Pyocyanin (5-methyl-1-hydroxyphenazine) Produced by *Pseudomonas aeruginosa* as Antagonist to Vibrios in Aquaculture: Overexpression, Downstream Process and Toxicity

Thesis submitted to the

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in

MARINE BIOTECHNOLOGY

Under the Faculty of Marine Sciences
Department of Marine Biology, Microbiology & Biochemistry

By

Priyaja P

Reg.No.2877

NATIONAL CENTRE FOR AQUATIC ANIMAL HEALTH

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

KOCHI 682016, KERALA, INDIA

January-2012

Certificate

This is to certify that the research work presented in this thesis entitled **"Pyocyanin (5-methyl-1-hydroxyphenazine) produced by *Pseudomonas aeruginosa* as antagonist to vibrios in aquaculture: overexpression, downstream process and toxicity"** is based on the original work done by Ms. Priyaja P under our guidance, at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682016, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy** and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Dr. Rosamma Philip
(Research Guide)
Assistant Professor
Department of Marine Biology,
Microbiology and Biochemistry
School of Marine Sciences
Cochin University of Science and
Technology, Kerala, India

Prof. I.S. Bright Singh
(Co-Guide)
Coordinator,
National Centre for Aquatic Animal
Health (NCAAH),
Cochin University of Science and
Technology, Kerala, India

Cochin – 682 016

January 2012

Declaration

I hereby do declare that the work presented in this thesis entitled **"Pyocyanin (5-methyl-1-hydroxyphenazine) produced by *Pseudomonas aeruginosa* as antagonist to vibrios in aquaculture: overexpression, downstream process and toxicity"** is based on the original work done by me under the guidance of Dr. Rosamma Philip, Assistant Professor, Department of Marine Biology, Microbiology and Biochemistry and Co-Guidance of Prof.I.S. Bright Singh Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682 016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

Cochin 682 016

January 2012 **Priyaja P**

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"Rather than individual souls, we perhaps have one great soul – the soul of the entire Universe, and this is the sum total of all the little particles that makes us what we are"

……Thomas Edison

I bow before that Universal power at work in our bodies, minds and spirits which tends towards perfection and wholeness……

This thesis is dedicated to amma……

Priyaja P

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Chapter 1

GENERAL INTRODUCTION

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General Introduction

Aquaculture is the fastest growing food sector and multibillion dollar industry on global scale, looked upon as the high protein resource to meet the nutritional requirements of the increasing population. The trend in aquaculture development is towards intensification (Bondad-Reantaso et al., 2005). However, the commercial production objectives by intensive aquaculture are hampered by diseases caused by viruses, bacteria, fungi, parasites and other undiagnosed and emerging pathogens. In this scenario antibiotics occupied the central stage as the most common control strategy of diseases, which over a period of time resulted in antibiotic resistance and horizontal transmission of resistant genes from fish pathogens to humans (Angulo, 2000). In this context several alternate strategies for the prevention and control of diseases in aquaculture have been put forth, such as vaccines, immunostimulants and probiotics (Gomez et al., 2007). Among them, probiotics offer themselves as one of the most promising prophylaxis in lieu of antibiotics in fish and shrimp aquaculture (Verschuere et al., 2000; Balcázar, 2006; Rahman et al., 2011).

As the application of probiotics in aquaculture is a promising strategy, their mode of action has been a subject of investigation and has been found multifactorial

and strain specific (Touhy et al., 2003). Enhanced colonization of the probiotics and resistance to the invasion of pathogens and their direct inhibitory effects upon the pathogens are likely to be the modes of action by which reduction in the incidence of diseases have accomplished (Gomez et al., 2007). Probiotic strains have inhibited pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms. These include creating a hostile environment for pathogens by the production of inhibitory compounds such as bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide, formation of ammonia and diacetyl, alteration of pH values by the production of organic acids (Verschuere et al., 2000), competition for essential nutrients and adhesion sites (Vine et al., 2004), supply of essential nutrients and enzymes resulting in enhanced nutrition of the cultured animal (Wang et al., 2000), direct uptake of dissolved organic matter mediated by the bacteria (Dalmin et al., 2001), modulating interaction with the environment and the development of beneficial immune responses (Gullian et al., 2004).

In screening for potential probiotics, most of the researchers have employed identification of inhibitory activity *in vitro* initially (Dopazo et al., 1988; Westerdahl et al., 1991; Sugita et al., 1996a, b; Bly et al., 1997; Sugita et al., 1997a, b, 1998, 2002; Burgess et al., 1999; Jorquera et al., 1999; Spanggaard et al., 2001; Chythanya et al., 2002; Hjelm et al., 2004a, b). The production of an inhibitory substance has been shown to work very well in probiotics and this screening method has been instrumental in identifying fabulous number of probiotics in aquaculture (Irianto and Austin, 2002b; Lategan and Gibson, 2003; Vaseeharan et al., 2004; Lategan et al., 2004a, b).

There are many commercial probiotic products available, especially in shrimp aquaculture. Nevertheless, the search for more potential probiotic strains adapted, to the dynamics of an aquaculture production system is highly imperative, to arrive at the identification of the most appropriate ones as the final product. *Bacillus subtilis* and *B.*

licheniformis have been used commercially as probiotics in aquaculture, mainly for prawns (Moriarty, 2003). Meanwhile, *Pseudomonas* which has formed one of the components in a few probiotic preparations , has acted as an antagonistic probiotic to marine prawns causing growth inhibition of a number of pathogens such as *Salmonella*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *V. harveyi*, *V. fluvialis*, *Photobacterium damselae* (previously *V. damsela*), *V. vulnificus* and *Aeromonas* spp. (Daly et al., 1973; Goatcher and Westhoff, 1975; Oblinger and Kreft, 1990; Chythanya et al., 2002; Vijayan et al., 2006, Preetha et al., 2010). Furthermore, pseudomonads are common components of the nonpathogenic microbiota of fish and have been widely studied for biocontrol purposes in aquaculture (Smith and Davey, 1993; Bly et al., 1997; Gram et al., 1999, 2001).

1.1. Pseudomonads

Pseudomonas is a diverse genus of Gammaproteobacteria with more than 60 species exhibiting varied life styles in a wide range of environments, including soil, water, plant surfaces, and animals (Gross and Loeper, 2009). Members of the genus *Pseudomonas* inhabit a wide variety of environments, which is reflected in their versatile metabolic capacity and broad potential for adaptation to fluctuating environmental conditions and are of great interest because of their importance in plant and human diseases, and their growing potential in biotechnological applications (Silby et al., 2011). They are well known for their ubiquity in the natural world, capacity to utilize a striking variety of organic compounds as energy sources and production of a remarkable array of secondary metabolites (Gross and Loeper, 2009).

The functional and environmental range of *Pseudomonas* spp. showed that the common ancestor of *Pseudomonas* had encountered a wide range of abiotic and biotic environments that led to the evolution of a multitude of traits and lifestyles with significant overlap among species (Silby et al., 2011). The remarkable ecological and metabolic diversity of *Pseudomonas* spp. is reflected in the genomes

of these bacteria with genome size varying from 4.6 to 7.1 Mega bases with 4237 to 6396 predicted genes, and the GC contents ranging from 57.8 to 66.6%. The genomic diversity is particularly apparent from the relatively small size of the core genome that is shared among *Pseudomonas* species.

Though *Pseudomonas* spp. have been extensively studied as plant and animal pathogens, their biotechnological potential is increasingly getting realized by recent advances in genomic sciences. The Pseudomonads have an astonishing capacity of secondary metabolite production which plays important roles in their diverse life styles, nutrient acquisition, virulence, and defense against competitors and predators confronted in natural habitats. Although the biosynthetic pathways for the *Pseudomonas* metabolites have much in common with those of the well-studied Actinomycetes, they also exhibit unusual features. Consequently, the study of secondary metabolism in *Pseudomonas* spp. has led to the discovery of novel biosynthetic mechanisms (Gross and Loeper, 2009) which have promising biotechnological applications.

The terrestrial isolates of *Pseudomonas* have been studied extensively, but only a few marine isolates of this genus have been described that produce novel bioactive substances (Isnansetyo and Kamei, 2009). The chemical structures of the bioactive substances from marine *Pseudomonas* are diverse, including pyroles, pseudopeptide pyrrolidinedione, phloroglucinol, phenazine, benzaldehyde, quinoline, quinolone, phenanthren, phthalate, andrimid, moiramides, zafrin and bushrin with diverse mechanisms of action, heterogeneous structures and diverse applications (Isnansetyo and Kamei, 2009). The genetic and ecological diversity of *Pseudomonas* suggest that marine isolates are potential sources of bioactive metabolites.

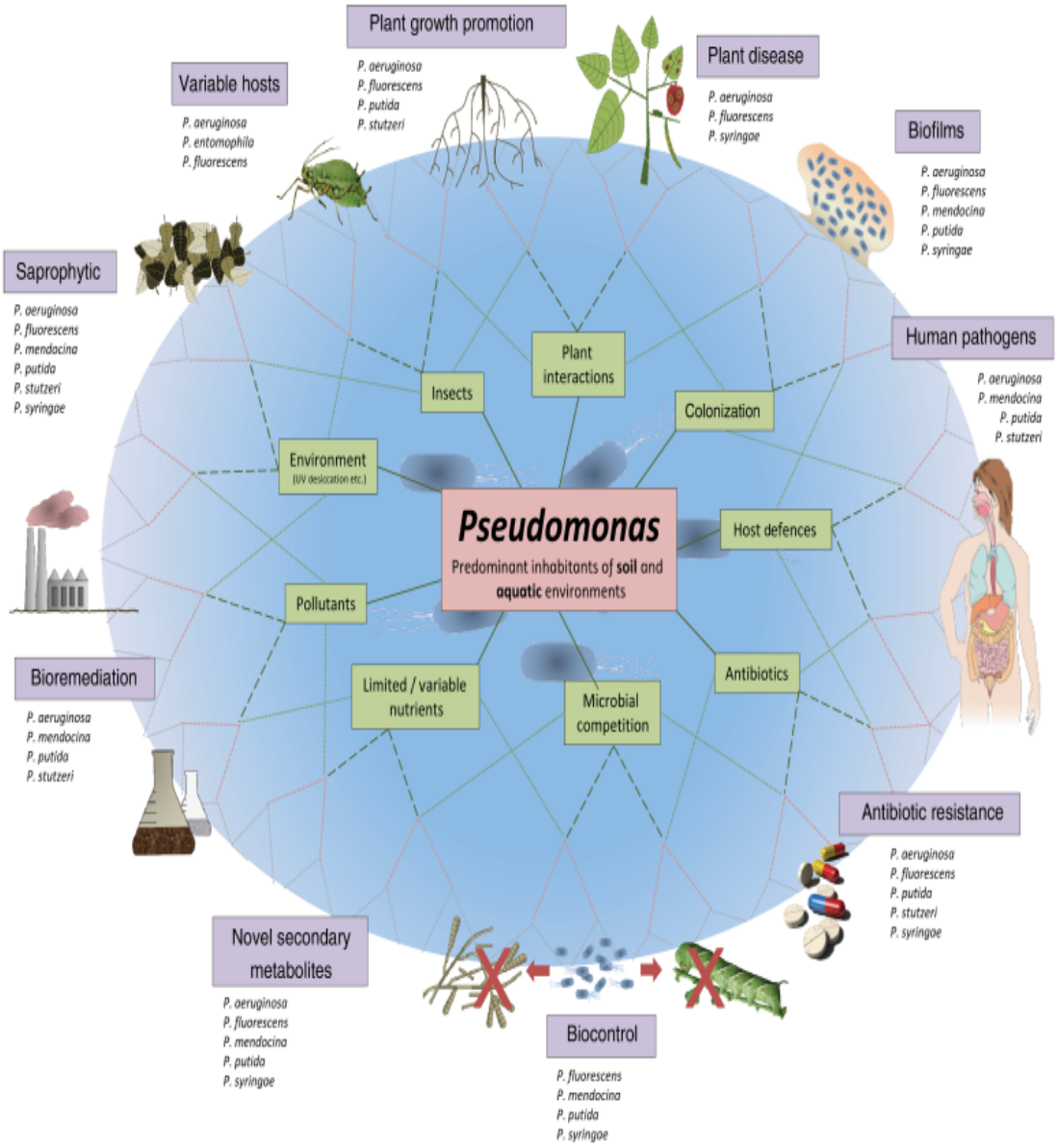

Fig.1 The functional and environmental range of *Pseudomonas* spp. (Silby et al., 2011)

1.1.1. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative aerobic rod belonging to the family Psuedomonadaceae. It is an aquatic and soil bacterium that can infect a range of organisms and an opportunistic human pathogen capable of infecting different tissues and sites (Lyczak et al., 2000). Though they are specially noted for their pathogenicity, the recent research and genomic studies have thrown light into the unique property of these organisms to produce a wide array of secondary metabolites with varieties of potential biotechnological applications. *Pseudomonas aeruginosa*

has a large number of paralogous groups (distinct gene families), indicating that its genome has evolved through genetic expansion (Stover et al., 2000), which may suggest adaptation to colonization and competitive fitness in a diverse range of ecological niches. The function of many genes may become apparent only when analysed within more complex environments than generally used in laboratories, as has been argued in the case of environmentally induced genes in *P. fluorescens* (Rainey, 1999; Silby and Levy, 2004).

The ecological diversity of *Pseudomonas* is related to its great metabolic versatility. *Pseudomonas aeruginosa* could utilize long chain length alkanes and survive in heavy oil without any change in their morphology and size for at least five years in oil contaminated coasts (Chaerun et al., 2004). It was reported that the biodegradability of crude oil by *Pseudomonas aeruginosa* was dependent on the rhamno lipid productivity of the organism in culture (Tang et al., 2007). *Pseudomonas* spp. can produce a variety of bioactive compounds that can control shrimp pathogenic bacteria such as *Vibrio* spp. with no harm to shrimp (Chaythanya et al., 2002; Liu et al., 2000; Vijayan et al., 2006, Preetha et al., 2010). It is well recognized that *Psuedomonas* spp. can produce bioactive compounds such as phenazine compounds, quinolones, hydrogen cyanide, 2-4- diacetylphloroglucinol, pyoluteorin and pyrrolnitrin (Ge et al., 2004; Mashburn-Warren et al., 2009; Preetha et al., 2010). Kumar et al. (2005) identified phenazine 1- carboxamide as the compound present in a *Psuedomonas aeruginosa* strain with broad spectrum antifungal activity and biofertlizing traits. *Pseudomonas* spp. has been found to successfully suppress rice disease under saline soil conditions (Rangarajan et al., 2003).

1.1.1.1 Environmental vs clinical isolates of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa strains of clinical origin have been differentiated from environmental strains as a result of their ability to use paraffin as sole carbon source (Smits et al., 2003). Wide mutational diversity was observed in the chromosomal porin gene (oprD) in clinical and environmental isolates compared to laboratory strains (Pirnay et al., 2002) and there was high variability in sequences and intra and possibly inter species recombination within oprD gene. The defective chromosomal oprD mutations emerging *in vivo* in clinical isolates were found to be

far more versatile than those selected *in vitro* from a laboratory strain carrying the plasmid borne PAOI oprD gene. OprD is important for bacterial adaptability to changing environments and it is known that such contingency genes are highly mutable compared with the housekeeping genes which are relevant for basic bacterial metabolism and structure and which mutate at an expected low frequency (Moxon et al., 1994).

Horizontal gene transfer has an important role in bacterial adaptation to different habitats and it has been found that *P. aeruginosa* can exchange large DNA blocks that integrate at specific sites (Arora et al., 2001; Kiewitz and Tummler, 2000; Kresse et al., 2003; Larbig et al., 2002; Liang et al., 2001; Romling et al., 1997; Schmidt et al., 1996). Morales et al. (2004) identified that horizontal gene transfer may play more important role than point mutations on the adaptation of *P. aeruginosa* to different habitats.

1.1.2. Antagonism of *Pseudomonas aeruginosa*

Antagonistic property of *Pseudomonas aeruginosa* to various pathogenic microorganisms have been extensively studied and is applied 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 (Chythanya et al., 2002; Preetha et al., 2010) and also as bioaugmentors in bioremediation programmes. (De Meyer et al., 1999; Chaerun et al., 2004; Hasanuzzaman et al., 2004; Tang et al., 2007). The biotechnological applications of antagonistic organisms producing inhibitory compounds are very promising in aquaculture (Vaseeharan and Ramasamy, 2003; Hjelm et al., 2004; Ravi et al., 2007), because of their ability to control the pathogenic bacterial population in a system without affecting the total chemical or biochemical balance of the environment (Kesarcodi-Watson et al., 2008; Tinh et al., 2008).

It has been reported that some marine *Pseudomonas* isolates have the same biosynthetic capabilities as their terrestrial counterparts. *P. aeruginosa* isolated from an Antarctic sponge, *Isodictya setifera*, produced six diketopiperazines and two phenazine alkaloids (Jayatilake et al., 1996) that are with antibiotic properties against

Gram-positive bacteria, *Bacillus cereus*, *Micrococcus luteus*, and *S. aureus*. *P. aeruginosa*, isolated from a mangrove environment, also produced phenazines (Saha et al., 2008) pyocyanin and pyorubrin with antibacterial activity against *Citrobacter* sp. as well as hemolytic activity in a chick blood assay. Phenazine is commonly produced by terrestrial fuorescent *Pseudomonas* isolates (Leisinger and Margara, 1979) including terrestrial *P. aeruginosa.*

Dive (1973) found that the action of extra cellular pigment production by *P. aeruginosa* inhibited the growth and division of *Colpidium campylum.* The antifungal activity of pyocyanin produced by *P. aeruginosa* was studied by Costa and Cusmanao (1975) and Kerr et al. (1999) and the results showed that pyocyanin strongly inhibited *Candida albicans* and *Aspergillus funmigatus* and many yeast species pathogenic for man. They further concluded that there may be a role for pyocyanin in the prevention of pulmonary candidiasis in patients colonized by *P. aeruginosa*. The antibacterial activity of pyocyanin against different bacteria such as *Staphylococci* and *Vibrio* spp. were done by Arun Kumar et al. (1997) and Vijayan et al. (2006). Rattanachuay et al. (2010) reported that one of the most active component of the antivibrio compound of their isolate was a small molecular weight, heat stable, pH resistant, and mostly tolerant to a variety of enzymes such as lyzozyme, proteolytic, lipolytic and amylolytic enzymes. The major component was characterized as 2-heptyl-4-quinolone (Rattanachuay et al., 2011). Specific inhibition of *V. harveyi* by *P. aeruginosa* has been reported earlier by Torrento and Torres (1996), Chythanya et al. (2002) and Vijayan et al. (2006).

1.2. Vibriosis in aquaculture and its control

Vibriosis is a major disease caused by *Vibrio* spp. which are ubiquitous in aquaculture settings associated with all cultured species including fish, molluscs and crustaceans (Verdonck et al., 1997; Thompson et al., 2001; Vandenbergh et al., 2003; Jayaprakash et al., 2006). Signs of vibriosis include lethargy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bolitas negricans, bioluminescence, muscle opacity and melanization. In infected individuals the pathogen multiplies in the haemolymph (Jayabalan et al., 1996). In many cases,

vibrios are opportunists, only causing disease when the host organism is immune suppressed or otherwise physiologically stressed, with the frequency of infection often being attributable to intensive culture and adverse environmental conditions (Alderman and Hastings, 1998). Though almost all types of cultured animals can be affected by these bacteria, the most serious problems have been reported in penaeid shrimp culturing, and luminescent vibriosis has become a major constraint on shrimp production in South America and Asia (Austin and Zhang, 2006) resulting in low survival rates in hatchery or grow - out conditions. Pathogenic luminous bacteria infect the eggs and tissues of early larva, post larvae and juveniles of penaeid shrimps. *Vibrio harveyi* and *V. splendidus* are the predominant pathogens for luminescent vibriosis in shrimps (Baticados et al., 1990). Saulnier et al. (2000) reviewed that larval mortalities associated with vibriosis has been reported globally due to *Vibrio* species such as *V. harveyi, V. alginolyticus, V. damsela, V. parahaemolyticus, V.* v*ulnificus* and *V. penaeicida* that have been observed in nursery or grow-out ponds of *P.* v*annamei, P. monodon, P. japonicus* and *P. stylirostris*. Vibrio disease is described as vibriosis or bacterial disease, penaeid bacterial septicemia, penaeid vibriosis, luminescent vibriosis or red-leg disease (Aguirre-Guzma et al., 2004).

Though traditionally antibiotics have been used to prevent bacterial diseases in aquaculture, the massive use of antibiotics resulted in multiple resistance of luminescent vibrios to several antibiotics (Defoirdt et al., 2007). Consequently, antibiotics are no longer effective in treating luminescent vibriosis. Karunasagar et al. (1994) reported mass mortality in black tiger shrimp (*Penaeus monodon*) larvae caused by *V. harveyi* strains with multiple resistance to cotrimoxazole, chloramphenicol, erythromycin and streptomycin of which the first two had been regularly used as prophylactics.

The important alternative biocontrol measures directed towards luminescent vibrios are virbriophage therapy, killing of pathogen using short-chain fatty acids, virulence inhibition by disruption of quorum sensing and use of antagonistic probiotics. Attempts to use phages to control luminescent vibriosis have only recently been reported. Shivu et al. (2007) isolated seven phages from hatchery and

creek water and tested their lytic spectrum against 183 *V. harveyi* strains originating from different geographical regions and found that the phages lysed between 15% and 69% of the strains. Short-chain fatty acids (SCFAs) are known to inhibit the growth of pathogenic bacteria, and Defoirdt et al. (2006) reported that SCFA inhibited the growth of pathogenic luminescent vibrios in liquid medium. Defoirdt et al. (2007) found that the natural furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone blocks quorum-sensing-regulated gene expression in *V. harveyi* by decreasing the DNA-binding activity of the quorum-sensing response regulator LuxRVh.

Antagonistic probiotics have been successfully used to control vibriosis in aquaculture systems. It has been observed that in the beginning of the shrimp larval rearing period the *Pseudomonas* group dominates and subsequently members of the family Vibrionaceae take over (Singh, 1986, 1990). Therefore, if we could introduce probiotic isolates that could exclude the vibrios from the system in the beginning itself it should be possible to improve larval survival. There are many commercial probiotic products available, especially in shrimp aquaculture. *Bacillus* spp. are the most common probiotic that have been successfully used to control luminescent vibrios by the production of antibiotics (Moriarty, 1998; Rengpipat et al., (2003); Vaseeharan and Ramasamy, 2003; Decamp, et al., 2005). Another important possibility to control vibriosis is the antagonistic microalgae, however, much research is required in this area to demonstrate that green water or algae can indeed protect cultured animals from luminescent vibriosis.

1.3. *Pseudomonas* as a probiotic in aquaculture

Many bacterial isolates which are common members of the non pathogenic microflora of fish and shellfish culture systems have been shown to inhibit fish and prawn pathogens *in vitro* (Jayapraksh et al., 2005). Gomez-Gil (1995) and Verschuere et al. (2000) claimed that certain strains of bacteria associated with *Artemia* and prawn culture systems have the ability to control pathogens by means of competitive exclusion or by the production of inhibitory compounds. Pseudomonads are common inhabitants of the aquatic environment including shrimp culture ponds

(Otta et al.,1999) and are commonly associated with gills, skin and intestinal tract of live fish (Cahill, 1990). Smith and Devey (1993) noted that bathing Atlantic salmon presmolts in a strain of *Pseudomonas fluorescens* reduced subsequent mortality from stress-induced furunculosis. Gram et al. (1999) observed *in vitro* inhibition of *Vibrio anguillarum* by *Pseudomonas flourescens* and obtained lower mortalities in probiotic-treated fish, *Oncorhynchus mykiss*. *Pseudomonas* acts as a potential probiotic for marine prawn and has caused growth inhibition of a number of pathogens such as *Salmonella*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Vibrio fluvialis*, *Photobacterium demenselae*, *Vibrio vulnificus* and *Aeromonas* (Oblinger and Kreft, 1990; Chaithanya et al., 2002; Vijayan et al., 2006). Hai and Fotedar (2009) reported that *P. synxantha* and *P. aeruginosa* are most effective probiotic in inhibiting bacteria isolated from *Penaeus latisulcatus*.

1.4. Phenazines from *Pseudomonas aeruginosa*

Phenazines are heterocyclic nitrogen containing metabolites with antibiotic, antitumor and antiparasitic activity; synthesized by a limited number of bacterial genera including *Pseudomonas*, *Burkholderia*, *Brevibacterium* and *Streptomyces.* Among the naturally occurring phenazine compounds pyocyanin (Fordos, 1859), chlororaphine (Guignard and Sauvageau, 1894) and iodinin (Clemo and Mcllwain, 1938) are well studied. Phenazines represent every colour of the visible spectrum, with an intense peak in the range 250-290nm and a weaker peak at 350-400nm (Gerber, 1973). Certain bacterial producers are able to synthesize mixtures of as many as 10 different phenazine derivatives at one time. A number of strains of *Pseudomonas aeruginosa* were reported to produce more than one phenazine and growth conditions were found to affect the relative amounts of each metabolite.

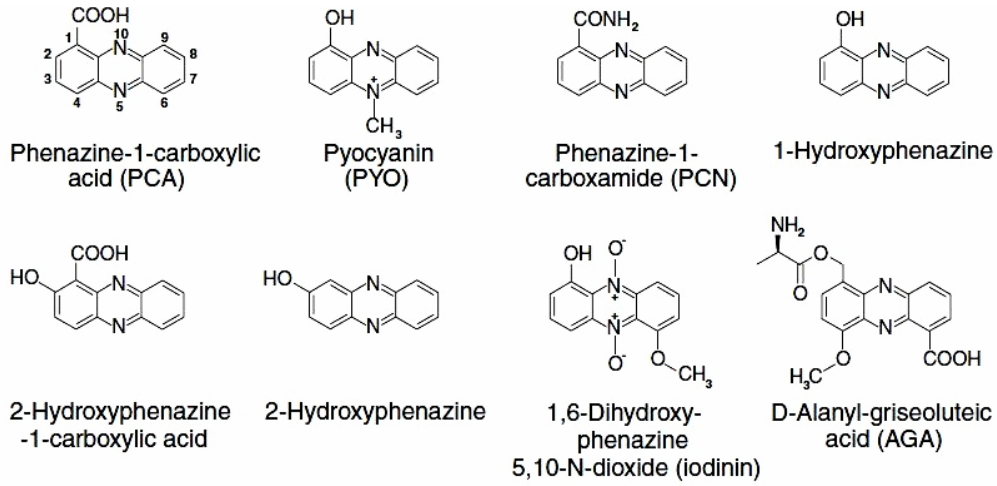

Fig.2 Some common phenazines of bacterial origin (Mavrodi et al., 2006)

All most all phenazines exhibit broad spectrum activity against various species of bacteria and fungi (Kavitha et al., 2005; Arun Kumar et al., 1997). This activity is connected with the ability of phenazine compounds to undergo oxidation-reduction transformations and thus cause the accumulation of toxic superoxide radicals in the target cells. Some phenazines can act as bacterial virulence factors. Phenazine pigments are mostly water soluble and are secreted into the medium. Thus, pyocyanin diffuses readily into agar-solidified media which become stained blue. Some are sparingly water soluble and precipitate.

Pseudomonas aeruginosa produce diverse group of phenazines including pyocyanin, phenazine-1-carboxylic acid (PCA), 1-hydroxyphenazine (1-OH-PHZ) and phenazine-1-carboxamide (PCN) (Chang and Blackwood, 1969; Turner and Messenger, 1986; Lesinger and Margraff, 1979). Phenazine-1-carboxamide (PCN) was first isolated from old cultures of *P. aeruginosa* by Birkofer (1947) using ether extraction and crystallization. This yellow compound was identical with oxychlororaphine, first reported to be produced by *Pseudomonas chlororaphis*. Chlororaphine, a crystalline emerald green complex was also isolated from old cultures of *P. aeruginosa* (Birkofer, 1947). Production of Phenazine-1-carboxyilic acid (PCA), in addition to its amide derivative, by *P. aeruginosa* was reported by Takeda (1958). Strains of *P.aeruginosa* producing a bright red water soluble pigment, pyorubin were initially described by Gessard (1917). It was found to be a characteristic of freshly isolated strains of the species and that old cultures lost the ability to produce both pyorubin and pyocyanin.

1.4.1. Pyocyanin, an anti-vibrio phenazine compound produced by *Pseudomonas aeruginosa*

In 1859, Fordos gave the name Pyocyanin ('blue pus') to the blue coloured phenazine produced by *Pseudomonas aeruginosa*. It was identified as an organic base, blue in alkaline aqueous solutions but red when acidified. Its chemical reduction to a colourless form, spontaneously reoxidised in air, was also described by Fordos (1860). He also established that pyocyanin slowly decomposed to a yellow substance, no longer basic in nature, now known to be 1-hydroxyphenazine. The bacterium responsible for pyocyanin production was first isolated by Gessard in 1882. However, the phenazine nature of pyocyanin was established by Wrede and Strack in 1924. Moreover, they examined its decomposition product hemipyocyanine, correctly deduced to be 1-hydroxyphenazine. Jensen and Holten (1949) studied the zwitter ion nature of the pigment by potentiometric studies and showed that pyocyanin in mixture with its reduced leuco derivative acted as a reversible redox system. At acidic pH, colour changes associated with progressive reduction of pyocyanin are red to yellow, to green, to colourless and at alkaline pH the colour change is from blue to colorless (Friedheim and Michaelis, 1931). Cultures of *Pseudomonas aeruginosa* were observed to reduce pyocyanin to its colourless form in the absence of air and depending upon the pH value, the colour variation according to the redox state of the system appeared to account for the shifting play of tints referred to as 'chameleon phenomenon'.

Production of pyocyanin by *P. aeruginosa* was identified to be sensitive to the phosphate concentration in the growth media (Frank and DeMoss, 1959; King et al., 1954). Moreover, Ingledew and Campbell (1969) concluded that phosphate deficiency triggered pyocyanin biosynthesis by *P.aeruginosa.* Burton et al. (1947) reported that amino acids could replace the peptone commonly claimed to be essential for good pigmentation and a medium containing glycerol, leucine, glycine or alanine and mineral salts was recommended for *P. aeruginosa*. The findings of Halpern et al. (1962) showed that production of the pigment continued for upto 10 hours after growth has ceased and was not inhibited by addition of chloramphenicol. However, the phenazine production was repressed during growth on media containing glucose or other readily utilizable carbon sources.

1.4.1.1 Applications of Pyocyanin

Pyocyanin producing *P. aeruginosa* environmental isolates have been recognized as putative biological control agents against phytopathogenic fungi and bacteria in agriculture (Anjaiah et al., 2003; Bano and Musarrat, 2003, Rangarajan et al., 2003), vibrios in aquaculture (Chythanya et al., 2002, Vijayan et al., 2006) and as bioaugmentors (Chaerun et al., 2004; Hasanuzzaman et al., 2004; Tang et al., 2007).

The antibiotic activities of pyocyanin against protozoans were studied by Dive (1973) and found that the action of extracellular pigment production by *P. aeruginosa* inhibited the growth and division of *Colpidium campylum*. Costa and Cusmanao (1975) and Kerr et al. (1999) explained the antifungal activity of pyocyanin and the strong antagonism against *Candida albicans* and *Aspergillus fumigatus*. It was also active against many yeast species pathogenic to human. They concluded that there may be a role for pyocyanin in the prevention of pulmonary candidiasis in patients colonized by *P. aeruginosa*. The antibacterial activity of pyocyanin against different bacteria such as *Staphylococci* and *Vibrio* sp. were done by Arun Kumar et al. (1997) and Vijayan et al., (2006). Anjaiah et al. (1998) reported that pyocyanin produced by *P. aeruginosa* isolate (SA44), from Chilli rhizosphere was found to induce a potent systemic acquired resistance reaction (SAR) against the leaf pathogen *Botrytis* in a bean system.

Morrison et al. (1978) found that pyocyanin's redox behaviour mimicked that of riboflavin and had been used as a model for flavins which themselves were found to be experimentally difficult to use in electrochemical and spectroscopic studies. Pyocyanin is a nitric oxide (NO) antagonist in various pharmacological preparations and has various pharmacological effects reported on eukaryotic and prokaryotic cells (Mashburn-warren et al., 1990; Vukomanovic et al., 1997).

Pyocyanin plays an important role as an electron transfer agent and catalyst in the phosphorylation reactions associated with the photosynthetic processes of bacteria and green plants (Zaugg, 1964). It could function as an extra-cellular respiratory pigment. 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.

Microbial Fuel Cells (MFCs) known as bio-electrochemical systems (BESs) enable conversion of chemical energy into electrical energy through the catalytic activity of microorganisms. It was found that high rate electron flow towards the microbial fuel cells anode was enabled through pyocyanin (Pham et al., 2008; Rabaey andVerstraete, 2005). Pyocyanin was not only used by *P. aeruginosa* to improve electron transfer, but enhanced electron transfer by other bacterial species as well. These findings have considerable implications with respect to the power output attainable in MFC.

1.4.1.2. Biosynthetic pathway of pyocyanin

In *P. aeruginosa* the phenazine biosynthetic pathway branches off from the shikimic acid pathway, which is also the source for metabolites such as the aromatic amino acids, siderophores and quinines (Chin et al., 2001). Usually the phenazine formation commences after the exponential phase of microbial growth along with associated aromatic amino acid biosynthesis. Shikimic acid acts as a precursor for the simultaneous biosynthesis of phenazines. DAHP (3-deoxy-7-phosphoheptulonate) synthase is the first enzyme of the shikimate pathway and catalyses the condensation of phosphoenol pyruvate and erythrose -4-phosphate.

The first phenazine structure in the biosynthetic pathway is believed to be phenazine-1, 6-dicarboxilic acid and is formed by the symmetrical condensation of two molecules of chorismic acid (Leisinger and Margraff, 1979). In this step, firstly, the amination of chorismic acid converts chorismic acid to 2-amino-2-deoxyisochorismic acid (ADIC) by aminodeoxyisochorismate (ADIC) synthase. ADIC formed is converted to trans-2, 3-dihydro-3-hydroanthranilic acid (DHHA). The condensation of two identical DHHA molecules is then required to form the phenazine ring system. The molecules react with each other by nucleophilic addition, dehydration and tautomerization to give 5, 10-dihydroanthranilic acid which is oxidized to phenazine-1-carboxylic acid (PCA). Romer and Herbert (1982) reported that glutamine was the primary nitrogen source for PCA biosynthesis and that the phenazine ring was constituted by a combination of two units of the same precursor. Pyocyanin is formed from phenazine-1-carboxylic acid (Parsons et al., 2007) by hydroxylative decarboxylation mechanism and the 5-methylphenazine-1-carboxylic acid betaine was shown to be the precursor in this reaction.

