Bioprospecting of novel antimicrobial metabolites from *Bacillus subtilis* MBTDCMFRI Ba37 and *Pseudomonas aeruginosa* MBTDCMFRI Ps04 of tropical estuarine habitats of Cochin, India and its application in fish health management

Thesis submitted to the

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

In partial fulfillment of the requirements

for the Degree of

Doctor of Philosophy

in

Marine Microbiology

Under the

Faculty of Marine Sciences

By

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February 2016

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Certificate

This is to certify that the thesis entitled ***"Bioprospecting of novel antimicrobial metabolites from Bacillus subtilis MBTDCMFRI Ba37 and Pseudomonas aeruginosa MBTDCMFRI Ps04 of tropical estuarine habitats of Cochin, India and its application in fish health management",*** *is a bonafide record of research work carried out by* ***Mrs. Anusree V. Nair (Reg. No. 4098)*** *under my supervision in the Marine Biotechnology Division, Central Marine Fisheries Research Institute (CMFRI), Kochi, in partial fulfillment of the requirements for the degree of* ***Doctor of Philosophy*** *in* ***Marine Microbiology****, Cochin University of Science and Technology, Kochi. This thesis, as a part or whole has not been presented before, for the award of any degree, diploma or associateship in any University. I further certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis.*

February 2016
Kochi – 18

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(Supervising Guide)
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Declaration

I hereby do declare that the thesis entitled ***"Bioprospecting of novel antimicrobial metabolites from Bacillus subtilis MBTDCMFRI Ba37 and Pseudomonas aeruginosa MBTDCMFRI Ps04 of tropical estuarine habitats of Cochin, India and its application in fish health management",*** *is a genuine record of research work done by me under the supervision of* ***Dr. K. K. Vijayan,*** *Director, Central Institute of Brackishwater Aquaculture, Chennai, 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 of any University or Institution.*

Kochi-18
February 2016

Anusree V. Nair
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Acknowledgement

First and foremost, praises and thanks to the Almighty GOD who has been giving me everything to accomplish this thesis. I bow before you for the gracious love and blessing showered on me.

With profound respect, I express my sincere gratitude and thank my honored guide, Dr. K. K Vijayan, Director, CIBA for his infallible guidance, untiring efforts, valuable suggestions and constant encouragement for the successful completion of my research work. Apart from guiding me, he has unwearyingly been a continuous source of moral support to me. I am thankful to him for having trust in me to work independently and yet was always there to make sure that I was on the right track. I realized that I was fortunate to work with one of the best mentor a student could ever wish for. I thank him honestly for offering me an opportunity to carry out research work under his supervision. I earnestly thank him for everything and for considering me for fellowships through the externally funded projects he strives to get from time to time during the course of this work.

I gratefully thank Dr. A. Gopalakrishnan, Director, CMFRI for providing all the facilities to work in the institute and for all the support extended during the study. I am also thankful to the former Director, Dr. G. Syda Rao, for allowing me to carry out my doctoral programme.

I will be forever thankful to Dr. P Vijayagopal, Head, Marine Biotechnology Division for providing all the help and facilities to work in the department. I express my profound gratitude for his valuable advice, suggestion and support during this endeavor.

I am immensely thankful to Dr. P.C Thomas, former Principal scientist and SIC, HRD cell CMFRI for the kind support and constant encouragement extended throughout the investigation. I am also thankful to Dr. Boby Ignatius, SIC and other staffs of HRD cell for the help and support throughout my research work.

I extend my sincere thanks to all the scientist of Marine Biotechnology Division, especially Dr. Kajal Chakraborthy, Mr. N. K Sanil , Dr.Pradeep M.A, Dr. Sandhya Sukumaran, and Dr. V Srinivasa Raghavan for their advice and suggestion during my research. I also thank Dr. Raja Swaminathan (NBFGRI) for all the help and support to carry out cytotoxicity studies.

I express my sincere gratitude to Dr. M.P Paulton, Mr. Nandakumar Rao and Mr. K.K Surendran for their whole hearted help and suggestions rendered throughout the study. I

also thank Mrs.P. Vineetha, Mr. Girish and Mr. A.K Shaji of MBTD for their good wishes and help.

I am thankful to all the members of Doctoral and Research Committee for their valuable suggestions. My sincere gratitude to research expert committee member, Dr. A.Mohammed Hatha, Associate Professor, School of Marine Sciences, CUSAT for his valuable suggestions and encouragement to carry out this work.

I express my profound gratitude to Dr. Shanta Achuthankutty, Former Chief Scientist, Microbiology Department, CSIR-National Institute of Oceanography, for her valuable advice, suggestion and support during this endeavour. The discussions with her have greatly influenced the development of my scientific aptitude and the experience I have gained working with her does not limit itself to academics alone. Her criticisms and insight comments during our discussions were always valuable and will be remembered forever. I take this opportunity to convey my respect and indebtedness to her.

I wish to acknowledge Dr. Meera Menon and Mrs. Preetha K for the whole hearted help and support rendered for the work and editorial inputs for completing the thesis.

As we spent long hours in the lab we tend to foster relationships which go far beyond than mere lab mates, I am fortunate to have a great team with some delightful individuals during these years and thanks to all of them.

I heartily thank Mr. Leo Antony for always being together for any informative and critical discussions, valuable suggestions and support which has made my investigations more valuable and interesting. This work would have been impossible without his constant support and guidance. My special thanks to Ms. Sandhya S V, Ms. Adithya C, Mr. Sayooj, B, Mr. Shamal P and Mr. Subin C S for their constant support and corporation throughout.

My whole-hearted thanks to all my friends in Marine Biotechnology Division especially, Mrs. Suja Gangadharan, Mr. Bineesh, Ms. Saira, Mrs. Jazeera K, Mr. Arun Kumar, Mrs. Esha Arshad, Mr. Iyyappa Raja, Mr. Shihab Ismail, Mr. Reynold Peter, Mr. Vineesh, Mrs. Levina, Mr. Nevin, Mr. Wilson, Mrs. Archana, Ms. Pinky Kaur for their great help and constant encouragement in carrying out my work.

I further sincerely express my gratitude to all scientists, staffs and scholars of CMFRI for providing all their supports during the tenure

Special thanks to my seniors Dr. Neetha Joseph, Dr. Jiya Jose, Mr. Francis Xavier, Mr. Raj Kumar and Mr.Shyam for their corporation and timely support.

I am extremely happy to recollect the support given by, Dr. P. A. Vikas, Technical officer, KVK, CMFRI, Dr. Lijo John, Assistant Director (Tech), Export Inspection Council of India, Ernakulam, Mr. Renjith, Sub - Inspector of Fisheries, Regional Shrimp Hatchery, Azhikode for their help and support extended during this period.

I owe my thanks to OIC Library , other staffs members of Library, administration staffs, canteen, security and all other members of CMFRI for their sincere help and cooperation extended during the course of my study.

Financial support to a student is always a necessity and I greatly acknowledge "Application of microorganisms in agriculture and allied sectors (AMAAS)", ICAR for the financial assistance in the form of SRF fellowship to carry out this research work.

I deeply acknowledge the scientists especially Dr. M. S. Moni, Dr. C. Baby and all other staffs of SAIF, IIT Madras, Dr. Sabareesh, VIT, Vellore and staffs of STIC- CUSAT for their help and support extended towards the structural evaluation of the compounds.

I sincerely express my gratitude to Dr. Anil Krishna, Managaing Director, Elixir extracts, Kinfra Park, Muvattupuzha for allowing me to do the spray drying and granulation technique. Further I am grateful to Mr. Abhilash P and other staffs of Elixir for their timely support.

I am extremely thankful to Dr. Suhair , Director, Regional Shrimp Hatchery, Azhikode for permitting me to carry out the feeding trail experiment and constant support for this work and extended my thanks to all staffs of hatchery.

It gives me immense pleasure to express my love and gratitude to my beloved family members for their understanding and giving me more than what I deserved. It is their constant prayers that enabled me to reach the present position in life and I am sure that they would feel proud of this effort.

I find no appropriate words to express my feelings and gratitude to my beloved husband, Dr. Praveen N.K and little darling Harigovind N. Kartha, who has made this arduous journey more pleasant. Their unconditional love, care and deep understanding was my inspiration and the driving force which gave me the strength to complete my Ph.D work in ease. Words fail me to express the sort of heavenly benediction they have been to me.

Finally I thank all those who have helped me directly or indirectly in the successful completion of my thesis.

Anusree V. Nair

.....Dedicated to my AMMA

(Your blessings & deep love always inspired me to work hard and to overcome all the difficulties throughout my life and motivated me to achieve the goals beyond my expectations)

Contents

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Abbreviations

3`	:	three prime DNA end
5`	:	five prime DNA end
°C	:	degrees Celsius
γ	:	Gamma
%	:	Percentage
µg	:	Microgram(s)
µL	:	Microlitre(s)
µM	:	Micromolar
γ_r	:	Rocking Vibration
ν	:	Stretching Vibration
δ	:	Bending Vibration
ν_s	:	Symmetric Stretching Vibration
ν_a	:	Asymmetric Stretching Vibration
BLAST	:	Basic local alignment search tool
BHT	:	Butylated Hydroxytoluene
bp	:	Base pairs
CFU	:	Colony forming unit
COSY	:	Correlation Spectroscopy
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide
DCM	:	Dichloromethane
DEPT	:	Distortionless Enhancement By Polarization Transfer
DM	:	Dry matter
EtOAc	:	Ethyl Acetate
EDTA	:	Ethylenediaminetetraacetic acid

et.al.	:	And others
FCS	:	Fetal calf serum
FT-IR	:	Fourier Transform Infra Red
GC-MS	:	Gas Chromatography-Mass Spectrometry
GA	:	Glycerol alanine
g	:	Gram(s)
h	:	Hours
HMBC	:	Heteronuclear Multiple Bond Correlation
HSQC	:	Heteronuclear Single Quantum Coherence
IR	:	Infra Red
K/A	:	Alkaline/acid
LC-MS	:	Liquid Chromatography-Mass Spectrometry
LB	:	Luria Bertani
m/z	:	Mass-to-Charge Ratio
MeOH	:	Methanol
min	:	Minute(s)
mg	:	Milligram(s)
mL	:	Millilitre(s)
mm	:	Millimetre(s)
mM	:	Millimolar
Mp	:	Microbial product
MBM	:	Modified bacillus medium
MHA	:	Mueller Hinton Agar
MS	:	Mass Spectroscopy
NA	:	Nutrient Agar
NFE	:	Nitrogen free extract
NCBI	:	National Center for Biotechnology Information

ng	:	Nanogram(s)
NMR	:	Nuclear Magnetic Resonance
ID- NMR	:	1-Dimensional-NMR
2D-NMR	:	2-Dimensional-NMR
NOESY	:	Nuclear Overhauser Effect Spectroscopy
OD	:	Optical Density
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
pH	:	Hydrogen Ion Concentration
ppt	:	parts per thousands
ppm	:	Parts Per Million
R_t	:	Retention Time
R_f	:	Retardation Factor
rDNA	:	Ribosomal DNA
RNA	:	Ribonucleic acid
rpm	:	Revolutions per minute
sec	:	Second(s)
SW	:	Sea water
TLC	:	Thin layer chromatography
TMS	:	Tetramethylsilane
TSB	:	Trptone soya broth
TSI	:	Triple sugar iron
TE	:	Tris EDTA
ZMA	:	Zobell Marine Agar

Chapter 1

GENERAL INTRODUCTION

General Introduction

Aquaculture is an important economic activity the world over and about 90% of the global production is contributed by Asian countries. Global aquaculture has a persistent goal to maximize the production with optimal profit. Even though the aquaculture is growing at a rapid rate, the practice of aquaculture faces many challenges in its developing path. Intense cultivation in high densities significantly affect the environment by issues of used - up farm water discharges and heavy waste accumulation through hyper nutrification due to excessive feeding and high dietary nutrient composition (Liao and Mayo 1974; Boyd 1985). This has brought stress to the rearing environment and diseases to the cultured species and subsequently decreased the overall productivity due to mass mortality which has led to significant loss to the industry (Irie et al. 2005; Cruz et al. 2012; FAO 2012). Thus, it is necessary to face the challenges and find solutions in order to make aquaculture sustainable.

Disease is the result of interaction between the host, pathogen and external environment. At the onset of disease, the harmonious interplay between pathogens and non-pathogens is disturbed result in unhealthy host and thereby infection occurs (Verschuere et al. 2000; Schulze et al. 2006; Zhou et al. 2009; Nemutanzhela et al. 2014). Bacteria, fungi, viruses and parasites are the major pathogens affecting aquaculture industry. Among different aquaculture pathogens, bacteria play a major role. It can survive and flourish independently in any aquatic environment. The bacterial fish infections are mainly from the species of

Aeromonas, *Vibrio*, *Flavobacterium*, *Edwardsiella*, *Yersinia*, *Pseudomonas*, *Streptococcus*, *Renibacterium*, *Piscirickettsia*, *Mycobacterium* etc. The common bacterial disease symptoms are external reddening and haemorrhage in the peritoneum, body wall and viscera, generically referred to as haemorrhagic septicaemia. The progress of disease leads to ulcerative lesions and mortality of untreated cultured animals (Pridgeon and Klesius 2012). Among the bacterial diseases, vibriosis is caused by a major bacterial fish pathogen *Vibrio* spp. which is ubiquitous in all aqua cultured species (Jayaprakash et al. 2005; Thompson et al. 2010). In tropical countries, penaeid shrimp culture is highly affected by *V. harveyi* which causes their mass mortality (Austin & Zhang 2006). Another *Vibrio* species which affects the shrimp culture are *V. alginolyticus*, *V. damsela*, *V. parahaemolyticus*, *V. vulnificus*, *V. splendidus* and *V. penaeicida* (Saulnier et al. 2000).

