purified pyocyanin revealed that at 5mg l\(^{-1}\), the growth of *Vibrio harveyi* was inhibited by arresting the cell multiplication at 10\(^3\) CFU ml\(^{-1}\) (Fig. 8). However, at 1.0 and 2.5mg l\(^{-1}\), the cell count increased to >10\(^9\) CFU ml\(^{-1}\). Moreover, concentrations above 5mg l\(^{-1}\) were bacteriocidal and the bacterial count was reduced to 45±15 CFU ml\(^{-1}\).

5.3.4. Stability testing and shelf life determination of purified pyocyanin

Stability testing and shelf life determination confirmed the antagonistic activity against *V. harveyi* MCCB111 by the purified pyocyanin stored for two years at -20°C (Fig. 9). This finding suggested that the purified pyocyanin can be stored for two years without losing its inhibitory effect.

5.3.5. Feasibility of the process using genetically modified *P. aeruginosa* PA-pUCP-Phz\(^{++}\) strain

The yield of purified pyocyanin from the designed downstream using the genetically modified strain PA-pUCP-Phz\(^{++}\) was found to be 45mg l\(^{-1}\) of the broth. Meanwhile, the recovery of purified pyocyanin from the wild strain *Pseudomonas aeruginosa* MCCB117 was only 25±5mg l\(^{-1}\). Accordingly, following the above downstream process the purified pyocyanin recovered from genetically modified strain PA-pUCP-Phz\(^{++}\) could be enhanced to 80±5% higher than that of the wild strain. The approximate production cost of the downstream process was calculated to be less than Rs. 3000/- for one litre culture supernatant from which 45mg purified pyocyanin could be obtained showing the feasibility of the process for large scale production of pyocyanin.

5.4. Discussion

The downstream processing of pyocyanin produced by the genetically modified strain PA-pUCP-Phz\(^{++}\) resulted in 80±5% higher yield than that from the wild strain of *Pseudomonas aeruginosa* MCCB117 following the same protocol. Though the biochemical engineering approaches have been reported for increased pyocyanin production (Preetha et al., 2007), no previous report of genetic engineering of *Pseudomonas aeruginosa* for enhanced pyocyanin production could be observed. There are reports of genetic engineering of *Pseudomonas* sp. for
increased biocontrol effect under field conditions. Timms-Wilson et al. (2000) observed that insertion of genes responsible for phenazine-1-carboxylic acid (PCA) biosynthesis into the chromosome of a plant-growth-promoting rhizobacterium Pseudomonas fluorescens significantly improved the ability of the wild-type P. fluorescens to reduce damping-off disease of pea seedlings caused by Pythium ultimum, even under conditions of heavy soil infestation. Huang et al. (2004) observed that transformation of Pseudomonas fluorescens with genes for biosynthesis of phenazine-1-carboxylic acid improved biocontrol of rhizoctonia root rot and in-situ antibiotic production. In the present study we could establish higher yield of pyocyanin by the genetically engineered PA-pUCP-Phz++ strain.

We could establish the purity of pyocyanin produced by the recombinant strain based on the retention time (15 minute) which was the same as observed for both the pyocyanin standard and the pyocyanin from the genetically modified strain. UV-visible spectroscopic analysis of the purified pyocyanin in both chloroform and 0.1N HCl as solvents showed comparable absorption maxima with the standard. The 1H NMR spectrum of the purified pyocyanin showed peaks in the aromatic region (δ 7-9) identical to that of the pure pyocyanin showing the purity of the product and is in accordance with the previous reports (Preetha et al., 2010; Rao and Suresh Kumar, 2000). Mass spectroscopic analysis of the purified compound and pyocyanin standard demonstrated a protonated molecular ion at m/z 211 further confirming the purity of the compound.

In the present study, the purified pyocyanin from the recombinant strain inhibited the growth of Vibrio spp. such as V. harveyi, V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. fluvialis, V. mediterranei, V. proteolyticus and V. nereis with the diameter of the zone of inhibition ranging from 13.5 to 31 mm. This confirmed that the pyocyanin produced can be used as an aquaculture drug in place of conventional antibiotics. Luminescent vibrios are important pathogens in aquaculture that can affect almost all cultured animals. Luminescence has been reported as an important property of virulent vibrios and bioluminescence is a phenotype controlled by the V. harveyi quorum sensing system in vitro (Bassler et al., 1993). Light production is directly proportional to the metabolic activity of the
bacterial population and any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence. Disruption of quorum sensing, the quorum quenching by means of small signal molecules, has been proposed as the alternative strategy to control infections caused by antibiotic-resistant bacteria in aquaculture (Defoirdt et al., 2004, 2008). Luminescence inhibition assay of purified pyocyanin showed inhibition even at a low concentration of 2mg l\(^{-1}\) establishing that very low concentrations of pyocyanin is effective in controlling vibrio to a safe level.