Phoshoenol pyruvate + Erythrose-4-phosphate
↓
3-deoxy-D-arabino-heptulosonate
↓
5-dehydroquinic acid
↓
5-dehydroshikimic acid
↓
5-phosphoshikimic acid
↓
3-enol pyruvyl-5-phosphoshikimic acid
↓
Chorismic acid
↓
2-amino-2-deoxyisochorismic acid (ADIC)
↓
Trans-2,3-dihydroxyanthranilic acid (DHHA)
↓
2,3-dihydro-3-oxo-anthranilic acid
↓
Phenazine-1-dicarbolxylic acid (PCA)
↓
5-methyl phenazinium-1-carboxylic acid betaine
↓
5-methyl-1-hydroxy phenazinium betaine (Pyocyanin)

Fig.3 Biosynthetic pathway of pyocyanin

1.4.1.3. Genetics of pyocyanin biosynthetic pathway

Pseudomonas aeruginosa contains a complex phenazine biosynthetic pathway consisting of two homologous core loci (*phz*$A_1B_1C_1D_1E_1F_1G_1$ and *phz*$A_2B_2C_2D_2E_2F_2G_2$) responsible for the synthesis of phenazine-1-carboxilic acid (PCA), the precursor of pyocyanin. Mavrodi et al. (2001) reported that these seven genes are sufficient for synthesis of PCA and two additional genes *phzM* and p*hzS* encoding unique enzymes are involved in the conversion of PCA to pyocyanin. Knockout mutant analysis on these genes also proved its contributory effect on pyocyanin production (Chieda et al., 2008; Mavrodi et al., 2001).

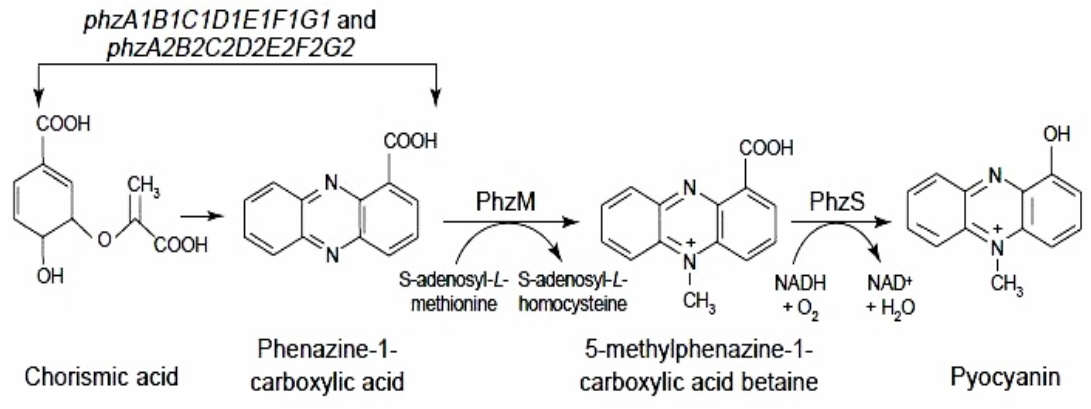

Fig.4 Synthesize of pyocyanin from chorismate by the *phz* operons, as well as by *phzM* and *phzS*, which catalyze the final steps in the synthesis (Lau et al., 2004).

In *P. aeruginosa*, the two copies of conserved seven-gene operon *phzABCDEFG* are transcribed as a single mRNA and are localized within a 6.8kb Bg/11-Xbal fragment from the phenazine biosynthesis locus at positions 4,713,795 to 4,720,062 in *P. aeruginosa* PAO1 genome; a well conserved ribosome binding site precedes each gene. In this operon, the start and stop codons of open reading frames *phzC1*, *phzD1* and *phzE1* overlap possibly reflecting translational coupling. *phzM* is preceded by a putative ribosome binding site, GAGAGA and spans positions 4,713,098 to 4,712,094. *phzM* are transcribed divergently and are separated by 695 bp. *PhzS* is located 236 bp downstream from *phzG1* and spans positions 4,720,300 to 4,721,508 of the *P. aeruginosa* PAO1 genome and is preceded by a well conserved ribosome binding site, AAGGAA.

In the seven-gene operon *phzE* converts chorismate to 2-amino-2-deoxyisochorismic acid (ADIC). Isochorismatase producing *phzD* converts ADIC to trans-2, 3-dihydro-3-hydroanthranilic acid (DHHA). The first phenazine nucleus is formed by the condensation of two molecules of trans-2, 3-dihydro-3-hydroanthranilic acid (DHHA) by the products of *phzF* and *phzG* genes and the *phzA* and *phzB* stabilize a multienzyme phenazine biosynthetic complex. The sequence data showed that the phzC protein from *Pseudomonas fluorescens* is a typical type 11 enzyme. Being expressed late in growth, *phzC* could function to divert common carbon metabolites into the shikimate pathway providing the high levels of chorismic acid needed to support the synthesis of PCA. *phzM* and *phzS* which convert PCA to

pyocyanin encode putative phenazine-specific methyltransferase and flavin containing monooxygenase enzymes, respectively.

The *phzC*, *phzD* and *phzE* are similar to enzymes of the shikimic acid pathway and together with *phzF* are absolutely required for phenazine synthesis. *phzG* is similar to pyridoxamine-5'-phosphate oxidases and probably is a source of cofactor for the PCA synthesizing enzymes. Products of the *phzA* and *phzB* genes are highly homologous to each other and may be involved in the stabilization of a putative PCA synthesizing multienzyme complex. *phzF* exhibits significant structural similarity to members of the diaminopimelate epimerase (*DapF*) fold family of proteins. *phzM* encodes a 334-residue protein with a calculated molecular mass of 36.4 kDa. *phzS* encodes a 402-aminoacid protein with a molecular mass of 43.6 kDa and is similar to bacterial monooxygenases. Polypeptides corresponding to all the *phz* genes were identified by analysis of recombinant plasmids by Mavrodi et al., (1998).

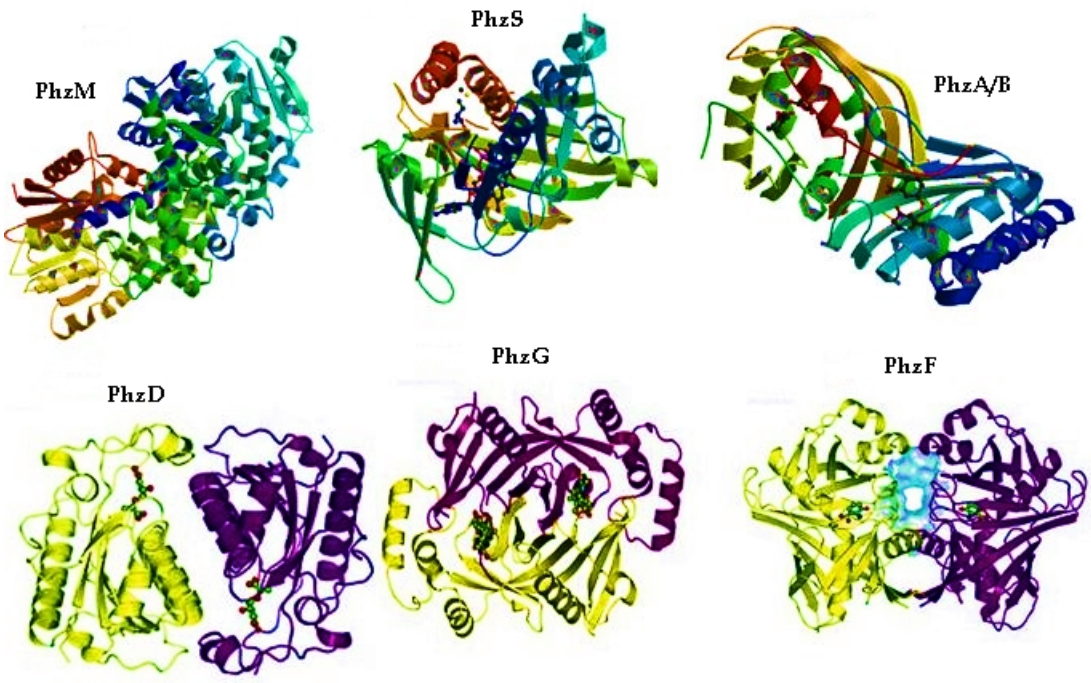

Fig.5 Structure of phenazine polypeptides involved in pyocyanin biosynthetic pathway Mavorodi et al., (1998).

1.4.1.4. Mode of action of pyocyanin in target cells.

The potent antagonistic action of pyocyanin was identified as the result of its unique redox potential by accepting a single electron, yielding a relatively stable

anion radical and readily undergoing a redox cycle. During respiration, pyocyanin becomes reduced and univalently reduces oxygen to the toxic superoxide radical. Accordingly, the antibiotic action of pyocyanin might actually be an expression of the toxicity of the O_2^- and of H_2O_2 produced in increased amounts in its presence (Hassan and Fridovich., 1980). The resistance of various bacteria to pyocyanin would therefore be dependent upon the levels of superoxide dismutase and catalase possessed by the organism and on the presence of oxygen (Baron and Rowe, 1981).

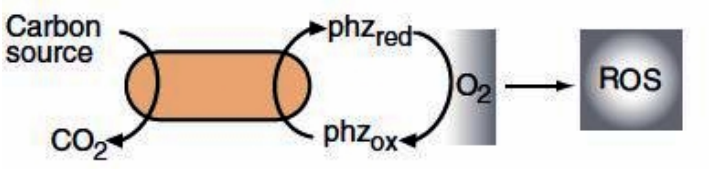

Fig. 6 Schematic of phenazine reduction and auto-oxidation. Reduced phenazines are oxidized abiotically by oxygen, generating reactive oxygen species (Price-Whelan et al., 2006).

Hassan and Fridovich (1980) described a mechanism for the toxicity of pyocyanin whereby electron flow from biological pathways is diverted to increase the production of intracellular O_2 reduction products, leading to cell death. It is of particular interest that *P. aeruginosa*, a "strict" aerobe, is itself insensitive to pyocyanin and seemingly escapes free-radical injury during production of or exposure to this compound (Hassett et al., 1992). Physiological studies have shown that *P. aeruginosa* resists the toxicity of this compound with increased superoxide dismutase and catalase activities under pyocyanin-producing conditions (Price-Whelan et al., 2007). Baron and Rowe (1981) reported that the antimicrobial action of pyocyanin was bactericidal in nature and the effect was dependent on pyocyanin concentration. This was in contradiction to the findings of Waksman and Woodruff (1942) in which they categorize pyocyanin as a bacteriostatic agent.

1.4.1.5. Environmental degradation, inactivation and detoxification of pyocyanin molecule.

Environmental degradation of the residual pyocyanin is an important factor as the compound has to be applied in aquaculture system. Yang et al. (2007) reported the biodegradation of phenazine-1-carboxylic acid (PCA) - the precursor of pyocyanin by soil organisms *Sphingomonas* sp. DP58 which consume PCA as the

sole source of carbon and nitrogen and completely degrade it within 40 hours. Biological degradation of pyocyanin is favored by the presence of phenolic character in the compound and phenolics are excellent substrates for peroxidases. The oxidation of pyocyanin leads to its inactivation and become non-toxic and the reaction is irreversible (Reszka et al., 2004). Besides, Hill and Johnson (1969) reported the microbial transformation of phenazines by *Aspergillus sclerotiorum* and Chen et al. (2008) conducted the study on intermediates or metabolites produced during of PCA biodegradation.

The study on photosensitized oxidation and inactivation (Reszka et al., 2004) showed that pyocyanin could be partially inactivated through photochemical oxidation and the resulting product(s) is a poorer free radical generator and therefore a less efficient stimulant of oxidative processes. These results suggest that photosensitization could be a potentially useful method for inactivation and possibly for detoxification.

1.5. Significance of the work and objectives

As mentioned in the above sections, pyocyanin is a versatile and multi-functional phenazine, widely used as a bio-control agent. Besides its toxicity in higher concentration, it has been applied as bio-control agents against many pathogens including the *Vibrio* spp. in aquaculture systems. The exact mechanism of the production of pyocyanin in *Pseudomonas aeruginosa* is well known, but the genetic modification of pyocyanin biosynthetic pathways in *P. aeruginosa* is not yet experimented to improve the yield of pyocyanin production. In this context, one of the aims of this work was to improve the yield of pyocyanin production in *P. aeruginosa* by way of increasing the copy number of pyocyanin pathway genes and their over expression. The specific aims of this work encompasses firstly, the identification of probiotic effect of *P. aeruginosa* isolated from various ecological niches, the overexpression of pyocyanin biosynthetic genes, development of an appropriate downstream process for large scale production of pyocyanin and its application in aquaculture industries. In addition, this work intends to examine the toxicity of pyocyanin on various developmental stages of tiger shrimp (*Penaeus*

monodon), *Artemia* nauplii, microbial consortia of nitrifying bioreactors (Packed Bed Bioreactor, PBBR and Stringed Bed Suspended Bioreactor, SBSBR) and *in vitro* cell culture systems from invertebrates and vertebrates. The present study was undertaken with a vision to manage the pathogenic vibrios in aquaculture through eco-friendly and sustainable management strategies with the following objectives:

1. Identification of *Pseudomonas* isolated from various ecological niches and its antagonism to pathogenic vibrios in aquaculture.
2. Saline dependent production of pyocyanin in *Pseudomonas aeruginosa* originated from different ecological niches and their selective application in aquaculture
3. Cloning and overexpression of *Phz* genes encoding phenazine biosynthetic pathway for the enhanced production of pyocyanin in *Pseudomonas aeruginosa* MCCB117
4. Development of an appropriate downstream process for large scale production of pyocyanin from PA-pUCP-Phz^{++}; Structural elucidation and functional analysis of the purified compound
5. Toxicity of pyocyanin on various biological systems

Chapter 2

IDENTIFICATION OF *PSEUDOMONAS* ISOLATED FROM VARIOUS ECOLOGICAL NICHES AND ITS ANTAGONISM TO PATHOGENIC VIBRIOS IN AQUACULTURE

Contents

2.1. Introduction

The genus *Pseudomonas* encompasses arguably the most diverse and ecologically significant group of bacteria on the planet. Members of the genus are found in large numbers in all of the major ecological niches (terrestrial, freshwater and marine) and also form intimate associations with plants and animals. This universal distribution suggests a remarkable degree of physiological and genetic adaptability (Ramezanpour, 2011; Spiers et al., 2000). Stanier et al. (1966) published a detailed study on the taxonomy of *Pseudomonas* spp., subjected to particular emphasis on biochemical, physiological and nutritional characters. Kersters et al. (1996) explained the past and current taxonomic organization of *Pseudomonas* on the basis of the rRNA relationship. Moreover, chemo-taxonomical studies of the pseudomonads have been performed by several scientists (Moss et al., 1972; Ikemoto et al., 1978; Oyaizu and Komagata, 1983; Stead, 1992; Vancanneyt et al., 1996). Besides, Pseudomonads have been studied by polyphasic taxonomic approaches, especially using methods for analysing micro-organisms at the molecular level and many of the organisms originally described as species of the genus *Pseudomonas* have been reclassified (Anzai et al., 2000).

Among the genus the most extensively studied subgroup of aerobic pseudomonads consists of the fluorescent pseudomonads such as *P. aeruginosa*, *P. fluorescence* and *P. putida*; primarily characterized by their ability to produce water-soluble, yellow-green fluorescent pigments. Moreover, *P. aeruginosa* shows a high degree of internal phenotypic uniformity (Stanier et al., 1966). Among the subgroup, *Pseudomonas aeuruginosa* is a ubiquitous and remarkably versatile Gram-negative bacterium found in association with plants, animals, water, and soil. *Pseudomonas aeruginosa* is noted for its metabolic versatility and its exceptional capability of thriving in a great number of seemingly dissimilar ecological niches and ability to adapt and colonize a wide variety of ecological environments (Goldberg, 2000; Kiewitz and Tummler, 2000). Apart from the wide distribution in various ecological niches, it is ubiquitously distributed as part of the normal bacterial flora of the intestine, mouth and skin of live fish (Cahill, 1990). There seems to be a consensus about the fact that *P. aeruginosa* clinical isolates are genotypically, chemotaxonomically and functionally indistinguishable from environmental isolates from various ecological niches suggesting a common recent origin of these strains (Pirnay et al., 2002). The early biochemical studies of Stanier (Stanier et al., 1966) had proven the physiological diversity of *P. aeruginosa*. However, the complete population structure of *P. aeruginosa* is still under discussion (Morales et al., 2004; Wiehlmann et al., 2007; Pirnay et al., 2009)

P. aeruginosa has been recently recognized as a potential antagonistic bacterium against pathogenic vibrios in aquaculture (Pai et al., 2010; Vijayan et al., 2006) due to its metabolite, pyocyanin (Preetha et al., 2010). Moreover *P. aeruginosa* has been applied in aquaculture as effective probiotics and as an alternative to antibiotics against pathogenic *V. harveyi* (Preetha et al., 2010; Hai et al., 2009a, b; Pai et al., 2010). The present study deals with a detailed description of the phenotypic and genotypic characterization of *Pseudomonas aeruginosa* isolated from various ecological niches such as marine, brackish water and freshwater and its antagonism to pathogenic vibrios in aquaculture.

2.2. Materials and Methods

2.2.1. 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.

2.2.2. Phenotypic characterization

All the isolates were examined for phenotypic characters (Cowan and Steel, 1974) such as Grams staining, oxidation-fermentation reaction, motility, production of indole, arginine dihydrolase, cytochrome C oxidase, catalase, fluorescent pigment, lecithinase, hydrolysis of glucose, citrate utilization, subjected for Sudan black test, and examined for growth at 4°C and 42°C.

2.2.2.1. Motility assay

Motility was tested in soft agar medium having the following composition (per liter).

Yeast extract	1g
Peptone	5g
Agar	3g
pH	7.2 ±0.2

The medium was melted and poured into tubes in 3ml aliquots and autoclaved at 15 lbs for 15 minutes. Stab inoculated the medium and incubated at 28 ± 0.5°C for 24 to 48 hours. Rhizoidal growth from the line of inoculation towards the peripheral area was considered as the sign of motility. A thick growth along the line of inoculation was considered negative.

2.2.2.2. Oxidation Fermentation reaction

This characteristic is usually determined by inoculating the organisms into deep agar media supplemented with 1% glucose in the culture tubes. Cultures were stabbed and streaked with an inoculation nichrome needle after solidification of agar.

Acidic changes at or near the surface indicated that the substrate was oxidized by aerobic bacteria, whereas the development of uniform acidity throughout the tube showed that facultative anaerobic organisms were both oxidizing and fermenting the substrate. Anaerobic bacteria that ferment only the substrate usually produced an acidic reaction in the lower part of the tube initially, but acidic materials might diffuse upwards to give an appearance of acid production throughout the tube.

MOF medium (HiMedia Laboratories, India) was employed for the present work. Transferred 2.2g of MOF medium to 100ml of water, added 1.5g agar and autoclaved at 15lbs for 15 minutes. Added 1% glucose to the sterile basal medium and transferred into 4ml aliquots aseptically into sterile tubes and autoclaved at 10lbs for 10 minutes. Converted to slants with a long butt. The tubes were stabbed and streaked and incubated at 28±0.4°C.

The results were recorded as follows:

O - Oxidation (yellow colouration in the butt)

F - Fermentation (yellow colouration throughout the tube)

F/G - Fermentation with gas production

Alk / N - alkaline reaction (pink or purple colouration in the slant and no reaction in the butt)

2.2.2.3. Kovac's Oxidase test (Cytochrome oxidase activity)

This test is used to examine whether the culture is capable of producing cytochrome oxidase enzyme. The detection of cytochrome oxidase activity is used as a differentiating test mainly for the aerobic and facultatively anaerobic groups of Gram negative bacteria. The enzyme cytochrome oxidase is capable of oxidizing paraphenylenediamine of the test solution tetramethyl-p-phenylene diamine dihydrochloride into indophenol, a blue coloured compound with which the activity is detected.

According to the methods recommended by Kovac's (1956), the organisms were freshly grown on nutrient agar slants. A platinum loop was used to pick a colony and make a compact smear on a filter paper moistened with 1% aqueous solution of tetramethyl-p-phenylene diamine dihydrochloride (TPDD). A positive result was recorded when the smear turned violet within 10 seconds, indicating the formation of indophenol.

2.2.2.4. Production of Indole

Certain bacteria produce indole by decomposition of tryptophan, which is present in tryptone broth. This liberated indole reacts with Kovac's reagent to produce red colour at the top of the medium (Cowan and Steel, 1965).

Composition of test medium (per litre)

Tryptone	15g
NaCl	5.0g
pH	7.2 ±0.2

Dispensed the medium into 3ml aliquots into tubes, autoclaved at 15lbs for 15 minutes. Inoculated and incubated for 48 hours at room temperature (28±1°C. To each tube added about 0.5ml of Kovac's reagent.

Preparation of Kovac's reagent:

ρ - dimethyl amino benzaldehyde	5g
Con.HCl	25ml
Amyl alcohol	75ml

2.2.2.5. Arginine dihydrolase test

The ability of certain organisms to produce an alkaline reaction in arginine containing medium under relatively anaerobic conditions has been used by Thornley (1960). The alkaline reaction is thought to be due to the production of ornithine, CO_2 and NH_3 from arginine. Thornley's medium has the following composition per litre:

Peptone	1g
NaCl	5g
K_2HPO_4	0.3g
Agar	3g
L(+)-arginine hydrochloride	10g

The above ingredients were dissolved in distilled water and the pH was adjusted to 6.7. Added indicator solution, dispensed the medium in 2ml aliquots in culture tubes, over laid with liquid paraffin and sterilized at 10lbs for 10 minutes. The test organisms were stab inoculated into the medium through the liquid paraffin layer. Color changes were recorded after incubation at 28 ± 0.5°C for 7 days, the color change from yellowish orange to red were considered positive.

2.2.2.6. Catalase test

The principle of this test is that when organisms containing catalase are mixed with hydrogen peroxide (H_2O_2), gaseous oxygen is released.

The test organisms are grown on a slope of nutrient agar. A thick smear of the organism was made from a 24 hour culture on a clean slide and a drop of hydrogen peroxide was placed on it. Immediate formation of gas bubbles indicated the liberation of oxygen and positive catalase test.

2.2.2.7. Production of fluorescent pigment

Test for the ability to produce specific water-soluble fluorescent pigments and phenazine pigments are very useful determinative character among aerobic pseudomonads. King et al. (1954) developed *Pseudomonas* Agar (King A medium)

for enhancing phenazine pigments production, and *Pseudomonas* Agar F (King B medium) for enhancing fluorescein production

Composition of ' King A ' (g l^{-1})

Bacto- peptone	20g
Bacto-Agar	20g
Glycerol	10ml
K_2SO_4	10g
$MgCl_2$	1.4g
pH	7.2

Composition of ' King B ' (gl^{-1})

Protease- peptone	20g
Bacto-Agar	20g
Glycerol	10ml
K_2SO_4	1.5g
$MgCl_2$	1.5g
pH	7.2

They were prepared as culture plates, and inoculated the *Pseudomonas* cultures by streaking, and incubated at room temperature (28±1°C). The plates were examined for pigmentation after 24, 48, and 72 hour.

2.2.2.8. Production of lecithinase

Bacterial phospholipases (lecithinases) decomposes phospholipid complexes that occur as emulsifying agents in serum and egg yolk (Holding and Collee, 1971). The enzymatic activity breaks the emulsion and liberates free fats so that turbidity is produced.

The test organisms were cultured on nutrient agar medium having the following composition per litre:

Peptone	5.0g
Beef extract	5.0g
Yeast extract	1.0g
NaCl	20.0g
Agar	20.0g
pH	7.2±0.2

An aliquot of 4% sterile fresh egg yolk emulsion (HiMedia Laboratories, India) was added to the sterile nutrient medium at 55°C just before the plates were poured. The test organisms were spot inoculated heavily and incubated at 27°C for 24 to 48 hours. Phospholipase production was characterized by a zone of turbidity in the medium surrounding each colony.

2.2.2.9. Hydrolysis of glucose

Carbohydrate fermentation was demonstrated by the production of acid or acid and gas (CO_2 and/ or H_2) in Hugh and Leifson's basal liquid medium in test tubes.

Hugh and Leifson's medium composition (per litre)

Peptone	2.0g
NaCl	5.0g
K_2HPO_4	0.3g
Phenol red (1% aqueous solution)	30 ml
pH	7.3±0.2

The carbohydrates were added to a final concentration of 0.1% (w/v). Acid production was readily observed by incorporating appropriate pH indicator into the medium (e.g. phenol red). The basal medium was first autoclaved at 15 lbs for 15 minutes along with plugged tubes. All the carbohydrates were added to the sterile basal medium to a final concentration of 0.1% (w/v). The medium was dispensed into the sterile tubes aseptically and was autoclaved at 10lbs for 10 minutes.

The tubes were inoculated with an inoculation needle and incubated at 28±0.5°C for 3 days and the results recorded. The production of acid induced change in the phenol red indicator was noted, as pink to yellow under acidic condition. The following carbohydrates (sugars and sugar alcohols) were used for the production of acid.

Pentoses	Arabinose, xylose, rhamnose
Hexoses	Glucose, fructose, mannose, galactose
Disaccharides	Sucrose, maltose, lactose, trehalose, cellobiose
Trisaccharides	Raffinose
Polysaccharides	Starch, inulin, dextrin, glycogen
Polyhydric alcohols	Glycerol, adonitol, mannitol, sorbitol, inositol
Glycosides	Salicin, aesculin

2.2.2.10. Citrate utilization test

This test demonstrates the ability of the microbes to utilize the test compound citrate as sole source of carbon and energy. Utilization of citrate and growth in citrate agar results in an alkaline reaction, which changes the colour of the medium, provided. In Simmon's citrate agar medium, bromothymol blue indicator is used which changes from green to bright blue on utilization of citrate. The composition of Simmon's citrate agar medium per litre (pH 6.9) is as follows:

Sodium citrate	0.2g
$MgSO_4$. $7H_2O$	0.02g
NaCl	15.0g
$(NH_4)_2HPO_4$	1.0g
K_2HPO_2	1.0g
Bromothymol blue	0.02g
Agar	20.0g

Simmon's citrate agar medium was prepared in the form of agar slopes in tubes. The slope was inoculated by streaking over the surface with a loopful of culture and was incubated for 3-4 days. Colour change from green to bright blue indicated that the culture was positive.

2.2.2.11. Sudan black test

Thin smear of each culture from the broth were made on a clean glass slide and was heat fixed. This slide was immersed in a filtered solution of 0.3% (w/v) Sudan black-B (in 70% ethyl alcohol) and incubated for 15-20 minutes at room temperature. Drained off the staining solution, washed briefly in tap water and removed excess water with tissue paper. Then the slide was immersed in xylene and blot dried with absorbent paper. Finally, the microscopic slide was counter-stained for 10 seconds with (0.5% w/v) aqueous safranin, rinsed with tap water, blot dried and examined under a microscope. The polyhydroxybutyrate (PHB) granules in cells stain blue-black while the cell walls stain pink.

2.2.2.12. Growth at 4°C and 42°C.

Growth at 4°C and 42°C was tested by observing growth in ZoBell's marine broth 2216E (HiMedia Laboratories, India) at pH 7.2. The medium was dispensed in 3ml aliquots in to tubes and sterilized at 15Ibs for 15 minutes. These broth media were inoculated with 24 hour grown cultures and incubated at 42°C in water bath, and another set at 4°C for 24 hour. The growth was detected visually by observing turbidity.

2.2.3. Molecular characterization

Molecular identification of *Pseudomonas* sp. was carried out by the sequence analysis of 16S rRNA gene and the distinctiveness of all the isolates were ascertained by sequencing a 1500bp fragment amplified from the genomic DNA.

2.2.3.1. Extraction of total DNA

Total genomic DNA extraction was carried out following the method of Lee et al. (2003) with slight modifications. An aliquot of 2ml cell suspension was taken, centrifuged at 8000rpm, 4°C for 10 minutes, discarded the supernatant and

resuspended the pellet is in 1ml TE (10mM Tris-Cl, pH 8.0, 1mM EDTA) buffer, mixed thoroughly, centrifuged, discarded the supernatant and harvested the cells. The above step was repeated. Resuspended the cells in 1ml lysis buffer (0.05M Tris –Cl, pH 8.0, 0.1M NaCl, 0.05M EDTA, 2% SDS, 0.2% PVP, 0.1% β mercapto ethanol) and 10µl proteinase K (20mg ml^{-1}) and incubated at 37°C for 1 hour and then at 55°C for 2 hours. Subsequently, added 1 volume phenol: chloroform : isolamyl alcohol (25:24:1 v/v) and the tubes were placed on a flat rocking platform and mixed thoroughly and kept for 5 minutes. Phases were separated by centrifugation at 15,000rpm for 15 minutes. Transferred the upper aqueous layer to a new tube and added equal volumes of chloroform : isoamyl alcohol (24:1 v/v). Centrifuged at 15,000rpm for 15 minutes. Repeated the step twice. Transferred the aqueous layer to a new 1.5ml micro centrifuge tube and added 0.1 volume sodium acetate (3M, pH 5.2) and 1volume ice cold absolute ethanol. DNA was precipitated by keeping at -20°C overnight, recovered by centrifugation at 15,000 rpm for 15 minutes, washed the DNA pellet by adding 200µl ice cold 70% ethanol, centrifuged and discarded the supernatant. Repeated the step twice. Dried the pellet in air, and suspended finally in 50-100µl of Milli-Q water.

2.2.3.2. PCR amplification of the 16S rRNA gene

Amplification of the 16S rRNA gene was performed using universal primers NP1F : GAG TTT GAT CCT GGC TCA and NP1R- ACG GCT ACC TTG TTA CGA CTT, which were complementary to the conserved regions at the 5' and 3' ends of the 16S rRNA gene corresponding to the 9-27 and 1477-1498 of the *Escherichi coli* 16S rRNA gene (Reddy et al., 2000). The bacterial DNA (75ng) was amplified by the PCR in a total volume of 25µl containing: Taq DNA polymerase – 1µl, 10x buffer – 2.5µl, 3mM $MgCl_2$ – 1.5µl, 10pmol of each of the two primers – 1µl, 200µM each of dNTP – 2µl, Template and Milli Q. The amplification was carried out in a thermal cycler (Eppendorf, Germany) programmed for 94°C for 5 minutes 35 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 30 seconds, extension at 68°C for 20 seconds, and final extension of 10 minutes at 68°C.

2.2.3.3. Cloning with pGEM-T Easy vector

Amplified 16S rRNA gene PCR product was ligated with pGEM-T Easy vector. The 10µl ligation mixture containing 0.5µl pGEM-T Easy vector, 3.5µl PCR product, 5µl ligation buffer, 0.5µl ligase enzyme and MilliQ was incubated at 4°C, overnight.

2.2.3.4. Transformation into *E. coli* JM109

Thawed the competent cells (*E.coli* JM109) by placing on ice for 5-10 minutes, added 10µl of each ligation reaction to a sterile 15ml culture tube already on ice, transferred 100µl of competent cells into the 15ml tubes (containing ligation mix) on ice, gently flicked the tubes to mix and placed them on ice for 20 minutes. Heat shocked the cells for 90 seconds in a water bath at exactly 42°C. Immediately returned the tubes to ice for 2 minutes, added 600µl Super Optimal broth with Catabolite repression (SOC; Composition for 10ml: Tryptone-0.2 g, yeast exytract-0.05g, NaCl-0.005g, 1M KCl- 100µl, 2M $MgCl_2$-50µl, 1M glucose-200µl. $MgCl_2$ and glucose were added just before transformation) to the tubes containing cells transformed with ligation reactions and incubated for 2 hours at 37°C with shaking at 220-230 rpm. Plated 100 µl of each transformation culture onto Luria-Bertani (LB) plates in triplicates containing ampicillin (100µg ml^{-1}), isopropyl-β-d-thiogalactopyranoside (IPTG, 100mM), 5-bromo-4- chloro-3-indolyl β-d-galactoside (X-gal, 80µg ml^{-1}) and incubated the plates overnight at 37°C.

2.2.3.5. Colony PCR

The white colonies were selected and patched on ampicillin/IPTG/X-gal plates to reconfirm the transformation. All the individually streaked colonies were subjected for colony PCR using vector primers designed from either side of the multiple cloning site of the vector so that whatever be the product formed primer could amplify it from either side. The 25µl reaction mixture containing 2.5µl 10x buffer, 2.5µl dNTP (2.5mM), 1µl Taq polymerase (0.5U $µl^{-1}$), pinch of colony, 1µl of T7 and SP6 primers each and the mixture was made up to 25µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95°C for 5 minutes followed by holding at 80°C, 35 cycles of denaturation at 94°C for 15

seconds, annealing at 57°C for 20 seconds, extension at 72°C for 1 minute, followed by final extension at 72°C for 10 minutes. The PCR products (10µl) was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using Bio-Rad gel Documentation system (Biorad, USA).

2.2.3.6. Propagation of confirmed colony

After confirmation, the transformed *E.coli* JM10 containing cloned vector was propagated into 10ml LB ampicillin (100µg μl^{-1}) medium at 37°C at 230rpm.

2.2.3.7. Plasmid extraction and purification

The plasmid was extracted and purified using Genelute plasmid miniprep kit (Sigma). An aliquot of 5ml culture after overnight incubation was pelletised at 12,000 x g for 1 miunte. The pellet was resuspended in 200µl resuspension solution containing RNase A and lysed by adding 200µl lysis buffer. An aliquot of 350µl neutralization solution was added and centrifuged at 12,000 x g for 10 minutes to remove the cell debris. The lysate was loaded into GenElute HP Miniprep binding column which is inserted in a microcentrifuge tube and centifuged at 12,000 x g for 1 minute. Plasmid DNA bound to the column was washed twice to remove the endotoxins, salt and other contaminants. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100µl MilliQ water and centrifuged at 12,000 x g for 1 minute. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis, and by determining the ratio of the absorbance at 260nm to 280nm(260/280nm) in a UV-VIS spectrophotometre (Shimadzu).