The existing conventional methods of application of chemotherapeutics (chemical and antibiotic treatments) were proved to be unsustainable and unfriendly to environment as the pathogens develop drug resistance, the effluent discharged contains antibiotics and chemicals residues. These have negative impact on the environment and biological filtration systems (Barker 2000). In order to overcome these challenges, an alternate environmental friendly and sustainable strategy to control the bacterial disease problems in aquaculture and to enhance water quality is an urgent pre requisite (Verschuere 2000; Gomez - Gil et al. 2000; Kesarcodi-Watson et al. 2008). A number of alternatives are proposed to replace the chemotherapeutics viz; vaccines, immunomodulatory agents, bacteriophages and their lysins, antimicrobial peptides (AMPs), pro, pre and synbiotics, plant extracts, bacterial quorum sensing (QS) inhibitors, biofilm and virulence, and feed enzymes (Cheng et al. 2014; Mohapatra et al. 2013). Earlier definition given to probiotics was as live microbial adjunct which can

impart positive effects on a host animal (Verschuere et al. 2000). Presently the probiotics is defined as the metabolites of live or dead bacterial cells, function as immunostimulants (Taoka et al. 2006). The use of biological agents to control bacterial diseases have gained wide spread acceptance and has emerged as a potential tool in treating the infections (Zhou et al. 2009; Cruz et al. 2012; Cheng et al. 2014). Most common bacteria which were considered as potential probiotics belong to genera the lactic acid bacteria, *Bacillus*, *Pseudomonas*, *Bifidobacterium, Roseobacter, Pseuodoalteromonas, Vibrio, Nitrobacter, Aeromonas*, *Carnobacterium*, *Clostridium*, *Debaryomyces*, *Micrococcus*, *Roseobacter*, *Saccharomyces*, *Streptococcus*, *Vibrio*, *Weissella* and other photosynthetic bacteria were reported to be useful in aquaculture (Irianto and Austin 2003; Jayaprakash et al. 2005; Nair et al. 2012). They are widely acknowledged for their inhibitory activity against pathogens by the production of beneficial primary and secondary metabolites. These microorganisms act as a promising source of biologically active molecules and could play a vital role in the discovery of novel pharmaceutical compounds (Penesyan et al. 2009). So the biocontrol agents offer a key solution to the challenges in aquaculture system and the strains isolated had many applications in both biotechnology and aquaculture.

Modes of Action

Probiotic bacteria either directly or in the form of certain metabolites, derived out of it, influences microbial balance in the aquatic environment through different mechanisms. The presence of these probionts either in its active or inactive form contribute to health status of the host (Irianto and Austin 2003; Salinas et al. 2006; Taoka et al. 2006). Live biocontrol agents can proliferate and can inhibit the pathogenic growth by colonizing within the host or environment. They stimulate the host immune system and control diseases. It has also been reported that fish fed with live biocontrol agents showed increased

disease resistance or improved host survival by stimulating the host immune system (Balcazar et al. 2006; Pai et al. 2010). Later on the term probiotic in aquaculture was redefined as microbial supplements, not necessarily alive or the metabolites of live or dead bacterial cells that have beneficial effects on host health. The main advantage in using dead organisms is the safety aspect wherein, the risk of foreign bacteria escaping into the open environment (Salinas et al. 2006; Taoka et al. 2006) is controlled. It was observed that the dead or inactivated bacterial cells also stimulated the innate immune system of aquatic animals (Irianto and Austin 2003; Irianto et al. 2003).

There are various modes of action postulated are as follows:

- Production of inhibitory compounds
- Competition for nutrients or energy
- Competition for adhesion sites
- Source of nutrients and enzymatic contribution to digestion
- Improvement of water quality
- Immune response enhancers
- Growth inhibition of pathogens
- Restoring the environmental stability

Selection and Screening of Biocontrol Agent

The main purpose of searching a biological agent is to bring healthier environment for the host, by controlling the proliferation of pathogens. The candidate may be indigenous or exogenous, but it should survive and reside in the aquatic environment and supplement beneficial outputs *in vivo*. For screening the biocontrol agents, it is important to choose the indigenous isolates (from the host or the same environment) as the same may perform better than

the isolates of completely foreign origin (Verschuere et al 2000; Mohapatra et al. 2013). The selection of specific candidates requires the background information, sorting of locations, purification of the isolates and evaluation with *in vitro* and *in vivo* screening methods (Gomez-Gil et al. 2000; Vine et al. 2006; Lauzon et al. 2008). Additional selection criteria which have to be considered is the biosafety and the pathogenicity with the target species, along with the downstream processing methods like production, processing, administration and their robustness in the environment (Lalloo 2010; Lalloo et al. 2010).

Selection criteria to obtain successful biological agents consist of :

- acquiring strains with antagonistic properties against aquatic animal pathogens
- taxonomic identification and collection of background information on the strains
- *in vitro* ability of the putative probionts to inhibit and/or outcompete pathogens
- *in vivo* ability of the probiotics to confer protection from disease due to the pathogen
- no pathogenicity / toxicity of selected probionts to host animals, non target species, other animals in the environment, and humans
- an economic cost-benefit analysis

Commercial Applications

The application of biocontrol agents has got wide acceptance in aquaculture industry because, they are indigenous, and have beneficial effects like antimicrobial activities, digestive enzyme production, stimulation of host

immune system and have a vital role in detritus degradation and recycling (Hong et al. 2005; Lalloo et al. 2008; Bandyopadhyay and Mohapatra 2009; Cutting 2011). The common commercial preparations contain one or more live microbes applied either in liquid or dry forms as feed additive or directly to the culture ponds (Sahu et al. 2008; Shen et al. 2010; Cruz et al. 2012; Xie et al. 2013). The viability and functionality of the probiotics can be enhanced by optimizing the fermentation conditions. Development of functional feed is a new dimension, where diets are formulated with dietary ingredients along with the additives such as potential antioxidants, enzymes, probiotics etc. to improve the efficiency of feed which in turn helps to increase growth and health of aqua cultured animals, enhanced the immune system and physiological benefits than conventional feeds (Ibrahem 2015 and Soto et al. 2015). In recent years, inactivated probiotic preparation through dietary supplementation is gaining more importance due to its ease in application and absence of side effects (Irianto and Austin 2003; Salinas 2006).

India is bestowed with vast aquatic resources harbouring very rich microbial biodiversity with immense biotechnological potential. Even then, we were lagging behind in utilizing these indigenous resources as probionts. This has resulted in the large scale import of probiotics formulated with exotic strains. Observing the potential market for probiotics in India, many companies began to set up their manufacturing units solely depended on exotic strains. Aquatic environments (freshwater, brackish and marine) are known to harbour diverse groups of microbes which synthesize several bioactive molecules with antimicrobial properties. The sediments are considered as 'hot spots' of microbial activity in the ocean. The bacteria attached to surfaces produce more inhibitory compounds than free living forms to protect their habitat niche (Long and Azam 2001). These sediment bacteria were involved in the ecological and

biogeochemical role in ecosystems (Tanaka and Rassoulzadegan 2004). Compared with terrestrial organisms, secondary metabolites produced by aquatic microorganisms have more novel and unique structures owing to the complex living circumstances and diversity of species (Zheng et al. 2005). These metabolites are found to have wide pharmaceutical and biotechnological applications (Long and Azam 2001; Zheng et al. 2005; Nithya and Pandian 2010). Estuaries are the complex ecosystem which is ecologically and economically important as they act as a transition zone between marine and freshwater systems (Peduzzi and Herndl 1992; Mann and Wetzel 1995). The tropical estuarine habitats of Cochin, located at 9º 30' - 10º 10' N and 76º 10' - 76º 29' E along the southwest coast of India, is one among the largest wetland rich in fine sediments and organic matters, supporting highly diverse flora and fauna with assorted ecological systems (Menon et al. 2000; Srinivas et al. 2003). The need of the hour is to explore the indigenous resources of novel organisms from our aquatic microbial flora to be used as biocontrol agents in aquaculture and to develop an appropriate technology for its practical application.

Objectives of the Study

Due to the limitations in using antibiotics and chemicals, the scope of using biocontrol agents to inhibit the pathogens and recycle the culture environment looks promising. More detailed and focused studies are necessary for the betterment of future aquaculture and its application in wide ecosystem. Meanwhile it would be useful to explore the bioactive compounds from antagonistic bacteria. Care should be taken to utilize the microbial product in aquatic feeds and to validate their efficiency. Thus, the probiotics may help to substitute the application of drugs and chemicals in animal nutrition which indicates a bright future for biocontrol agents.

Therefore the objectives of the study are

- To study the diversity of bacteria with antagonistic properties from various ecological habitats of Cochin estuary
- To develop a molecular screening tool for the detection of genus *Bacillus* and *Pseudomonas*
- To characterize the candidate antagonistic bacteria
- To optimize the protocols for production of bioactive metabolites
- To isolate and identify the active compounds
- To develop a microbial product and to evaluate its efficacy as an aquaculture supplement

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Chapter 2

DIVERSITY AND CHARACTERIZATION OF ANTAGONISTIC BACTERIA

Contents

2.1 Abstract

Mortalities due to pathogenic bacteria are a major problem in aquaculture, especially in larval rearing systems. Use of antibiotics to overcome this problem is not an option any more due to increasing antibiotic resistance among pathogens. The present study aimed to understand the diversity of bacteria with antagonistic properties in the tropical estuarine habitats of Cochin, located along the southwest coast of India, and to use them as an alternative to antibiotics in aquaculture. Among 4870 isolates screened, approximately 1% showed significant antibacterial activity against six common aquaculture pathogens belonging to the genera *Aeromonas* and *Vibrio*. The antagonistic bacteria were identified as *Bacillus* (81%) and *Pseudomonas* (19%) using biochemical and 16S rRNA gene sequence homology. The isolates showing stable and higher level of antibacterial activity were subjected to enzymatic expression profile, antibiotic resistance pattern and abiotic stress tolerance assays. As a result, five *Pseudomonas* spp. and four *Bacillus* spp., were identified as promising antagonistic isolates that could be exploited as probionts or microbial products (MP's), to control bacterial diseases in aquaculture rearing systems.

2.2 Introduction

Aquaculture production has grown steadily over the past decade contributing 46% of the global fish supply (FAO 2010). Diseases however form a major limiting factor with larval mortalities due to pathogenic *Vibrio* and *Aeromonas* infections being one of the chief causes (Balcazar and Rojas-Luna 2007). Good husbandry techniques, usage of chemical additives, disinfectants, antimicrobials and vaccines are primarily practiced to control bacterial infections in aquaculture (Wang et al. 2008). However, the increased use of antibiotics in aquaculture systems leads to complications such as increased stress among aquatic animals, development of drug resistance among fish and subsequently human pathogens via transfer of genes from drug resistant microbes (Wang et al. 2008; Heuer et al. 2009). Apart from this, the residues which are left behind, adversely affect the environment and public health (Defoirdt et al. 2007). Vaccination or immunization for the control of bacterial diseases is of limited practicality in shellfish due to non-specific immune response (Vine et al. 2006; Defoirdt et al. 2007). Individual vaccination in finfish too is infeasible under intensive culture environments (Toranzo et al. 2009). In this backdrop, use of live bacteria or in the form of heat killed microbial product (MP) with antagonistic properties emerged as an alternate strategy to control bacterial disease problems, increasingly accepted as a standard practice in aquaculture (Vine et al. 2006; Kesarcodi-Watson et al. 2008). Probiotics reduce bacterial infections by producing inhibitory compounds (Vijayan et al. 2006; Vaseeharan and Ramasamy 2003; Preetha et al. 2010; Pai et al. 2010), increase growth and survival of the host by supplementing various essential enzymes/nutrients (Vine et al. 2006; Kesarcodi-Watson et al. 2008; Boonthai et al. 2011) and may also act as immune modulators (Pai et al. 2010). The selection of probiotic bacteria is influenced by various factors including the type of species, water activity,

temperature, hydrogen-ion concentration (pH) and osmotic pressure (Kesarcodi-Watson et al. 2008). Absence of transmissible antibiotic resistance genes is also an important factor in selecting probionts (Saarela et al. 2000). Antibiotic susceptibility pattern varies greatly between different species indicating the necessity for susceptibility testing of each probiotic strain (Felten et al. 1999).

Indigenous microorganisms are natural candidates for biocontrol strategies because of their adaptations to local environmental constraints, hence are more likely to establish themselves in a particular habitat (Ortega-Morales et al. 2009). Marine microorganisms are well-known for the production of novel bioactive metabolites (or natural products) which is believed to be a requirement for their survival in the sea to counter acute competition from other species. These metabolites are found to have wide pharmaceutical and biotechnological applications (Long and Azam 2001; Zheng et al. 2005; Nithya and Pandian 2010). The tropical estuarine habitats of Cochin, located along the southwest coast of India, are rich in fine sediments and organic matter, supporting highly diverse flora and fauna with assorted ecological systems (Menon et al. 2000). The present study explored the diversity of culturable bacteria with antagonistic properties in the sediment and sub-surface water of Cochin estuary for developing strategies to control bacterial diseases in aquaculture.

2.3 Materials and Methods

2.3.1 Study Area and Sample Collection

The estuarine habitats of Cochin extend between 9º30′ – 10º 10′ N and 76º 10′ – 76º 29′ E with its northern boundary at Azhikode and the southern at Thanneermukkam bund on the southwest coast of India (Jose et al. 2011). The collection sites (Fig. 2.1) were: Station 1 - Mangalavanam (mangrove

ecosystem), station 2 - Vypeen Barmouth (high saline, high influx of sea water), station 3 - Vypeen Harbour (fish landing centre; anthropogenic effect), station 4 - Njarakkal (shrimp farm), station 5 - Sattar Island (Oyster farm near Munambam, northern end of estuary), station 6 - Eloor ferry (industrially polluted area), station 7 - Marine science Jetty (polluted due to human interaction) and station 8 - Perandoor canal (highly polluted with sewage discharge and fresh water influence).