To confirm the viability of the pyocyanin produced for commercial application in biological systems, the dose-dependent activity, shelf life of the product and economic feasibility were determined. The purified pyocyanin inhibited the growth of *Vibrio harveyi* at 5mg l\(^{-1}\) by arresting the cell multiplication at 10\(^3\) CFU ml\(^{-1}\), with less effect at lower concentration and bacteriocidal effect at higher concentrations. Stability testing and shelf life determination confirmed the antagonistic activity against *V. harveyi* MCCB111 by the purified pyocyanin stored for two years at -20°C. These results suggested that the purified pyocyanin can be stored for years without losing its inhibitory property. The analysis of economic feasibility of pyocyanin production from PA-pUCP-Phz\(^{++}\) strain showed that the production was significantly cost effective compared to commercially available pyocyanin product. The cost of purified pyocyanin (# P0046) from M/s Sigma Life Sciences is approximately Rs. 1, 23,819. 00 (50mg) whereas the approximate production cost of the developed downstream process was calculated to be less than Rs. 3000.0 for 45mg purified pyocyanin.

The results of the present study conclude that pyocyanin produced by the recombinant PA-pUCP-Phz\(^{++}\) strain gave very high yield (80±5% higher than that of the wild strain) compared to the wild strain and pure pyocyanin could be obtained by the down stream process as confirmed by the UV-Visible absorption maxima and structural elucidation using HPLC, \(^1\)H NMR and mass spectroscopic studies. The broad spectrum anti-vibrio activity of the purified compound, inhibition of luminescence, long shelf life and cost-effective production established that genetically modified PA-pUCP-Phz\(^{++}\) strain can be used for commercially feasible
large scale production of pyocyanin for various applications and specifically as an alternative to antibiotics in aquaculture.

Table 1  Absorption maxima (nm) of purified pyocyanin in CHCl₃ and 0.1N HCl

<table>
<thead>
<tr>
<th></th>
<th>Purified pyocyanin</th>
<th>Pyocyanin standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>In CHCl₃</td>
<td>699,529,310,254.5</td>
<td>691,529,306,255.5</td>
</tr>
<tr>
<td>In 0.1N HCl</td>
<td>553,390,284,246,224</td>
<td>555,388,284,247,225</td>
</tr>
</tbody>
</table>

Table 2  Antagonistic activity of purified pyocyanin against different Vibrio spp. measured as the diameter of the inhibition zone

<table>
<thead>
<tr>
<th>Vibrio spp.</th>
<th>Zone Diameter (in mm)</th>
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<tbody>
<tr>
<td>V.alginolyticus</td>
<td>27.5±0.71</td>
</tr>
<tr>
<td>V.fluvialis</td>
<td>26.5±0.71</td>
</tr>
<tr>
<td>V.mediterranei</td>
<td>26±1.41</td>
</tr>
<tr>
<td>V.nereis</td>
<td>27.5±0.71</td>
</tr>
<tr>
<td>V.parahaemolyticus</td>
<td>23.5±0.71</td>
</tr>
<tr>
<td>V.proteolyticus</td>
<td>17.5±0.71</td>
</tr>
<tr>
<td>V.vulnificus</td>
<td>38±1.41</td>
</tr>
<tr>
<td>V.harveyi</td>
<td>28.5±0.71</td>
</tr>
</tbody>
</table>

Fig. 1  Production of pyocyanin from Pseudomonas aeruginosa PA-pUCP-Phz⁺⁺ in ZoBell’s broth

Fig. 2  Phase separation of pyocyanin to form a blue pyocyanin-chloroform lower layer and an upper aqueous layer.
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Fig. 3 Column purification of pyocyanin. Blue colour indicates pyocyanin fraction.

Fig.4 HPLC of pyocyanin: A) Pyocyanin from *Pseudomonas aeruginosa* PA-pUCP-Phz++ B) Pyocyanin standard.
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Fig. 5 Nuclear Magnetic Resonance (¹H NMR) spectra of pyocyanin extracted from Pseudomonas aeruginosa PA-pUCP-Phz⁺⁺.

Fig. 6 Mass spectra of pyocyanin from Pseudomonas aeruginosa PA-pUCP-Phz⁺⁺ and pyocyanin standard.
Fig. 7 Pyocyanin induced inhibition on bioluminescence of *Vibrio harveyi*.

Fig. 8 Growth of *Vibrio harveyi* at different concentrations of purified pyocyanin.
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Fig. 9 Antagonistic activity (zone of inhibition) of purified pyocyanin against V. harveyi MCCB111.

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