2.2.3.8. Sequencing

The purified plasmid was sequenced in ABI 3700 sequencer at M/s Microsynth, Switzerland. The sequencing primers used were the T7 and SP6.

2.2.3.9. 16S rRNA gene sequence similarity and Phylogenetic analysis

The sequences obtained were matched with Genbank database using the BLAST algorithm (Altshul et al, 1990). The near complete 16S rRNA gene sequences of all the five isolates were multiple aligned using the ClustalW algorithm (Thompson et al., 1994).

Genetic distances were obtained by using Kimura's 2-parameter model (Kimura, 1980), and a phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) using the software MEGA4 (Tamura et al., 2007).

2.2.4. Antagonism to selected pathogenic vibrios in aquaculture

Antagonistic activity against pathogenic *Vibrio* spp. using the filter sterilized supernatants of five different cultures and the reference strain were carried out. The *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. Mediterranean* (BCCM-LMG 11258), *V. proteolyticus* (BCCM-LMG 3772), and *V. nereis* (BCCM-LMG 3895) were used and the antimicrobial effects were screened by disc diffusion test. 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 $Ab_{600,}$ and 500µl was swabbed on to ZoBell's marine agar plates. An aliquot of 1ml culture each of *Pseudomonas* spp. was removed from each flask, centrifuged at 10000 × g for 15 minutes and 20µl of the supernatant was spotted on to the 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 hour and zones of inhibition recorded using HI antibiotic scale (HiMedia Laboratories, India). The experiments were conducted in triplicates with all the five isolates.

2.3. Results

2.3.1. Phenotypic characterization

All five isolates of *Pseudomonas* along with the type strain were subjected for colony characterization, Gram staining and motility test. The Gram-negative rods, motile which produced characteristic pigments were subjected to biochemical tests such as oxidation-fermentation reaction, production of indole, arginine dihydrolyse, cytochrome C oxidase, catalase, fluorescent pigment, lecithinase, hydrolysis of glucose, citrate utilization, Sudan black test, and examined for growth at 4°C and 42°C (Table 1). All the five isolates showed uniform biochemical characteristics of

Pseudomonas aeruginosa. The results were compared with the type strain *Pseudomonas* MTCC741 to confirm its phenotypic identity. Each identified isolate was coded and stored in the microbial culture repository at the National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology, Kerala, India.

2.3.2. Molecular characterization

All the five isolates along with the type strain were subjected for molecular characterization through the sequence analysis of 16S rRNA gene. Good quantity (300-500µg/mL) and quality (260/280 ratio between 1.9- 2.0) of genomic DNA was obtained from all the isolates (Fig. 1). PCR amplification product of 1.5kb size was produced as shown in Fig. 2. The PCR products were cloned in to pGEMT vector and sequenced after the confirmation of the presence of the insert by colony PCR using T7 and SP6 vector primers, which produced a product size of 1.7kb (Fig. 3).

The 16S rRNA gene sequences of the isolates when matched with Genbank using the BLAST algorithm were 99% similar to *P. aeruginosa*. The sequences were submitted under the accession numbers EF062514 (MCCB102), EF053508 (MCCB103), EF062511 (MCCB117), EF062512 (MCCB118), and EF062513 (MCCB119). The phylogenetic tree based on the 16S rRNA gene sequences showed that the all the five environmental isolates formed a distinct cluster away from the clinical strain MTCC741 (Fig. 4).

2.3.3. Antagonism to selected pathogenic vibrios in aquaculture

Filter sterilized cell free supernatant of the isolates inhibited growth of *Vibrio* spp such as *V. harveyi* , *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. fluvialis, V. mediterranean, V. proteolyticus* and *V. nereis* with the inhibitory zones ranging from 12 to 18 mm when tested by disc diffusion assays (Table 2). The difference in the diameter of the zones of inhibition correlated with the quantity of pyocyanin produced by individual isolates.

2.4. Discussion

Although the bacterial isolates can be most definitively identified by 16S rRNA gene sequencing, phenotypic characterizations also contribute substantially to the determination of their coherence at generic and species level. It is very important to note that despite the satisfactory results of molecular characterization, minute errors in the sequencing or existence of cross over contamination might contribute extreme difficulty in distinguishing the organisms from related species. This makes the phenotypic characterization important in bacterial identification. Therefore, in the present study phenotypic characterizations were also given equal importance as16S rRNA gene sequence analyses in the identification of the isolates.

Among the phenotypic characters looked in to, the capability of aerobic pseudomonads to produce specific pigments is a very useful determinative character. However, synthesis of the two commonest types of pigments found in these bacteria (water-soluble fluorescent pigments and phenazine pigments) is highly dependent on the composition of the medium (King et al., 1954). In these experiments, among the two media (King A and King B) the former favoured the production of phenazine pigments and the latter the production of fluorescent pigment. These media were devised specifically for the differentiation of *Pseudomonas aeruginosa* from non-phenazine producing fluorescent pseudomonads (Stanier et al., 1966). In the present study all the isolates produced phenazine pigments in King A medium and fluorescent pigment in King B medium.

It is well known that pseudomonads are indole negative due to the lack of the mechanisms of tryptophan dissimilation. However, in this reaction there is a chance of mis-identification due to the cross reaction with pyocyanin which gives pink color up on addition of Ehrlich reagent to the culture. Moreover, there is no reliable biochemical evidence for the occurrence of tryptophanase among aerobic pseudomonads (Stanier et al., 1966). In the present study, all the isolates were indole negative

The characteristic feature of fluorescent pseudomonads is the anaerobic conversion of arginine to ornithine, ammonia and carbon dioxide through the action of arginine dihydrolase. This determines the taxonomic value of arginine dihydrolase production as a general character to distinguish aerobic pseudomonads from other Gram-negative eubacterial rods (e.g. coliform bacteria). All isolates used in this study were positive to arginine dihydrolase.

In the cytochrome oxidase test, broth cultures of all five isolates gave a positive reaction within 15 to 30 seconds. This is due to the high concentration of cytochrome oxidase associated with the cell membrane of *P. aeruginosa* (Gaby and Hadley, 1957). The ability to secrete extracellular hydrolytic enzymes (proteases and lecithinases) is common to *Pseudomonas aeruginosa.* In the present study the production of extracellular proteases was evidenced by the hydrolysis (halo zone) of lecithinase by lecithovitellin solution.

P. aeruginosa contains powerful catalase enzyme (Elkins et al., 1999; Gaby and Hadley, 1957) which was evidenced by nascent oxygen release when all the five isolates were reacted with hydrogen peroxide. Being strict aerobe, catalases are used to protect them from oxidative free radicals (Elkins et al., 1999).

Another character used for distinguishing *Pseudomonas aeruginosa* from other pseudomonads is the thermal death point. Under this assay ability of the isolates to grow at 42ºC and their inability to grow at 4ºC are examined. All the five isolates grew abundantly at 42ºC and none at 4ºC.

In the present study, based on the biochemical characterization all the five isolates from various ecological niches could be identified as *Pseudomonas* and based on 16S rRNA gene sequence, 99% homology could be established to *Pseudomonas aeruginosa*. The recent advances in genome sequencing of bacteria in general and of pseudomonads in particular had shed light into their taxonomy, adaptability and metabolic versatility. At present, there are 18 complete *Pseudomonas* genomes listed in NCBI's Entrez database, with another 72 listed as

being draft assemblies or incomplete. Of these 90, 15 are *P. aeruginosa* isolates and 38 are pathovars of *P. syringae* (Silby et al., 2011).

Antagonistic property of *Pseudomonas aeruginosa* to various pathogenic microorganisms have been extensively studied and is applied 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 (Chythanya et al., 2002) and also as bioaugmentors in bioremediation programmes (De Meyer et al., 1999; Chaerun et al., 2004; Hasanuzzaman et al., 2004; Tang et al., 2007). Pyocyanin, the blue-green chloroform-soluble phenazine pigment from *Pseudomonas aeruginosa* has been identified as a broad-spectrum antibiotic (Preetha et al., 2010, Arunkumar et al., 1997), anti fungal (Costa and Cusmana, 1975; Kerr et al., 1999), and antiprotozoal agent (Dive, 1973). The antagonism of *P. aeruginosa* against pathogenic vibrios in aquaculture (Pai et al., 2010; Vijayan et al., 2006) has been identified due to its metabolite, pyocyanin (Preetha et al., 2010). Moreover *P.aeruginosa* has been applied in aquaculture as effective probiotics and as an alternative to antibiotics against pathogenic *V.harveyi* (Preetha et al., 2010; Hai et al., 2009a, b; Pai et al., 2010). The isolates investigated here stand out as potent antagonists of *Vibrio* spp. such as *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. fluvialis, V. mediterranean, V. proteolyticus* and *V.harveyi* and thus it can be used for aquaculture applications as probiotics especially against *V.harveyi*.

On the basis of these evidences it is postulated that all the five isolates from various ecological niches belongs to the species *Pseudomonas aeruginosa* and all are antagonistic to selected pathogenic vibrios in aquaculture.

Table 1 Biochemical properties of *Pseudomonas* isolates.

Properties	Test strain - MCCB102, MCCB103 MCCB117, MCCB118, MCCB119	Reference strain -MTCC741
Colony Morphology	Small, oval, translucent, green pigmented colony	Small, oval, translucent, green pigmented colony
Gram staining	Gram negative rods	Gram negative rods
Motility	Motile	Motile
MOF/ OF Test	Oxidative	Oxidative
TSI test	Alkaline with H_2Sproduction	Alkaline with H_2Sproduction
Catalase test	Positive	Positive
Citrate test	Positive	Positive
Kovac's oxidase test	Positive	Positive
Indole test	Negative	Negative
Sudan black test	Negative	Negative
Fluorescent pigment production	Positive	Positive
Arginine dihydrolase test	Positive	Positive
Gelatin production	Positive	Positive
Lecithinase production	Positive	Positive
Growth at 4°C	Negative	Negative
Growth at 42°C	Positive	Positive

Table 2 Antagonistic activity of supernatant from the isolates against various pathogenic *Vibrio spp.*

***Vibrio* spp**	**Diameter of Zone (in mm)**					
	MCCB102	**MCCB103**	**MCCB117**	**MCCB118**	**MCCB119**	**MTCC741**
V.alginolyticus	16 ± 1.41	15 ± 1.41	16 ± 0.71	13.5 ± 0.71	14.5 ± 0.71	13 ± 1.41
V.fluvialis	18.5 ± 2.12	17.5 ± 2.12	17.5 ± 0.71	15.5 ± 0.71	15 ± 0	16.5 ± 0.71
V.mediterranei	18 ± 0	14.5 ± 0.71	16 ± 0.71	14 ± 1.41	14.5 ± 0.71	13.5 ± 0.71
V.nereis	15.5 ± 0.71	15 ± 1.41	15.5 ± 0.71	14 ± 0	13.5 ± 0.71	13 ± 1.41
V.parahaemolyticus	15.5 ± 0.71	14 ± 0.00	15.5 ± 0.71	13.5 ± 0.71	13.5 ± 2.12	13.5 ± 0.71
V.proteolyticus	13.5 ± 0.71	13 ± 1.41	13.5 ± 0.71	1.41 ± 0	12.5 ± 0.71	11.5 ± 0.71
V.vulnificus	31 ± 1.41	29.5 ± 0.71	31 ± 1.41	28.5 ± 0.71	29.5 ± 0.71	28.5 ± 0.71
V.harveyi	17.5 ± 0.71	14.5 ± 0.71	17 ± 0	13.5 ± 0.71	14.5 ± 0.71	13.5 ± 0.71

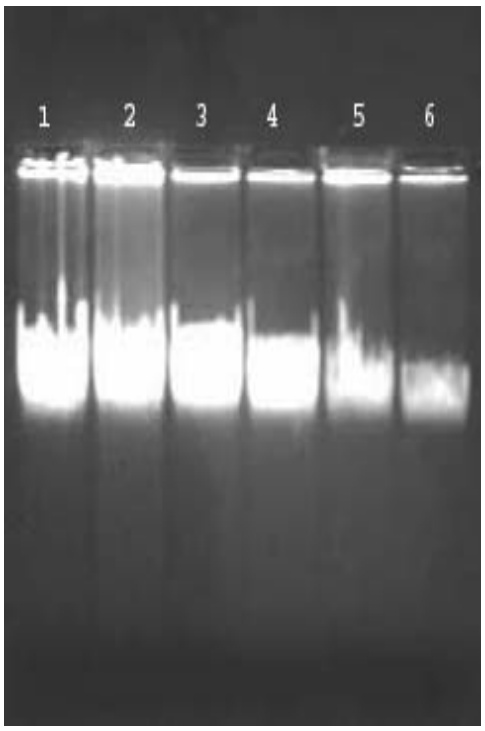

Fig. 1 DNA extracted from *Pseudomonas* isolates 1). MCCB102, 2). MCCB103, 3. MCCB 117, 4).MCCB118, 5).MCCB119, 6) .MTCC 741.

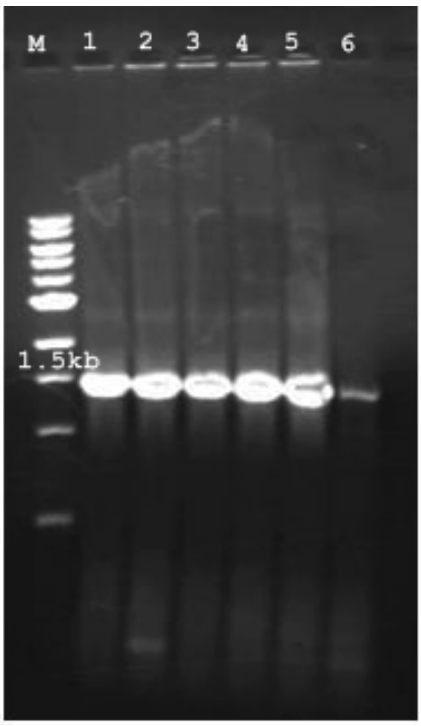

Fig. 2 PCR Amplified 16S rRNA gene of *Pseudomonas* isolates 1). MCCB102, 2). MCCB103, 3). MCCB117, 4). MCCB118, 5). MCCB119, 6). MTCC741.

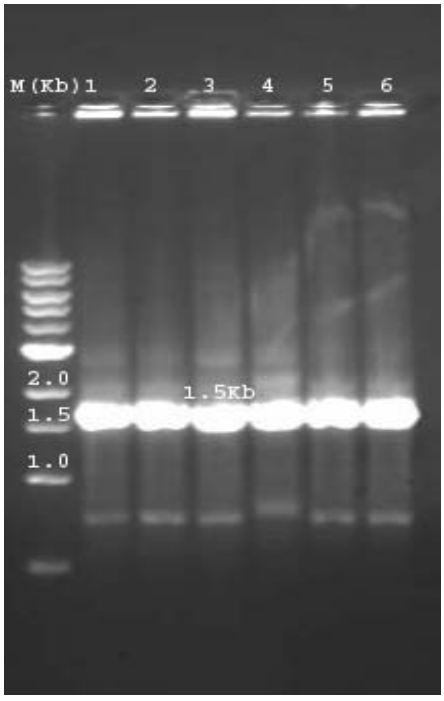

Fig. 3 Colony PCR of the clones carrying 1.5kb insert of 16S rRNA gene from. 1) MCCB102 2) MCCB103 3) MCCB117 4) MCCB118 5) MCCB119 6) MTCC741

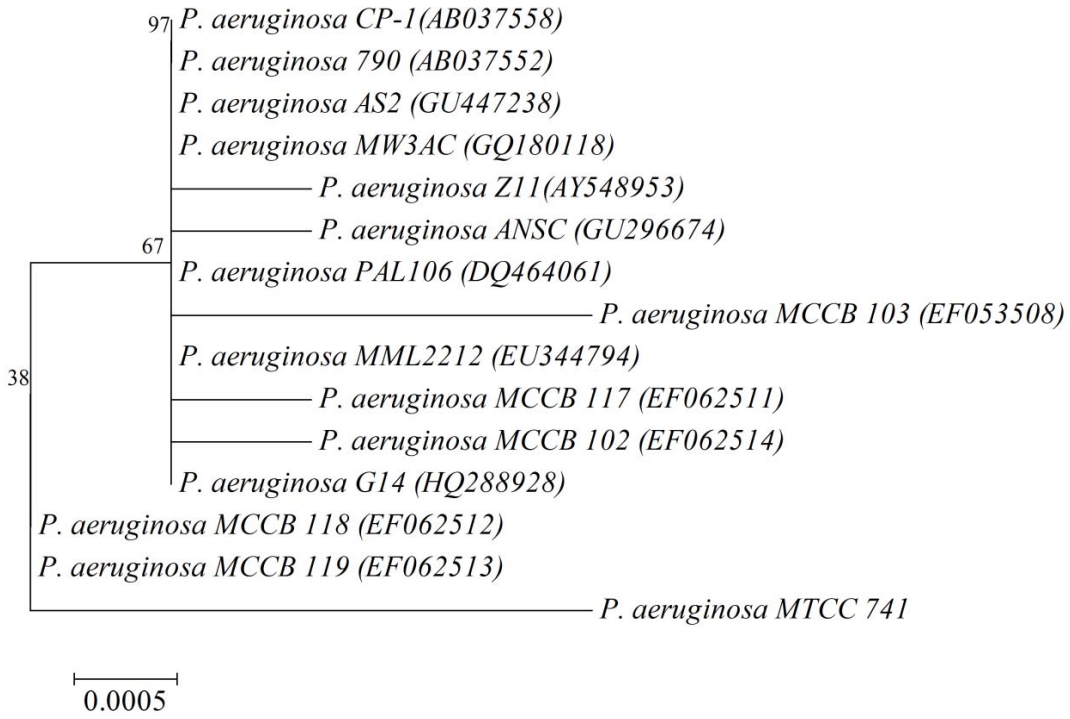

Fig.4 Phylogenetic tree based on 16S rRNA gene sequences of different isolates of *Pseudomonas aeruginosa* compared with sequences available in the GenBank. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates).

Chapter 3

SALINE DEPENDENT PRODUCTION OF PYOCYANIN IN *PSEUDOMONAS AERUGINOSA* ORIGINATED FROM DIFFERENT ECOLOGICAL NICHES AND THEIR SELECTIVE APPLICATION IN AQUACULTURE

Contents

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;

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 *Vibryo 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), anti fungal (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 (Ab_{600})

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 5-10g l^{-1}. Modified ZoBell's marine broth (0.5% peptone, 0.1% yeast extract) prepared with 5g l^{-1} salinity seawater served as the control. The growth, pyocyanin production and antagonism of *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 *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 70g 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 40g l^{-1}, the brackish water isolates (MCCB102, MCCB103) ceased to produce pyocyanin with salinities above 30g l^{-1}. The freshwater isolate (MCCB119) did not produce pyocyanin with salinities above 20g l^{-1}. Maximum pyocyanin production of marine and brackish water isolates occurred at a salinity of 10g l^{-1}, however, with the freshwater isolate and reference strain at 5g 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 (25.3mg l^{-1}) of pyocyanin followed by the marine isolate MCCB117 (21.8mg 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™, 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 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 *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 *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. More over this is the first report on the application of *P. aeruginosa* isolated from marine environment of 200m (MCCB118) 500m (MCCB117) depth.

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

Salinity (g l^{-1})	Diameter of Zone (in mm)					
	MCCB102	MCCB103	MCCB117	MCCB118	MCCB119	MTCC741
0	13.5±0.70	14±0.00	15.5±0.70	12±1.41	10±0.0	11±1.41
5	16±0.70	16±0.70	17±0.00	14±1.41	12.5±0.70	12±1.41
10	18±1.41	17±0.00	17±0.00	13.5±0.70	11.5±2.12	11±1.41
15	17±1.41	16±0.70	16.5±0.70	13.5±0.70	10.5±0.70	11.5±0.70
20	16.5±2.12	15±0.00	16.5±0.70	13±0.00	-	10.5±0.70
25	15.5±0.70	13±0.70	15.5±0.70	12.5±0.7	-	-
30	12.5±0.70	12±0.70	15±1.41	12±0.0	-	-
35	-	-	15±1.41	11±0.0	-	-
40	-	-	12.5±0.70	10.5±0.70	-	-
45	-	-	10.5±0.70	-	-	-

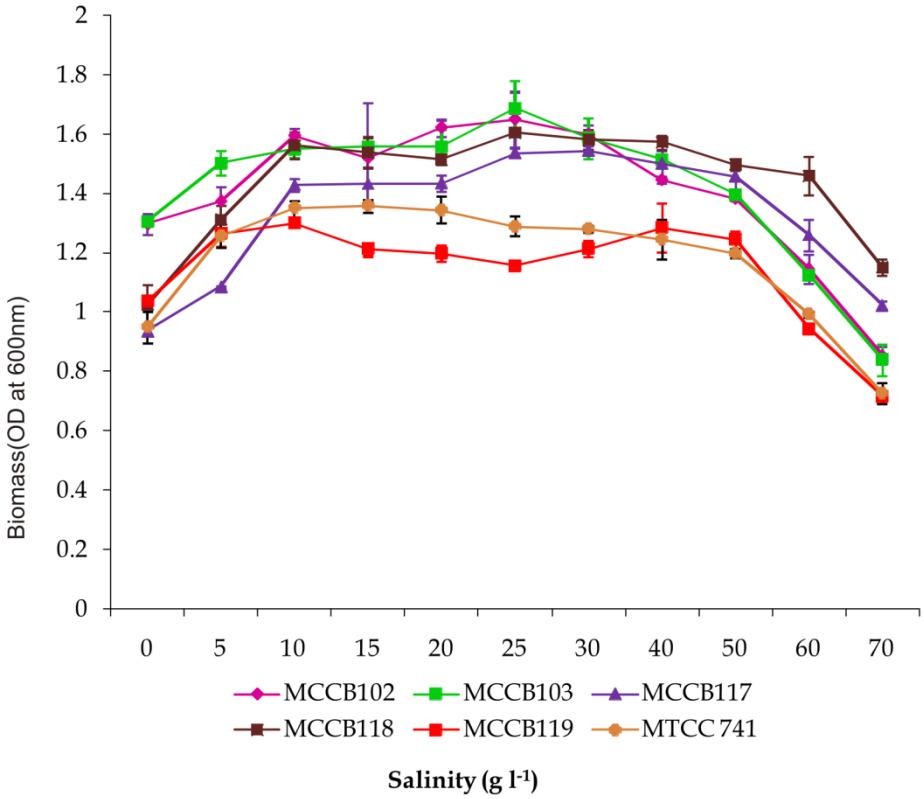

Fig.1 Growth of *Pseudomonas* isolates at different salinities.

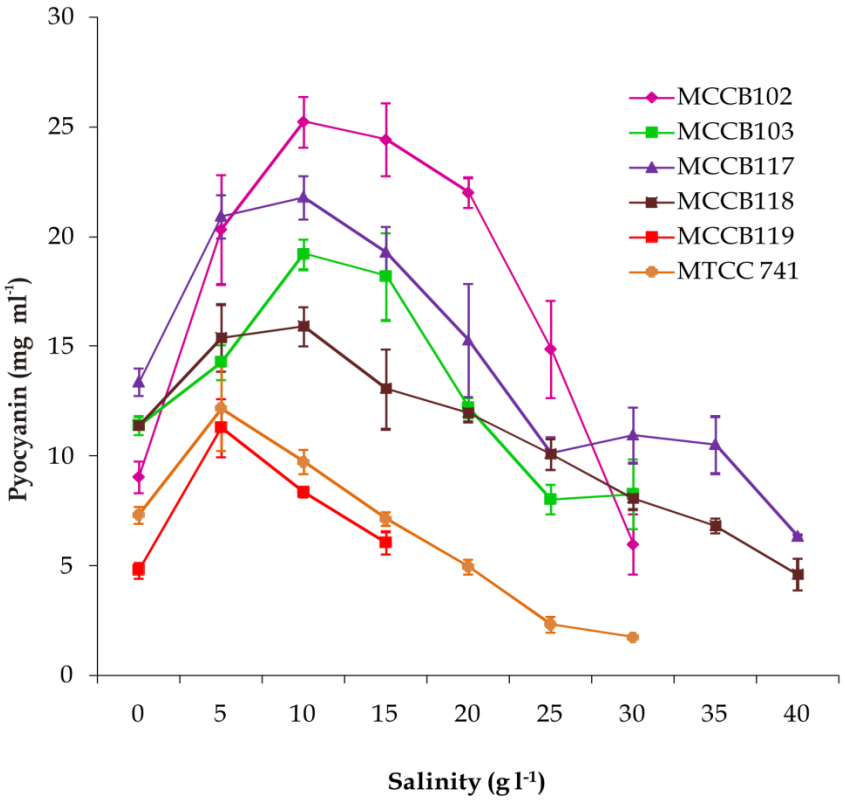

Fig.2 Pyocyanin production by *Pseudomonas* isolates at different salinities.

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.

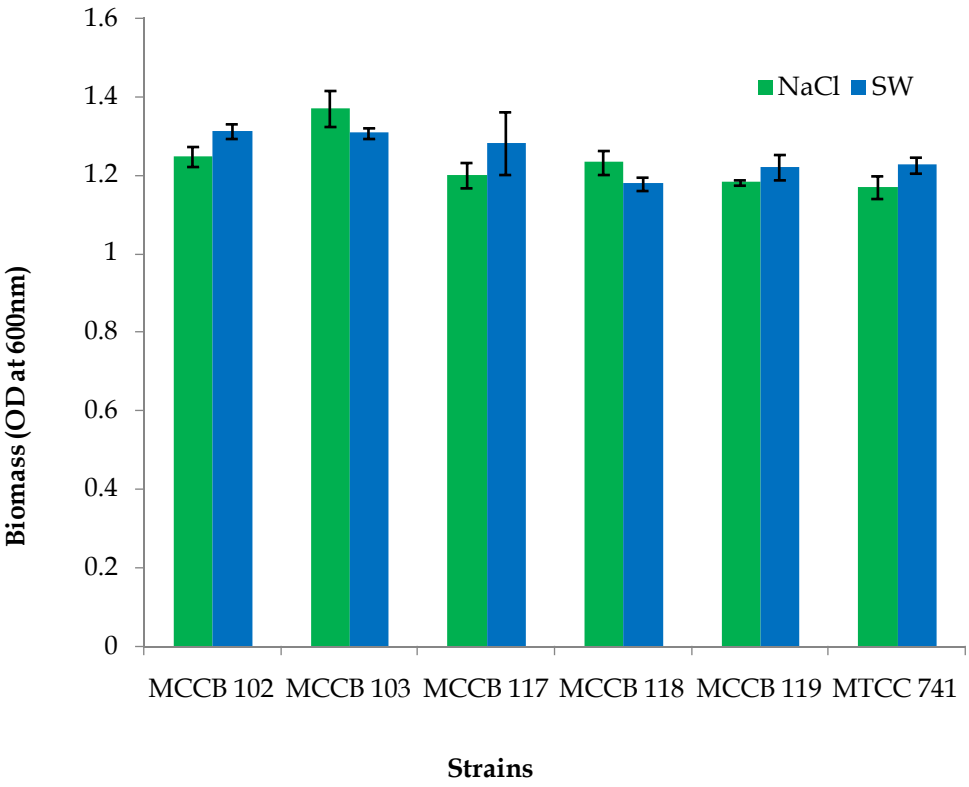

Fig. 4 Growth of *Pseudomonas* isolates in seawater based/NaCl supplemented growth media.

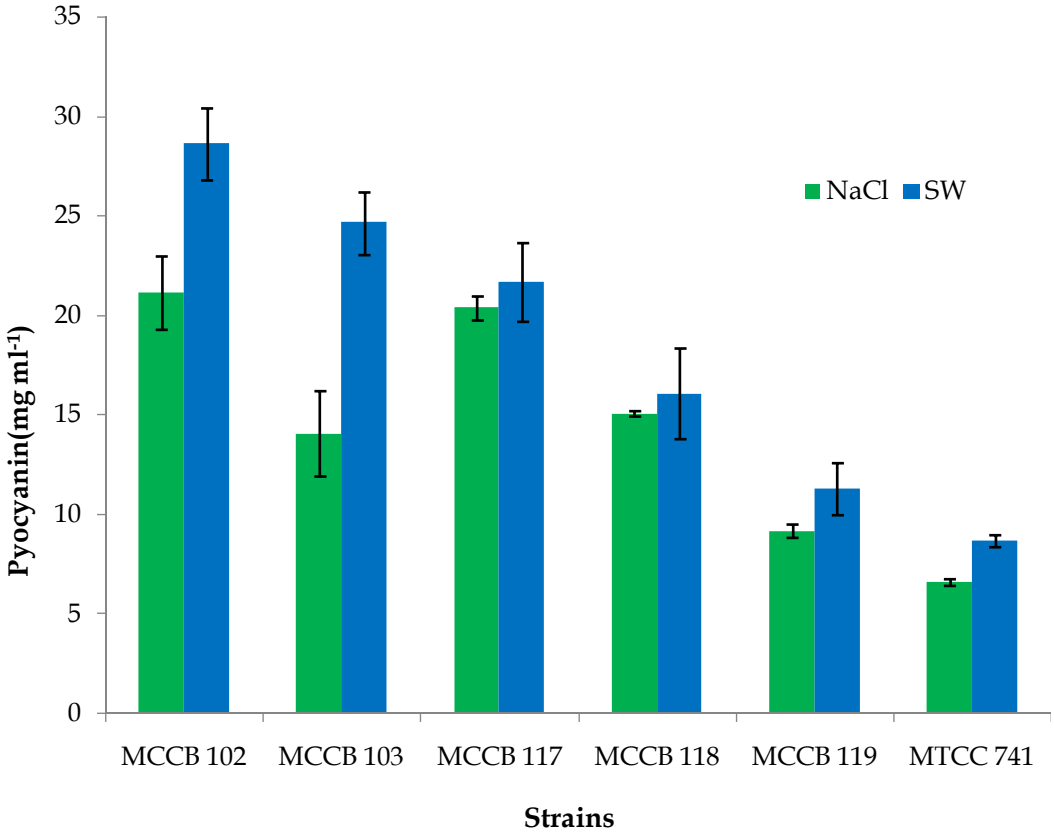

Fig. 5 Pyocyanin production by *Pseudomonas* isolates in seawater based/NaCl supplemented growth media.

Chapter 4

CLONING AND OVEREXPRESSION OF *Phz* GENES ENCODING PHENAZINE BIOSYNTHETIC PATHWAY FOR THE ENHANCED PRODUCTION OF PYOCYANIN IN *PSEUDOMONAS AERUGINOSA* MCCB117

Contents

4.1. Introduction

Phenazines are heterocyclic nitrogen containing metabolites with antibiotic, antitumor and antiparasitic activity; synthesized by fluorescent *Pseudomonas* and some other bacterial genera. In microorganisms, the phenazine biosynthetic pathway branches off from the shikimic acid pathway (Fig. 1) which is also the source for metabolites such as the aromatic amino acids, siderophores and quinines (Chin et al., 2001).

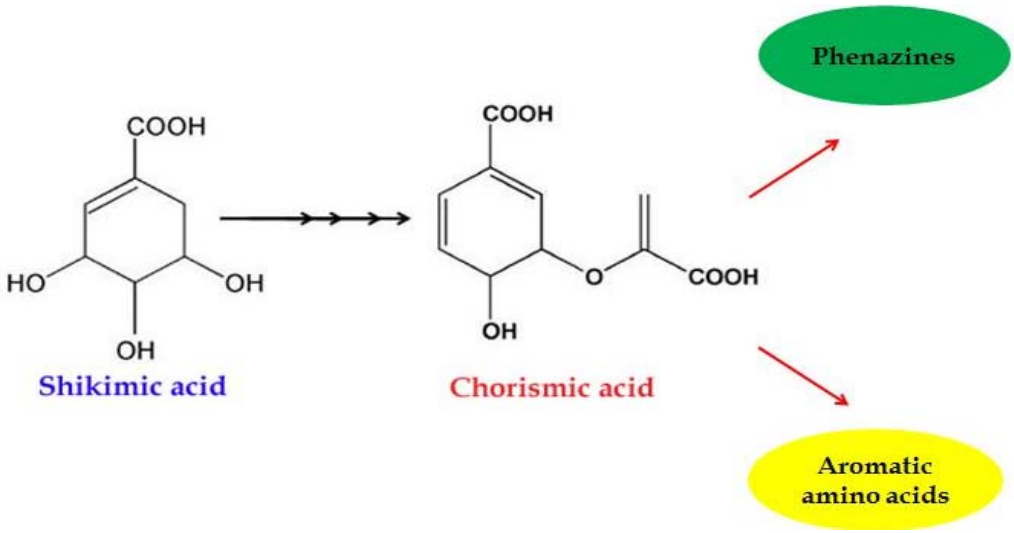

Fig.1 Shikimate to chorismate then to phenazines and aromatic amino acids.

Genes encoding the phenazine biosynthetic pathway are arranged in one operon, *phzABCDEFG* (Fig. 2), in all pseudomonads except the species *P. aeruginosa* which possesses two copies of these "core" biosynthetic genes responsible for the synthesis of phenazine-1-carboxilic acid (PCA) from chorismic acid (fig. 3).

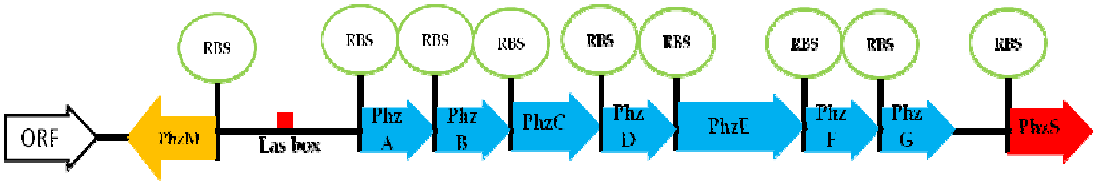

Fig. 2 Pyocyanin biosynthesis genes loci.

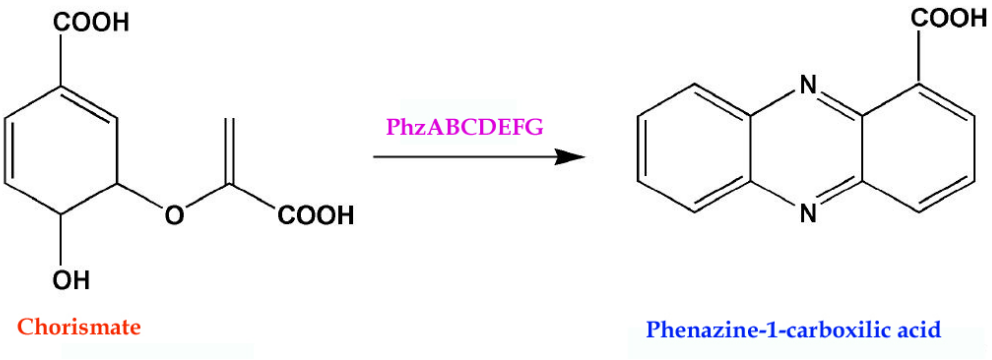

Fig. 3 Chorismate to phenazine-1- carboxylic acid by *phzABCDEFG* operon.