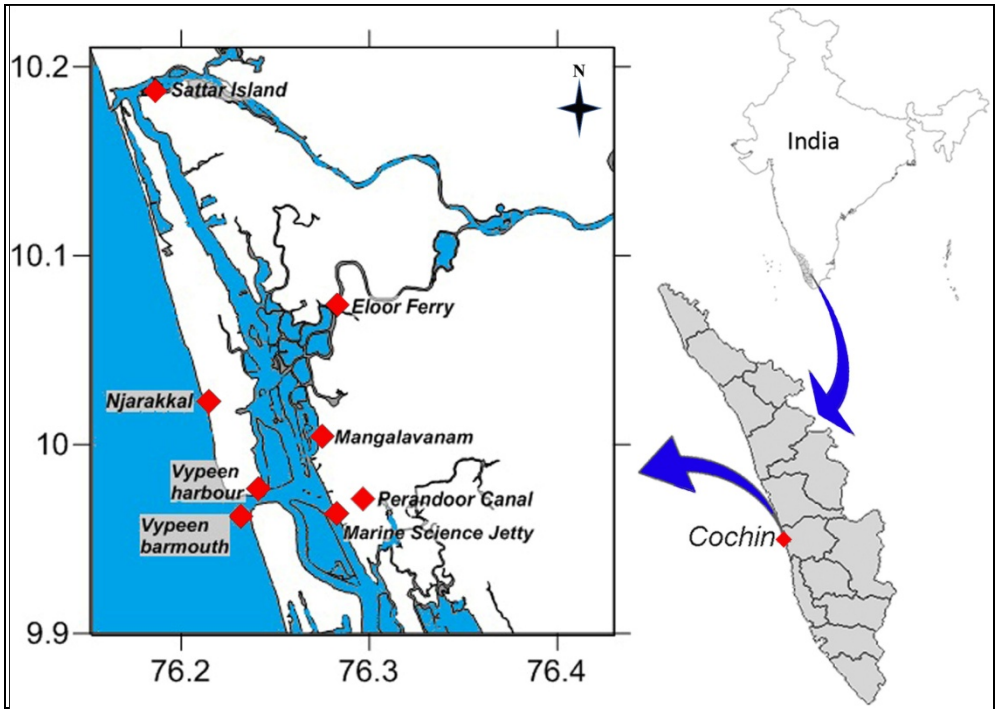

Fig. 2.1: Map showing the sampling locations in Cochin estuary located at southwest coast of India

From each station, sediment and sub-surface water samples were collected monthly for a period of twelve months from June 2009 - May 2010. Samples were collected using scoopers and buckets into sterile polypropylene bottles. These were transported in an ice box to the laboratory and processed within 3 - 5 h after collection.

2.3.2 Isolation of Bacteria

Culturable bacteria were retrieved using ZoBell marine agar 2216, nutrient agar with 50% sea water and nutrient agar supplemented with 1% sodium chloride (Himedia). Ten-fold serially diluted sample was plated on the above media in duplicate and incubated at 30ºC for 24 - 48 h. After incubation, colony counts were recorded and colonies with distinctive morphologies were purified for antibacterial assay.

2.3.3 Test Microorganisms

Antagonistic potential of the isolates was tested against six common aquaculture bacterial pathogens. The test organisms used were *Vibrio harveyi* 101, *V. anguillarum* A1, *V. alginolyticus* 101 (*courtesy* Central Institute of Brackishwater Aquaculture, Chennai), *Aeromonas hydrophila* (*courtesy* National Centre for Aquatic Animal Health, Cochin University of Science and Technology), *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (*courtesy* Microbial Type Culture Collection, Chandigarh, India). The bacteria were taken from the preserved slants before each assay and inoculated in freshly prepared broth to attain uniform growth.

2.3.4 Antibacterial Assay

The selected bacterial isolates were examined for antagonistic activity against the test pathogens using a spot diffusion assay. The pathogens were grown to log phase in nutrient broth with 1% sodium chloride for 18 - 24 h (10^8 CFU/mL). They were then swabbed on Mueller-Hinton agar (Himedia) with 1% sodium chloride/ 50% seawater over which the purified isolates (7 per dish) were spotted (~3 mm diameter) using sterile toothpicks. The plates were then incubated at 30ºC for 24 - 48 h and the zone of inhibition observed around the isolates was recorded. The bacteria showing antibacterial activity were preserved in nutrient agar slants and in 20% glycerol stocks at -80ºC until further use.

2.3.5 Identification of Bacteria

Bacteria with antagonistic properties were identified using biochemical methods (Krieg and Holt 1984) and by 16S rRNA gene sequencing. Characterisation of *Bacillus* into species level was carried out using the methods illustrated by Reva et al. (2001). Total genomic DNA was extracted from bacterial cultures grown in nutrient broth using phenol-chloroform extraction method (Sambrook and Russell 2001) and quantified using Biophotometer (Eppendorf, Germany). The 16S rRNA gene from the genomic DNA was amplified using universal primers; NP1F 5'–GAGTTTGATCCTGG CTCA-3' and NP1R 5'-ACGGCTACCTTGTTACGACTT-3' (Pai et al. 2010). The PCR reaction mixture (25 µl) comprised of bacterial DNA (50 ng), 10 pmol each of the two oligonucleotide primers (Genei, India), 2.5 mM of each deoxynucleoside triphosphate (Finnzymes), 1.5 U of Taq polymerase (Sigma) and 2.5 µl of 10X buffer. The amplification was carried out in Veriti thermal cycler (Applied Biosystems, Germany) with initial denaturation at 94ºC for 5 min, followed by 35 cycles of 94ºC for 30 s, 60ºC for 30 s and 72ºC for 90 s and a final extension of 72ºC for 10 min. The amplified products were purified (HiPurA PCR product purification kit, Himedia) and sequenced.

2.3.6 Phylogenetic Analysis

The 16S rRNA gene sequence were analysed and the relative phylogenetic positions was determined by searching GenBank database using BLASTn algorithm (Altschul et al. 1997). Multiple sequence alignment was performed with CLUSTALW (Thompson et al. 1994). A phylogenetic tree was constructed from evolutionary distances using neighbor-joining DNA distance algorithm (Saitou and Nei 1987). Tree topologies were evaluated by bootstrap analysis of 1,000 data sets with MEGA5 (Tamura et al. 2011) to validate the reproducibility of the branching pattern. This algorithm calculates distance matrix using a Kimura 2- parameter. The 16S rRNA gene sequence of two *Pseudomonas* isolates (Ps06 and Ps07) was not included in the analysis,

as sequencing reactions of those isolates repeatedly failed (3 trials) with NP1F, the primer NP1R was used for sequencing the products. The sequences obtained were submitted in GenBank for accession.

2.3.7 Characterization of Antagonistic Isolates

Based on the genus, geographical origin and wide range of antimicrobial activity, the isolates were shortlisted and subjected to various *in vitro* assays as follows.

2.3.7.1 Enzymatic Assay

The selected isolates were screened for hydrolytic exoenzymatic activities using an agar diffusion method. These tests were done using nutrient agar with 1% sodium chloride (Himedia) amended with the specific substrate such as starch for amylolytic activity (Sanchez-Porro et al. 2003), skimmed milk for proteolytic activity, tributyrin for lipolytic activity (Sirisha et al. 2010), gelatin for gelatinase activity, carboxy methyl cellulose for cellulase activity (Kasana 2008), phenolphthalein phosphate agar (Himedia) for phosphatase activity and urea agar (Himedia) for urease activity, respectively. Activity was detected after incubation at 30 $\pm$ 2ºC for 24 h, as growth, or a zone around the colony with/without the addition of respective reagents.

2.3.7.2 Abiotic Stress Tolerance Assay

The isolates were screened to study their tolerance to varying concentrations of salinity, temperature and pH. The tolerance to salinity was tested in a medium containing peptone, yeast extract supplemented with respective concentrations of salt (NaCl) at 0%, 2%, 5%, 10%, 15%, 20%, 25% and 30% (w/v) (Yeon et al. 2005). The temperature resistance of the isolates was studied on nutrient agar plates by growing them in a wide range of temperatures (20^{o}C - 60^{o}C). The pH tolerance was examined by growing the isolates in nutrient agar medium with different pH gradients 4 -10 (adjusted using 0.1N HCl and 0.1N NaOH).

2.3.7.3 Antibiotic Resistance Pattern

Antibiotic resistance pattern of the antibacterial isolates were determined by the standard disc diffusion method using commercially available antibiotics (Bauer et al. 1966). The antibiotics used were Penicillin (P-10U), Bacitracin (B-10U), Erythromycin (E-15mcg), Tetracycline (T-30mcg), Streptomycin (S-10mcg), Ciprofloxacin (Cf-5mcg), Chloramphenicol (C-30mcg), Gentamicin (G-10mcg), Ampicillin (A-10mcg) and Kanamycin (K-10mcg) (Himedia, India).

2.4 Results

2.4.1 Isolation and Enumeration of Bacteria

From eight locations of Cochin estuary, 4870 bacteria were isolated during the collection period (June 2009 - May 2010), out of which 2960 isolates were from sediment samples and 1910 were from sub-surface water samples. The average number of culturable heterotrophic bacteria in sediment and sub-surface water samples from the different locations was 6.6 x 10^5 CFU/g and 6.7 x 10^4 CFU/mL, respectively. Among the collection sites, sediment samples from the Mangalavanam mangroves and water samples from the polluted Perandoor canal recorded the highest bacterial counts 9.9 x 10^5 CFU/g and 1.53 x 10^5 CFU/mL, respectively (Fig. 2.2). The lowest count for sediment (3.2 x 10^5 CFU/g) and water samples (2.4 x 10^4 CFU/mL) was obtained from Eloor ferry (Fig. 2.3). During the collection period, maximum heterotrophic bacterial counts were recorded in June – September and the lowest was observed in October – February. Among the sediment samples collected, the highest count 1.2 x 10^6 CFU/g was recorded in June whereas the lowest 4.1 x 10^5 CFU/g was observed in February. Similarly, among the water samples analyzed, maximum bacterial count 9.1 x 10^4 CFU/mL was recorded in August, while minimum 2.5 x 10^4 CFU/mL was obtained during December.

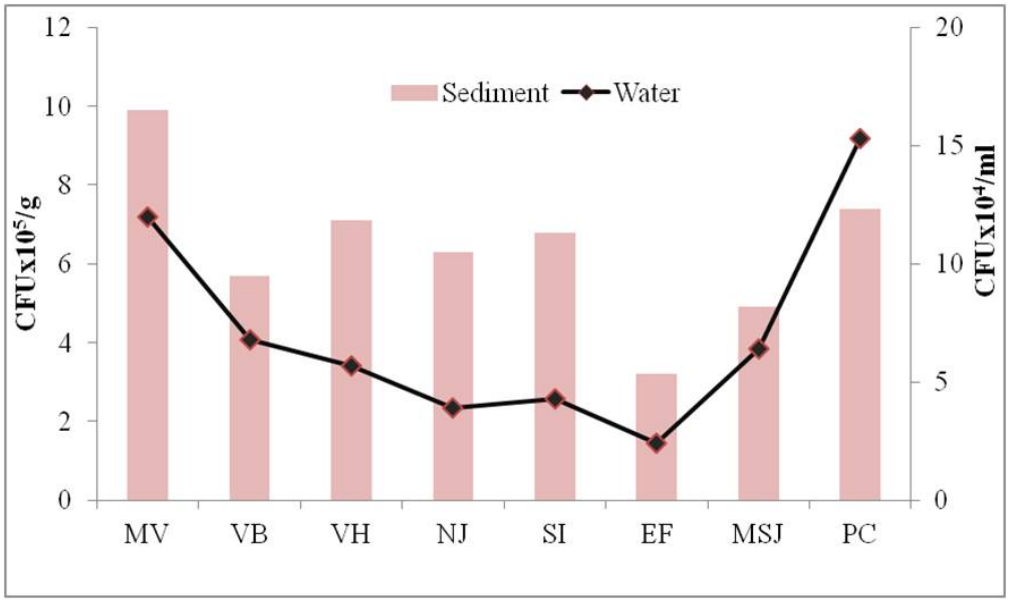

Fig. 2.2 Average heterotrophic bacterial counts in CFU/g and CFU/mL obtained from eight different collection sites with respect to sediment and water samples

Legends: [MV - Mangalavanam; VB - Vypeen Barmouth; VH - Vypeen Harbour; NJ - Njarakkal; SI - Sattar Island; EF - Eloor Ferry; MSJ - Marine Science Jetty; PC - Perandoor Canal]

2.4.2 Antimicrobial Screening

The isolates were first screened against *Vibrio anguillarum* A1, *V. vulnificus* MTCC1145 and *Aeromonas hydrophila.* Of the 4870 isolates, 83 strains showed good inhibitory activity with a zone of inhibition of 10 mm or greater, against at least 2 pathogens. These 83 strains were further tested against *V. harveyi* 101, *V. parahaemolyticus* MTCC451 and *V. alginolyticus* 101, in which 48 isolates showed activity against at least four test pathogens. Among the above 48 isolates, 31 (64.6%) were obtained from sediment samples and 17 (35.4%) from sub-surface water samples. Majority of the bacteria with significant antagonistic properties were isolated from Mangalavanam (12) and Perandoor canal (11), while Eloor ferry and Vypeen harbour (2) yielded the least.

2.4.3 Identification and Phylogeny

The biochemical tests ascertained that the isolates having antibacterial activity were predominantly from the genera *Bacillus* and *Pseudomonas*. Although much less pronounced, genera such as *Enterobacter* and *Vibrio* showing antibacterial activity were also obtained. The 16S rRNA gene sequences of the 48 isolates were searched for homology using BLAST analysis. The fallouts confirmed that the majority of the isolates were from the phyla Firmicutes (81%), followed by Proteobacteria (19%) representing the families Bacillaceae and Pseudomonadaceae (Fig. 3). The isolates from the family Bacillaceae were represented by *Bacillus subtilis*, *B. amyloliquefaciens* and *B. pumilus*, while all the isolates of Pseudomonadaceae were found to be *Pseudomonas aeruginosa*. The accession numbers of the sequences submitted in GenBank are shown in the Fig. 2.4.

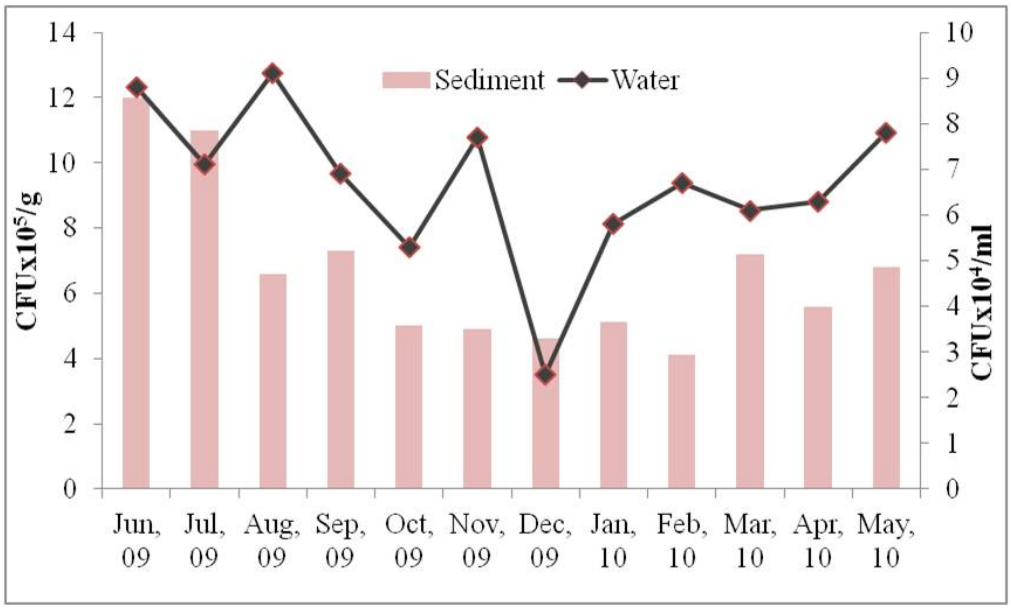

Fig. 2.3 Average heterotrophic bacterial counts in CFU/g and CFU/mL obtained during the collection period with respect to sediment and water samples.

Phylogenetic analysis, based on the partial sequences of 16S rRNA gene alignments of the bacterial isolates with antagonistic activity, showed that they shared 98-100% identity with known species. Among the 39 isolates of *Bacillus* spp., 3 strains of *B. pumilus* (MBTDCMFRI Ba01, MBTDCMFRI Ba33 and MBTDCMFRI Ba39) were clustered into a single clade, while *B. subtilis* and *B. amyloliquefaciens* were merged in sub-clades. All *Pseudomonas* isolates (MBTDCMFRI Ps01 - Ps05, MBTDCMFRI Ps08 - Ps09) were clustered together into a single separate clade and showed 97 - 99% similarity to *Pseudomonas aeruginosa* in the BLAST alignments.

2.4.4 Antibacterial Assay

A total of 48 antagonistic isolates obtained from estuarine regions of Cochin formed significant zones of inhibition ranging from 10-28 mm, of which 39 strains belong to *Bacillus* spp. and 9 *Pseudomonas* spp. Among these isolates 9 *Pseudomonas* strains (MBTDCMFRI Ps01 - MBTDCMFRI Ps09) and 4 *Bacillus* strains (MBTDCMFRI Ba01, MBTDCMFRI Ba29, MBTDCMFRI Ba37 - Ba38) were effective against all the 6 test aquaculture pathogens (Table 2.1, Fig. 2.5a & 2.5b). Fifteen *Bacillus* strains showed activity against all pathogens except *V. alginolyticus* 101 while the remaining 20 strains showed antagonistic activity against a minimum of four pathogens. Among all the potential isolates obtained from *Bacillus* spp., ≥ 95% showed activity against *Vibrio harveyi* 101, *V. anguillarum* A1, *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451. Only 75% of *Bacillus* strains showed activity against *Aeromonas hydrophila* and 50% against *V. alginolyticus* 101.

Table 2.1 Description and spectrum of antibacterial activity of the bacterial isolates from Cochin estuary

ISOLATE CODE	IDENTIFICATION	SAMPLING LOCATION	ZONE OF INHIBITION (mm)					
			A	B	C	D	E	F
MBTDCMFRI Ba01	*Bacillus pumilus*	Mangalavanam	12	9	10	15	13	11
MBTDCMFRI Ba06	*Bacillus amyloliquefaciens*	Vypeen barmouth	21	14	24	15	14	-
MBTDCMFRI Ba07	*Bacillus amyloliquefaciens*	Vypeen harbour	21	16	19	13	15	-
MBTDCMFRI Ba08	*Bacillus amyloliquefaciens*	Njarakkal	21	16	20	15	17	-
MBTDCMFRI Ba09	*Bacillus amyloliquefaciens*	Njarakkal	28	17	21	-	-	21
MBTDCMFRI Ba13	*Bacillus subtilis*	Mangalavanam	22	11	-	19	22	21
MBTDCMFRI Ba15	*Bacillus subtilis*	Njarakkal	17	14	21	-	17	19
MBTDCMFRI Ba17	*Bacillus amyloliquefaciens*	Vypeen barmouth	15	12	20	-	15	18
MBTDCMFRI Ba22	*Bacillus amyloliquefaciens*	Mangalavanam	15	14	19	-	15	19
MBTDCMFRI Ba23	*Bacillus amyloliquefaciens*	Perandoor canal	14	12	21	-	14	16
MBTDCMFRI Ba25	*Bacillus subtilis*	Mangalavanam	24	12	-	11	-	24
MBTDCMFRI Ba26	*Bacillus subtilis*	Eloor ferry	16	12	-	13	16	17
MBTDCMFRI Ba27	*Bacillus subtilis*	Perandoor canal	15	14	-	14	15	18
MBTDCMFRI Ba28	*Bacillus subtilis*	Perandoor canal	13	10	-	-	13	20
MBTDCMFRI Ba29	*Bacillus subtilis*	Marine science jetty	15	12	11	13	15	20
MBTDCMFRI Ba30	*Bacillus subtilis*	Marine science jetty	17	10	-	11	17	20
MBTDCMFRI Ba31	*Bacillus subtilis*	Mangalavanam	22	-	14	-	11	8
MBTDCMFRI Ba32	*Bacillus subtilis*	Perandoor canal	14	11	13	-	10	11
MBTDCMFRI Ba33	*Bacillus pumilus*	Njarakkal	18	17	15	-	17	17
MBTDCMFRI Ba34	*Bacillus subtilis*	Perandoor canal	18	11	12	-	-	12
MBTDCMFRI Ba35	*Bacillus subtilis*	Perandoor canal	22	-	12	-	12	12
MBTDCMFRI Ba36	*Bacillus amyloliquefaciens*	Perandoor canal	17	15	-	9	19	18
MBTDCMFRI Ba37	*Bacillus subtilis*	Mangalavanam	20	10	10	18	20	15
MBTDCMFRI Ba38	*Bacillus subtilis*	Mangalavanam	19	10	10	16	19	15
MBTDCMFRI Ba39	*Bacillus pumilus*	Njarakkal	11	10	-	22	11	13
MBTDCMFRI Ps01	*Pseudomonas aeruginosa*	Vypeen barmouth	25	12	19	13	21	20
MBTDCMFRI Ps02	*Pseudomonas aeruginosa*	Vypeen barmouth	23	12	20	12	18	19
MBTDCMFRI Ps03	*Pseudomonas aeruginosa*	Vypeen barmouth	23	13	21	13	20	20
MBTDCMFRI Ps04	*Pseudomonas aeruginosa*	Marine science jetty	22	15	21	14	19	20
MBTDCMFRI Ps05	*Pseudomonas aeruginosa*	Marine science jetty	23	14	20	13	18	18
MBTDCMFRI Ps06	*Pseudomonas aeruginosa*	Vypeen harbour	20	14	19	13	19	17
MBTDCMFRI Ps07	*Pseudomonas aeruginosa*	Njarakkal	19	15	20	14	20	19
MBTDCMFRI Ps08	*Pseudomonas aeruginosa*	Mangalavanam	18	14	20	11	20	20
MBTDCMFRI Ps09	*Pseudomonas aeruginosa*	Marine science jetty	21	15	18	13	16	16

A - *Vibrio vulnificus* MTCC1145; B - *Vibrio parahaemolyticus* MTCC 451; C- *Aeromonas hydrophila*; D - *Vibrio alginolyticus* 101; E- *Vibrio harveyi* 101 and F- *Vibrio anguillarum* A1. The measurement indicates the inhibition zone (mm) formed around the isolates. The assays were carried out in triplicates through spot diffusion method. The values may differ $\pm$ 1mm from the size mentioned.

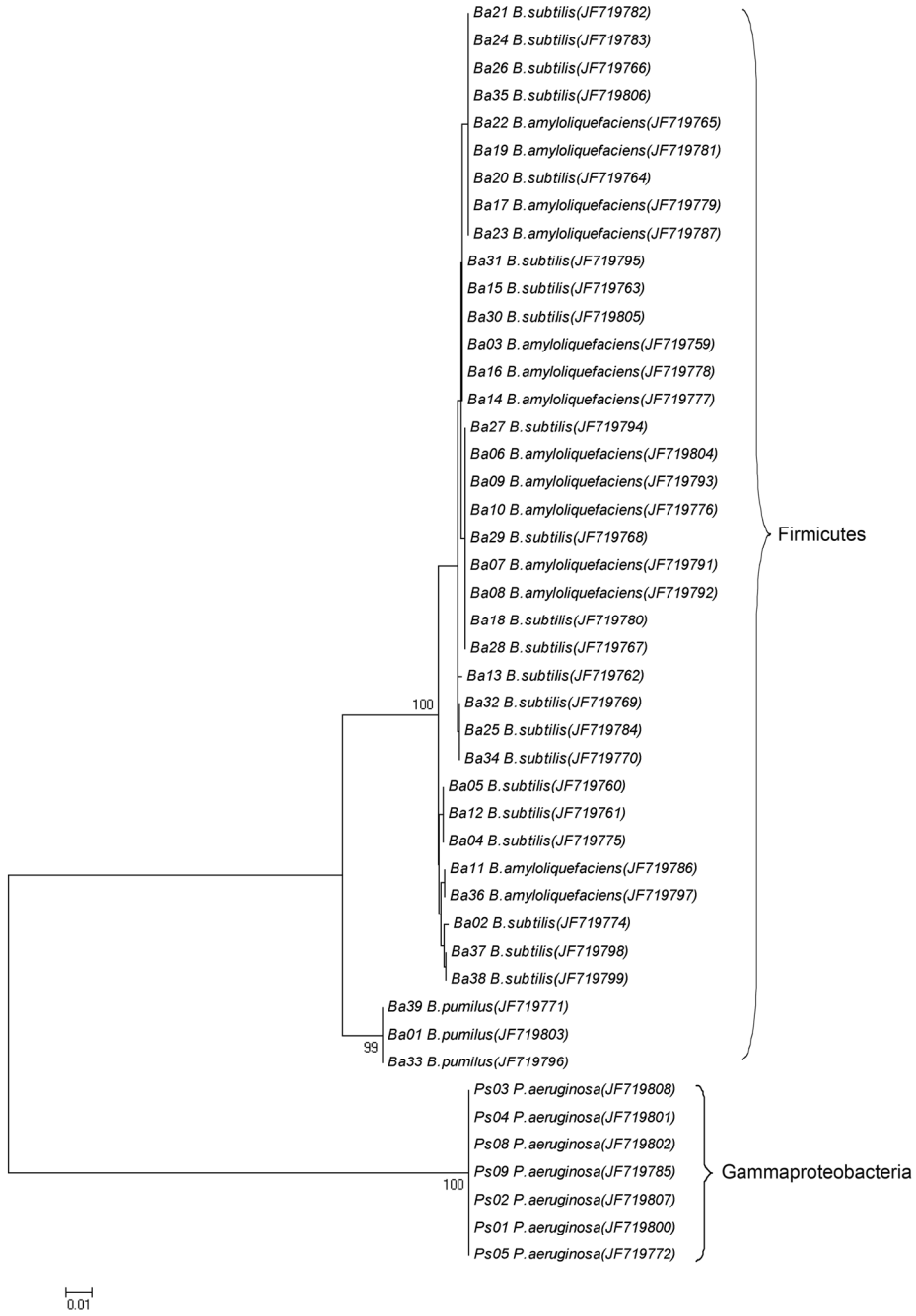

Fig. 2.4 Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequence of antagonistic bacterial isolates and the reference strains with MEGA 5 software

Legends: Isolate code for all strains (Ba01-Ba39, Ps01-Ps05 and Ps08-Ps09) starts with MBTDCMFRI as given in Table 1.