The conversion of PCA to pyocyanin is mediated by two novel phenazine-modifying genes, *phzM* and *phzS* (fig. 4).

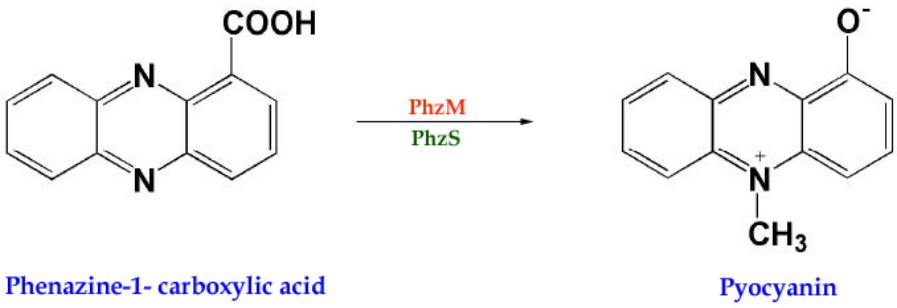

Fig.4 phenazine-1- carboxylic acid to pyocyanin.

In *P. aeruginosa* PA01 genome (Fig. 5a), *phzABCDEFG* operon spans positions 4,713,795 to 4,720,062 (6267 base pairs (bp)) and the *PhzM* spans positions 4,713,098 to 4,712,094 (1004bp) and are transcribed divergently and

separated by 695bp, (Fig. 5). The *PhzS* gene is located 236bp downstream from *PhzG1* and spans positions 4,720,300 to 4,721,508 (1208bp) (Mavrodi et al., 2001).

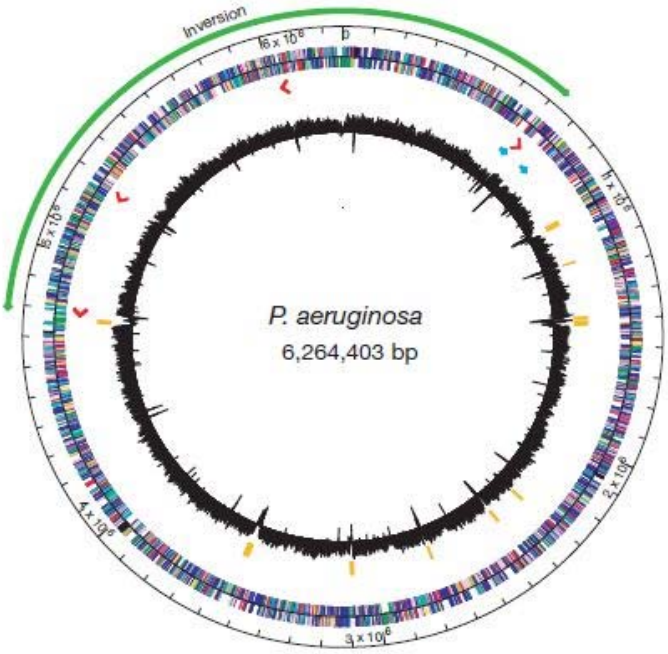

Fig.5a Circular representation of the *P. aeruginosa* PA01 genome (Stover et al., 2000)

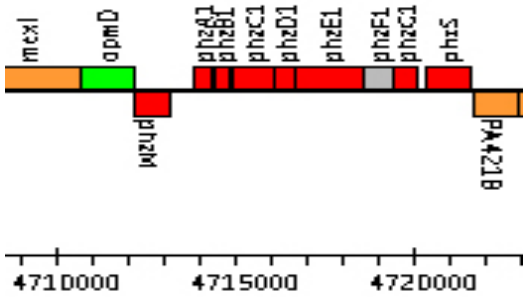

Fig.5b Position of pyocyanin biosynthesis genes in *P. aeruginosa* PA01complete genome (www.pseudomonas.com).

As the first step in pyocyanin biosynthetic pathway, *phzE* gene involves amination of chorismic acid to 2-amino-2-deoxyisochorismic acid (ADIC) through aminodeoxy isochorismate (ADIC) synthase enzyme (Fig. 6).

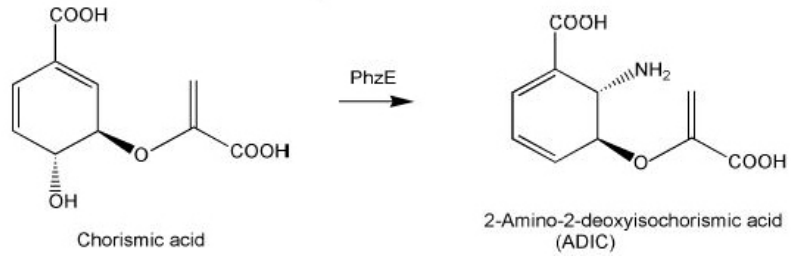

Fig. 6 Biosynthesis of ADIC from Chorismate by *PhzE* gene.

ADIC formed is then converted by *phzD*, an isochorismatase to trans-2,3-dihydro-3-hydroanthranilic acid (DHHA) (Fig. 7).

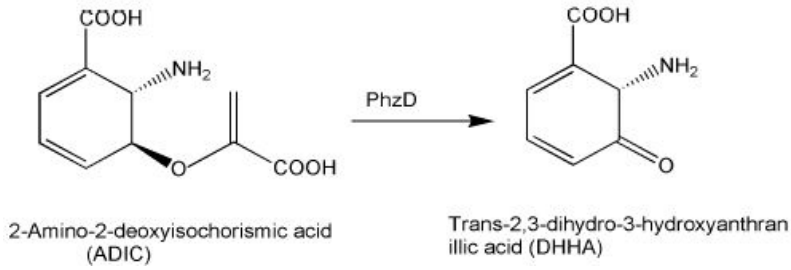

Fig.7 Conversion of ADIC to DHHA by *PhzD* gene.

The products of *phzF* and *phzG* synthesize the first phenazine ring system, phenazine-1, 6-dicarboxilic acid (Leisinger and Margraff, 1979) by the condensation of two identical DHHA molecules, from which PCA is refined by *phzA* and *phzB* genes (fig. 8). The *phzC* gene expresses late in this pathway to facilitate the common carbon metabolites to the shikimate pathway, from which the phenazine biosynthesis branches off.

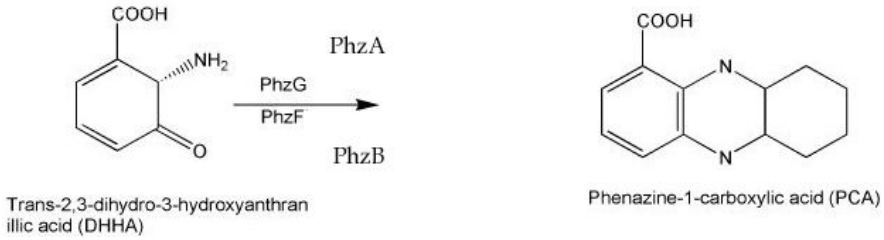

Fig.8 Biosynthesis of Phenazine-1, 6-dicarboxilic acid by *PhzG,F,A* and *B* genes.

The two novel phenazine-modifying genes, *phzM* and *phzS* that convert PCA to pyocyanin encode the putative phenazine-specific methyltransferase and flavin containing monooxygenase enzymes, respectively (Mavrodi et al., 1998, 2001). PCA is first acted upon by the enzyme *PhzM*, a methyltransferase, and gets converted to 5-

methylphenazine-1-carboxylic acid betaine by transfer of a methyl group. This is followed by the action of the enzyme *PhzS*, a FAD-dependent monooxygenase, which involves the hydroxylative decarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin. Pyocyanin can exist in either oxidized or reduced form, the later being an unstable form of pyocyanin that reacts rapidly with molecular oxygen (Fig. 9).

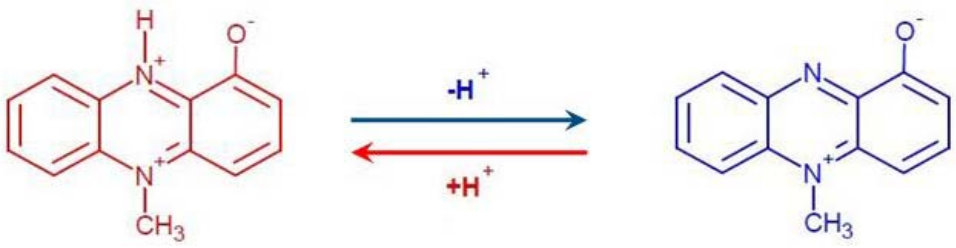

Fig. 9 Pyocyanin structure at two different protonation states.

Enhanced biosynthesis of pyocyanin in pseudomonads through modified growth media has been well explained (Frank and DeMoss, 1959; King et al., 1954) Ingledew and Campbell, 1969; Burton et al., 1947; Halpern et al., 1962) and the multi factorial statistical screening of media components was illustrated (Preetha et al., 2010). Moreover, Ingledew and Campbell (1969) concluded that the phosphate deficiency triggered pyocyanin biosynthesis. However, the overexpression of gene (s) encoding pyocyanin biosynthesis by increasing its copy number has not yet been performed in *P. aeruginosa*.

Considering the importance of pyocyanin and the existing low production potential of wild strain prompted us to undertake the present study to overexpress the genes encoding pyocyanin biosynthesis (approximately 10kb) in a wild strain *Pseudomonas aeruginosa* MCCB117 and development of genetically modified *Pseudomonas aeruginosa* -PA-pUCP-Phz^{++}.

4.2. Material and methods

4.2.1. Extraction of genomic DNA from *Pseudomonas aeruginosa* MCCB117

Genomic DNA extraction was carried out following the method of Lee et al. (2003) with some modifications. An aliquot of 2ml cell suspension was taken, centrifuged at 8000rpm, at 4°C for 10 minutes, discarded the supernatant and

resuspended the pellet is in 1ml TE buffer (10mM Tris-Cl (pH 8.0), 1mM EDTA), mixed thoroughly, centrifuged, discarded supernatant and harvested the cells. The above step was repeated. Resuspended the cells in 1mL lysis buffer (0.05M Tris –Cl (pH 8.0), 0.1M NaCl, 0.05M EDTA, 2% SDS, 0.2 % PVP, 0.1% β mercapto ethanol) and 10µl proteinase K (20mg ml^{-1}) was added and incubated at 37°C for 1 hour and then at 55°C for 2 hours. Subsequently, added 1 volume phenol : chloroform : isoamyl alcohol (25:24:1 v/v) and the tubes were placed on a flat rocking platform and mixed thoroughly and kept for 5 minutes. Phases were separated by centrifugation at 15,000rpm for 15 minutes. Transferred the upper aqueous layer to a new tube and added equal volumes of chloroform : isoamyl alcohol (24:1 v/v). Centrifuged at 15,000rpm for 15 minutes, repeated the step twice. Transferred the aqueous layer to a new 1.5ml MCT and added 0.1 volume sodium acetate (3M, pH 5.2) and 1 volume ice cold absolute ethanol. DNA was precipitated by keeping at -20°C overnight, recovered by centrifugation at 15,000rpm for 15 minutes, washed the DNA pellet by adding 200µl ice cold 70% ethanol, centrifuged and discarded the supernatant. Repeated the step twice. Dried the pellet in air, and suspended finally in 50-100µl of Milli-Q water.

4.2.2 Primers designed for the amplification of pyocyanin biosynthetic genes

Primers for the amplification of genes encoding pyocyanin biosynthetic pathway were designed using Gene tool software. The pyocyanin gene sequences were taken from the whole genome sequence of *Pseudomonas aerugenosa* (www.pseudomonas.com). The primers were designed to amplify the complete genes involved in pyocyanin biosynthetic pathway. For this, the forward primer NP89F: 5'TTCGTCAACGTCATCGCGCTCTACAAG3' was designed 100bp upstream to *PhzM* gene (including the partial sequence of *OPMD* gene in the same loci) and the reverse primer NP89R: 5'TGGCCTGCCCGGTGTTGCTAGCGTGG 3' was designed 30bp downstream to *PhzS* gene from the gene loci with a product size >10kb. In addition, NP139F: 5'CGAAACCGCCG GGGCCAAC3' and NP139R: 5'GCGCTTGGGTACTTCTCGGGTT3' were designed for the amplification of *PhzM* gene and NP62F: 5'GCGGTAAACGGTCATCCATCCC3' and NP62R:

5'GGTGGCCTGCCCGGTGTTGCTA3' were designed for the amplification of *PhzS* gene for the confirmation of insert gene in the vector.

4.2.3. Amplification of pyocyanin biosynthetic genes using long PCR from *Pseudomonas* MCCB117

For the amplification of genes encoding pyocyanin biosynthetic pathway, long PCR was carried out using Finnzyme- Phusion High-Fidelity PCR kit with slight modifications. The 20µl reaction mixture consists of 4µl Phusion HF buffer (5x), 0.4µl dNTP (200µM each), 0.2µl phusion DNA polymerase (0.02U μl^{-1}), 1.5µl template DNA (75ng), 1µl (10pmol μl^{-1}) of each forward and reverse primer (NP89F and NP89R) and the mixture was made up to 20µl with MilliQ. The gradient PCR programme used for the amplification of the complete genes was programmed for 98°C for 1 minute, followed by 25 cycles of 98°C for 5 seconds, annealing at 53°C for 15 seconds, extension at 72°C for 5 minutes, followed by final extension at 72°C for 10 minutes. An aliquot of 10µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using Bio-Rad gel documentation system.

4.2.4. Plasmid used for cloning the pyocyanin gene

Pseudomonas specific vector pUCP24 (Schweizer, 1991; West et al., 1994) was used for cloning the pyocyanin gene which was kindly supplied by Dr. Herbert Schweizer, Colorodo University USA. The vector in the host *E.coli*-DH5α has resistance for gentamycin for which *Pseudomonas* is sensitive (Fig. 10).

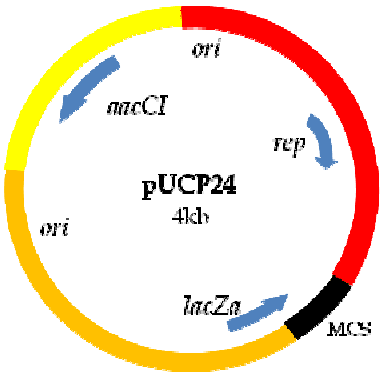

Fig.10 pUCP24 vector map.

4.2.5. Extraction and purification of pUCP24 plasmid

E.coli-DH5α with pUCP24 plasmid was inoculated into 10ml LB broth containing 15µg ml^{-1} gentamycin and incubated at 37°C with shaking at 220rpm. Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences). An aliquot of 2ml culture after overnight incubation was pelletised at 12,000 x g for 1 minute. The pellet was resuspended in 200µl resuspension solution containing RNase A and lysed by adding 200µl lysis buffer. An aliquot of 350µl neutralization solution was added and centrifuged at 12,000 x g for 10 minutes to remove the cell debris. Lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centifuged at 12,000 x g for 1 minute. Plasmid DNA bound to the column was washed twice with wash solution to remove the endotoxins, salt and other contaminants. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100µl 10mM Tris-Cl and centrifuged at 12,000 x g for 1 minute and stored at -20ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance reading at 260nm/280nm in a UV-VIS spectrophotometer (Shimadzu,Japan).

4.2.6. Restriction digestion of pUCP24 and treatment with Calf Intestinal Phosphatase (CIP)

The purified pUCP24 plasmid was restriction digested with *SmaI* (New England Biolabs) enzyme to linearize the plasmid with blunt-ends for T-tailing. An aliquot of 20µl reaction mixture containing 5µl plasmid, 0.5µl enzyme (*SmaI*-20,000U ml^{-1}) 2µl reaction buffer and 12.5µl MilliQ water was incubated for 1 hour at 25°C followed by heat inactivation at 65ºC for 20 minutes. The restriction digestion (linearization) was confirmed by 1% agarose gel electrophoresis. Restriction digested plasmid was CIP (Calf Intestinal Phosphatase) treated to remove the phosphate groups to prevent self ligation. The reaction mixture containing 20µl plasmid, 0.1µl CIP enzyme and 5µl buffer was incubated at 37ºC for 1 hour followed by heat inactivation at 65ºC for 20 minutes.

4.2.7. Gel purification of linearised and CIP treated pUCP24 plasmid

After CIP treatment the plasmid was run on 1% agarose gel and the resulting band was excised and purified using Promega Wizard SV Gel and PCR clean up system. To the excised gel in a 1.5ml microcentrifuge tube, added 10µl membrane binding solution per 10mg of gel slice, vortexed and incubated at 50-65°C until gel slice is completely dissolved. Transferred the dissolved gel mixture to the binding column provided and incubated at room temperature for 1 minute, centrifuged at 16,000g for 1 minute and discarded the flow-through. Added 700µl membrane wash solution to the column, centrifuged at 16,000*g* for 1 minute and discarded the flow-through. This step was repeated with 500µl membrane wash solution and centrifuged for 5 minutes followed by 1 minute centrifugation after discarding the wash solution to ensure the complete removal of ethanol from the column. To elute the linearised and CIP treated pUCP24 plasmid, the column was transferred to a fresh collection tube, added 50µl of Nuclease-Free MilliQ water, incubated at room temperature for 1 minute, centrifuged at 16,000g for 1 minute and stored at -20°C. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance reading at 260nm/280nm in a UV-VIS spectrophotometre (Shimadzu).

4.2.8. T-Tailing of purified plasmid (pUCP24) and A-Tailing of the amplified PCR product (pyocyanin biosynthetic pathway genes)

Gel purified pUCP-24 plasmid (linearized and CIP treated) was T-tailed to ligate with the A-tailed PCR product. An aliquot of 50µl purified plasmid was subjected to T-tailing with 1µl dTTP (final conc. 0.2mM), 1µl Taq polymerase (1 unit) and 5µl 10X buffer. The reaction was carried at 72°C for 2 hours. The T-tailed plasmid was run on 1% agarose gel, excised the band and gel purified using Promega Wizard SV Gel and PCR clean up system kit. The purified product (5µl) was run on 1% agarose gel electrophoresis for the confirmation of retrieved product and stored at -20°C till use. The amplified PCR product (pyocyanin biosynthetic pathway genes) was A-tailed to ligate with the T-tailed vector. Two µl of PCR product was subjected to A-tailing with 10µl reaction mixture containing 1µl Taq polymerase (5

units), 5µl dATP (0.2mM), 1µl 10X buffer and 1µl MilliQ. The reaction was carried at 70°C for 30 minutes.

4.2.9. Ligation of A-tailed pyocyanin biosynthetic pathway genes with the T-tailed pUCP24 vector

The T–tailed and purified pUCP24 plasmid was ligated with the A-tailed PCR amplified pyocyanin biosynthetic pathway gene. The 10µl ligation mixture containing 2µl T–tailed PUCP24, 3µl A-tailed PCR product, 1µl 10X buffer, 0.5µl T_4DNA Ligase enzyme and 3.5µl MilliQ water was incubated at 16°C, overnight.

4.2.10. Transformation of pUCP24 vector into *E.coli* DH5α by heat shock method

To the 10µl ligation mixture in a 5ml ice cold screw cap tube, added 50µl competent *E.coli* DH5α cells, mixed gently and incubated on ice for 20 minutes. Heat shock was given for 90 seconds at exactly 42°C. The tube was returned to ice for 2 minutes. Added 600µl Super Optimal broth with Catabolite repression (SOC; Composition for 10ml: Tryptone-0.2 g; yeast exytract-0.05g; NaCl-0.005g, 1M KCl-100µl; 2M $MgCl_2$-50µl; 1M glucose-200µl. $MgCl_2$ and glucose were added just before transformation) and incubated for 2 hours at 37°C with shaking at 230rpm. After incubation, 200µl each was plated onto LB gentamycin (15µg ml^{-1}) plate containing IPTG and X-gal (LB medium-2g $100ml^{-1}$; agar- 2g $100ml^{-1}$) and incubated for 24 hours at 37°C. Individual colonies developed (white) were inoculated into 100ml LB gentamycin broth and incubated at 37°C with shaking for plasmid extraction.

4.2.11. Plasmid extraction (Midi preparation)

The plasmid was extracted and purified using AxyPrep plasmid midiprep kit (Axygen). An aliqot of 50ml of overnight LB culture was centrifuged at 3,000g for 8 minutes to pellet the *E.coli* DH5α cells. The pellet was then resuspended in 4.5ml resuspension solution containing RNase A and lysed by adding 4.5ml lysis buffer and mixed gently. An aliquot of 4.5ml pre-chilled neutralization buffer was then added, mixed gently and incubated at room temperature for 5 minutes followed by the addition

of 4.5ml of pre-chilled DNA binding buffer, mixed gently and centrifuged at 6,000g at 4°C for 10 minutes. Lysate was loaded into Midiprep syringe filter fixed on vacuum connected Sigma Vacuum Manifold. The plunger was carefully inserted into the syringe filter and pushed slowly with a steady motion to discharge the filtrate into Midiprep plasmid column and switched on the vacuum source till the complete lysate passed through the column. Added 7ml of first wash buffer and then 8ml desalting buffer and drew the solution through the Midiprep plasmid column by vacuum. Column was removed from the assembly and placed in to a 1.5ml microcentrifuge tube, added 300µl desalting buffer and centrifuged at 12,000g for 2 minutes. To elute the plasmid DNA, the column was transferred to another fresh collection tube, added 300µl eluent (2.5mM Tris-HCl, pH 8.5) to the centre of the membrane, incubated at room temperature for 1 minute, centrifuged at 12,000g for 1 minute and stored at -20ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance at 260nm/280nm in a UV-VIS spectrophotometre (Shimadzu,Japan).

4.2.12. Restriction digestion.

The extracted plasmid was restriction digested with *Xba1* (New England Biolabs,USA) enzyme to confirm the plasmid size. An aliquot of 20µl reaction mixture containing 5µl plasmid, 0.5µl enzyme (20,000U ml^{-1}), 2µl reaction buffer (10X) and 12.5µl MilliQ water was incubated at 37 °C for 1 hour followed by heat inactivation at 65ºC for 20 minutes. The restriction digestion (linearization) was confirmed by 1% agarose gel electrophoresis.

4.2.13. Confirmation of insert orientation.

PCR amplification of the extracted plasmid using the forward primer (62F) of the *PhzS* gene (end gene of pyocyanin biosynthetic pathway genes) and the M13 reverse primer of the pUCP24 vector was performed to confirm the correct orientation of the insert. The 25µl reaction mixture containing 2.5µl 10x buffer, 2.5µl dNTP (2.5mM), 1µl Taq polymerase (0.5U μl^{-1}), 0.2µl plasmid DNA (75ng), 1µl of each M13 (Reverse) and 62F (forward) primer (10pmol μl^{-1}) and the mixture was made up to 25µl with Milli Q. The hot start PCR programme used for the

amplification was 95°C for 5 minutes followed by hold at 80°C, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 2 minutes, followed by final extension at 72°C for 10 minutes. Ten µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using Bio-Rad gel Documentation system.

4.2.14. Transformation of pUCP24 vector encoding pyocyanin biosynthetic gene(s) into wild strain *P. aeruginosa* (MCCB117) to get genetically modified PA-pUCP-Phz^{++}

The purified pUCP24 vector with pyocyanin biosynthetic gene insert was transformed into wild strain of *Pseudomonas aeruginosa* MCCB117 for generating multiple copies of the vector and overexpression of the pyocyanin gene. The transformation was done through electroporation. This genetically modified strain was designated as PA-pUCP-Phz^{++}.

4.2.14.1. Preparation of electrocompetent *P. aeruginosa* MCCB117 cells

P. aeruginosa MCCB117 was inoculated into 10ml of LB broth and incubated overnight at 37°C on a shaker incubator at 200rpm. An aliquot of 500µl of the overnight culture was transferred to 50ml of fresh LB-broth and incubated at 37°C with shaking (200rpm) until an optical density of 0.3-0.5 (600nm) was obtained. Centrifuged at 7000g for 10 minutes at 4 °C, discarded the supernatant. The pellet was resuspended and washed in an equal volume of ice-cold sterile 300mM sucrose. Repelletized the cells by centrifuging as before and discarded the supernatant, resuspended and washed the cells once again in 0.5 volume ice-cold water. The centrifugation step was repeated and the pellet was resuspended 0.01 volumes of ice-cold 300mM sucrose, chilled the cells on ice for 30 minutes and used for electroporation.

4.2.14.2. Electroporation of *P. aeruginosa* MCCB117

Electroporation of electrocompetent *Pseudomonas* MCCB117 was performed in accordance with the work of Smith and Iglewski with slight modifications (Smith

and Iglewski, 1989). Multiporator (Eppendorf, Germany) was used for the electroporation. The apparatus was set to bacterial mode at a voltage of 1200V for a time constant of 5.0 milliseconds. An aliquot of 3µl (10µg) of plasmid DNA (purified pUCP24 vector with pyocyanin biosynthetic gene insert) was added to 50µl of electrocompetent *Pseudomonas aeruginosa* MCCB117 cells kept on ice and mixed gently. This was transferred to a pre-chilled electroporation cuvette (2mm electrode gap) using a narrow pipette tip. The cuvette was placed into the sample holder and the electroporation apparatus was energized and the pulse was delivered and added 1ml of LB broth to the mixture. The electroporated cells were transferred to a sterile culture tube and incubated for 2 hours at 30°C on a shaker with moderate shaking (150rpm). After incubation, 200µl each was plated onto LB gentamycin plate (200µg ml^{-1}) containing IPTG and X-gal (LB medium-2g $100ml^{-1}$; agar- 2g $100ml^{-1}$) and incubated for 24 hours at 30°C. Individual colonies developed (white) was inoculated into 100ml LB gentamycin broth and incubated at 30°C with shaking for plasmid extraction.

4.2.15. Plasmid extraction from electroporated *P. aeruginosa (*MCCB117)

The plasmid was extracted and purified from *P. aeruginosa (*MCCB117) using AxyPrep plasmid midiprep kit (Axygen) as given above.

4.2.16. Confirmation of the inserted gene

PCR amplification of the extracted plasmid using the M13 forward primer of the pUCP-24 vector and the reverse primer (139R) of the *PhzM* gene was performed to confirm the presence of the insert with the vector. The 25µl reaction mixture containing 2.5µl 10x buffer, 2.5µl dNTP (2.5mM), 1µl Taq polymerase (0.5U μl^{-1}), 0.2µl extracted plasmid DNA (PA-pUCP-Phz^{++} - 75ng), 1µl (10pmol μl^{-1}) of each forward and reverse primer (M13F and 139R) was made up to 25µl with MilliQ. The hot start PCR programme used for the amplification was 95°C for 5 minutes followed by hold at 80°C, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 30 seconds, extension at 72°C for 2 minutes, followed by final extension at 72°C for 10 minutes. Ten µl of PCR products was analyzed by 1%

agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using Bio-Rad gel Documentation system.

4.2.17. Expression of pyocyanin gene in PA-pUCP-Phz^{++}

The transformed clones with pyocyanin biosynthetic gene (PA-pUCP-Phz^{++}) were patched on LB-gentamycin plate and sub-cultured into 25ml of ZoBell's broth (10g l^{-1}) containing gentamycin (200µg ml^{-1}). Wild type *P. aeruginosa* MCCB117 culture (un-transformed) in ZoBell's broth was used as control flask. After 24 hour incubation in a shaker incubator at 30°C, at 150rpm, the expressed pyocyanin was quantified by pyocyanin quantification assay.

4.2.18. Quantification of pyocyanin production in PA-pUCP-Phz^{++}

Pyocyanin production was quantified by extracting 5ml culture supernatant with 3ml of chloroform. This was then re-extracted into 1ml 0.2N HCl to give a pink-colored solution. Absorbance of this solution was measured at 520nm and the concentration in micrograms of pyocyanin produced per millilitre of culture supernatant was determined by multiplying the absorbance (at 520nm) by a factor 17.072 following Essar et al. (1990).

The clones which produced more pyocyanin in comparison with the control were selected and again confirmed for it's over production of pyocyanin by quantification assay along with control as mentioned above in triplicates under the same conditions in a shaker incubator at 30°C at 150rpm for 24 hours.

4.3. Results

4.3.1. DNA extraction

Good quantity (350µg ml^{-1}) and quality (260/280 ratio - 2.0) of genomic DNA was obtained from *Pseudomonas* MCCB117 (Fig. 11) by the method of Lee et al. (2003) with some modifications.

4.3.2. Amplification of pyocyanin biosynthetic genes using long PCR from *Pseudomonas* MCCB117

In order to clone the pyocyanin gene into pUCP24 vector, the genes encoding pyocyanin biosynthetic pathway was amplified by long PCR using Phusion™ High-Fidelity PCR kit (Finnzymes, Finland). For the complete amplification of pyocyanin biosynthetic genes forward primer NP89F was designed 100bp upstream to *PhzM* gene (including the partial sequence of *OPMD* gene in the same loci) and reverse primer NP89R was designed 30bp downstream to *PhzS* gene from the gene loci with a product size >10kb. The primers designed were analyzed for annealing temperature using gradient PCR and was fixed as 53°C (Fig. 12). The resulting fragment >10 kb long was visualized on agarose gel (Fig. 12). Amplified product of >10kb was used for cloning in to pUCP24 vector.

4.3.3. Restriction digestion of pUCP24 vector and treatment with Calf Intestinal Phosphatase (CIP)

pUCP24 vector extracted from *E coli*-DH5α was restriction digested with *SmaI* for blunt-ends, CIP treated and analyzed by agarose gel electrophoresis. The resulted band of 4kb (Fig. 13) was excised and purified using Promega Wizard SV Gel and PCR clean up system for further experiment.

4.3.4. Ligation of pyocyanin biosynthetic pathway genes to pUCP24 vector

The gel purified pUCP24 (linearized and CIP treated), T-tailed vector was ligated with the A-tailed PCR product (pyocyanin biosynthetic pathway genes) (Fig. 14)

4.3.5. Transformation into *E.Coli* DH5α cells

Heat shock method of DNA transformation was identified as the effective method of transformation in to *E.Coli* DH5α cells. The transformed white colonies were developed on LB gentamycin plate containing IPTG and X-gal plate after 24 hours of incubation at 37°C. These colonies were propagated in LB gentamycin broth for plasmid extraction.

4.3.6. Restriction digestion of cloned pUCP24 vector containing pyocyanin biosynthetic pathway gene.

The extracted plasmid was restriction digested with *Xba1* (New England Biolabs, USA) enzyme to confirm the plasmid size. Two bands were obtained, one at 4kb and other little above 10kb which confirmed the insertion of the pyocyanin biosynthetic pathway gene into the vector (Fig. 15).

4.3.7. Confirmation of insert orientation in pUCP24 vector.

The PCR amplification of the extracted plasmid using the forward primer (62F) of the *PhzS* gene and the M13 reverse primer of the pUCP24 vector, yielded a ~1400bp amplicon (Fig. 16) which confirmed the in-frame insert orientation.

4.3.8. Transformation of *Pseudomonas aeruginosa* MCCB117 to develop genetically modified *P. aeruginosa* PA-pUCP-Phz^{++}

Transformation of pUCP24 vector encoding pyocyanin genes into *Pseudomonas aeruginosa* MCCB117 could be achieved through electroporation techniques suggested by Smith and Iglewski, (1989) with slight modifications. The white colonies were developed on LB gentamycin plate containing IPTG and X-gal plate after 24 hours of incubation at 30°C. The genetically modified *Pseudomonas aeruginosa* MCCB117 was designated as *P. aeruginosa* PA-pUCP-Phz^{++}. The maximum electroporation efficiency was obtained at a voltage 1200V for a time constant of 5.0 milliseconds using multiporator (Eppendorf, Germany).

4.3.9. Plasmid extraction and confirmation of the inserted gene from electroporated *P. aeruginosa (*MCCB117)

The plasmid was extracted and purified from *P. aeruginosa (*MCCB117) using AxyPrep plasmid midiprep kit (Axygen) (Fig. 17). PCR amplification of the extracted plasmid using the M13 forward primer of the pUCP24 vector and the reverse primer (139R) of the *PhzM* gene was performed to confirm the presence of the insert with the vector. An amplified product of ~1100bp was analyzed by 1% agarose gel electrophoresis (Fig. 18)

4.3.10. Quantification of pyocyanin production in PA-pUCP-Phz^{++}

The white colonies (PA-pUCP-Phz^{++}) grown in LB-gentamycin (200µg ml^{-1}) plate were sub-cultured in ZoBell's marine broth (10gl^{-1}) and screened for pyocyanin production and compared with control (wild type). Out of 25 clones screened 5 of them which showed higher pyocyanin production (Fig. 19) were again screened in triplicates under same conditions in a shaker incubator. In this experiment the clones P12 (27.42mg l^{-1}) and P13 (48.15mg l^{-1}) showed higher pyocyanin production in comparison with that of the control (Fig.20) out of which the pyocyanin production by P13 was the highest (twice than that of the control). Accordingly, the clone P13 was used for the production of pyocyanin and further experiments.

4.4. Discussion

In the early 1900s, pyocyanin was identified in bacterial cultures and the phenazine-1- carboxylic acid (PCA) was long ago recognized as the biosynthetic precursor of pyocyanin (Parsons et al., 2007). *P. aeruginosa* produce a variety of redox-active phenazine compounds, including pyocyanin, phenazine-1- carboxylic acid (PCA), 1-hydroxyphenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) (Turner et al., 1986; Lesinger and Margraff, 1979). With these secondary metabolite production that function in microbial competitiveness, environmental isolates of *P. aeruginosa* have been widely applied as biological control agents against phytopathogenic fungi and bacteria in agriculture (Anjaiah et al., 2003; Bano and Musarrat, 2003; Rangarajan et al., 2003) vibrios in aquaculture (Chythanya et al., 2002; Vijayan et al., 2006) and as bioaugmentors (Chaerun et al., 2004; De Meyer et al., 1999) *P. aeruginosa* contains a complex phenazine biosynthetic pathway consisting of two homologous core loci (*phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*) responsible for synthesis of PCA. Mavrodi et al. (1998) reported that these seven genes are sufficient for synthesis of PCA and two additional genes (*phzM* and *phzS*) encoding unique enzymes are involved in the conversion of PCA to pyocyanin (Mavrodi et al., 2001). Knockout mutant analysis on these genes also proved its contributory effect on pyocyanin production (Chieda et al., 2008; Mavrodi et al., 2001).