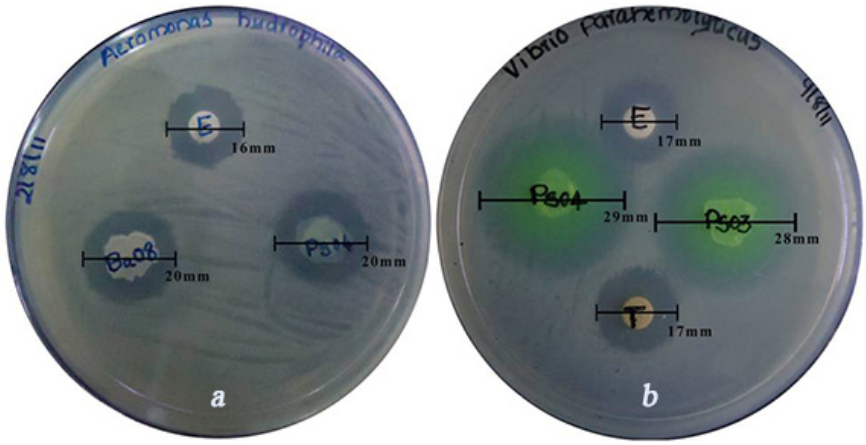

Fig. 2.5 **(a)** Antagonistic activity of the *Bacillus* sp. (MBTDCMFRI Ba08) and *Pseudomonas* sp. (MBTDCMFRI Ps04) compared with erythromycin (E) against *Aeromonashydrophila.* **(b)** Antagonistic activity of the *Pseudomonas* sp. (MBTDCMFRI Ps03 - Ps04) compared with erythromycin (E) and tetracycline (T) against *Vibrioparahaemolyticus*MTCC451.

2.4.5 Enzymatic Assay

Based on the pattern of inhibition, variation among strains and their sites of origin, 34 isolates (25 *Bacillus* spp. and 9 *Pseudomonas* spp.) among the 48 antagonistic isolates were selected for further screening, including enzymatic assays, abiotic stress tolerance and antibiotic resistance. All *Pseudomonas* strains (MBTDCMFRI Ps01- MBTDCMFRI Ps09) were positive for gelatinase, protease and lipase activity and negative for amylase, phosphatase and cellulase. Except *B. pumilus* (MBTDCMFRI Ba01, MBTDCMFRI Ba33 and MBTDCMFRI Ba39) all *Bacillus* strains exhibited amylase activity. Except MBTDCMFRI Ba33 and MBTDCMFRI Ba39, all *Bacillus* strains were urease positive. All *Bacillus* spp. exhibited cellulase and lipase activity and rest of the results are shown in Table 2.2. Amongst the 34 scrutinized isolates, four *Bacillus* isolates (MBTDCMFRI Ba07- Ba08, MBTDCMFRI Ba37 - Ba38) showed significant activity for all seven extracellular enzymes studied.

2.4.6 Abiotic stress Tolerance Assay

Assessment of abiotic stress tolerance of the selected isolates against salinity, temperature and pH was made. It was observed that a salinity gradient of 15-30 ppt in the growth medium supported confluent growth of all the isolates. *Bacillus* spp. MBTDCMFRI Ba01, MBTDCMFRI Ba33, MBTDCMFRI Ba39 and all 9 *Pseudomonas* spp. showed growth only up to 50 ppt. Rest of the isolates showed growth up to 100 ppt beyond which no growth was observed. The maximum temperature tolerance for *Bacillus* spp. and *Pseudomonas* spp. was 55ºC and 45ºC respectively, although both grew well between 25ºC and 45ºC. Any pH between 4 and 10 supported growth of all the isolates, where the range 5 - 9 supported the maximum growth (Table 2.2).

2.4.7 Antibiotic Resistance Pattern

All the selected strains of *Bacillus* spp. were 100% resistant to bacitracin but sensitive to other antibiotics tested. The sensitivity pattern for *Pseudomonas* spp. varied significantly. All *Pseudomonas* isolates were resistant to penicillin, bacitracin, erythromycin and ampicillin but sensitive to ciprofloxacin, chloramphenicol and gentamicin. Additionally, strains MBTDCMFRI Ps06 and MBTDCMFRI Ps09 were resistant to streptomycin and kanamycin, whereas, the other 7 strains were sensitive. Except MBTDCMFRI Ps01, MBTDCMFRI Ps03 and MBTDCMFRI Ps09, all other *Pseudomonas* strains were sensitive to tetracycline (Table 2.2).

Table 2.2 Profile of *in vitro* characterization of the antagonistic isolates

ISOLATE CODE	ENZYMATIC ASSAY							TOLERANCE ASSAY			ANTIBIOTIC SENSITIVITY ASSAY									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	T	U	V
MBTD CMFRI Ba06 MBTD CMFRI Ba07 MBTD CMFRI Ba08 MBTD CMFRI Ba09 MBTD CMFRI Ba17	+	+	+	+	+	+	+	4 -10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba25 MBTD CMFRI Ba37	++	+	+	++	+	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba28 MBTD CMFRI Ba38	+	+	++	+	+	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba31	+	+	++	+	++	++	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba35	+	+	++	+	++	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba30	+	+	++	++	+	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba13 MBTD CMFRI Ba15 MBTD CMFRI Ba26	+	+	-	+	+	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba23 MBTD CMFRI Ba27	++	+	-	+	+	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba29	+	+	-	+	+	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba32	+	+	-	++	++	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba34 MBTD CMFRI Ba36	+	+	-	+	++	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba22	+	+	++	-	+	+	+	4-10	20 - 55	0 - 100	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba01	+	+	-	+	-	-	+	4-10	20 - 55	0 - 50	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba33	+	-	-	+	-	+	+	4-10	20 - 55	0 - 50	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ba39	+	-	-	+	-	+	-	4-10	20 - 55	0 - 50	S	R	S	S	S	S	S	S	S	S
MBTD CMFRI Ps01	+	+	-	+	-	+	-	4-10	20 - 40	0 - 50	R	R	R	R	S	S	S	S	R	R
MBTD CMFRI Ps07	+	+	-	+	-	+	-	4-10	20 - 40	0 - 50	R	R	R	S	S	S	S	S	R	R
MBTD CMFRI Ps03	+	+	-	++	-	+	-	4-10	20 - 40	0 - 50	R	R	R	R	S	S	S	S	R	R
MBTD CMFRI Ps08	+	+	-	++	-	+	-	4-10	20 - 40	0 - 50	R	R	R	S	S	S	S	S	R	R
MBTD CMFRI Ps02 MBTD CMFRI Ps04 MBTD CMFRI Ps05	+	-	-	+	-	+	-	4-10	20 - 40	0 - 50	R	R	R	S	S	S	S	S	R	R
MBTD CMFRI Ps06	+	-	-	++	-	+	-	4-10	20 - 40	0 - 50	R	R	R	S	R	S	S	S	R	S
MBTD CMFRI Ps09	+	-	-	++	-	+	-	4-10	20 - 40	0 - 50	R	R	R	R	R	S	S	S	R	S

A - Lipase, B - Urease, C - Phosphatase, D - Gelatinase, E - Amylase, F - Protease, G - Cellulase, H - Hydrogen ion concentration (pH), I -Temperature (°C), J - Salinity (ppt), K - Penicillin (10U), L - Bacitracin (10U), M - Erythromycin (15mcg), N - Tetracycline (30mcg), O - Streptomycin (10mcg), P - Ciproflaxacin (5mcg), Q - Chloramphenicol (30mcg), T - Gentamycin (10mcg), U - Ampicillin (10mcg), V - Kanamycin (30mcg), S-Sensitive, R - Resistant, [-] no enzymatic activity, [+] zone diameter - 10-20 mm and [++] zone diameter - 20-30 mm.

2.5 Discussion

In the present study heterotrophic bacteria with significant antibacterial properties were isolated from the tropical estuarine habitats of Cochin. The selected isolates were further identified and characterized for prospective use as bio-control agents against bacterial diseases in aquaculture. Broad classification of the isolates with antagonistic activity showed that they belong to the phyla Firmicutes and Proteobacteria (Class γ- Proteobacteria), which is in agreement with Nithya and Pandian (2010). Zheng et al. (2005) reported that *Bacillus*, *Pseudomonas*, *Vibrio*, *Alteromonas* and *Streptomycetes* were the common genera that produce antimicrobial metabolites. Later, Kennedy et al. (2009) also endorsed that the aforesaid microorganisms from marine sources provide numerous essential products such as antimicrobials, anti-inflammatory agents and antioxidants. The Mangalavanam mangroves yielded the highest bacterial diversity with antagonistic properties which could be due to enhanced availability of nutrients in the sediments and water of mangrove environment (Sahoo and Dhal 2009). Samples from Perandoor canal were also rich in cultivable bacteria, probably due to the discharge of organic wastes and sewage into it. The low diversity of bacteria in samples collected from the Eloor ferry could be attributed to the acute heavy metal pollution (Jose et al. 2011).

Phylogenetic analysis of the antibacterial isolates revealed two different bacterial groups, *Bacillus* and *Pseudomonas*. Wiese et al. (2009) reported that the members of genus *Bacillus* produce almost 800 metabolites including antimicrobial agents in the form of low molecular weight peptides. *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus*, isolated in this study, have been noted as good bio-control agents and have been often reported to produce secondary metabolites with antagonistic properties (Wulff et al. 2002). In the phylogenetic tree, *B. amyloliquefaciens* and *B. subtilis* clustered together,

revealing their relatedness. *B. amyloliquefaciens* is thought to have originated from *B. subtilis* therefore exhibiting similar phenotypic and genotypic characters (Priest et al. 1987). Species level identification and characterization into *B. amyloliquefaciens* and *B. subtilis* was achieved following the methods described by Reva et al. (2001). The genus *Pseudomonas* is also a well-known candidate for production of antimicrobial agents (Chythanya et al. 2002; Vijayan et al. 2006). Of the total active metabolites derived from microbes, more than 2.7% are obtained from *Pseudomonas* species (Berdy 2005).

The antibacterial activity exhibited by different isolates of the same species differed with each other in the plate assay. This is in support with the report of Vynne et al. (2011) that the inhibitory activity and secondary metabolite production would vary within the species level itself. The inhibitory effects of *Bacillus* spp. could be due to the production of antimicrobial peptides, volatile compounds or by altering the pH of the surrounding medium and by the utilization of essential nutrients (Bizani and Brandelli 2002; Vaseeharan and Ramasamy 2003). Similarly, the broad spectrum antagonistic activity of *Pseudomonas* spp. has been attributed to a number of factors, such as the production of phenazine compound, hydrogen cyanide or iron chelating siderophores and surface attachment inhibitors (Vijayan et al. 2006; Kennedy et al. 2009; Preetha et al. 2010; Pai et al. 2010). The present study also revealed the fact that the majority of the active antagonistic bacterial isolates were obtained from sediment samples rather than from water samples since the bacteria attached to surfaces tend to produce more inhibitory compounds than free living forms to protect their habitat niche (Long and Azam 2001).

Bacteria with antibacterial properties are widely used in aquaculture to control bacterial diseases by adopting different methods to introduce them into

the host and the system. As probionts/live agents, they can control pathogenic bacterial proliferation by colonizing surfaces, competitive exclusion and by producing inhibitory compounds (Kennedy et al. 2009). *Bacillus* spp. with antagonistic activity against bacterial pathogens are widely and successfully used as probiotics in aquaculture systems (Vaseeharan and Ramasamy 2006; Balcazar and Rojas-Luna 2007). Interestingly, inactivated bacterial cell preparations applied as feed additives also show positive effects in controlling bacterial infections in goldfish and rainbow trout (Irianto and Austin 2003; Irianto et al. 2003). Such application takes away the concerns of using live biocontrol agents such as *Pseudomonas aeruginosa* which is an opportunistic pathogen to humans, animals and plants (Khan et al. 2007).

Most of the antagonistic isolates obtained from the study were also capable of producing hydrolytic enzymes such as lipase, cellulase, gelatinase etc. which could support digestion and enhanced nutrient uptake of the host (Pai et al. 2010). It has been reported that bacteria may contribute to the digestion processes of bivalves by producing extracellular enzymes, and essential growth factors (Prieur et al. 1990). These characteristics further enhance the suitability of the selected bacteria to supplement the nutrition of the host animals (Boonthai et al. 2011). The inherent nature of the selected antagonistic bacterial strains to grow under a wide range of environmental conditions would help their better adaptation towards the host and the aquatic environment aiding their function as bio-control agents (Vine et al. 2006). It has also been reported that fish fed with live biocontrol agents showed increased disease resistance or host survival by stimulating host immune system (Taoka et al. 2006; Pai et al. 2010). Similarly, it was observed that the dead or inactivated bacterial cells also stimulated the innate immune system of aquatic animals (Irianto and Austin, 2003; Irianto et al. 2003).

Bacteria selected as prospective probionts should not carry the genes for antibiotic resistance (Saarela et al. 2000). The *Bacillus* spp. isolated in this study were sensitive to all the antibiotics tested, except bacitracin. This resistance can be considered null because this antibiotic is an active polypeptide molecule produced by most members of this genus (Bizani and Brandelli 2002). On the other hand, *Pseudomonas aeruginosa* strains isolated showed resistance to certain antibiotics, which is believed to be due to its efficient multidrug efflux system or the presence of various resistance factors (Lambert 2002). Resistances to antibiotics like penicillin, ampicillin, bacitracin and erythromycin were already reported in most of the gram negative bacteria (Serrano 2005). Some isolates obtained from the study were sensitive to all aminoglycoside antibiotics except kanamycin. Considering the above criteria the chance of transferring multiple antibiotic resistances to the pathogens is limited. Consequently, this study revealed that the selected antagonistic bacteria obtained from the estuarine regions of Cochin may function as probionts in aquaculture.

To summarize, bacterial strains isolated from the tropical estuarine habitats of Cochin representing the genera *Pseudomonas* (MBTDCMFRI Ps02, Ps04, Ps05, Ps07 and Ps08) and *Bacillus* (MBTDCMFRI Ba01, Ba29, Ba37 and Ba38) were able to resist and survive under diverse physiological stresses, produce multiple enzymes and possessed significant antibacterial activity against aquaculture pathogens. Further studies to exploit the antagonistic potential of these bacteria will allow their utilization as live probionts or inactivated microbial products as feed additives, providing an alternative to the use of commercial antibiotics for the control bacterial diseases in aquaculture.

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Chapter 3

MOLECULAR APPROACH FOR THE RAPID DETECTION OF *BACILLUS* AND *PSEUDOMONAS* GENERA USING COLONY MULTIPLEX PCR

Contents

3.1 Abstract

Bacillus and *Pseudomonas* are the dominant groups of bacteria known for their antagonistic potential against many plant and animal pathogens. Presently, exploration of these genera with antagonistic property, for disease management of aquaculture system is gaining more importance to overcome the use of antibiotics and related resistance issues. Rapid screening and identification of these genera, from diverse bacterial populations by conventional methods is laborious, cost intensive and time consuming. To overcome these limiting factors, in the present study, a colony multiplex PCR (*cm*PCR) method was developed and evaluated for the rapid detection of *Bacillus* and *Pseudomonas*. The technique amplifies the partial 16S rRNA gene of *Bacillus* and *Pseudomonas* with a product size of ~1100 and ~375 bp, respectively, using single forward (BSF2) and two reverse primers (PAGSR and BK1R). Reliability of the *cm*PCR method was confirmed by screening 472 isolates obtained from 10 different eco-stations, of which 133 isolates

belonged to *Bacillus* and 32 to *Pseudomonas*. The *cm*PCR method also helped to identify 6 different *Pseudomonas* spp. and 14 different *Bacillus* spp. from environmental samples. Of the total 472 isolates studied, 46 showed antagonistic activity, among which 63% were *Bacillus* and 17.4% were *Pseudomonas*. Thus, the newly developed molecular approach provides a quick, sensitive and a potential screening tool to detect novel, antagonistically important *Bacillus* and *Pseudomonas* genera for their use in aquaculture. Further, it can also act as a taxonomic tool to understand the distribution of these genera from wide ecological niches and their exploitation for diverse biotechnological applications.

3.2 Introduction

Aquaculture is the fastest growing food production sector in the world and accounts for approximately 47% of the world's total fish food supply (FAO 2012). Disease outbreaks due to pathogenic bacteria have presented a major challenge, which adversely affects the development of aquaculture based fish production (Gomez et al. 2000). The therapeutic options available in the control of bacterial diseases are the use of approved antibiotics, chemicals and vaccination, but these applications were limited due to the development of antibiotic resistances and lack of consistency. Nowadays, the use of antagonistic bacteria such as *Bacillus*, *Pseudomonas*, *Alteromonas* and *Flavobacterium* are also gaining importance in aquaculture industry to control bacterial diseases because of its ease in application and absence of side effects (Zheng et al. 2005; Balcazar and Rojas-Luna 2007; Vinoj et al. 2013). Our previous studies have clearly emphasized the importance of the genera *Bacillus* and *Pseudomonas*, with antagonistic activity and its application in aquaculture systems (Vijayan et al. 2006; Nair et al. 2012). These genera were also reported for its potential antimicrobial activity against many plant and

human pathogens (Nithya and Pandian 2010; Sharma and Kaur 2010).

Generally, the identification of bacteria by conventional methods is time-consuming, invariably mono-specific and also laborious, especially when screening a large number of field samples (Chen et al. 2012). The methods like fatty acid methyl ester (FAME) profiling (Bobbie and White 1980; Vaerewijck et al. 2001) and the API system based identification have been shown to be more useful than classical methods (Logan and Berkeley 1984). However, these phenotypical protocols are again laborious, time-consuming and are ineffective in rapid identification, with many non-specific results (Wattiau et al. 2001). The disadvantages associated with these techniques could be overcome by employing alternative DNA based detection methods which are generally faster, specific and more reliable in identification (Olive and Bean 1999; Cunningham 2002).

The 16S rDNA based identification of bacteria has been widely recognized in studying distinct phylogenetic relationships and are effective in developing numerous taxonomical tools (Stackebrandt and Goebel 1994; Maeda et al. 2006). Uniplex PCR based assays have also been reported to be useful in the identification of bacteria (Garbeva et al. 2004; Spilker et al. 2004) however, the rapid detection of multiple bacteria of interest in a single reaction is complicated as it requires simultaneous amplification of more than one locus, and realizing the required specificity (Maeda et al. 2006). Compared to individual PCR assays, multiplex PCR allows the amplification and detection of multi-targeted genes in a single reaction which minimizes the time, cost and effort required for identification of different groups of bacteria, especially when studying a large number of field samples (Chamberlain and Chamberlain 1994; Perry et al. 2007; Ozdemir 2009; Altinok 2011)

Direct colony PCR is a useful approach that can avoid the difficulties

encountered during the isolation of DNA. This method was found to be a quick, precise and cost effective tool for characterizing a large number of isolates from environmental samples (Kong 2005). Colony PCR was initially applied in bacteria (Hofmann and Brian 1991) followed by yeast (Ward 1992) and fungi (Van Zeijl et al. 1998), and eventually applied for detection purposes in higher organisms (Wan et al. 2011). Earlier reports about the genus-specific primers for *Bacillus* (Kwon et al. 2005; Wu X-Y et al. 2006) and *Pseudomonas* (Garbeva et al. 2004; Spilker et al. 2004), and the 16S rDNA information of these genera obtained from the NCBI database served as a base for developing a multiplex PCR assay for the simultaneous detection of both the genera. The present work was intended to develop a multiplex PCR assay in combination with colony PCR as a molecular screening tool for simultaneous identification of the genera *Bacillus* and *Pseudomonas* in a single reaction, and also to validate the reliability of the method to detect these genera from various ecological niches.

3.3 Materials and Methods

3.3.1 Bacterial Strains and Culture Conditions

Bacterial strains used in this study belonged to the Microbial Culture Collection, Marine Biotechnology Division (MCC-MBTD), Central Marine Fisheries Research Institute (CMFRI), Cochin, India (Table 3.1a). Apart from the above, 21 reference strains used in this study were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India and Central

Institute of Brackish water Aquaculture (CIBA), Chennai, India (Table 3.1b). Bacterial strains were preserved in nutrient broth as glycerol stocks under -80ºC for further use.

Table 3.1a List of various strains used in this study and their identification details by various methods

Species	Number of isolates (n)	Source	Identification		
			Biochemical	16S rDNA sequencing	*cm*PCR assay
Pseudomonas aeruginosa	9	Sediment/Water	*Pseudomonas* sp.	*Pseudomonas aeruginosa*	*Pseudomonas* sp. *
Pseudomonas fluorescens	2	Sediment	*Pseudomonas* sp.	*Pseudomonas fluorescens*	*Pseudomonas* sp. *
Pseudomonas mendocina	2	Fish/Microalgae	*Pseudomonas* sp.	*Pseudomonas mendocina*	*Pseudomonas* sp. *
Pseudomonas pseudoalcaligenes	1	Sediment	*Pseudomonas* sp.	*Pseudomonas pseudoalcaligenes*	*Pseudomonas* sp.
Pseudomonas putida	5	Sediment/Water	*Pseudomonas* sp.	*Pseudomonas putida*	*Pseudomonas* sp. *
Pseudomonas syringae	1	Water	*Pseudomonas syringae*	*Pseudomonas syringae*	*Pseudomonas* sp.
Pseudomonas sp.	5	Sediment	*Pseudomonas* sp.	*Pseudomonas* sp.	*Pseudomonas* sp.
Bacillus amyloliquefaciens	14	Sediment/Water	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens*	*Bacillus* sp. *
Bacillus aquimaris	3	Sediment	*Bacillus aquimaris*	*Bacillus aquimaris*	*Bacillus* sp.
Bacillus cereus	6	Fish/Sediment	*Bacillus* sp.	*Bacillus cereus*	*Bacillus* sp. *
Bacillus cibi	1	Sediment	*Bacillus cibi*	*Bacillus cibi*	*Bacillus* sp.
Bacillus firmus	1	Sediment	*Bacillus* sp.	*Bacillus firmus*	*Bacillus* sp. *
Bacillus flexus	2	Fish/ Water	*Bacillus* sp.	*Bacillus flexus*	*Bacillus* sp.
Bacillus fusiformis	1	Fish	*Bacillus* sp.	*Bacillus fusiformis*	*Bacillus* sp.
Bacillus horikoshii	1	Sediment	*Bacillus* sp.	*Bacillus horikoshii*	*Bacillus* sp.
Bacillus licheniformis	2	Fish	*Bacillus licheniformis*	*Bacillus licheniformis*	*Bacillus* sp. *
Bacillus marisflavi	6	Sediment	*Bacillus marisflavi*	*Bacillus marisflavi*	*Bacillus* sp.
Bacillus megaterium	3	Fish/Sediment	*Bacillus* sp.	*Bacillus megaterium*	*Bacillus* sp.
Bacillus pumilus	8	Sediment/Microalgae	*Bacillus pumilus*	*Bacillus pumilus*	*Bacillus* sp. *
Bacillus sphaericus	1	Sediment	*Bacillus sphaericus*	*Bacillus sphaericus*	*Bacillus* sp.
Bacillus subtilis	24	Sediment/Water	*Bacillus subtilis*	*Bacillus subtilis*	*Bacillus* sp. *
Bacillus sp.	2	Sediment	*Bacillus* sp.	*Bacillus* sp.	*Bacillus* sp.
Acinetobacter baumanii	1	Water	*Acinetobacter* sp.	*Acinetobacter baumanii*	No amplification
Acinetobacter sp.	2	Sediment	*Acinetobacter* sp.	*Acinetobacter* sp.	No amplification
Aeromonas veronii	1	Fish	*Aeromonas veronii*	*Aeromonas veronii*	No amplification
Aeromonas sp.	1	Sediment	*Aeromonas* sp.	*Aeromonas aquariorum*	No amplification
Alcaligenes faecalis	8	Sediment	*Alcaligenes* sp.	*Alcaligenes faecalis*	No amplification
Alcanivorax sp.	2	Water	*Alcanivorax* sp.	*Alcanivorax dieselolei*	No amplification
Arthrospira maxima	3	Water	*Arthrospira maxima*	*Arthrospira maxima*	No amplification
Brevibacterium sp.	2	Fish	*Brevibacterium* sp.	*Brevibacterium* sp.	No amplification
Citrobacter freundii	3	Fish	*Citrobacter* sp.	*Citrobacter freundii*	No amplification
Enterobacter sp.	3	Fish	*Enterobacter* sp.	*Enterobacter* sp.	No amplification
Enterococus faecium	1	Fish	*Enterococus* sp.	*Enterococus faecium*	No amplification
Escherichia coli	2	Water	*Escherichia* sp.	*Escherichia coli*	No amplification
Halomonas sp.	1	Fish	*Halomonas* sp.	*Halomonas aquamarina*	No amplification
Micrococcus sp.	1	Water	*Micrococcus* sp.	*Micrococcus* sp.	No amplification
Nocardiopsis sp.	1	Sediment	*Nocardiopsis* sp.	*Nocardiopsis* sp.	No amplification
Oceanomonas sp.	1	Sediment	*Oceanomonas* sp.	*Oceanomonas dourdoffi*	No amplification
Pedobacter sp.	1	Sediment	*Pedobacter* sp.	*Pedobacter* sp.	No amplification
Pseudoalteromonas sp.	2	Fish	*Pseudoalteromonas* sp.	*Pseudoalteromonas* sp.	No amplification
Shewanella sp.	3	Oyster /Water	*Shewanella* sp.	*Shewanella* sp.	No amplification
Streptomyces sp.	3	Sediment	*Streptomyces* sp.	*Streptomyces* sp.	No amplification
Vibrio alginolyticus	6	Water	*Vibrio* sp.	*Vibrio alginolyticus*	No amplification
Vibrio cholerae	2	Water	*Vibrio* sp.	*Vibrio cholerae*	No amplification
Vibrio fluvialis	8	Sediment	*Vibrio fluvialis*	*Vibrio fluvialis*	No amplification
Vibrio sp.	2	Water	*Vibrio* sp.	*Vibrio* sp.	No amplification

*An isolate from each of these species were cross-checked by sequencing the *cm*PCR product. The sequenced results matches with the results of 16S rDNA.