Enhanced biosynthesis of pyocyanin in Pseudomonads through modified growth media has been well explained (Frank and DeMoss, 1959; King et al., 1954)

Ingledew and Campbell, 1969; Burton et al., 1947; Halpern et al., 1962) and the multi factorial statistical screening of media components was illustrated (Preetha et al., 2010). Moreover, Ingledew and Campbell (1969) concluded that the phosphate deficiency triggered pyocyanin biosynthesis in *P. aeruginosa* . Cloning and expression of functional genes in *Pseudomonas aeruginosa* for the study of biosynthesis mechanism was well explained (Akochy et al., 2004; Urbauer et al., 2005) and transformation studies were made in *P. fluorescens* with genes for biosynthesis of PCA (Huang et al., 2004). Pierson and Thomashow (1992) performed the cloning and expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens*. However, the overexpression of gene(s) encoding pyocyanin biosynthesis by increasing its copy number in *P. aeruginosa* has not yet been performed. Moreover, the commercial interests in phenazine antibiotics were limited due to its inconsistent performance and lower yield from Pseudomonas (Mavrodi et al., 2006). In this context, to overexpress genes encoding pyocyanin biosynthetic pathway for the enhanced production of pyocyanin by increasing the pyocyanin gene copy number in wild *Pseusomonas aeruginosa* was performed by transformation with specific vector encoding the same gene.

In the present study, genes encoding pyocyanin biosynthetic pathway (>10kb) was cloned into pUCP24 (4035bp) vector for transformation into wild type *P. aeruginosa* MCCB117 to develop genetically modified *P. aeruginosa* PA-pUCP-Phz^{++}. As the product size was >10kb, long PCR was carried out for the PCR amplification. The expression vector pUCP24 was a pUC18/19 derivative (Schweizer et al., 1991; West et al., 1994) *Escherichia-Pseudomonas* shuttle vector constructed to enhance gene cloning in *P. aeruginosa*. pUCP24 was obtained by substitution of a 1352bp *ScaI-StuI* fragment in pUC18 with the blunt-ended 832bp encoding gentamycin resistance fragment from pUCGM vector. This contained 1.2kb *StuI-PstI* fragment required for replication of ColE1 replicons in *P. aeruginosa*. West et al. (1994) reported that the substitution of *bla* gene (ampicillin resistance) of pUC18 with Gm resistance (gentamycin) enhanced the utility in *P. aeruginosa* and reduced the size of pUCP24 from 4557bp (pUC18) to 4035bp.

Effective transformation of pUCP24 vector encoding pyocyanin genes could be achieved through electroporation techniques suggested by Smith and Iglewski (1989) with slight modifications. Maximum electroporation efficiency was obtained at a voltage 1200V for a time constant of 5.0 milliseconds using multiporator

(Eppendorf, Germany). Smith and Iglewski (1989) reported the maximum efficiency at 8kV cm^{-1} and the exponential decay constant of 5 milliseconds.

Expression (over) of inserted pyocyanin gene was identified by the production of pyocyanin in transformed clones in comparison with un-transformed control (wild). In the results, the transformed clone, P13 produced pyocyanin approximately double than that of the control. This (over) expression may be due to the increased copy number of pyocyanin biosynthetic pathway genes in transformed *P. aeruginosa* MCCB117. The structural and functional analysis of seven gene locus made by Mavrodi et al. (1998) identified that *phzABCDEFG* were responsible for the synthesis of phenazine-1-carboxylic acid (PCA) in *P. fluorescens* and *E.Coli* transformed with complete *PhZ* locus (*phzA-G*) expressed large amount of PCA. According to Turner and Messenger (1986), two modifications were needed to convert PCA to pyocyanin: addition of the N-methyl group, converting PCA to 5-methylphenazine-1-carboxylate betaine; and hydroxylative decarboxylation of the betaine to form pyocyanin. Moreover, Mavrodi et al. (2001) confirmed the essential role of *PhzM* and *PhzS* for the conversion of PCA to pyocyanin and demonstrated that the transformation of the PCA producing *P. fluorescens,* with *PhzM* and *PhzS* genes triggered synthesis of large amounts of pyocyanin. Recently, Parsons et al. (2007) made an extensive study on structural and functional analysis of the pyocyanin biosynthetic protein *PhzM* from *Pseudomonas aeruginosa* and further confirmed the gene involvement. By analyzing the functional genes for biosynthesis of pyocyanin and PCA, Mavrodi et al. (2001) reported that in *P. aeruginosa* PA01 genome *phzABCDEFG* operon (Positions 4,713,795 to 4,720,062) and the *PhzM* are transcribed divergently and separated by 695bp, spans positions 4,713,098 to 4,712,094 (Fig 4.2b). The *PhzS* gene located 236bp downstream from *PhzG1* and spans positions 4,720,300 to 4,721,508. In our experiment, we were able to transform the seven operon *phzABCDEFG* along with the *PhzM* and *PhzS* genes and obtain expression (over) of pyocyanin from *P. aeruginosa* PA-pUCP-Phz^{++}.

In conclusion, the investigation suggests that enhanced production of pyocyanin in *P. aeruginosa* PA-pUCP-Phz^{++} could be achieved by transformation of *Pseudomonas aeruginosa* MCCB117 using pUCP24 *Escherichia-Pseudomonas* shuttle vector encoding pyocyanin biosynthetic gene operon. Thus the genetically modified *P. aeruginosa* PA-pUCP-Phz^{++} could be used for the mass production of pyocyanin.

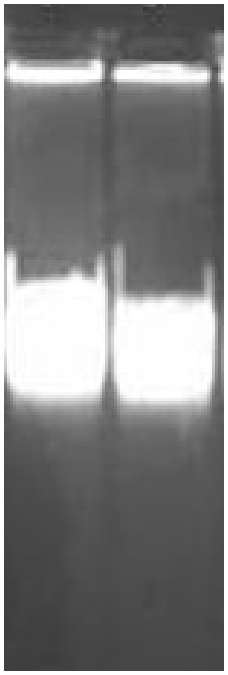

Fig.11 Genomic DNA extracted from *Pseudomonas aeruginosa* MCCB117.

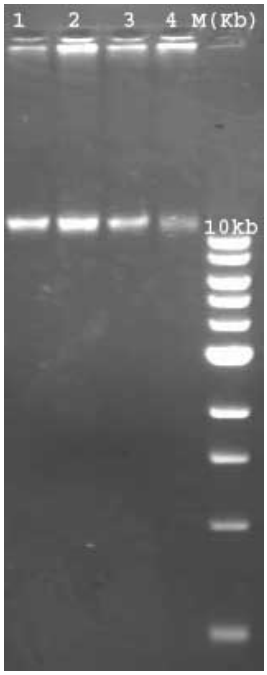

Fig.12 Amplicon of pyocyanin gene using long PCR at different melting temperature (Tm)- 1) 51ºC 2) 53ºC 3) 55ºC 4) 58ºC.

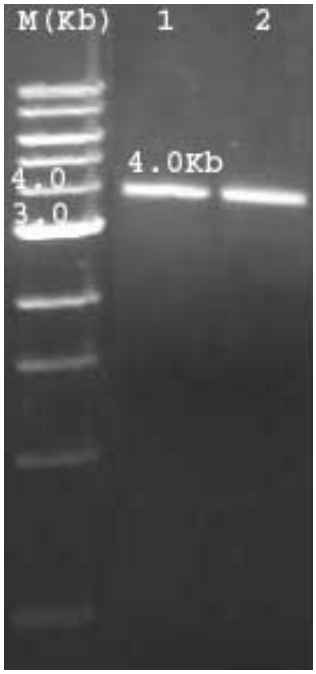

Fig.13 Restriction digested pUCP24 vector with *SmaI* (1 and 2).

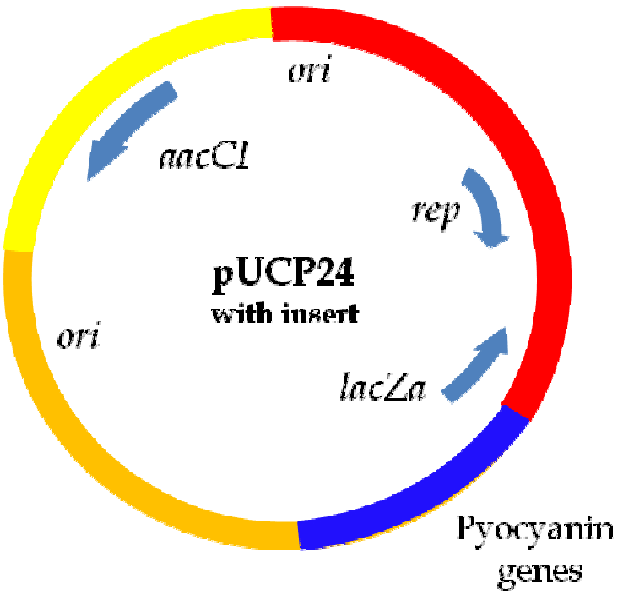

Fig.14 Schematic diagram of pyocyanin biosynthetic pathway gene (s) inserted into pUCP24.

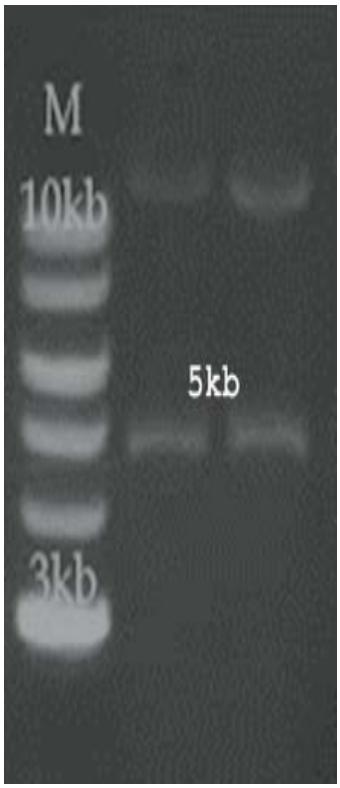

Fig.15 Restriction digested pUCP-24 along with insert using *Xba1.*

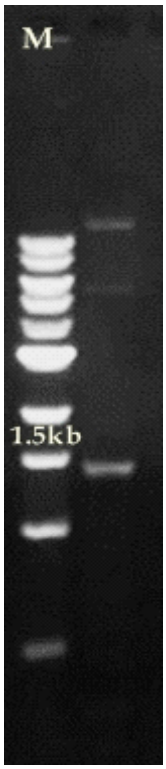

Fig.16 Amplification of *PhzS* gene using the M13 reverse primer of the pUCP24 vector and 62F forward primer of the *PhzS* gene.

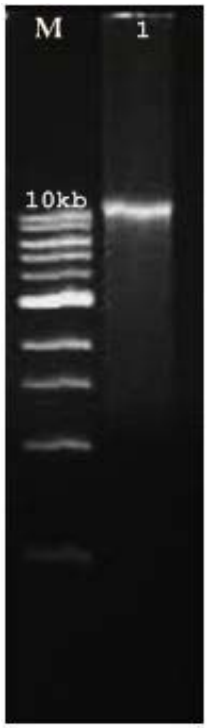

Fig.17 Plasmid pUCP24 extracted and purified from *P. aeruginosa* (MCCB117).

Fig.18 Amplification of *PhzM* gene using the M13 forward primer of the pUCP24 vector and 139 reverse primer of the *PhzM* gene.

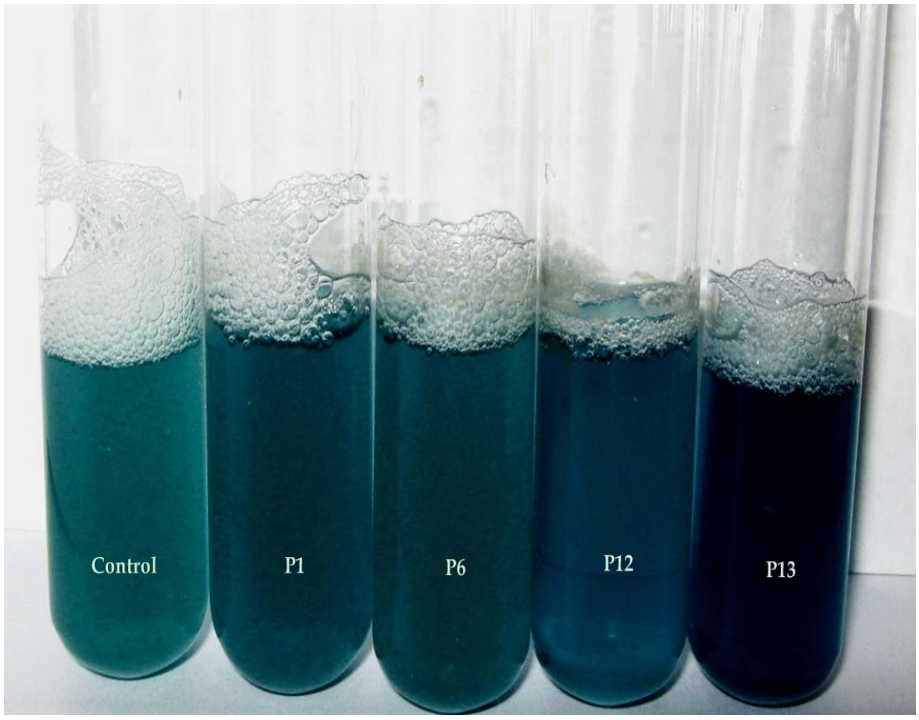

Fig.19 Selected clones of *P. aeruginosa* PA-pUCP-Phz^{++} with varying production of pyocyanin along with control. Tubes arranged in an increasing order of production.

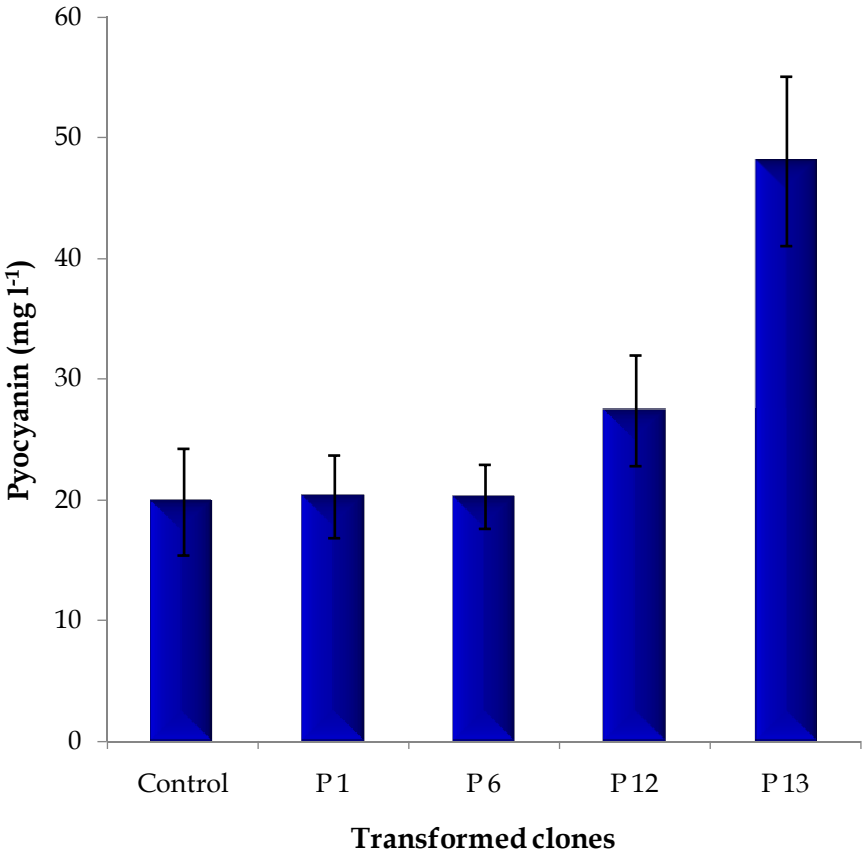

Fig. 20 Differential production of pyocyanin in transformed clones.

Chapter 5

DEVELOPMENT OF AN APPROPRIATE DOWNSTREAM PROCESS FOR LARGE SCALE PRODUCTION OF PYOCYANIN FROM PA-pUCP-Phz^{++}; STRUCTURAL ELUCIDATION AND FUNCTIONAL ANALYSIS OF THE PURIFIED COMPOUND

Contents

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 (Verrnon 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

et al., 2004). 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 *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 *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 *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 *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

into a 500ml vacuum rotary flask (BUCHI, Switzerland) and concentrated in a vacuum rotary evaporator at 40°C (BUCHI, Switzerland).

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-trifluroacetic acid (100: 0.04, v/v) and solvent B was acetonitrile-water-trifluroacetic 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. ^{1}H NMR spectral analysis

^{1}H 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. $CdCl_3$ 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 (10^3 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 bio-luminescence 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,

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 ^{1}H 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 ^{1}H 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 $CHCl_3$ and 0.1N HCl

	Purified pyocyanin	Pyocyanin standard
In $CHCl_3$	699,529,310,254.5	691,529,306,255.5
In 0.1N HCl	553,390,284,246,224	555,388,284,247,225

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

Vibrio spp.	Zone Diameter (in mm)
V.alginolyticus	27.5 ± 0.71
V.fluvialis	26.5 ± 0.71
V.mediterranei	26 ± 1.41
V.nereis	27.5 ± 0.71
V.parahaemolyticus	23.5 ± 0.71
V.proteolyticus	17.5 ± 0.71
V.vulnificus	38 ± 1.41
V.harveyi	28.5 ± 0.71

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.

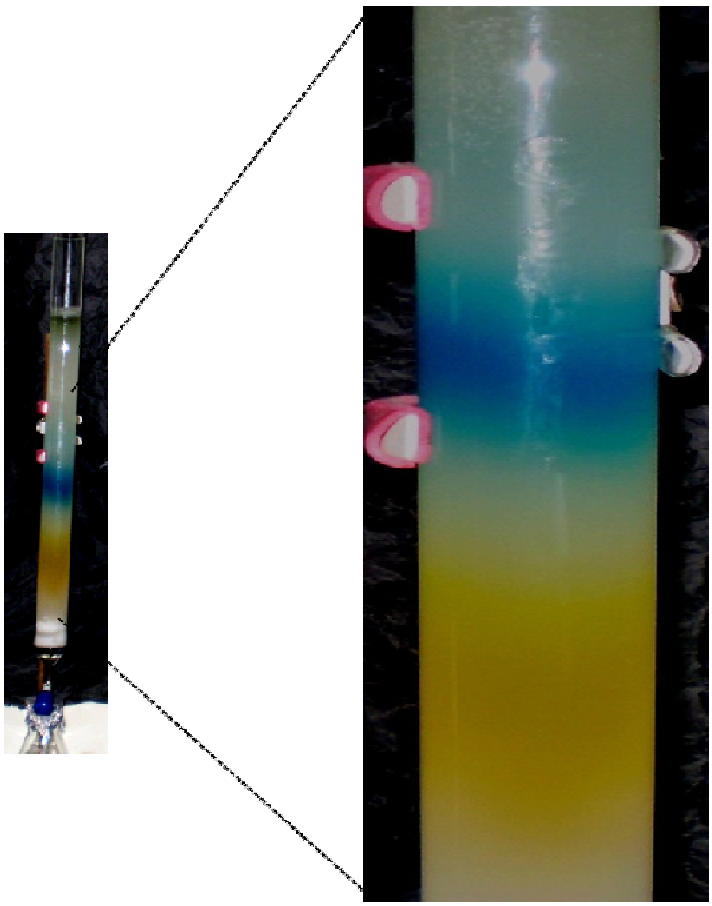

Fig. 3 Column purification of pyocyanin. Blue colour indicates pyocyanin fraction.

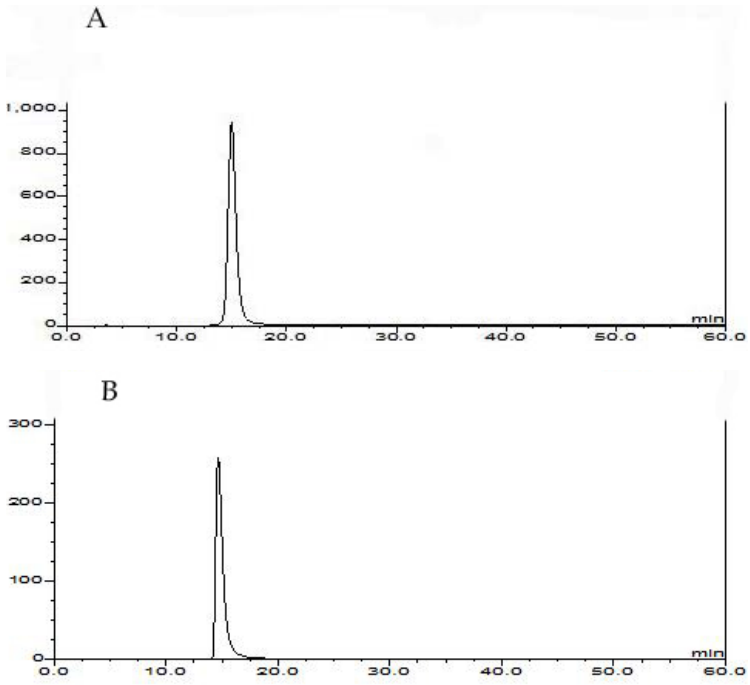

Fig.4 HPLC of pyocyanin: A) Pyocyanin from *Pseudomonas aeruginosa* PA-pUCP-Phz^{++} B) Pyocyanin standard.

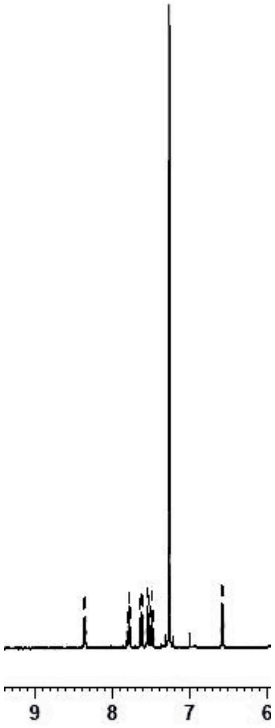

Fig.5 Nuclear Magnetic Resonance (^{1}H NMR) spectra of pyocyanin extracted from *Pseudomonas aeruginosa* PA-pUCP-Phz^{++}.

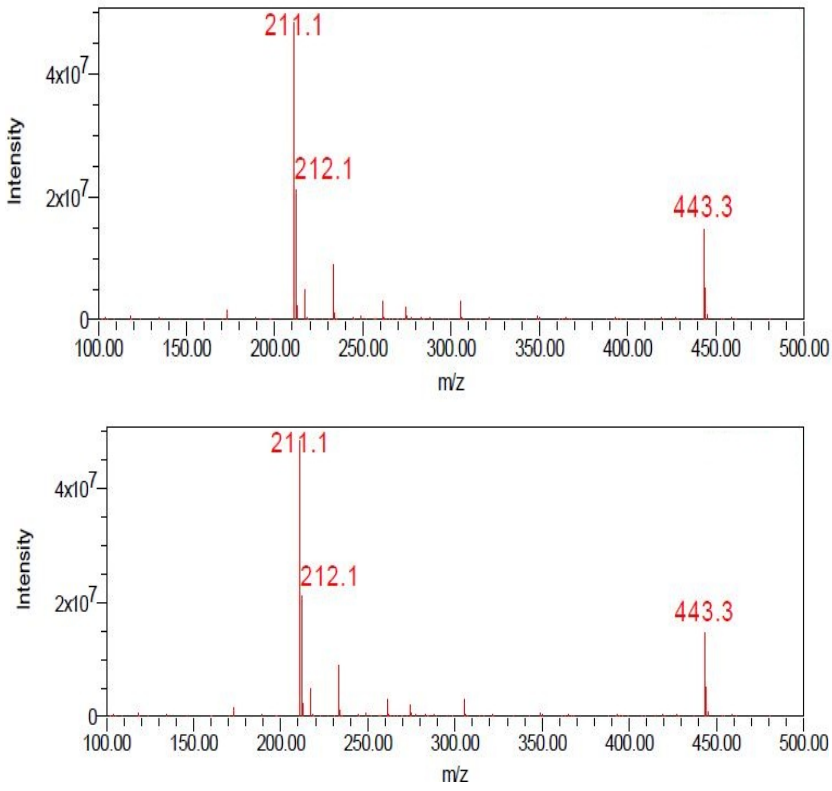

Fig. 6 Mass spectra of pyocyanin from *Pseudomonas aeruginosa* PA-pUCP-Phz^{++} and pyocyanin standard.

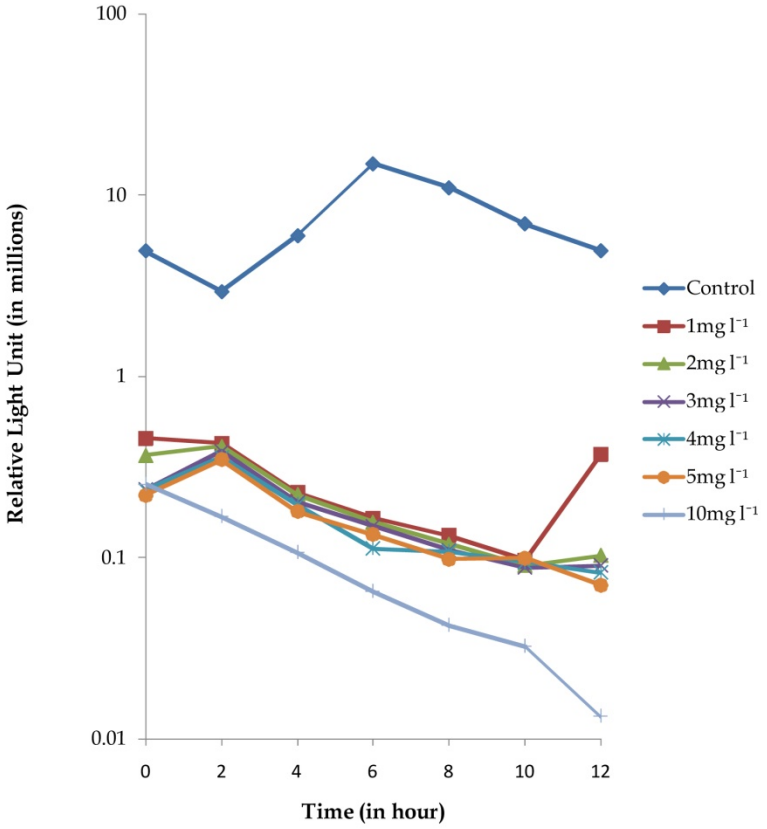

Fig. 7 Pyocyanin induced inhibition on bioluminescence of *Vibrio harveyi.*

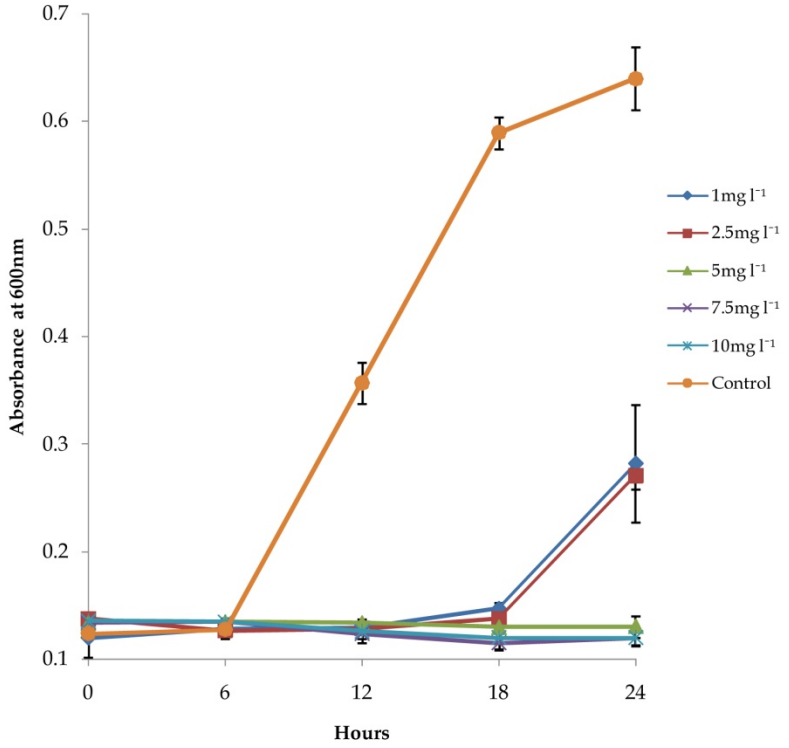

Fig. 8 Growth of *Vibrio harveyi* at different concentrations of purified pyocyanin.

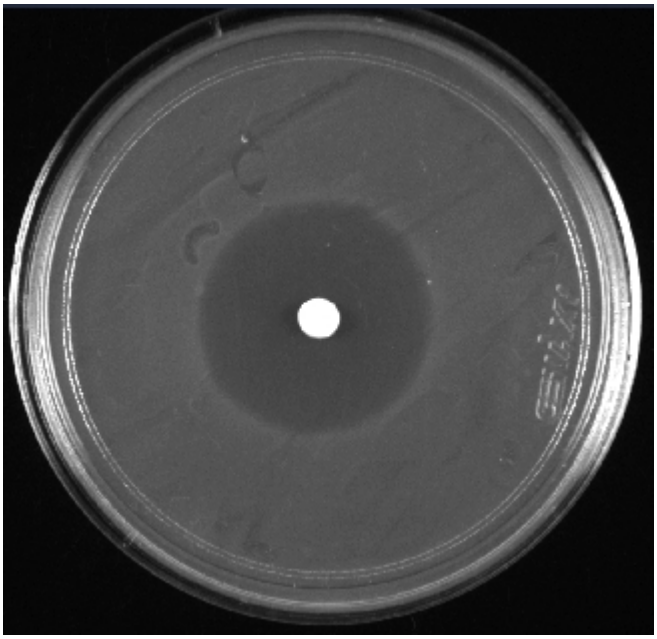

Fig. 9 Antagonistic activity (zone of inhibition) of purified pyocyanin against *V. harveyi* MCCB111.

Chapter 6

TOXICITY OF PYOCYANIN ON VARIOUS BIOLOGICAL SYSTEMS

Contents

6.1. Introduction

Pyocyanin (5-methyl-1-hydroxyphenazine) is a biologically active blue coloured phenazine pigment produced by certain species of *Pseudomonas.* Even though they have broad antibiotic activity against various bacteria, fungi and protozoa the toxicity on host animal are controversial. Nevertheless, the molecular mechanism and mode of action of pyocyanin in target organism has been studied extensively (Hassan and Fridovich, 1980).

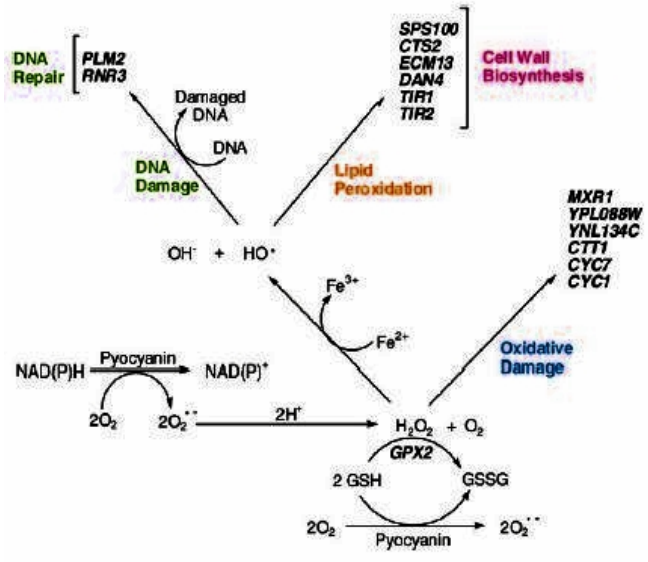

Fig.1 Pyocyanin metabolic pathway induces oxidative stress in yeast (Angell et al., 2006).

Moreover, Britigan et al. (1997) and Hassan and Fridovich, (1980) reported that the toxicity is thought to be caused by their ability to undergo redox cycling in the presence of various reducing agents and molecular oxygen, which leads to the accumulation of toxic superoxide and hydrogen peroxide and, eventually, to oxidative cell injury or death. Besides its toxicity in higher concentration, it has been applied as a bio control agent/probiotic against many pathogens including the *Vibrio* spp. in aquaculture systems (Preetha et al., 2010; Vijayan et al., 2006; Chythanya et al., 2002). However, one of the most important criteria of merit for a candidate to be used in biocontrol is that the organism should be nonpathogenic to the host (Rahman et al., 2011; Chythanya et al., 2002).

Bio-degradation and detoxification of residues are other important requirements for a compound to be applied in aquaculture systems. Yang et al. (2007) reported the biodegradation of phenazine-1-carboxylic acid (PCA) - the precursor of pyocyanin by soil organisms *Sphingomonas* sp. DP58 which consumed PCA as the sole source of carbon and nitrogen and completely degraded within 40 hours. Besides, Hill and Johnson, (1969) reported the microbial transformation of phenazines by *Aspergillus sclerotiorum* and Chen et al. (2008) conducted the study on intermediates or metabolites produced during PCA biodegradation.

The study on photosensitized oxidation and inactivation (Reszka et al., 2004) showed that pyocyanin could be partially inactivated through photochemical oxidation and the resulting product(s) is not an effective free radical generator and therefore a less efficient stimulant of oxidative processes. These results suggest that photosensitization could be a potentially useful method for inactivation and possibly for detoxification of pyocyanin.

Reszka et al. (2004) suggested that the biological detoxification of pyocyanin is favored by the presence of phenolic character in the compound and phenolics are excellent substrates for peroxidases. The oxidation of pyocyanin leads to its inactivation and become non-toxic and the reaction is irreversible (Price-Whelan et al., 2006). As the pyocyanin can be readily oxidized, and is susceptible to biodegradation; it can be applied as a drug eco-friendly in aquaculture system. Moreover, the control of fish and shellfish pathogenic vibrios, particularly using non-

pathogenic bacterial isolates and thereby the disease prevention has received much attention during the last decade (Rengpipat et al., 2000, Sugita et al., 1998). In this context, the dose-response study of pyocyanin from environmental isolates of *Pseudomonas aeruginosa* in various biological systems are of great importance to evaluate its toxicity and thus for selective applications.