Table 3.1b Reference strains used in the study

Species	Strain code	Source	Identification	
			Biochemical and 16S rDNA sequencing	Multiplex PCR assay
Pseudomonas aeruginosa	MTCC1688	Microbial Type Culture Collection, Chandigarh, India	*Pseudomonas aeruginosa*	*Pseudomonas* sp.
P. fluorescens	MTCC103		*P. fluorescens*	*Pseudomonas* sp.
Bacillus subtilis	MTCC441		*Bacillus subtilis*	*Bacillus* sp.
B. amyloliquefaciens	MTCC1270		*B. amyloliquefaciens*	*Bacillus* sp.
B. cereus	MTCC430		*B. cereus*	*Bacillus* sp.
B. licheniformis	MTCC6824		*B. licheniformis*	*Bacillus* sp.
Aeromonas hydrophila	MTCC1739		*Aeromonas hydrophila*	No amplification
Vibrio vulnificus	MTCC1145, MTCC1146		*Vibrio vulnificus*	
V. alginolyticus	MTCC4439		*V. alginolyticus*	
V. parahaemolyticus	MTCC451		*V. parahaemolyticus*	
V. anguillarum	O1& A1	Central Institute of Brackishwater Aquaculture, Chennai, India	*V. anguillarum*	No amplification
V. harveyi	101, 102, LB203, LB208, LB166 & LB209		*V. harveyi*	
V. alginolyticus	101 & 102		*V. alginolyticus*	

3.3.2 Oligonucleotide Design/ Primer Selection

Generic level variations existing in 16S rDNA sequences were utilized for designing specific primers. Genus specific oligonucleotides were designed for *Bacillus* and *Pseudomonas* from the 16S rDNA sequences (72 *Bacillus* spp. and 92 *Pseudomonas* spp.) available in the GenBank database, including the submissions from our own collections. Multiple alignments of 16S rDNA sequences of *Bacillus*, *Pseudomonas* and its phylogenetically related strains were carried out with ClustalW using BioEdit software (version 7.1.3.0) (Hall 1999). The conserved sites from the aligned sequences were identified and the target specific primers were designed manually with an average length of 18 - 22 bp having an annealing temperature range between 55 - 62ºC. The performance of the designed primers was validated with the published primers as combinations. The oligonucleotide primers used were synthesized commercially (Sigma, India), and were suspended in sterile milliQ water and stored at -20ºC until use (Table 3.2).

Table 3.2 Genus specific primers used in this study to identify the genera *Bacillus* and *Pseudomonas* spp.

PRIMERS	SEQUENCE (5'-3')	LENGTH (nt)	PRODUCT SIZE	REFERENCES
Target Genus - *Pseudomonas*				
PsF	TTA GCT CCA CCT CGC GGC	18	960bp	Garbeva et al. [11]
PsR	GGT CTG AGA GGA TGA TCA GT	20		
PA-GS-F	GAC GGG TGA GTA ATG CCT A	19	618bp	Spilker et al. [30]
PA-GS-R	**CAC TGG TGT TCC TTC CTA TA**	**20**		
PSF1	GGT CTG AGA GGA TGA TCA G	19	Present study	
PSF2	ACA CTG GAA CTG AGA CAC GG	20		
PSF6	CGG AAT TAC TGG GCG TAA A	19		
PSR1	CGT GGA CTA CCA GGG TAT CTA	21		
PSR6	GCC GTA AGG GCC ATG ATG A	19		
PSR7	ATT ACT AGC GAT TCC GAC TTC	21		
Target Genus – *Bacillus*				
Bac-F	CGG CGT GCC TAA TAC ATG CAA G	22	1200bp	Kwon et al. [19]
Bac-R	GGC ATG CTG ATC CGC GAT TAC TA	23		
B-K1/F	TCA CCA AGG CRA CGA TGC G	19	1114bp	Wu et al. [39]
B-K1/R	**CGT ATT CAC CGC GGC ATG**	**18**		
BSF1	ACA CTG GGA CTG AGA CAC G	19	Present study	
BSF2	**TAC GGG AGG CAG CAG TRG G**	**19**		
BSF6	GAG GAA CAC CAG TGG CGA A	19		
BSR1	CCA GGG TAT CTA ATC CTG T	19		
BSR2	CCG TCA ATT CCT TTG AGT TT	20		
BSR5	GTT GCG CTC GTT GCG GGA	18		

3.3.3 DNA Extraction

Multiplex PCR optimization was initially carried out with purified bacterial DNA. Bacterial cells (5 ml) grown (18-24 h old) were pelletized by centrifugation at 8000 rpm for 10 min. The cells were re-suspended in 450 µl TEG (25 mM TrisHCl; 10 mM EDTA; 50 mM glucose) buffer (pH 8) containing lysozyme (5 mg/ml). The suspension was vortexed thoroughly and mixed with 35 µl of 10% SDS. The tubes were then incubated on ice for 10 min, followed by the addition of 5 µl of proteinase K (20 mg/ml). This was

further incubated at 60ºC in a water bath for 60 min. After the completion of cell lysis, the DNA was purified with the standard phenol/chloroform extraction method (Sambrook and Russell 2001). The isolated DNA was quantified using Biophotometer (Eppendorf, Germany). Purified bacterial DNA was dissolved in 30 µl of TE buffer and stored at -20ºC for future use.

3.3.4 Uniplex PCR

All the 20 primers were individually tested and optimized for the conditions to amplify the genus *Bacillus* and *Pseudomonas*. The total reaction volume (25 µl) comprised of bacterial DNA (50 ng), 10 pmol each of two oligonucleotide primers, 2.5 mM of each deoxynucleoside triphosphate (Finnzymes), 1.5 U of Taq polymerase (Sigma) and 2.5 µl of 10X PCR buffer. Annealing temperature was standardized to a range of 48 - 62ºC. The amplification was carried out in a Veriti thermal cycler (Applied Biosystems, UK) with an initial denaturation at 95ºC for 5 min, followed by 35cycles of 94ºC for 30 s, 59ºC for 30 s and 72ºC for 45 s and a final extension of 72ºC for 10 min. In each PCR reaction a set of negative bacterial control (*Aeromonas hydrophila* MTCC1739, *Alcaligenes faecalis* MBTDCMFRI Af01, *Enterobacter* sp. MBTDCMFRI Eb01, *Enterococcus* sp. MBTDCMFRI Ec01, *Escherichia coli* DH5alpha, *Shewanella* sp. MBTDCMFRI Sh02 and *Vibrio vulnificus* MTCC1145) and a negative reaction control (without template DNA) was also maintained. The amplified products were separated by electrophoresis in 1.5% agarose gel and visualized under UV transilluminator by staining with ethidium bromide. The size (bp) of the amplified product was calculated by comparing it with standard molecular weight DNA marker (Step-up 100 bp DNA ladder, Merck, India) using the software Image Lab version 3 (Bio-Rad Laboratories, Inc., USA).

3.3.5 Multiplex PCR

Based on the specificity and product size obtained from uniplex reactions, two pairs of primers were selected for multiplex PCR optimization. Variables such as annealing temperatures (gradient from 48ºC - 62ºC, with an interval of 2ºC), $MgCl_2$ concentration (1.5 mM - 3 mM) and primer concentrations were optimized with the ability to produce good intensity and specific bands with each targeted DNA. The reaction mixture composition and the amplification conditions are same as in uniplex PCR except for the addition of $MgCl_2$, combination of multiple primers and a change in annealing temperature. A negative reaction control (without template DNA) and a positive control (50 ng/µl) of purified DNA of reference strains (*Bacillus subtilis* MTCC441 and *Pseudomonas aeruginosa* MTCC1688) were also included in each batch of the PCR reaction. The amplified products were detected in 1.5% agarose gel stained with ethidium bromide by gel electrophoresis.

3.3.6 Colony Multiplex PCR

This protocol is based on the crude DNA obtained from boiling water bath method described by Wan et al. (2011). Briefly, the single bacterial colony (18-24 h old) was picked with an autoclaved toothpick and mixed with 100 µl of TE buffer. The mixture was heated in a boiling water bath at 100°C for 10 min. Cells were vortexed and centrifuged at 10000 rpm for 2 min. One microlitre of supernatant was used as template for a 10 µl PCR reaction mix. Thermal cycling conditions and reaction mixture preparation were similar to that of multiplex PCR conditions.

3.3.7 Sensitivity Assay

Sensitivity of the multiplex PCR assay was tested for both purified and crude DNA obtained from bacterial suspensions. The ability to detect the lowest levels for multiplex PCR amplification (limits of detection) was performed in duplicates. Sensitivity of the multiplex PCR assay was evaluated using a series

of targeted genomic DNA by decimally diluting the purified template DNA (concentration 50 ng) in sterile water. From the dilutions, 1µl each were used as DNA template to carry out the multiplex (combinational primers) assay. Similarly, for detecting the sensitivity with the crude DNA, a single colony was picked from the cultures (18-24 h old) grown on nutrient agar and the bacterial suspensions were prepared by dissolving it in 1 ml of sterile saline (0.85% NaCl) then serially diluted (up to 10^{-7} fold). These dilutions (1 µl per reaction volume) were used to determine the concentration (count) of each bacterium to get amplified in *cm*PCR assay. The concentration of each bacterium was determined by surface plating (100 µl) of appropriate dilutions into nutrient agar plates (Himedia, India). The plates were kept in incubation at 30ºC for 18 - 24 h. The counts were taken from each plate and the corresponding CFU/µl present in each dilution was calculated for the reaction.

3.3.8 Specificity Assay

The specificity of colony multiplex PCR in identification of the genus *Bacillus* and *Pseudomonas* was examined with purified and crude DNA obtained from bacterial colonies (separately or in combination) representing different genera. The assay was carried out with the isolates of different genera obtained from the MCC-MBTD, CMFRI by keeping *Bacillus* and *Pseudomonas* as positive controls (Table 3.1a).

3.3.9 Screening of Environmental Isolates

To evaluate the usefulness of the developed *cm*PCR assay, the protocol was applied to screen the bacteria isolated from a wide range of environments.

The sediment samples were collected from 8 different stations (Chettuva, Puthuvype and Mangalavanam - mangrove associated environments; Edavanakkad, Mulavukad, Njarakkal and Andhakaranazhi - fish farming environments and Perandoor - polluted canal), water samples from 2 stations [oyster hatchery (Krishi Vigyan Kendra, Njarakkal) and

ornamental fish hatchery (West coast, Andhakaranazhi)], and also from oyster (*Crassostrea madrasensis*) and fishes from farming environments were used in the study (Fig 3.1). The sample processing and isolation protocols were carried out as described as Nair et al. (2012). Briefly, bacteria were isolated by serially diluting the samples and plated in Zobell marine agar. After 24 - 48 h of incubation, the colonies with distinct morphology were selected and purified. The efficacy of the multiplex PCR protocol in the detection of environmental isolates were confirmed by performing simultaneous assay using both purified DNA and colony PCR method. The isolates which were identified using 16S rDNA were preserved as glycerol stocks under -80ºC.

Fig 3.1 Map showing the sample collection sites from the southwest coast of India

3.3.10 Detection of Antagonistic Activity

Antagonistic activity of the isolated bacteria was screened against aquaculture pathogens by using the spot diffusion method (Nair et al. 2012). Briefly, targeted bacteria were spotted over pre-swabbed plates with aquaculture pathogens *viz*., *Vibrio vulnificus* MTCC1145, *V. harveyi* 101, *V. anguillarum* O1, *V. parahaemolyticus* MTCC451 and *V. alginolyticus* 101. After incubation, the bacteria having notable antagonistic potential (zone of clearance of 10 mm or greater observed around isolates) were selected, identified and preserved for further use.

3.3.11 Identification of Bacteria

The consistency and specificity of *cm*PCR was verified by selecting 160 isolates at random (amplified and non-amplified strains in multiplex PCR assay) for identification using standard biochemical (Krieg and Holt 1984) and molecular (16S rDNA) (Nair et al. 2012) methods. The amplified products were purified (HiPurA PCR product purification kit, Himedia) and sequenced. Sequences of 16S rDNA fragments were imported to BLASTn (Altschul et al. 1990) for similarity searching with available sequences in the GenBank database at NCBI. The sequences obtained were submitted in the GenBank for accession. Similarly, the products obtained through *cm*PCR were also sequenced and the results were cross checked.

3.4 Results

3.4.1 Uniplex PCR Optimization

A total of 10 primer sets was selected for this study, of which 6 primer sets were designed using the information already available in the GenBank and 4 primer pairs were reported elsewhere. Among these, 5 primer sets were

intended to amplify *Pseudomonas* spp. and rest for *Bacillus* spp. (Table 3.2). Uniplex PCR optimization employing each primer pair produced amplified products ranging from 115 - 1200 bp in size. The primer pairs which yielded amplicons sufficient to differentiate the genera *Bacillus* and *Pseudomonas* were picked for optimizing multiplex PCR. Among the 20 primers used, four pairs of primers (PSF2 & PSR1, PAGSF & PAGSR, BSF2 & BSR5 and BK1F & BK1R) were chosen for multiplex PCR with an optimal annealing temperature at 59ºC (Fig 3.2 & 3.3).

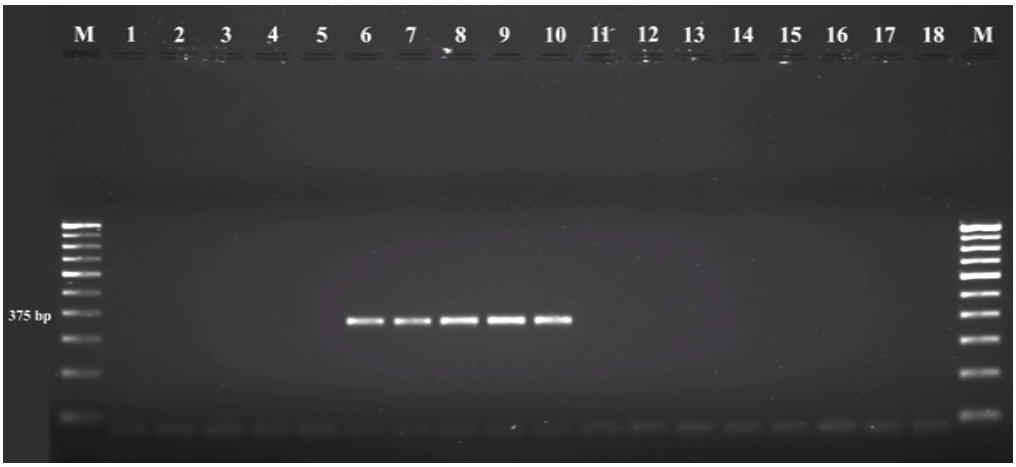

Fig 3.2 The specificity of the uniplex PCR assay developed for the detection of *Pseudomonas* spp. using PSF2 and PAGSR

Legend: Lane M - molecular size marker (100 bp ladder); lane 1 - *Bacillussubtilis* MTCC441; lane 2 - *Bacillus amyloliquefaciens* MTCC1270; lane 3 - *Bacillus licheniformis* MTCC6824; lane 4 - *Bacillus cereus* MTCC430; lane 5 - *Bacillus pumilus* MBTDCMFR1 Ba01; lane 6 - *Pseudomonas aeruginosa* MTCC1688; lane 7 - *Pseudomonas fluorescens* MTCC103; lane 8 - *Pseudomonas putida* MBTDCMFRI Ps20; lane 9 - *Pseudomonas mendocina* MBTDCMFRI Ps21; lane 10 - *Pseudomonas* sp. MBTDCMFRI Ps18; lane 11 - *Aeromonas hydrophila* MTCC1739; lane 12 - *Vibrio vulnificus* MTCC1145; lane 13 - *Enterobacter* sp. MBTDCMFRI Eb01; lane 14 - *Escherichia coli* DH5alpha; lane 15 - *Enterococcus* sp. MBTDCMFRI Ec01; lane 16 - *Alcaligenesfaecalis* MBTDCMFRI Af01; lane 17 - *Shewanella* sp. MBTDCMFRI Sh02; lane 18 - negative control of the reaction (without template).