Cytotoxicity is considered an important index for evaluating safety of antimicrobials and management of chemicals prior to their administration in aquaculture. For its accomplishment cell lines could be used which out-rightly eliminated animal experimentation, as part of the bioassays as is being followed now (Jose et al., 2010) and is considered as *in vitro* models for toxicity studies (Allen et al., 2005). Such an approach could forecast their *in vivo* effects as well as assisting their optimization for field level application. This is specifically because under field conditions realization of their negative impacts happens to be quite cumbersome (MacGowan et al., 2001)

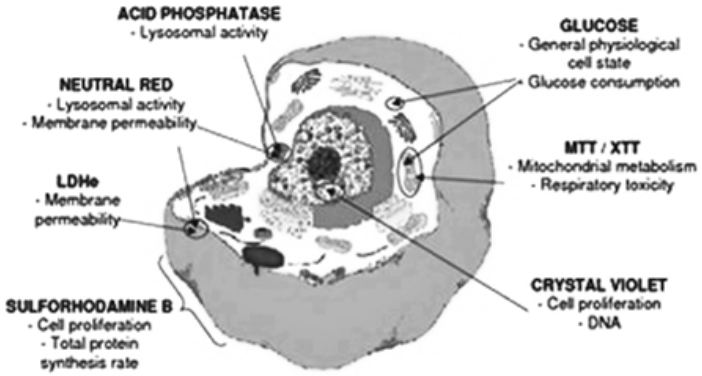

Fig. 2 Cyototoxicity assays and associated targets in cell (www.xenometrix.ch)

Cytototoxicity assays have been developed which use different parameters associated with cell death and proliferation. Moreover, the most convenient, modern assays have been optimized for the use of microtitre plates (96-well format) to allow many samples to be analyzed rapidly and simultaneously (Weyermann et al., 2005). In this study five cytotoxicity assays were performed and the oxidative damage was correlated with the accumulation of hydrogen peroxide (H_2O_2) in the growth medium. The leakage of intra cellular lactate dehydrogenase (LDH) was measured by extra cellular LDH assay, mitochondrial dehydrogenase activity was measured by

reduction of 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), lysosomal activity by neutral red uptake assay (NRU), protein inhibition assay by affinity of electrostatic binding of protein with Suforhodamine B dyes (SRB) and the inhibition in cell metabolism was measured by glucose assay.

Pyocyanin induced toxicity in cell lines and primary cell cultures has been well documented: Studies of pyocyanin-induced acute ROS production and subsequent oxidative stress in A549 alveolar epithelial cells (Gloyne et al., 2011; Muller et al., 2009; Muller, 2006; Yunxia et al., 2003), toxicity in 1321N1 astrocytoma cells (McFarland et al., 2011), induction of neutrophil apoptosis (Usher et al., 2002), toxicity in primary heamocyte culture of *P. monodon* (Preetha et al., 2010) are the examples of its toxicity studies. However, the *in vitro* toxicity studies and the IC_{50} value of pyocyanin from environmental isolates in various biological system including human cell lines have not yet to be accomplished. Regardless of its *in vitro* toxicity, these findings give an account of the concentration which has to be applied in aquaculture system to control pathogenic *Vibrio* spp.

In the previous chapter description of pyocyanin extracted from genetically modified *Pseudomonas* PA-pUCP-Phz^{++}, its purification and structural elucidation have been given. Besides, the MIC (Minimum Inhibitory Concentration) of the purified compound against *Vibrio harveyi*, (bacteriostatic at 5mg l^{-1}, bactiriocidal at 10mg l^{-1} have also been presented. Meanwhile, in the present study, pyocyanin-induced toxicity on brine shrimp larvae, various larval stages of *Penaeus monodon* and toxicity in human cell line, in insect and fish cell lines have been presented. Moreover, considering the importance of recirculating aquaculture system (RAS) integrated with nitrifying bioreactors such as activated packed bed bioreactor (PBBR) and stringed bed suspended bioreactors (SBSBR) for maturation and shrimp larval production, inhibitory action of pyocyanin on nitrification of the nitrifying bacterial consortia has also been examined.

6.2. Materials and Methods

6.2.1. Brine shrimp lethality assay

Toxicity of pyocyanin was tested using *Artemia salina* nauplii (Brine shrimp). Dried cysts were hatched (1g cyst per liter) in filtered seawater at 27-30°C with strong aeration, under a continuous light regime. Approximately 12 hour after hatching, the phototrophic nauplii were collected with a pipette and concentrated in a small vial. Each test consisted of exposing groups of 20 nauplii to various concentrations (0, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500mg l^{-1}) of pyocyanin. The toxicity was determined after 12 and 24 hours of exposure by counting the number of survivors and calculating the percentage of mortality. Larvae were considered dead if they did not exhibit any movement of appendages. All data were subjected to Probit analysis using SPSS software (SPSS 17.0, SPSS Inc., Chicago, USA).

6.2.2. Larval lethality assay

Toxicity in different stages of life cycle of *P.monodon* such as nauplius, zoea, mysis and post larvae (PL-5 and PL-15) were tested by exposing appropriate numbers to various concentrations of pyocyanin (Preetha et al., 2007; Chythanya et al., 2002). The toxicity was determined after 12 hour of exposure by counting the number of survivors and calculating the percentage of mortality. All data were subjected to Probit analysis using SPSS software (SPSS 17.0, SPSS Inc., Chicago, USA).

6.2.3. Cytotoxicity of pyocyanin on various cell lines

Cytotoxicity assays are the *in vitro* bioassay methods used to predict toxicity of substances to various tissues or animals. Three cell lines such as from insects (Sf9), fish (RTG-2) and human beings (L-132) were selected for the study. Fish cell line was used as the anticipated application was in aquaculture system, insect cells for testing toxicity in the cell line related (nearly) to crustaceans and the human cell lines for testing the toxicity in humans to assess its toxicity while handling the compound during application. All cell lines were exposed to pyocyanin for a fixed time interval (24 hour) and the induced cytotoxicity among the cell lines were

compared and the IC_{50} values were calculated by Probit analysis using SPSS software (SPSS 17.0, SPSS Inc., Chicago, USA).

6.2.3.1. Cell lines and its growth condition

The insect cell line, Sf9, originated from *Spodoptera frugiperda* pupal ovarian tissue were maintained at 28°C in Grace's insect medium with 2mM L-glutamine, 500mg l^{-1} calcium chloride, 2.8g l^{-1} potassium chloride, 3.33g l^{-1} lactalbumin hydrolysate supplemented with 10% fetal bovine serum and antibiotic mixture containing 100µg ml^{-1} streptomycin and 100IU ml^{-1} penicillin. The growth medium was changed once in every 2-3 days.

Fish cell line, RTG-2, originated from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue was maintained at 25°C in Minimum essential medium (MEM) with 2mM L-glutamine and Earle's balanced salt solution adjusted to contain 0.35g l^{-1} sodium bicarbonate, 0.1mM non-essential amino acids and 1mM sodium pyruvate supplemented with 10% fetal bovine serum and antibiotic mixture containing 100µg ml^{-1} streptomycin and 100IU ml^{-1} penicillin. The growth medium was changed once in a week.

Human embryonic lung epithelial cell line, L-132, was maintained at 37°C in Dulbecco's modification of minimum essential medium (DMEM) with 2mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5g l^{-1} sodium bicarbonate, 0.1mM non-essential amino acids and 1mM sodium pyruvate supplemented with 10% fetal bovine serum and antibiotic mixture containing 100µg ml^{-1} streptomycin and 100IU ml^{-1} penicillin. The growth medium was changed three times in a week.

6.2.3.2. Cytotoxicity assays

After 24 hours exposure to each of the cell lines, combined colorimetric assays for the quantification of the membrane integrity, mitochondrial metabolism, lysosomal integrity and activity, total protein synthesis rate of cells, glucose metabolism and free radical accumulation in response to pyocyanin were evaluated. All assays were performed sequentially on the same cell line samples in triplicates. This experimental set-up eliminated all other sources of variability due to external

factors (passage number, cell density and exposure time). Accordingly, differences in IC_{50} between assays reflected more accurately the differential response of cellular functions to toxicants. Six different parameters were evaluated to assess the toxicity. The leakage of intra cellular lactate dehydrogenase (LDH), reduction of 2,3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), uptake of neutral red (NR), and affinity of electrostatic binding of protein with Suforhodamine B dyes (SRB) were evaluated with a commercial kit (Cytotox-PAN I, Xenometrix, Germany). Whereas glucose metabolism and accumulation of free radicals were evaluated with GOD-PAP end-point glucose assay kit (Biolab Diagnostics, India) and hydrogen peroxide assay kit (BioVison, USA) respectively.

6.2.3.2.1. Exposure to pyocyanin and sequential assay procedure for six parameters

Cell cultures in 96 well plates were developed from each cell line by adding 0.2ml cell suspension in growth medium containing approximately 5 $x10^5$ cells ml^{-1} and incubating for 12 hours at appropriate temperature. Different concentrations of purified pyocyanin prepared in growth medium were added to the wells to attain final strength ranging from 6.25 to 200 for XTT (mitochondrial activity), neutral red up take (plasma membrane damage) and SRB (protein synthesis) , 0 to 200 for LDH and H_2O_2, and 25 to 200 for glucose consumption in triplicate for each concentration. Cells without pyocyanin were kept as control. After 24 hours incubation the wells were observed under phase contrast microscope (Leica, Germany) and sequential cytotoxicity assays were performed. Supernatant from each well along with replicates and control were collected for LDH, glucose and hydrogen peroxide assays with a sample volume 20, 3 and 50µl respectively. The remaining growth medium in the wells were removed, washed with PBS and added 200µl fresh medium along with 50µl pre-warmed XTT solution, incubated for 4 hours in dark. The same cells were used for SRB and neutral NR tests.

6.2.3.2.1.1. Extra cellular lactate dehydrogenase enzyme assay (LDH)

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme rapidly released into the cell culture supernatant upon damage of the plasma membrane. The LDH

assay, therefore, is a measure of membrane integrity. Thus the extra cellular lactate dehydrogenase activity considered as a parameter for cytotoxicity assay due to impaired cell membrane. In this assay the LDH catalyze the conversion of lactate to pyruvate through the reduction of NAD^+ to $NADH/H^+$ and the NADH reduce the tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride to form water-soluble formazan dye. Formation of formazan is directly proportional to membrane damage (extracellular LDH activity) (Decker and Lohmann-Matthes, 1988; Korzeniewski and Callewaert, 1983)

An aliquot of 20µl supernatant from each well along with replicates and control were added with 240µl LDH II (NADH solution) and LDH III (pyruvate solution) mixture with 16ml and 3.4ml respectively. Immediately after the addition, the reading was measured kinetically at 340nm for 25 minutes at 37ºC. The kinetic readings were compared with control and the response of cells against various concentrations of pyocyanin was measured. The extracellular LDH enzyme activity was calculated as nanomoles NADH consumed / min / ml and calculated as follows: (Cytotox - PAN I , Xenometrix, Switzerland).

$$\text{NADH consumption} = \frac{\text{OD/min} \times 0.260 \times 1000}{6.2 \times 20}$$

Where, 0.260 ml: reaction volume in ml

1000µl: allows calculating the result in ml

6.2: millimolar extinction coefficient of NADH at 340nm

20µl: volume taken for the assay

Extracellular LDH enzyme was measured by taking the kinetic reading for 10 minutes at 37ºC calculated the percentage of enzyme activity by comparing the activity in growth medium used.

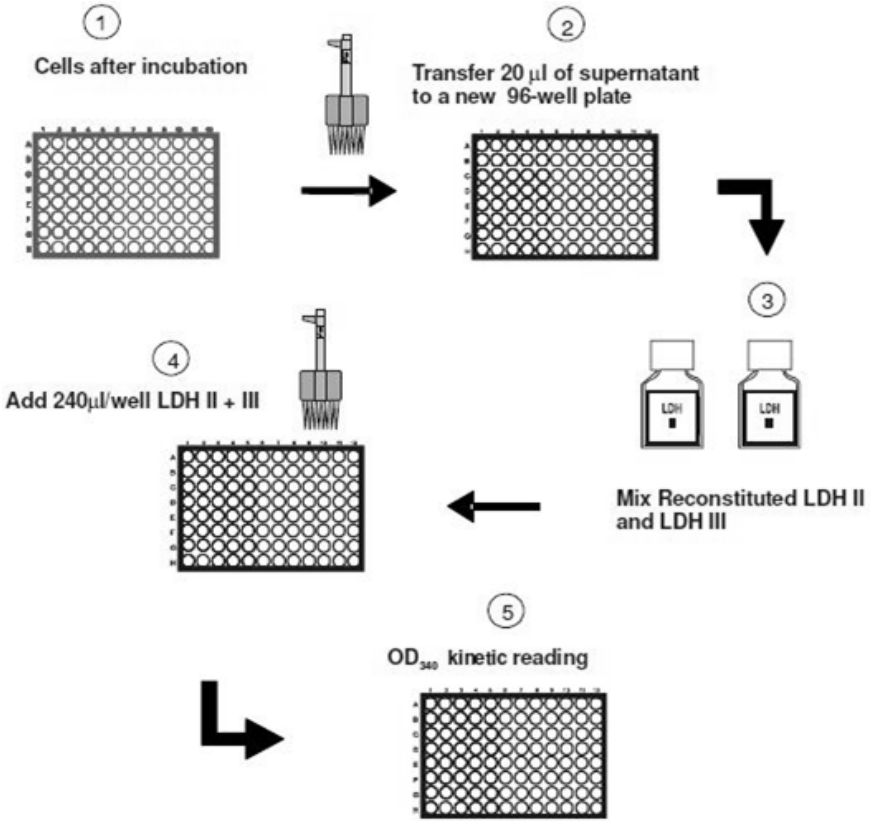

Fig.3 Schematics of LDH assay

6.2.3.2.1.2. XTT assay

The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (2, 3-bis [2- methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide, XTT) with the mitochondria of metabolically active cells. The reduction of the tetrazolium salt by mitochondrial dehydrogenase within the cells produces a water soluble formazan product. This reagent allows direct absorbance readings, therefore eliminating a solubilization step and shortening the assay procedure (Scudiero et al., 1988). Whilst the use of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) produce a non-soluble formazan compound which necessitated dissolving the dye in order to measure it (Mosmann, 1983), the use of XTT produces a soluble dye.

After 4 hours incubation with 50µl pre-warmed XTT solutions at 37ºC, 28ºC and 25ºC for human, insects and fish cells respectively, mixed the formazan formed in each wells very carefully by pipeting. Absorbance was measured at

480nm in a microplate reader (TECAN Infinite Tm, Austria) with a reference wave length at 690nm. The percentage inhibition on the metabolic activity (mitochondrial activity) of cells were calculated and depicted. The calculation was done using the formula:

[100- (Average absorbance at a particular concentration of the compound/Average absorbance in control cells without the compound) x 100)].

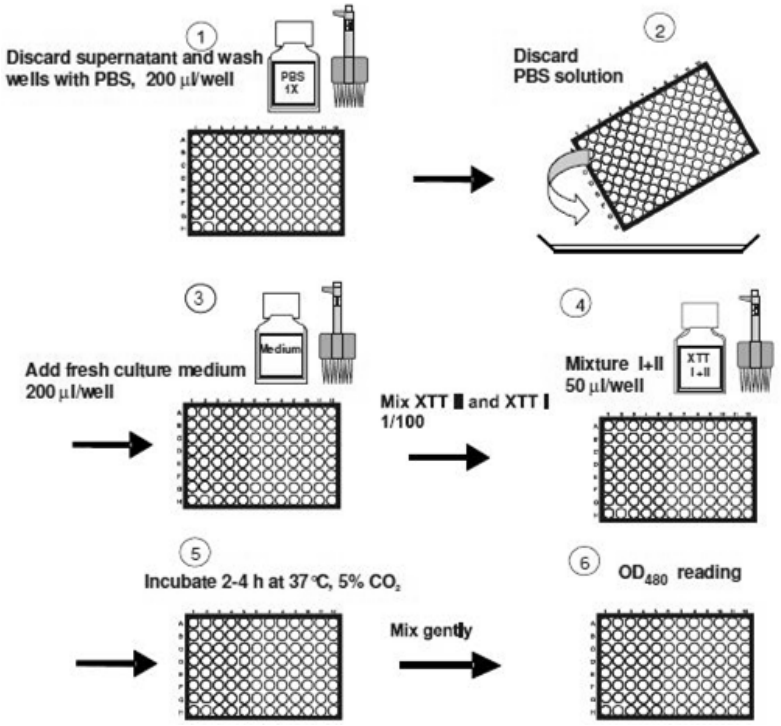

Fig. 4 Schematics of XTT assay

6.2.3.2.1.3. Neutral red uptake assay (NRU)

Neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride) uptake assay is a survival/ viability test based on the ability of living cells to incorporate the neutral red dye on their lysosomes, where it accumulates on membrane-intact cells. The estimation of dye extracted from the cells after their exposure to pyocyanin is indirectly proportional to the pyocyanin-induced toxicity. The amount of dye incorporated can be measured spectroscopically at 540nm (Borenfreund and Puerner, 1984; DeRenzis and Schechtman, 1973).

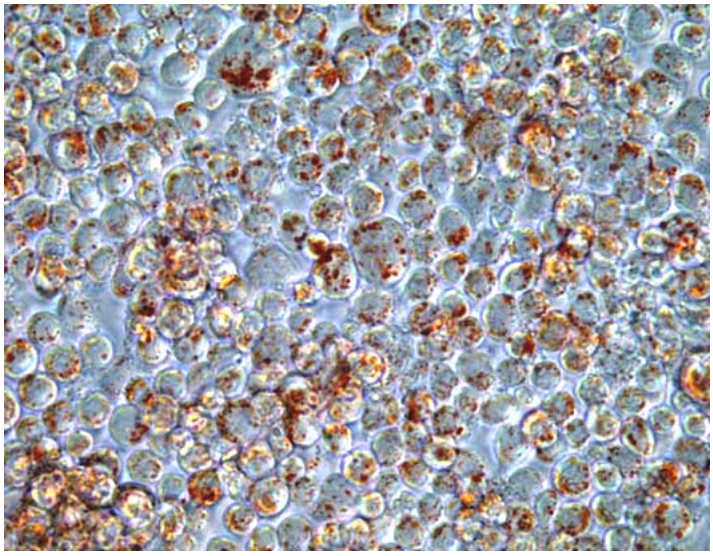

Fig.5 Neutral red accumulated in control cells

Following the sequential assay procedure, XTT solution from each well was discarded and washed with 300µl of wash solution (Neutral red assay solution I). Added 200µl neutral red labeling solution diluted (1:200) with growth medium and incubated for 3 hours at appropriate temperature depending on the cell line (25ºC, 28ºC and 37ºC for RTG-2, Sf9 and L-132 cell lines respectively). Discarded the labeling solution and added 100µl fixing solution (neutral red assay solution III) to each well and discarded after 1 minute, added 200µl solubilization solution (neutral red assay solution IV) to each well and incubated for 15 minutes at room temperature. After incubation, mixed gently using a multichannel pipette and the absorbance were measured at 540nm in a micro plate reader (TECAN Infinite Tm, Austria) with a reference wavelength at 690nm. The percentage damage on cell membrane (lysosomal activity) were calculated and depicted. The calculation was done using the formula- [100- (Average absorbance of accumulated neutral red in cells at a particular concentration of the compound/Average absorbance of accumulated neutral red in control cells without the compound) x 100)].

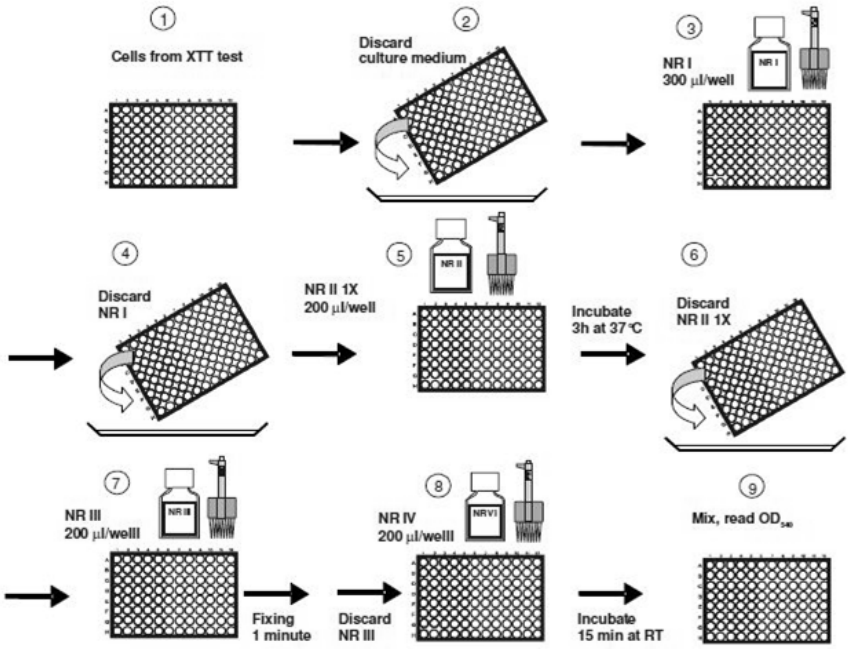

Fig.6 Schematics of neutral red uptake assay

6.2.3.2.1.4. Sulforhodamine B assay (SRB)

Sulforhodamine B is a bright pink aminoxanthene dye with two sulfonic groups that binds to basic amino acid residues under mild acidic conditions, and dissociate under basic conditions. The Sulforhodamine B assay is one of the most widely used methods for in vitro cyctotoxicity studies. However, SRB method does not distinguish between viable and dead cells and are independent of cell metabolic activity. (Vichai and Kirtikara, 2006; Skehan et al., 1990)

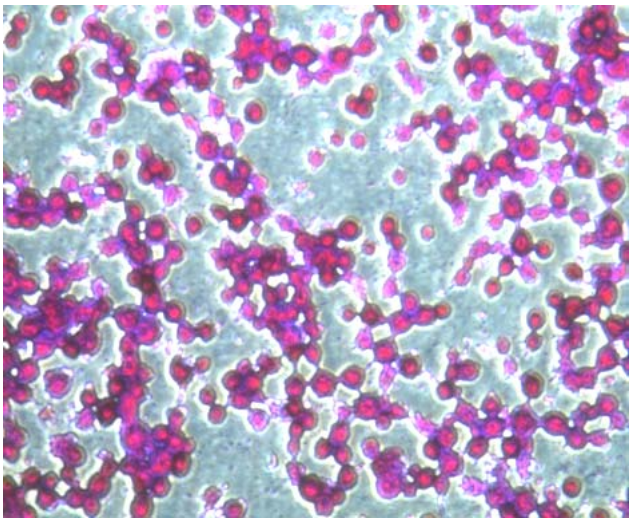

Fig. 7 Sulphorodamine B combined with cellular protein

In the sequential assay procedure, the wells used for NR test were used for SRB test also. The solubilization (Neutral red assay solution IV) solution from Neutral red uptake assay procedure was carefully removed, with the cells remaining attached to the bottom of the wells. Washed with 300µl wash solution (SRB I) and added 250µl fixing solution (SRB II), incubated the plate for 1 hour at 4ºC. Washed the cells 3 times with 200µl Milli Q and added 50µl labeling solution (SRB III), incubated for 15 minutes at room temperature. Washed 2 times with 400µl rinsing solution (SRB IV) and air dried the cells. Dissolved the air dried cells with 200µl solubilization solution (SRB V), incubated for 1 hour at room temperature. After incubation the solution was mixed gently and the absorbance was read at 540nm with a reference wavelength at 690nm. Percentage inhibition of various cells at each concentration of pyocyanin was calculated based on the formula, percentage inhibition/activity of cells = [100- (Average absorbance of cells at a particular concentration of the compound/Average absorbance of control cells without the compound) x 100)] (Vichai and Kirtikara, 2006).

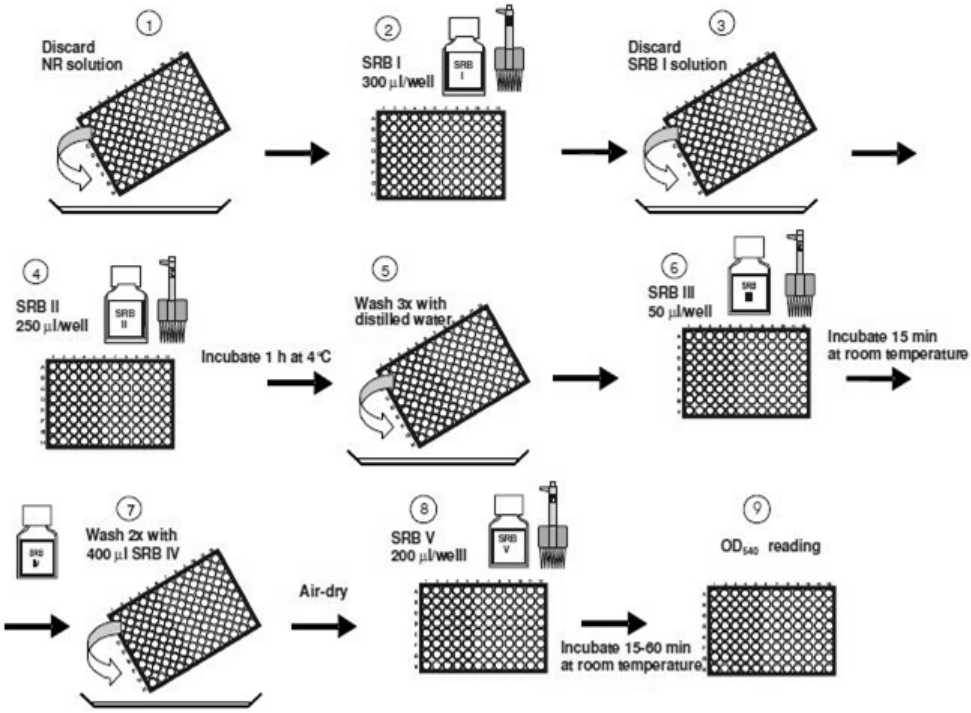

Fig. 8 Schematic of Sulforhodamine B test

6.2.3.2.1.5. Assay for glucose metabolism

Healthy cells continuously consume glucose from their culture medium for metabolic activities. Continuous monitoring of the glucose concentration in the medium after the passage of the cell culture should make it possible to draw conclusions concerning the metabolic state of the cultivated cells (von Woedtke et al., 2002). Glucose assay allow determining the physiological state of cultured cells by measuring glucose consumption. By knowing the initial glucose concentration in the medium, its consumption rate and an IC_{50} value of the test compound were assessed by measuring glucose levels in the culture medium after incubation in the absence and presence of a test compound. Differences of glucose consumption in pyocyanin treated and control cells reflected changes in their metabolic state induced by pyocyanin toxicity. This procedure utilizes the coupled activities of glucose oxidase (GOD) and peroxidase (POD). The amount of oxidized dye in the supernatant was measured spectrophotometrically.

An aliquot of 3µl exposed cells supernatant from each wells and control wells were used for glucose assay. Supernatants were mixed with 300µl working buffer solution prepared by mixing enzyme reagent (0.125mM Amino-4-antipyrine, 30,000U l^{-1} glucose oxidase, 10,000U l^{-1} peroxidase and 100mM phosphate buffer) with 100ml assay buffer (16mM sodium phenolate). Mixed well and incubated for 15 minutes at 37ºC. Absorbance of test and standard (100mg% glucose) were measured at 500nm in a microplate reader (TECAN Infinite Tm, Austria) against blank and control. Inhibition on glucose consumption compared to control cells was calculated. Glucose concentration in mg % was calculated by the formula: OD of test x100/OD of glucose standard.

6.2.3.2.1.6. Determination of pyocyanin-generated hydrogen peroxide

Under aerobic conditions *in vitro*, pyocyanin results in the formation of the reactive oxygen species, superoxides, and hydrogen peroxide (H_2O_2). H_2O_2 is an uncharged species that penetrates membranes; therefore, H_2O_2 stress arises inside cells whenever H_2O_2 is present in their extracellular environment. Generally, millimolar doses of pyocyanin is required to induce toxicity in cells and this

concentration is far beyond the physiological relevance (Imlay, 2008). Moreover, H_2O_2 itself does not inflict any DNA damage (Angell et al., 2006)

In this assay, an aliquot of 50µl supernatant from each well was mixed with 50µl hydrogen peroxide assay mixture containing 46µl assay buffer, 2µl OxiRed™ probe solution, 2µl horse radish peroxidase (HRP) enzyme (BioVison, USA). Mixed well and incubated at room temperature for 10 minutes. Absorbance was measured at 570nm.

6.2.4. Effect of pyocyanin on nitrifying bacterial consortia used in SBSBR and PBBR

The effect of pyocyanin on nitrification process was conducted on a nitrifying bacterial consortia used in SBSBR (Stringed Bed Suspended Bio-Reactor) and PBBR (Packed Bed Bio-Reactor). Consortia were generated in nitrifying bacterial production unit maintained in three different salinities (0, 15 and 30g l^{-1}). The consortia from three different salinities were inoculated into respective saline condition supplemented with 10mg l^{-1} $(NH_4)2SO_4$, 2mg l^{-1} KH_2PO_4 (Watson, 1965) with a pH 8.5 in a 250ml culture flask, incubated in dark at room temperature with shaking at 100rpm. The nitrification process was monitored daily by checking the oxidation of NH_4 –N to NO_2-N to NO_3-N. The pH was maintained by supplementing sodium carbonate and evaporation loss by supplementing with sterile distilled water. The incubation was continued till the culture started effective nitrification and added pyocyanin to the final concentration of 10ppm to the culture flasks in triplicates. Culture flasks without pyocyanin in triplicates were used as control. After 24 hours incubation NH_4-N removal, NO_2-N build up and removal and NO_3-N build up were analyzed.

6.2.4.1. Assay for NH_4-N removal/oxidation

Removal/oxidation of NH4-N in the culture flasks containing nitrifying bacterial consortia were estimated by Phenate method (Solorzano, 1969). In this method, ammonia in the culture flask was allowed to react with hypochlorite in moderately alkaline solution (pH 8.0 to 11.5), to form monochloramine, which in presence of phenol, catalytic amounts of nitroprusside ions and excess of

hypochlorite, formed indophenol blue. The intensity of blue color is directly proportional to the concentration of ammonia present.

An aliquot of 1 ml test sample along with control were taken in test tubes and made up to 10 ml with double distilled water. Added 0.4ml phenol solution (20g of crystalline phenol in 200ml 95% v/v ethyl alcohol), 0.4ml of sodium nitroprusside solution (1.0g sodium nitroprusside in 200ml deionised water), and 1.0ml of oxidizing solution (100g Sodium citrate and 5g sodium hydroxide in 500ml deionized water with 25ml Sodium hypochlorite) and incubated for 1hour at room temperature. The absorbance was measured at 640nm against blank. A series of standards of NH_4Cl (4.714mg NH_4Cl dissolved in 100ml double distilled water gave 10μg m l^{-1}) were prepared and the factor value calculated. From this ammonia nitrogen was estimated as follows.

Ammonia nitrogen (NH_4^+-N) in mg l^{-1} = Factor Value x Absorbance of the sample

6.2.4.2. Assay for NO_2-N removal/oxidation

Under acidic condition (pH 2.0 to 2.5) nitrite ion (NO_2^- as nitrous acid (HNO_3) reacts with sulphanilamide to form a diazonium salt, which combine with N- (1-naphthyl)-ethylene diamine dihydrochloride (NED dihydrochloride) to form a bright coloured pinkish red azo dye. The colour produced is directly proportional to the amount of nitrite present in the sample (Strickland and Parson, 1968).

An aliquot of 1 ml test sample along with control were taken in a test tube and made up to 10 ml with double distilled water. Added 0.2ml of sulphanilamide (5g sulphanilamide in a mixture of 50ml conc. HCl and about 450ml distilled water) and 0.2ml of NED (0.5g NED dissolved in 500 ml distilled water) and the absorbance was measured at 543nm against blank after 8 minute. A series of standards (4.925mg $NaNO_2$ dissolved in 100ml gave 10μg ml^{-1}) were prepared and the factor value calculated. From this nitrite nitrogen was found out as follows.

Nitrite in the sample in mg l^{-1} = Factor value x Absorbance of the sample

6.2.4.3. Assay for NO_3-N build up

Nitrate build up was determined by converting nitrate to nitrite using phenol – NaOH mixture and $CuSO_4$ - hydrazine sulphate mixture. Reagents were added and incubated in the dark for 18 hours, added acetone, complexed with sulphanilimide and NED **(**Bendschneider and Robinson, 1952**).**

An aliquot of 1ml test sample along with control were taken in a test tube and made up to 10ml with double distilled water. Added 0.4ml of buffer reagent (Phenol solution (46g dry AR phenol in 1000ml distilled water) and NaOH (30g in 2000ml (1:1 ratio)), 0.2ml hydrazine sulfate (14.5g $N_2H_4.H_2SO_4$ in 2000ml distilled water) and $CUSO_4$ (0.1g AR $CuSO_4.5H_2O$ in 1000ml distilled water (1:1 ratio) incubated in dark at room temperature for 18-24 hours. Added 0.4ml acetone, 0.2ml of sulphanilamide and 0.2ml of NED mixed well and incubated for 8 minute at room temperature, the absorbance was measured at 543nm against blank. A series of standards (6.0707mg $NaNO_3$ dissolved in 100ml gave 10µg ml^{-1}) and the factor value calculated. From this nitrate nitrogen was found out as follows.

Concentration of nitrate in sample in mg l^{-1} = [(x-y) x 100/ efficiency]

Where x = Absorbance of nitrate x Factor value of nitrite

y = corresponding concentration of nitrite

Efficiency = (A/B) x 100

Where A = Observed concentration of standard (absorbance x factor value of nitrite), B = Original concentration of standard prepared.

6.3. Results

6.3.1. Brine shrimp lethality assay

Pyocyanin-induced toxicity on brine shrimp was studied over a period of 12 and 24 hours by exposing 20 brine shrimp larvae to different concentrations of pyocyanin. Percentage mortalities amongst groups of brine shrimp exposed to pyocyanin for 12 and 24 hours are shown in Fig. 9. From Probit analysis, LC_{50} values calculated were 321.45 ± 38.33mg l^{-1} and 217.48 ± 43.19mg l^{-1} for exposure times of 12 and 24 hours, respectively.

6.3.2. Larval lethality assay

For assessing the toxicity of pyocyanin in penaeid as the model animal, different stages of the life cycle of *P.monodon* were selected. The LC_{50} values were found to be 24±6.6mg l^{-1} for nauplius, 7±2.4mg l^{-1} for zoea and 10.69±4.6mg l^{-1} for mysis. For post larval stages, it was 20.35±5.9 mg l^{-1} for PL-5 and 33.92±6.7 mg l^{-1} for PL -15. These results show that zoea and mysis stages were more susceptible to pyocyanin-induced toxicity than the nauplius and post larval stages (Fig. 10).

6.3.3. Cytotoxicity of pyocyanin on various cell lines

In Sf9 cells, pyocyanin at higher concentrations (175 and 200ppm) caused morphological changes such as clumping and necrosis, on observing microscopically (Fig. 17). IC_{50} value of pyocyanin on inhibition of mitochondrial dehydrogenase was 106.39±13.92mg l^{-1} and plasma membrane damage was 107.77±28.14mg l^{-1} respectively (Fig. 11, 12). Moreover, the IC_{50} value on protein synthesis was found to be a little higher with a concentration 133.67±23.6mg l^{-1} (Fig. 13). At 25mg l^{-1}, pyocyanin caused 3.9% inhibition on mitochondrial activity, 19.6% plasma membrane damage and 2.1% inhibition on protein synthesis in Sf9 cells. At lower concentration (6.25ppm) the toxicity was undetectable. Whereas at 200 mg L^{-1} these value were 65.5%, 67.5% and 56% respectively.

In RTG-2 cells, necrosis and cell rounding were observed only at higher concentrations above 150mg l^{-1} (Fig. 18). IC_{50} values of pyocyanin were 146.19±28.78mg $l^{-1,}$ 109.83±11.28mg l^{-1} and 76.64±9.99mg l^{-1} for inhibition of mitochondrial dehydrogenase, plasma membrane damage and inhibition of protein synthesis respectively (Fig. 11, 12, 13). Pyocyanin - induced toxicity in RTG-2 at 25mg l^{-1} concentration of pyocyanin was 4.8% inhibition on mitochondrial activity, 6.2% plasma membrane damage, and 20.8% inhibition on protein synthesis. At lower concentration (6.25ppm) the toxicity was undetectable. However, toxicity observed at the maximum concentration of pyocyanin tested (200mg l^{-1}) was 68.8% inhibition on mitochondrial activity, 67.7% plasma membrane damage and 77.9% inhibition on protein synthesis.

Human embryonic lung epithelial cell line (L-132) was more prone to pyocyanin-induced toxicity compared to the insect (Sf9) and fish (RTG-2) cell lines (Fig. 19). IC_{50} value of pyocyanin on inhibition of mitochondrial dehydrogenase activity was 112.01±23.73mg l^{-1} (Fig. 11). Meanwhile, the IC_{50} value of pyocyanin induced damage on plasma membrane was 21.79±14.23mg l^{-1} and the same on inhibition of protein synthesis with 32.57±16.52mg l^{-1}. (Fig. 12, 13). At a concentration of 25mg l^{-1} pyocyanin, 3.9% inhibition on mitochondrial activity, 47.3% plasma membrane damage and 26.6% inhibition on protein synthesis were observed in L-132 cells. At lower concentration (6.25ppm) the toxicity was negligible.Whereas at 200mg l^{-1} the values were 64.8%, 72.8% and 91.7% respectively.

In addition, determination of plasma membrane damage induced by pyocyanin (contact time 24h) through extracellular LDH activity (in terms of % reduction in NADH) showed only 16.52%, 19.81% and 43.96% damage at a concentration 25mg l^{-1} and 77%, 68.42% and 80% damage at 200mg l^{-1} in RTG-2, Sf9 and L-132 cells respectively (Fig. 14). At lower concentration the toxicity observed was negligible. Besides, control cells showed 9.32%, 1.13% and 14.82% extracellular LDH activity as the results of normal metabolic activity for RTG-2, Sf9 and L-132 cells respectively.

6.3.3.1. Effect of pyocyanin-induced toxicity on glucose metabolism

Pyocyanin-induced inhibition on glucose metabolism was observed in human cell lines having the IC_{50} value 19.1±11mg l^{-1}. In fish cell line it was 21.1±5.8mg l^{-1} and in Sf9 cells, the toxicity was multifold lesser with higher IC_{50} value of 70.6±28.7mg l^{-1} (Fig. 15). At 200mg l^{-1}, pyocyanin inhibited glucose metabolism by 100% in L-132 cells, 98.2% in RTG-2 cells and 70.5% in Sf9cells. However, at 25mg l^{-1} the values were 65.5%, 59.4% and 38.4% respectively.

6.3.3.2. Pyocyanin induced hydrogen peroxide production

Exposure of cells (L-132, RTG-2 & Sf9) to pyocyanin resulted in the formation of H_2O_2 in a dose-dependent manner. Base level production of H_2O_2 by untreated cells was determined to be 0.026µM for L-132 and Sf9 cells and 0.024µM

for RTG-2 cells respectively. An increase of 46.2%, 53.8% and 54.2%, of H_2O_2 concentration from the base level was observed in Sf9, L-132 and RTG-2 cells in the presence of pyocyanin at a concentration 25mg l^{-1} with a value of 0.038µM, 0.04µM and 0.037µM respectively. The production of H_2O_2 at 200mg l^{-1} was 0.06µM, 0.064µM and 0.056µM with a percentage increase of 130.8%, 146.2% and 133.3% for Sf9, L-132 and RTG-2 cells respectively (Fig. 16).

6.3.4. Effect of pyocyanin on nitrifying bacterial consortia used in SBSBR and PBBR

Pyocyanin did not inhibit nitrification in the three nitrifying bacterial consortia maintained at 0, 15 and 10g l^{-1} salinities (Fig. 20, 21, 22). The ammonia – nitrogen was completely utilized by all the consortia by the 2nd day in both the controls and tests (Fig. 14). Results of one way ANOVA showed that there was no significant difference in the ammonia, nitrite and nitrate concentrations between the experimental and control sets.

6.4. Discussion

Environmental isolates of *P. aeruginosa* and the inhibitory compound produced by them (pyocyanin) have been accepted as probiotics as well as antagonistic compound against pathogenic *Vibrio* spp. in aquaculture systems (Preetha et al., 2010; Hai et al., 2009a, b; Pai et al., 2010). In favour of this concept, the degradation potential and the environmental detoxification of pyocyanin and its precursors have been well documented (Hill and Johnson, 1969; Chen et al., 2008; Reszka et al., 2004). Reszka et al. (2004) and Price-Whelan et al. (2006) identified that phenazine-1-carboxylic acid (PCA) got completely degraded within 40 hours by soil organism *Sphingomonas* sp. DP58 (Yang et al., 2007). However, the pyocyanin-induced toxicity is still a controversial issue as the studies have been mainly performed with clinical isolates focusing on its dimensions clinically (Gloyne et al., 2011; Muller et al., 2009). In this context, determination of the toxicity of pyocyanin from the environmental isolates and its IC_{50} values in various biological systems including human cell lines give a deeper understanding on the concentration at which it can be applied in aquaculture system to control pathogenic vibrios. This will pave

way for the safe and environment friendly application of pyocyanin as the drug of choice either as prophylaxis or therapy in aquaculture systems.

Brine shrimp nauplii and shrimp larvae and post larvae have been used for lethality/toxicity assays in dose-response manner (Vinayak et al., 2011; Harwing and Scott et al., 1971). In literature no study has ever been reported on the toxicity of pyocyanin on biological systems other than the study conducted by Chythanya et al. (2002) on the toxicity of chloroform extract of *Pseudomonas* 1-2 in *P. monodon* post-larvae PL-18 and Preetha et al. (2010) on *in vitro* toxicity studies of pyocyanin from *Pseudomonas* MCCB102 in primary haemocyte culture of *P. monodon*. Vijayan et al. (2006) studied the pathogenecity of environmental isolates of *Pseudomonas* PS-102 and reported that it did not cause any lethality to shrimp larvae (Pl-9) upon challenge even at a dosage of 10^7 cells, and a higher LD_{50} to BALB/c mice (10^9 cells) suggested its safety to mammalian system. Subsequently, this isolate was deposited with the Culture Collection of National Central for Aquatic Animal, Cochin University of Science and Technology, India, as *Pseudomonas* MCCB102 and the same could be used by Preetha et al. (2010) and concluded that the inhibitory compound produced by the organism was pyocyanin. In the present study, *Pseudomonas* MCCB102 has been identified as *Pseudomonas aeruginosa* (Refer Chapter-2). This has been commercialized as the probiotics preparation PS - 1^{TM} (www.ncaah.org ,NCAAH, India) for shrimp aquaculture systems against pathogenic *Vibrio* spp.

The LC_{50} values of pyocyanin on brine shrimp nauplii exposed for 12 and 24 hours were 321.45 ± 38.33mg l^{-1} and 217.48 ± 43.19mg l^{-1} respectively. At higher concentration (450 and 500mg l^{-1}) within 24 hours of exposure the toxicity was 100%, indicating that the toxicity was concentration and time dependent. However, this is multifold higher in concentration than the required one to treat pathogenic *Vibrio* in general and *Vibrio harveyi* in particular. In larval lethality assay, zoea and mysis stages were found to be more susceptible to pyocyanin toxicity with LC_{50} value 7±2.4mg l^{-1} and 10.69±4.6mg l^{-1} respectively. Whereas, nauplii and postlarval stages were able to withstand higher concentrations of pyocyanin having LC_{50} values of 24±6.6mg l^{-1}, 20.35±5.9mg l^{-1} and 33.92±6.7mg l^{-1} respectively in nauplii and the post larval stage 5 (PL-5) and 15 (PL -15).

Pyocyanin-induced toxicity in human cell lines has been studied extensively. Muller (2006) reported that 23.8 to 47.6mg l^{-1} (5–10µM) pyocyanin arrested cell growth and resulted in the development of a morphological phenotype consistent with cellular senescence in A549 cells and all cells treated with pyocyanin at 47.6mg l^{-1} (10µM) were to be the senescent phenotype and were stable for 7 days. Moreover, exposure to pyocyanin at 119mg l^{-1} (25µM) resulted in detachment of cells from their substratum, shrinkage, and blabbing of cell membranes, morphological characteristics of apoptosis and leading to cell death (Muller, 2006). In our study, pyocyanin at very higher concentrations (175mg l^{-1} and 200mg l^{-1}) only caused significant morphological changes such as clumping, and necrosis as visualized microscopically in all cell lines tested (Sf9, RTG-2 and L-132). Human cell membrane was found to be more susceptible to oxidative damage induced by pyocyanin in comparison with the insect and fish cell lines. The IC_{50} values on plasma membrane damage for Sf9 cells and RTG-2 cells were almost similar (106.39±13.92mg l^{-1} and 109.83±11.28mg l^{-1}respectively), whilst for L-132 it was 21.79±14.23mg l^{-1}. In addition, pyocyanin induced plasma membrane damage could be compared with the extracellular LDH activity which showed a percentage activity of 16.52%, 19.81% and 43.96% at a concentration of 25mg l^{-1} pyocyanin for RTG-2, Sf9 and L-132 cells respectively.

Measurement of cellular metabolic activity through mitochondrial dehydrogenase system revealed that it required higher pyocyanin concentration to inhibit the mitochondrial dehydrogenase activity. RTG-2 showed highest IC_{50} value of 146.19±28.78mg l^{-1} on mitochondrial dehydrogenase. Whilst, insect cell line (Sf9) and human cell line (L-132) showed a similar pattern of inhibition having IC_{50} values 107.77±28.14mg l^{-1} and 112.01±23.73mg l^{-1} respectively. Even though at higher pyocyanin concentration Sf9 resisted pyocyanin induced inhibition on protein synthesis with an IC_{50} value 145.94±25.38mg l^{-1} the human cell line L-132 showed more susceptibility with higher damage on protein synthetic mechanism (IC_{50} 32.57±16.52mg l^{-1}) than that of fish cell line (IC_{50} 76.64±9.99mg l^{-1}).

In this study, pyocyanin induced toxicity in all cell lines tested could be linked to the hydrogen peroxide production in a dose-dependent manner and the lowering in glucose consumption. This supported the findings of Muller (2006) who pointed out that the toxicity was due to the oxidative stress/injury caused by

pyocyanin mediated hydrogen peroxide production in a dose-dependent manner and could be prevented/controlled by using antioxidants.

In the aquaculture industry, Recirculating aquaculture system (RAS) has emerged as the major environmentally sustainable solution (Kumar et al., 2010) to the environmental impact of aquaculture. One of the major limiting factors in such RAS operated prawn/shrimp hatcheries is the accumulation of NH_4^+-N and NO_2^- - N to toxic levels (Achuthan et al., 2006; Chen and Cheng, 1995). The most prominent requirement of any recirculating aquaculture system (RAS) is an efficient biofilter to prevent the accumulation of toxic metabolites such as ammonia and nitrite (Kumar et al., 2009b). Packed bed bioreactor (PBBR) and Stringed bed suspended bioreactor (SBSBR) are the nitrifying bio reactors designed for RAS based aquaculture systems (Kumar et al., 2009a,c; Kumar et al., 2010). In such systems once the pathogenic *Vibrio* enters it might become too difficult to be eliminated to ensure bio-security. Antibiotics and other chemotherapeutants would cause damage to the nitrifying bacterial consortia in the bioreactors. In this context, pyocyanin stand out as the drug of choice to eliminate the pathogen with out doing any harm to the nitrifying bacterial consortia used in PBBR and SBBR as at 10mg l^{-1} pyocyanin was found not to inhibit nitrification.

The present study revealed that pyocyanin did not inhibit nitrification in the three nitrifying bacterial consortia maintained at 0, 15 and 10g l^{-1} salinities. Ammonia – nitrogen could be completely utilized by all the consortia by the 2nd day in both the control and in the pyocyanin added cultures. The same was the situation of nitrite – nitrogen. The results of the statistical analysis showed that there was efficient nitrification with no significant difference in the ammonia, nitrite and nitrate concentrations between the experimental cultures and the control. It has to be pointed out that it is these nitrifying bacterial consortia which are used for activating nitrifying bioreactors for nitrification in aquaculture systems (Achuthan et al., 2006). The mass produced consortia (Kumar et al., 2009a) are used to activate commercialized Packed Bed Bioreactors (PBBR) and Stringed Bed Suspended Bioreactors (SBSBR) (Kumar et al., 2009b, c; Kumar et al., 2010). The results of the present study showed that the pyocyanin could be applied in recircualting aquaculture systems integrated with PBBR or SBBR to control *V. harveyi* as it does not inhibit nitrification of the consortia. However, more *in vivo* studies are required under real life situations to confirm the results.

The overall cytotoxicity study with pyocyanin from environmental isolates revealed that the IC_{50} values were multifold higher than the required concentration of pyocyanin to treat pathogenic *Vibrio* in general and *V. harveyi* in particular by applying to the culture system. In our study, MIC (bacteriostatic) of pyocyanin on *Vibrio harveyi* was 5mg l^{-1}, whilst at 10mg l^{-1} it turned out to be bacteriocidal. At these concentrations the toxicity detected in cell lines was either undetectable or negligible.

In conclusion, the study demonstrated the pyocyanin - induced toxicity in various biological systems and the effect on nitrifying bacterial consortia used in bioreactors integrated with recirculating aquaculture system. Despite the toxicity induced by pyocyanin from clinical isolates, the pyocyanin from environmental isolates can be used as the prophylactic agent to control pathogenic *Vibrio* spp. in aquaculture systems including the recirculating aquaculture system.

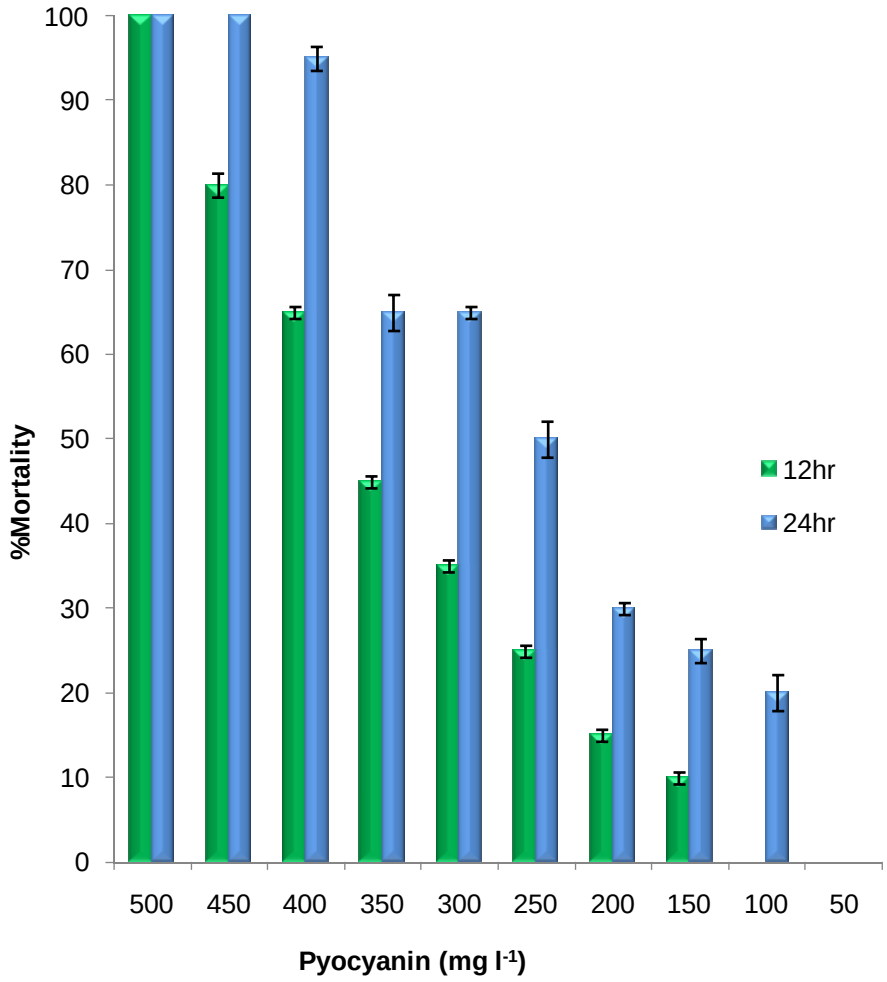

Fig.9 Brine shrimp lethality assay using *Artemia salina* nauplii. The results represent the mean ± standard deviation (n= 3).

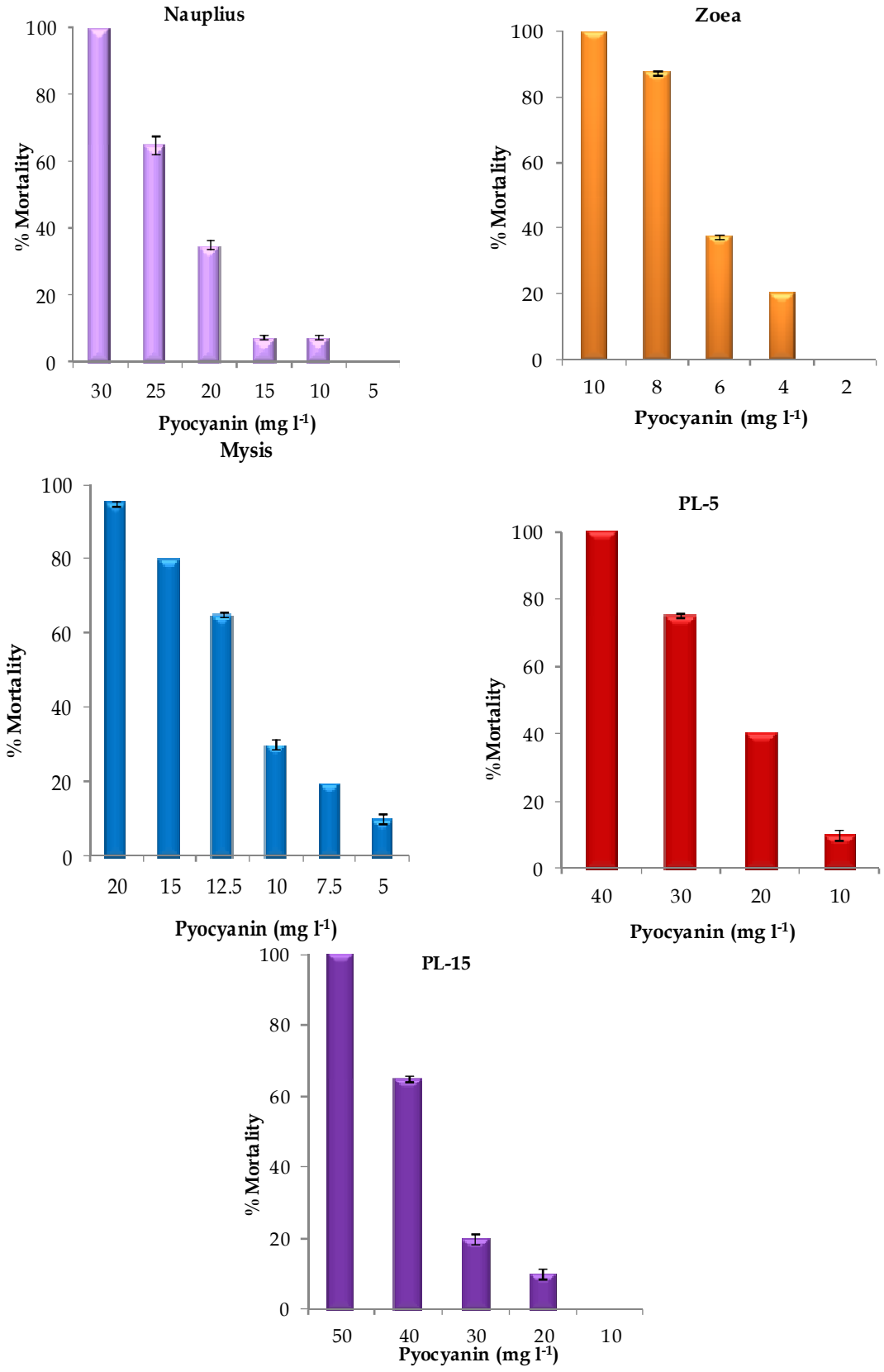

Fig.10 Larval lethality assay using nauplii, zoea,mysis and post-larvae (PL-5&15). The results represent the mean ± standard deviation (n= 3).

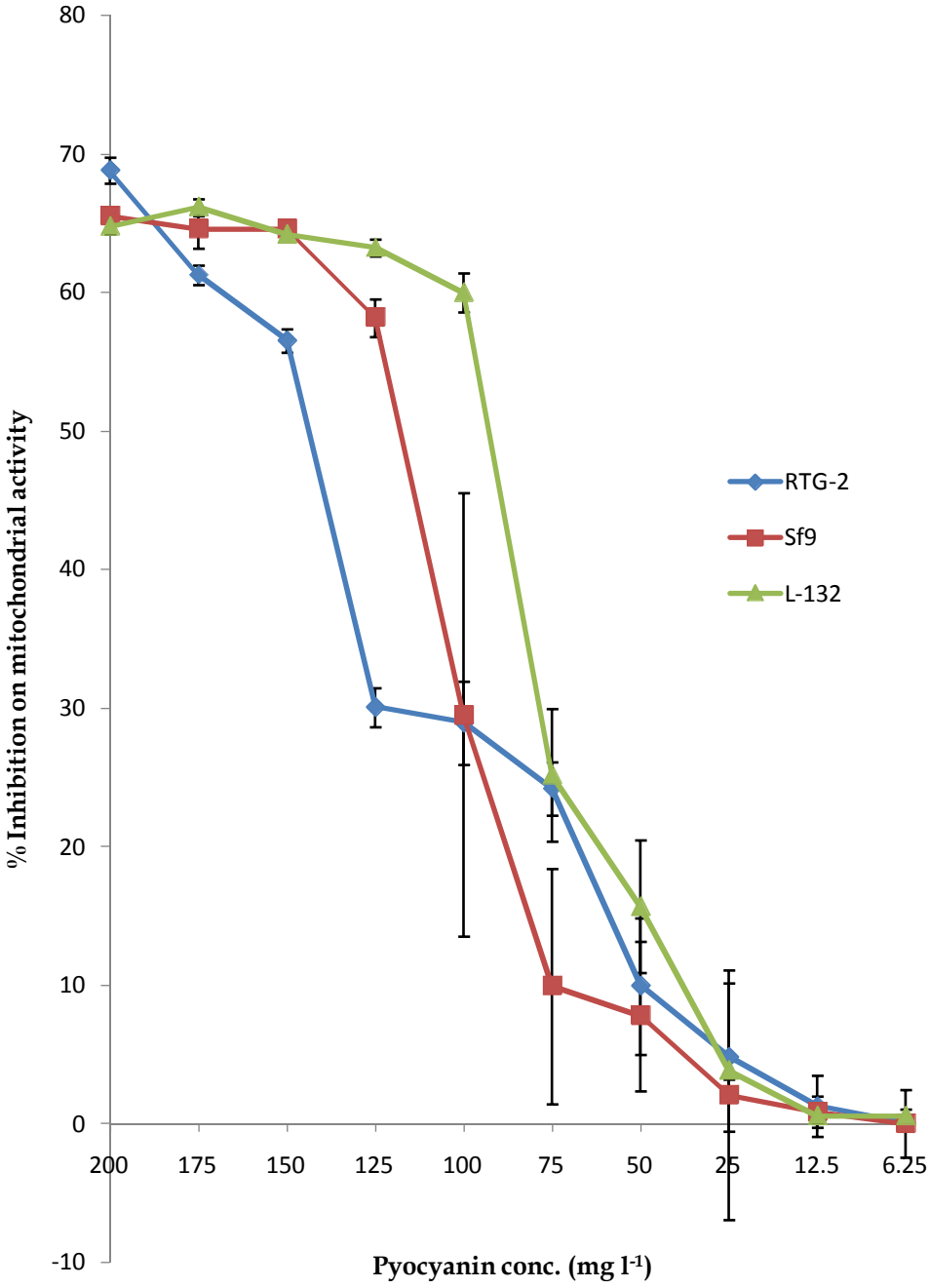

Fig. 11 Pyocyanin induced inhibition on mitochondrial dehydrogenase activity in different cell lines tested.

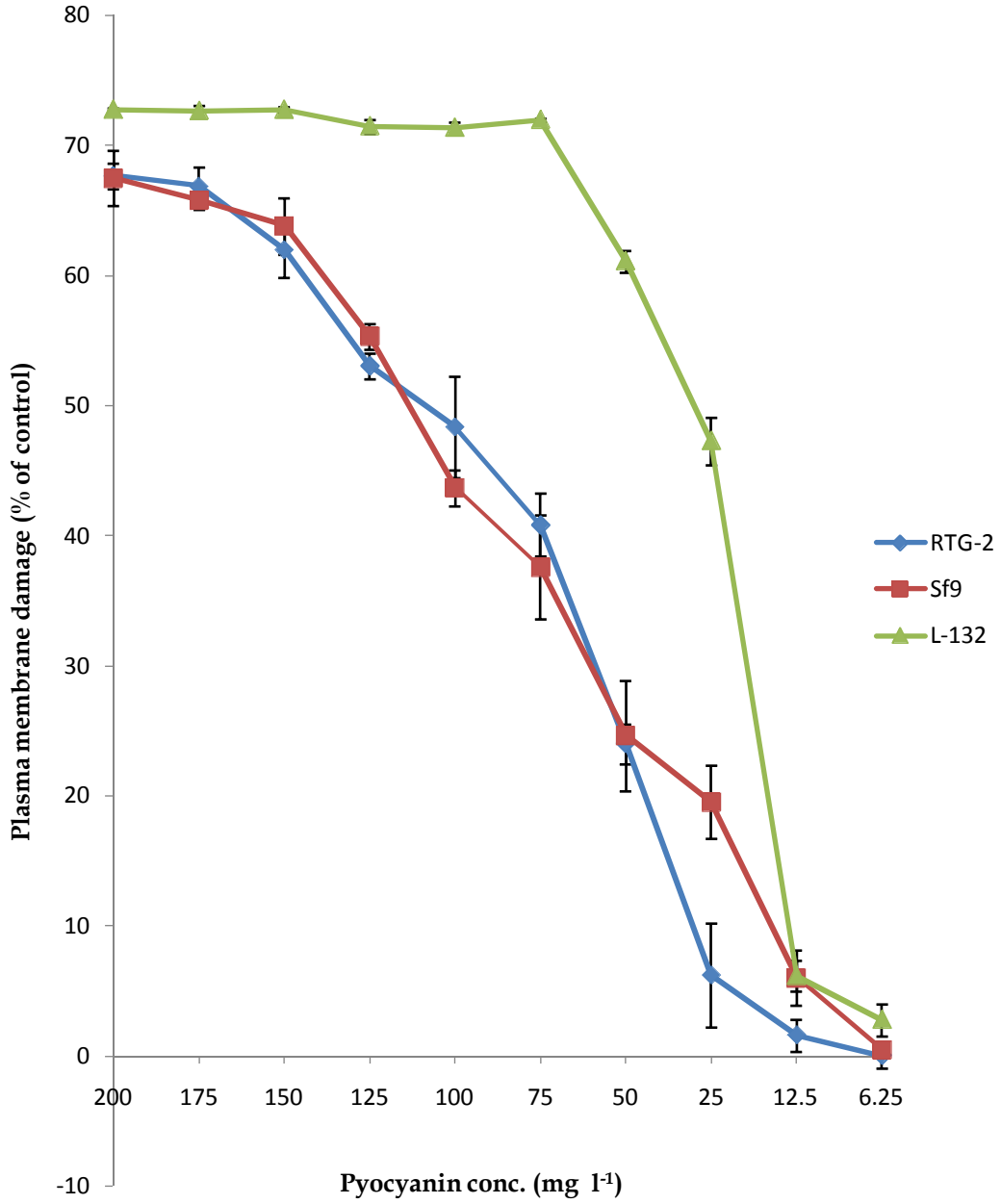

Fig.12 Pyocyanin induced oxidative damage on plasma membrane of different cell lines tested.

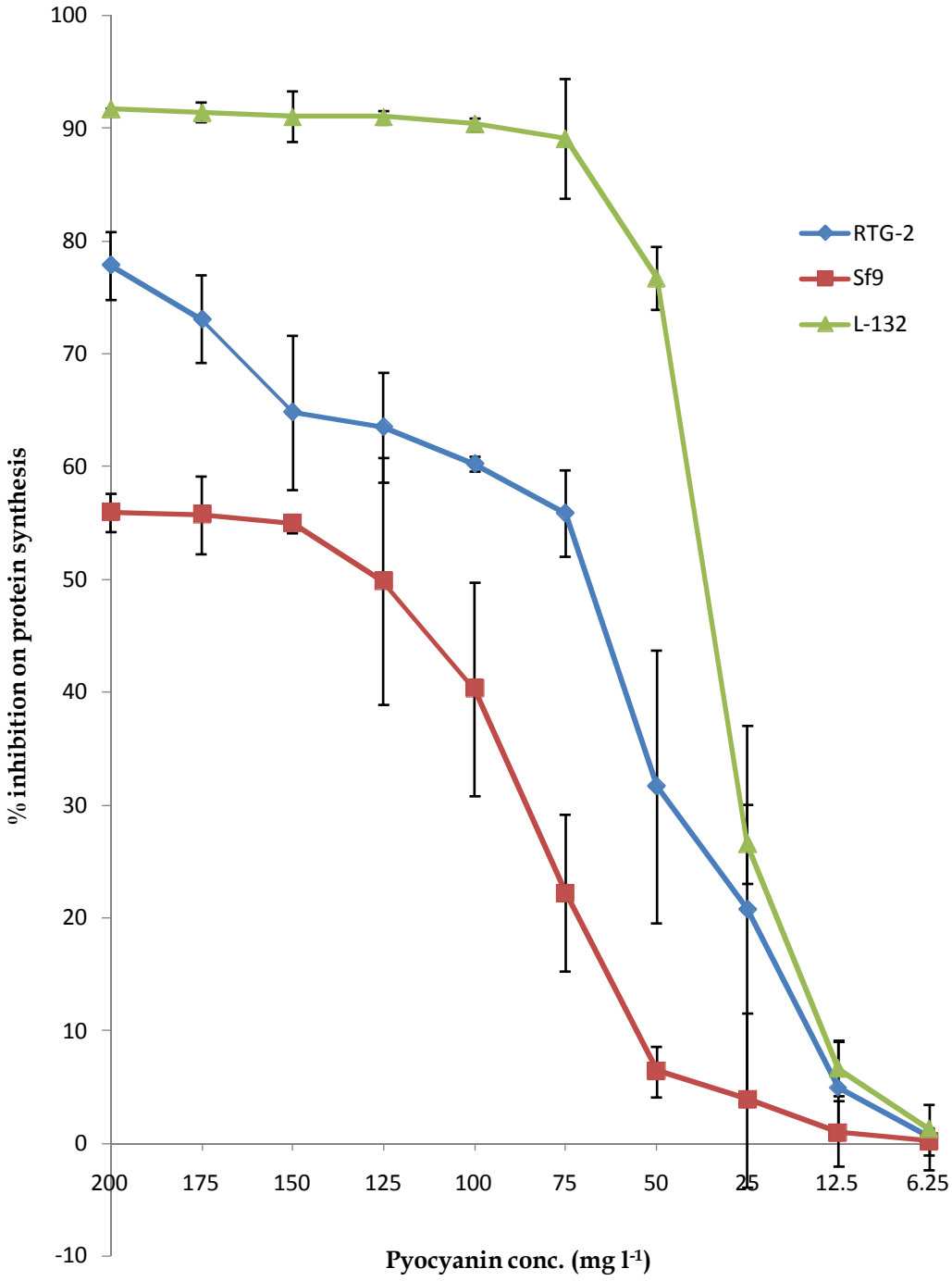

Fig.13 Pyocyanin induced inhibition on protein synthesis in different cell lines tested.

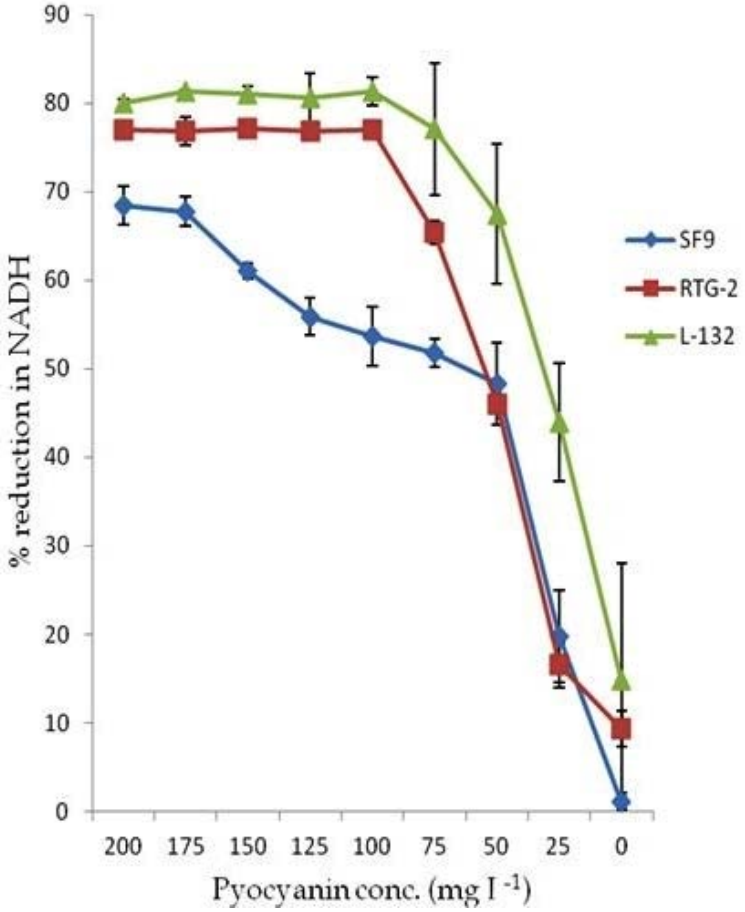

Fig.14 Extracellular LDH activity in terms of % reduction in NADH of different cell lines tested.

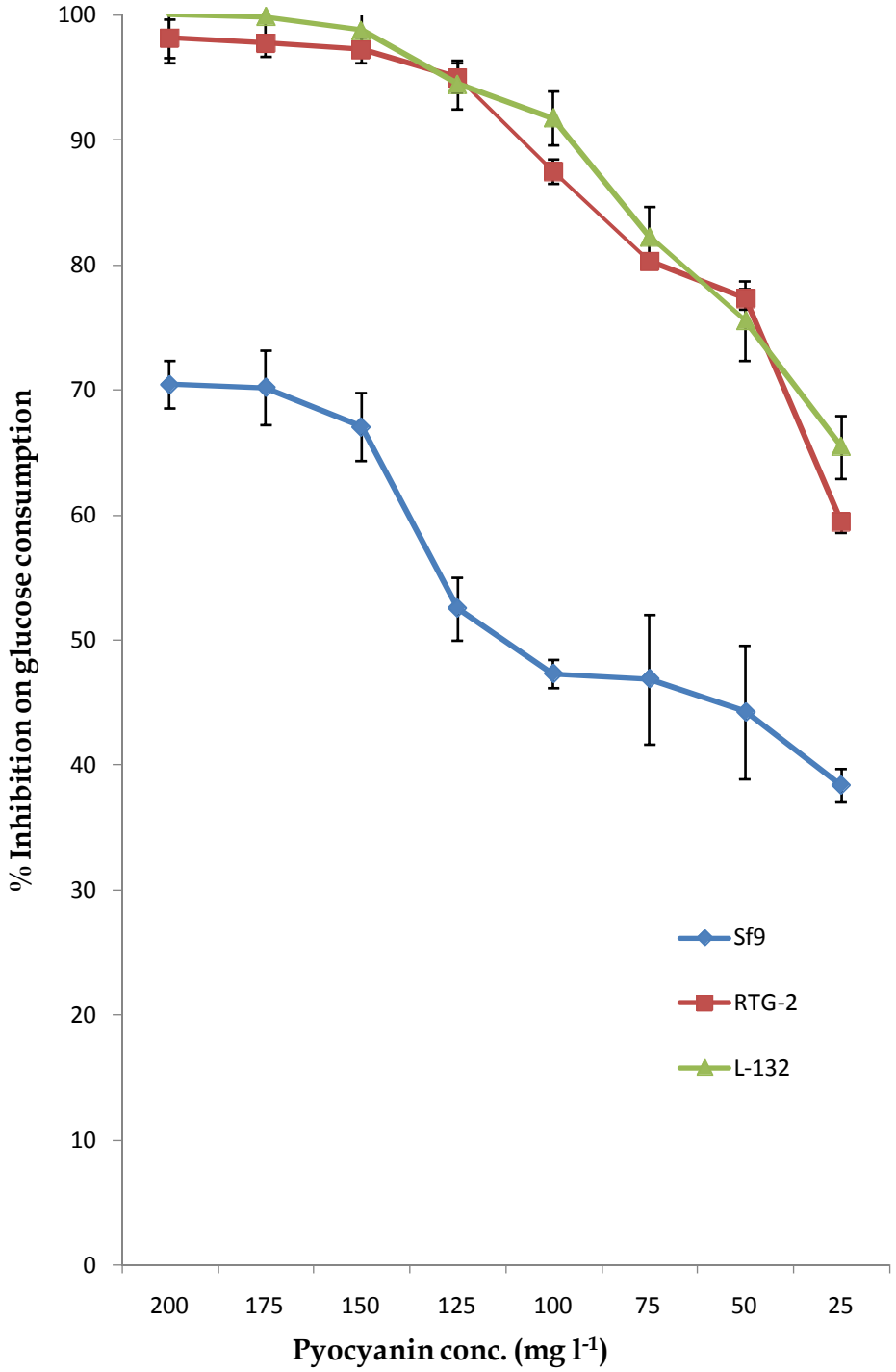

Fig. 15 Pyocyanin induced effect on glucose metabolism in different cell lines tested.

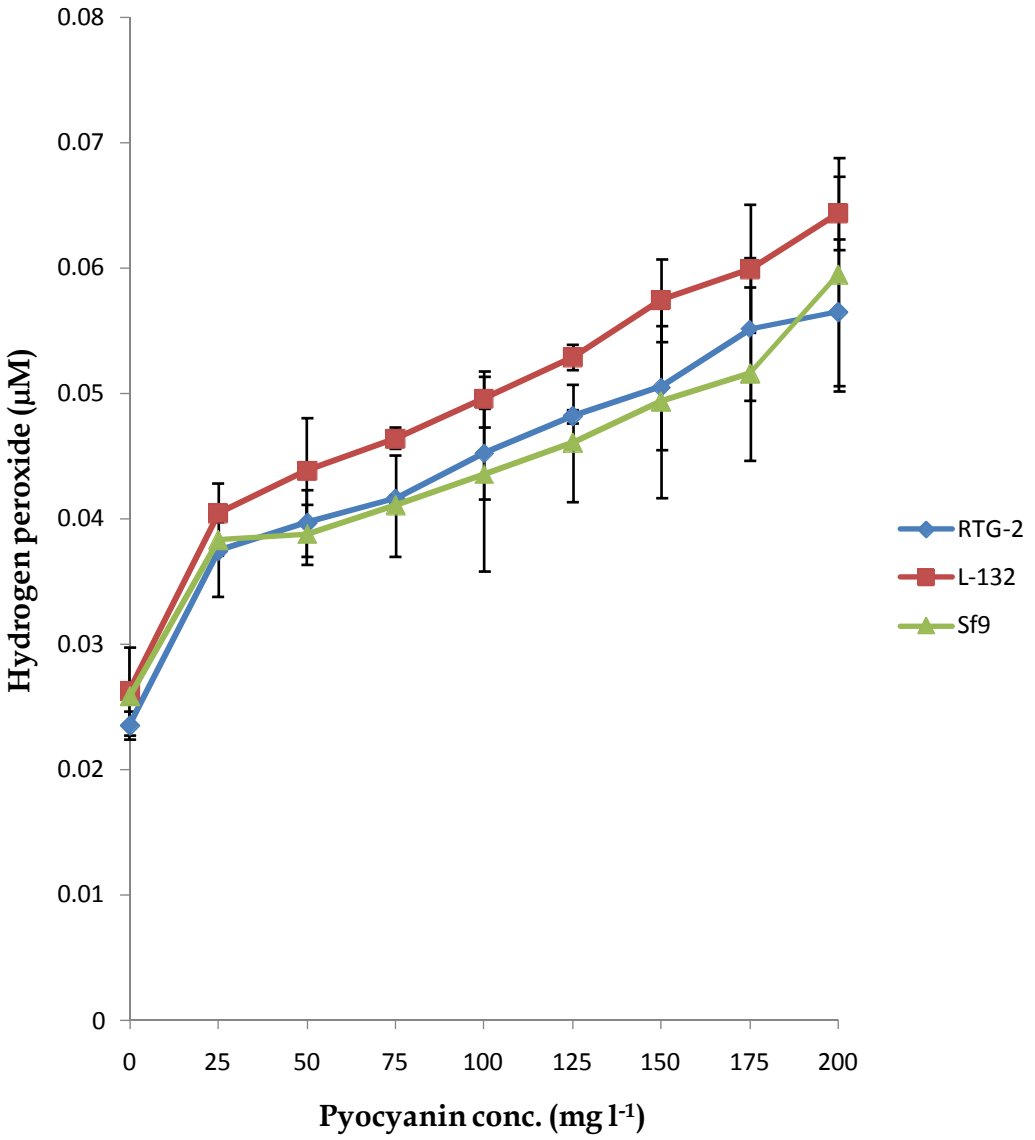

Fig. 16 Pyocyanin induced generation of H_2O_2 in dose dependent manner in different cell lines tested.

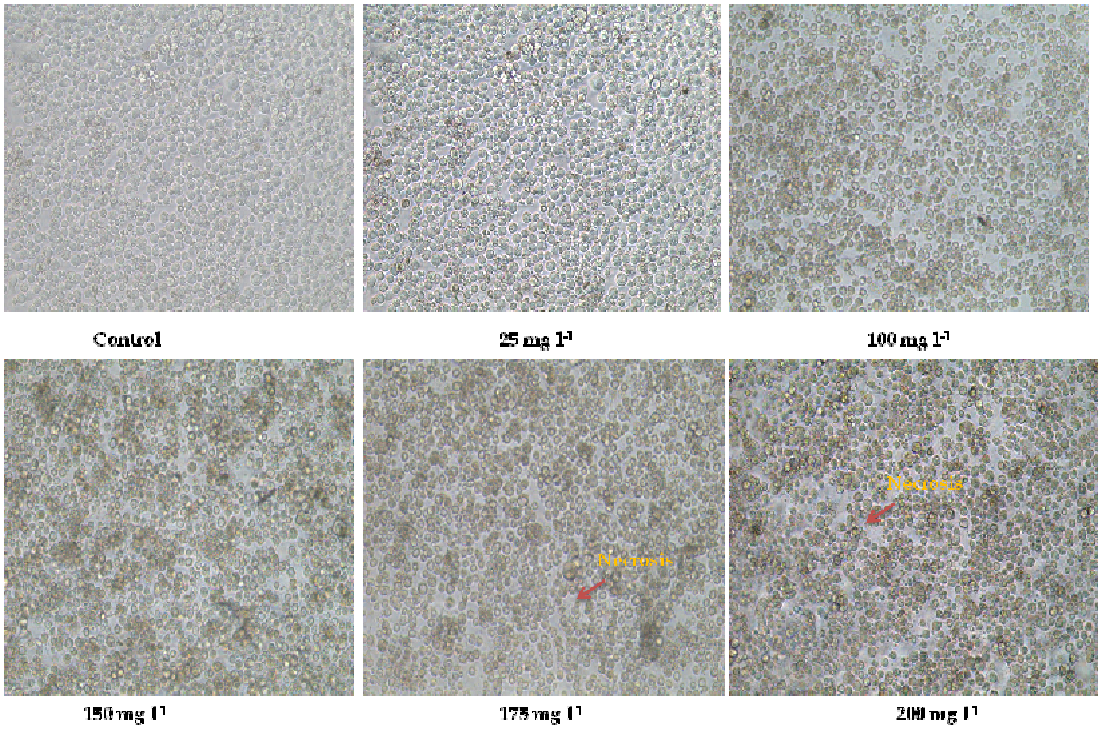

Fig.17 Pyocyanin induced toxicity in insect cell line (Sf9) 20x

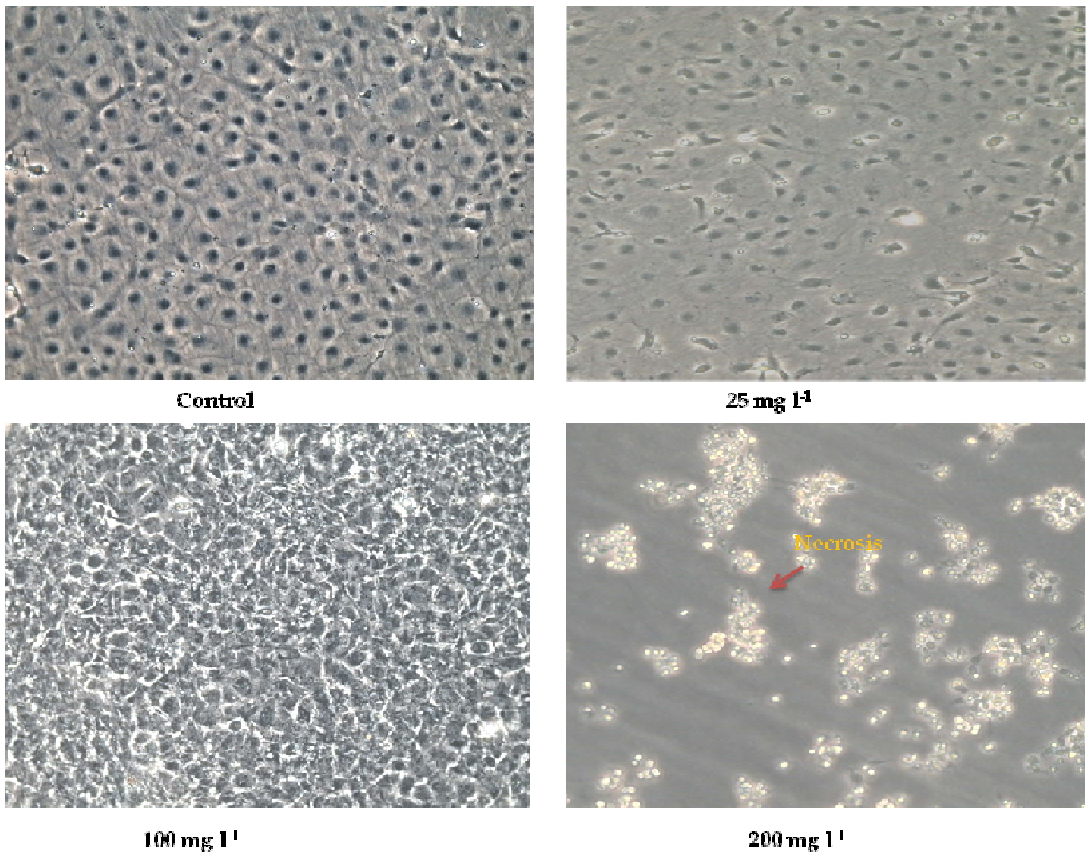

Fig.18 Pyocyanin induced toxicity in fish cell line (RTG-2) 20x

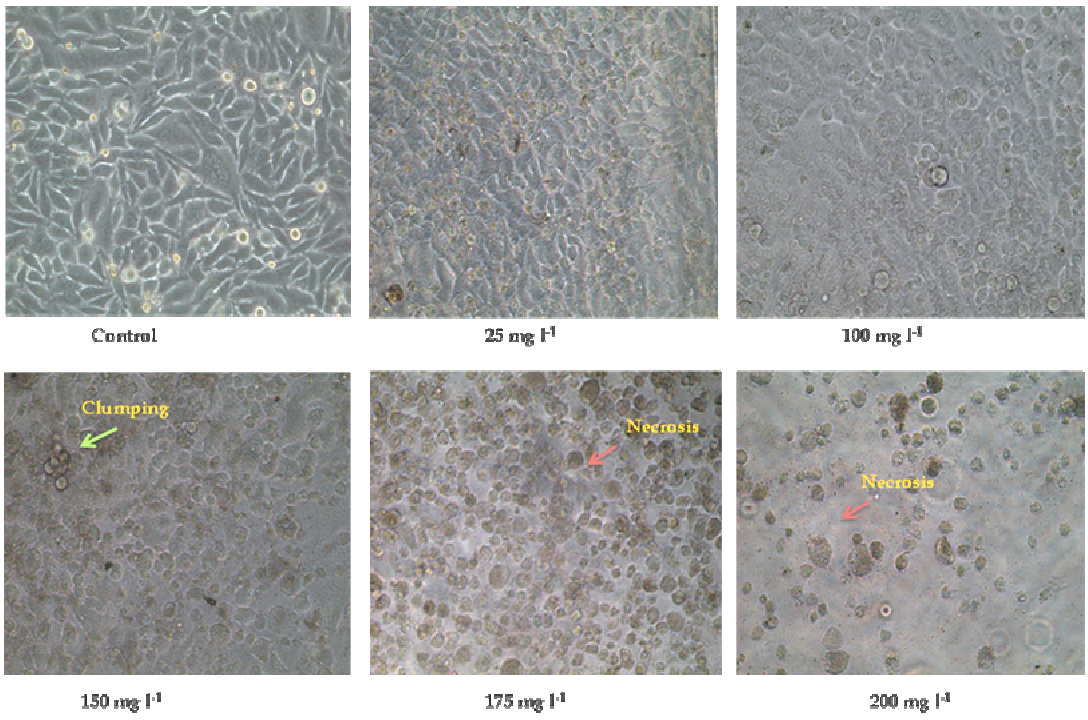

Fig.19 Pyocyanin induced toxicity in human embryonic lung epithelial cells (L-132) 20x

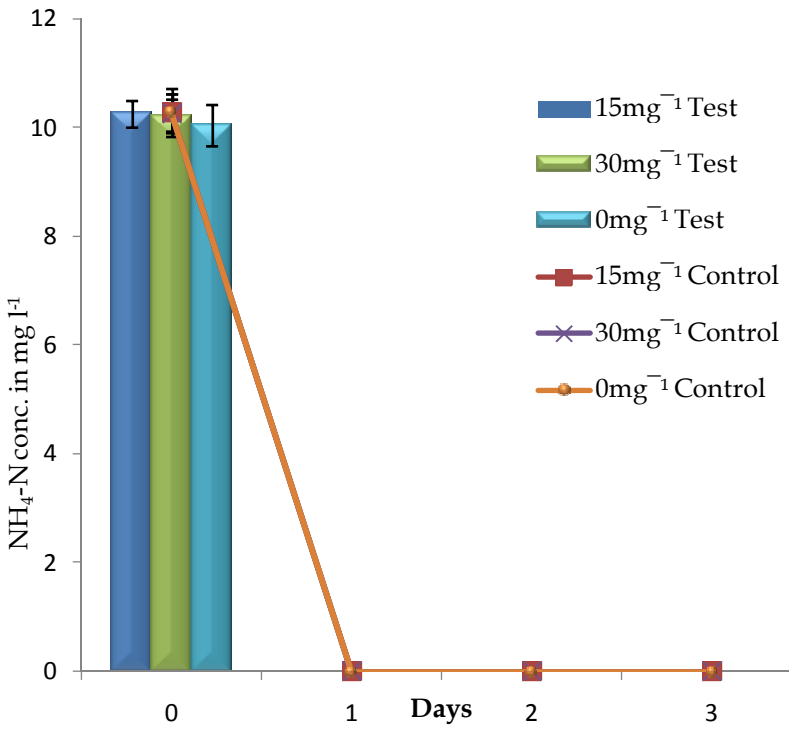

Fig. 20 Ammonia nitrogen removal by the nitrifying bacterial consortia at 10mg l^{-1} pyocyanin.

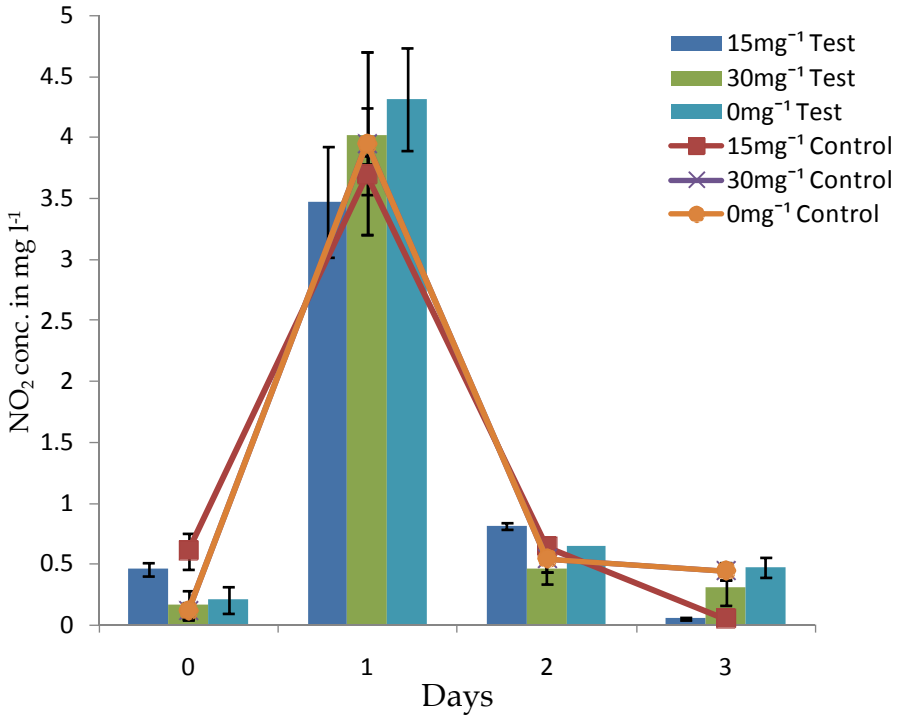

Fig. 21 Nitrite - nitrogen build-up and removal by the nitrifying bacterial consortia at 10mg l^{-1} pyocyanin.

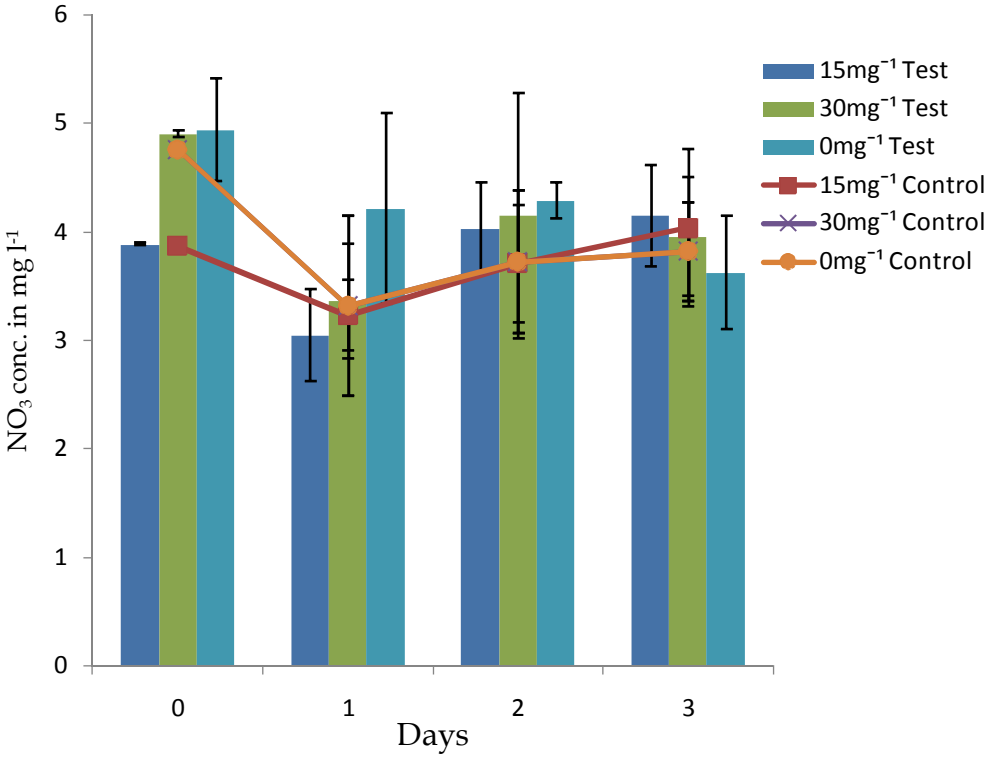

Fig. 22 Nitrate-nitrogen formation/build up by the nitrifying bacterial consortia at 10mg l^{-1} pyocyanin.

Chapter 7

CONCLUSION AND SCOPE FOR FUTURE RESEARCH

More than 360 species of aquatic animals are being cultured worldwide, among them around 25 are high value fishes traded globally (FAO, 2010). Among the cultured animals, penaeid shrimp has taken up predominant position in aquaculture industry attaining globally the sixth position in terms of value among all taxonomic groups of animals cultivated, and continues to be the largest single commodity in terms of value, accounting for 15 percent of the total internationally traded fishery products (FAO, 2006 and 2010). As aquaculture intensifies and diversifies, the biological hazards and risks to farmed animals also increase in number and diversity, with potentially serious consequences.

Diseases are recognized as a major constraint and also a limiting factor for sustainable shrimp farming. Similar to viral diseases, bacterial diseases are equally important in causing mass mortality in cultured shrimp. Among bacteria, *Vibrio* has been implicated as the causative organism, which may trigger mortalities up to 100% (Lightner., 1983). Moreover, *Vibrio harveyi* has been considered as the most important bacterial pathogen to penaeids both in hatchery and in grow out systems in India and in all South East Asian countries. The most common and prevalent mode of control of bacterial disease was the application of antibiotics both in prophylaxis and therapy which resulted in multiple antibiotic resistance and horizontal transmission of plasmids from aquaculture pathogens to those of humans. In this context the development of an alternative approach to address the problem was invariable which brought forth the concept of probiotics antagonistic to vibrios in general and *V. harvaeyi* in particular. In this thesis *Pseudomonas aeruginosa* has been identified as the probiotic and the bioactive compound and pyocyanin,

produced by it as the drug of choice against vibriosis to be applied in water either as prophylactic or as therapy.

The thesis deals with a detailed description of the identification of *Pseudomonas aeruginosa* isolated from various ecological niches and its antagonism to pathogenic vibrios in aquaculture. Subsequently the saline-dependent production of pyocyanin in *Pseudomonas aeruginosa* originated from different ecological niches and its selective application in aquaculture realm are dealt with. As the extent of production of pyocyanin by the wild strains was found to be lower, strain improvement by increasing the copy number of the pyocyanin gene was attempted on the marine isolate MCCB117 and accomplished with enhanced production and the genetically modified strain was named as *Pseudomonas* PA-pUCP-Phz^{++}. Subsequently, purification, structural elucidation and downstream process of pyocyanin could be accomplished besides a detailed study on the toxicology in animal models and in cell lines.

The application of *P. aeruginosa* as probiotics was under controversies as pyocyanin-induced toxicity was observed from clinical isolates (Gloyne et al., 2011 Muller et al., 2009). It was in this background the present study was conceptualized and executed focusing on the development of environmental isolates of *P. aeruginosa* as probiotics and the pyocyanin produced by them as the drug of choice in aquaculture against Vibriosis, even in recirculating aquaculture systems as alternatives to antibiotics.

The subject matter in this thesis has been divided under the following heads:

1. Identification of *Pseudomonas* isolated from various ecological niches and its antagonism to pathogenic vibrios in aquaculture.
2. Saline dependent production of pyocyanin in *Pseudomonas aeruginosa* originated from different ecological niches and their selective application in aquaculture.
3. Cloning and overexpression of *Phz* genes encoding phenazine biosynthetic pathway for the enhanced production of pyocyanin in *Pseudomonas aeruginosa* MCCB117.

4. Development of an appropriate downstream process for large scale production of pyocyanin from PA-pUCP-Phz^{++}; Structural elucidation and functional analysis of the purified compound.
5. Toxicity of pyocyanin on various biological systems

Overall achievements of this work are summarized as given below:

- *Pseudomonas aeruginosa* from various ecological niches such as freshwater brackish water, marine sediment were isolated and phenotypically and genotypically characterized, identified and deposited in GenBank with accession nos. EF062514 (MCCB102), EF053508 (MCCB103), EF062511 (MCCB117), EF062512 (MCCB118), EF062513 (MCCB119).
- The isolates identified from marine sediment MCCB117 and MCCB118, brackish water environment MCCB102 and MCCB103 and the freshwater MCCB119 were 98-99% similar in 16S rRNA sequence analysis indicating the ubiquitous nature of the organism.
- Salinity was found to regulate levels of pyocyanin produced by *Pseudomonas aeruginosa*, having 5-10g l^{-1} as the optimum.
- Despite their origin from different ecological niches, all isolates of *P. aeruginosa* grew uniformly at salinities ranging from 5 to 70g $l^{-1.}$ However, pyocyanin production varied distinctly among the isolates. The isolates of marine origin (MCCB117, MCCB118) produced detectable levels of pyocyanin with salinities up to 40g l^{-1}. The brackish water isolates (MCCB102, MCCB103) ceased to produce pyocyanin above 30g l^{-1}. The fresh water isolate (MCCB119) did not produce pyocyanin with salinities above 20g l^{-1}.
- The marine *Pseudomonas* isolate MCCB117 possessed ability to inhibit *Vibrio* spp. even when grown at a salinity of 40g l^{-1}.
- *P. aeruginosa* MCCB119 has been identified as the suitable organism for applying in fresh water zones, MCCB102 and 103 in brackish water and MCCB117 and 118 in marine environment.

- The genes coding for pyocyanin synthesis could be overexpressed in MCCB117 by increasing their copy number and renamed it as *Pseudomonas* PA-pUCP-Phz^{++}.
- The study has suggested that *Pseudomonas* PA-pUCP-Phz^{++} could be used to increase the yield of pyocyanin production by two fold.
- An appropriate downstream process for the large scale production of pyocyanin from PA-pUCP-Phz^{++} was developed to produce economically feasible compound for aquaculture applications.
- Purity of the compound was confirmed by HPLC, and structural elucidation by ^{1}H NMR spectroscopy and mass spectroscopy.
- The yield of purified pyocyanin from the designed downstream process using the genetically modified strain, PA-pUCP-Phz^{++}, was found to be 45mg l^{-1} and the yield was 80±5% than the wild strain.
- 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} and showed cidal effect at 10mg l^{-1}.
- Toxicity studies of pyocyanin extracted from PA-pUCP-Phz^{++} strain on various biological systems such as *in vitro* cell culture, animal models and bacterial consortia were performed.
- LC_{50} values calculated for pyocyanin induced toxicity on brine shrimp were 21.45 ± 38.3mg l^{-1} and 217.48 ± 43.19mg l^{-1} during the exposure times of 12 hour and 24 hour, respectively.
- Pyocyanin-induced toxicity studies on penaeid animal model using different larval stages of *P.monodon* were carried out. The LC_{50} values were 24±6.6mg l^{-1} for nauplius, 7±2.4mg l^{-1} for zoea and 10.69±4.6mg l^{-1} for mysis. In post larval stages it was 20.35±5.9mg l^{-1} for PL-5 and 33.92±6.7mg l^{-1} for PL -15.
- These results showed that zoea and mysis were more susceptible to pyocyanin-induced toxicity than the nauplii and postlarvae.

- In insect cell line, Sf9, IC_{50} value of the pyocyanin in plasma membrane damage, inhibition of mitochondrial dehydrogenase enzyme and protein synthesis were 106.39±13.92mg l^{-1}, 107.77±28.14mg l^{-1} and 133.67±23.6mg l^{-1} respectively.
- Necrosis and cell rounding of RTG-2 cells were observed only at higher concentration above 150mg l^{-1}. IC_{50} values of the pyocyanin were 146.19±28.78mg l^{-1}, 109.83±11.28mg l^{-1} and 76.64±9.99mg l^{-1} for the inhibition of mitochondrial dehydrogenase enzyme, plasma membrane damage and inhibition on protein synthesis respectively
- In human embryonic lung epithelial cell line (L-132) pyocyanin induced damage on plasma membrane and inhibition of protein synthesis with IC_{50} values 21.79±14.23mg l^{-1} and 32.57±16.52mg l^{-1} respectively were observed. However, the IC_{50} value on mitochondrial dehydrogenase activity was found to be 112.01±23.73mg l^{-1}.
- Pyocyanin-induced inhibition on glucose metabolism was found to be higher in human cell lines with LC_{50} 19.1±11mg l^{-1} followed by fish cell line (LC_{50} 21.1±5.8mg l^{-1}) and insect cell line 70.6±28.7mg l^{-1}.
- Pyocyanin induced toxicity was found to be very less in the cell lines of insect and fish than that in human. However, toxicity studies proved that the pyocyanin concentrations required to induce toxicity in cell line and in animal models were multifold higher than the required concentration for aquaculture application for inhibiting *Vibrio* spp.
- Pyocyanin induced toxicity on nitrifying bacterial consortia were performed to evaluate the effect on nitrification in the bioreactors used in RAS and found that it did not affect nitrification process.
- The study demonstrated the potential use of pyocyanin for removing invaded pathogenic *Vibrio* spp. in nitrifying bioreactors without affecting nitrification.
- In short, the pyocyanin from PA-pUCP-Phz^{++} strain could be used as prophylactic agents to control pathogenic *Vibrio* spp. in aquaculture systems

including the recirculating aquaculture systems. To control *Vibrio* spp. in general and *V. harveyi* in particular it would be sufficient to apply pyocyanin at a concentration of 5 – 10mg l^{-1} at which toxicity happens to be negligible.

- The *in vitro* toxicity studies and the IC_{50} value of pyocyanin derived from environmental isolates in various biological systems will pave way to the application of a safely and environmental friendly prophylactic drug as well as a probiotic in aquaculture system.

Scope for future research:

1. The mechanism of pyocyanin induced antagonism against invaded pathogenic *Vibrio* spp. in the nitrifying bioreactor.
2. Pyocyanin induced toxicity on biofilm produced by the nitrifying bacterial consortia and the mechanism of preventing the toxic effect if at all any.
3. Quorum quenching mechanism of pyocyanin.
4. Anti-viral property of pyocyanin.

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