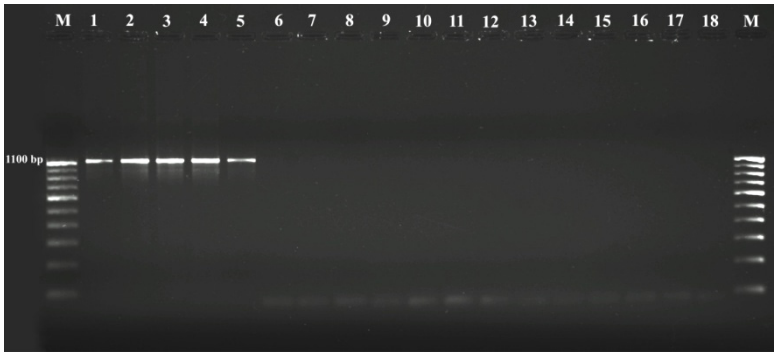

Fig 3.3 The specificity of the uniplex PCR assay developed for the detection of *Bacillus* spp. using BSF2 and BK1R

Legend: Lane M - molecular size marker (100 bp ladder); lane 1 - *Bacillus subtilis* MTCC441; lane 2 - *Bacillus amyloliquefaciens* MTCC1270; lane 3 - *Bacillus licheniformis* MTCC6824; lane 4 - *Bacillus cereus* MTCC430; lane 5 - *Bacillus pumilus* MBTDCMFR1 Ba01; lane 6 - *Pseudomonas aeruginosa* MTCC1688; lane 7 - *Pseudomonas fluorescens* MTCC103; lane 8 - *Pseudomonas putida* MBTDCMFRI Ps20; lane 9 - *Pseudomonas mendocina* MBTDCMFRI Ps21; lane 10 - *Pseudomonas sp*. MBTDCMFRI Ps18; lane 11 - *Aeromonas hydrophila* MTCC1739; lane 12 - *Vibrio vulnificus* MTCC1145; lane 13 - *Enterobacter* sp. MBTDCMFRI Eb01; lane 14 - *Escherichia coli* DH5alpha; lane 15 - *Enterococcus* sp. MBTDCMFRI Ec01; lane 16 - *Alcaligenesfaecalis* MBTDCMFRI Af01; lane 17 - *Shewanella* sp. MBTDCMFRI Sh02; lane 18 - negative control of the reaction (without template).

3.4.2 Multiplex PCR Optimization

Through multiplex PCR, successful amplification of the targeted region with highest sensitivity was obtained at annealing temperature 58ºC with 3 mM $MgCl_2$ concentrations. Among the selected primers, the primers PSF2, PAGSR, BSF2 and BK1R were chosen for optimization of multiplex reactions. The rest primers (PSR1, PAGSF, BSR5 and BK1F) were omitted due to the occurrence of non-specific bands and cross amplification with other genera during multiplex assays. The details of primer binding regions in the genera *Bacillus* and *Pseudomonas* were shown in Fig. 3.4. The non-specific band obtained from the mixture of selected two primer pairs was limited by removing single forward primer (PSF2). The precise amplification for multiplex PCR was attained with the use of a single forward (BSF2) and two reverse (BK1R and PAGSR) primers at a concentration of 0.5 pmol/µl. These primer combinations yielded a distinguishable product of ~1100 bp and ~375 bp for *Bacillus* spp. and *Pseudomonas* spp. respectively (Fig. 3.5).

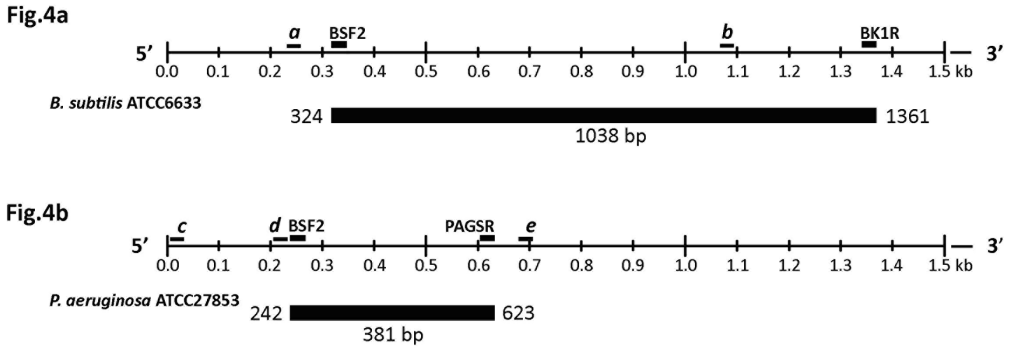

Fig.3.4 Map represents the relative binding position of the primers in 16S rDNA sequence of *Bacillus* and *Pseudomonas*.

Legend: Primers: a - BK1F, b - BSR5, c - PAGSF, d - PSF2 & e - PSR1 The lower bar represents the amplified product of *Bacillus subtilis* ATCC6633 (4a) and *Pseudomonas aeruginosa* ATCC27853 (4b) by using multiplex PCR primers.

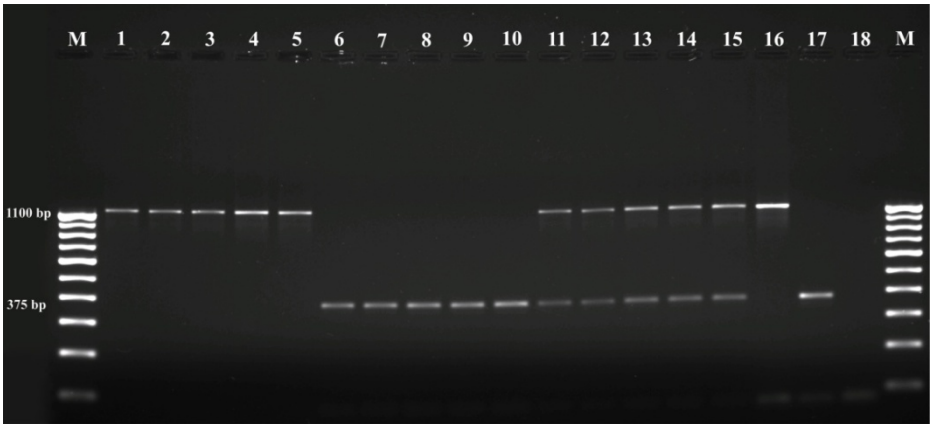

Fig 3.5 The specificity of the mPCR assay developed for the detection of the genera *Bacillus* and *Pseudomonas* by using unitemplate and multitemplate DNA. PCR product size obtained for *Bacillus* spp. and *Pseudomonas* spp. are ~1100 bp and ~375 bp respectively.

Legend: Lane M - molecular size marker (100 bp ladder); lane 1 - *Bacillus subtilis* MTCC441; lane 2 - *Bacillus amyloliquefaciens* MTCC1270; lane 3 - *Bacillus licheniformis* MTCC6824; lane 4 - *Bacillus cereus* MTCC430; lane 5 - *Bacillus pumilus* MBTDCMFR1 Ba01; lane 6 - *Pseudomonas aeruginosa* MTCC1688; lane 7 - *Pseudomonas fluorescens* MTCC103; lane 8 - *Pseudomonas putida* MBTDCMFRI Ps20; lane 9 - *Pseudomonas mendocina* MBTDCMFRI Ps21; lane 10 - *Pseudomonas* sp. MBTDCMFRI Ps18; lane 11-*Pseudomonas aeruginosa* MTCC1688 and *Bacillus subtilis* MTCC441; lane 12 - *Pseudomonas putida* MBTDCMFRI Ps20 and *Bacillus amyloliquefaciens* MTCC1270; lane 13 - *Pseudomonas fluorescens* MTCC103 and *Bacillus cereus* MTCC430; lane 14 - *Pseudomonas mendocina* MBTDCMFRI Ps21 and *Bacillus licheniformis* MTCC6824; lane 15 - *Pseudomonas* sp. MBTDCMFRI Ps18 and *Bacillus pumilus* MBTDCMFR1 Ba01; lane 16 - *Bacillus subtilis* MTCC441 and *Vibrio vulnificus* MTCC1145; lane 17- *Pseudomonas aeruginosa* MTCC1688 and *Enterococcus* sp. MBTDCMFRI Ec01; lane 18 - negative control of the reaction (without template).

Multiplex PCR amplification using purified and crude DNA gave similar product quality under the same reaction conditions. By using the crude DNA extraction and the current mPCR method, the identification of the genera *Bacillus* and *Pseudomonas* from an unidentified bacterial culture can be completed within 2 h.

3.4.3 Sensitivity Assay

The intensity of amplicon qualitatively decreased with the decrease in DNA concentration and also with CFU in dilutions (Fig 3.6). The detection limit for *Bacillus* spp. and *Pseudomonas* spp. varied considerably in both purified DNA and crude DNA. The detection limits of the genomic DNA (purified and crude) in the multiplex PCR varied among different bacterial species and are listed in Table 3.3.

Table 3.3 Limit of detection for various species identified using multiplex PCR sensitivity assay

S. No.	Bacteria	Detection Limit	
		pg/µl	CFU/µl
1.	*Bacillus amyloliquefaciens* MTCC1270	35	900
2.	*B. cereus* MTCC430	48	300
3.	*B. licheniformis* MTCC6824	46	580
4.	*B. pumilus* MBTDCMFRI Ba33	48	670
5.	*B. subtilis* MTCC441	51	1000
6.	*Pseudomonas aeruginosa* MTCC1688	5.3	9
7.	*P. fluorescens* MTCC103	3.3	7
8.	*P. mendocina* MBTDCMFRI Ps21	4.2	13
9.	*P. putida* MBTDCMFRI Ps20	3.1	3
10.	*Pseudomonas* sp. MBTDCMFRI Ps18	8.8	11

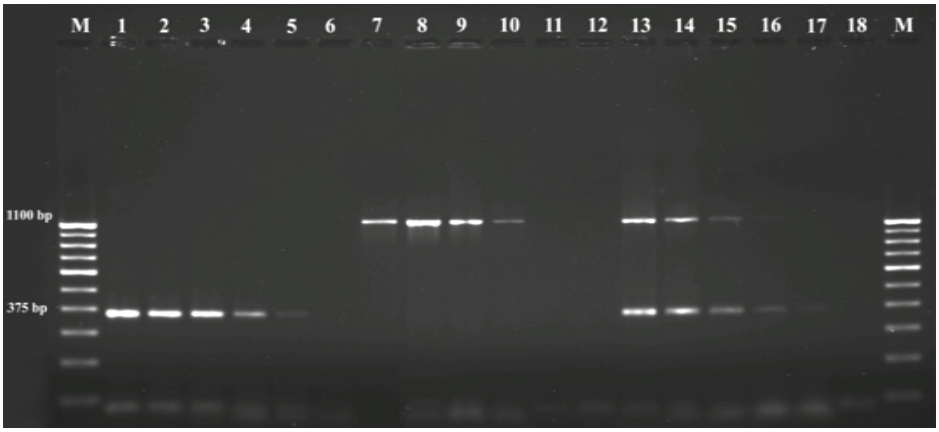

Fig 3.6 The sensitivity of detection of *Bacillus* sp. and *Pseudomonas* sp. by mPCR assay. PCR amplification was carried out using 10-fold-serially diluted template DNA of *Bacillus licheniformis* MTCC6824 and *Pseudomonas aeruginosa* MTCC1688. The size of the PCR products obtained were ~1100 bp and ~375 bp for the genera *Bacillus* and *Pseudomonas* respectively.

Legend: Lane M - molecular size marker (100 bp ladder); lane 1-6 - *Pseudomonas aeruginosa* MTCC1688 DNA serially diluted to 53 ng, 5.3 ng, 0.53 ng, 53 pg, 5.3 pg and 0.53 pg; lane 7-12 - *Bacillus licheniformis* MTCC6824 DNA serially diluted to 46 ng, 4.6 ng, 0.46 ng, 46 pg, 4.6 pg and 0.46 pg respectively; lane 13-17 - serially diluted template of *Bacillus licheniformis* MTCC6824 and *Pseudomonas aeruginosa* MTCC1688; lane 18 - negative control of the reaction (without template).

3.4.4 Specificity Assay

The specificity was determined by using the *cm*PCR protocol on 239 bacterial isolates from MCC-MBTD, CMFRI (Table 3.1a). The results were also confirmed with the reference strains mentioned in Table 3.1b. None of the non-targeted genera produced any cross reactive or non-specific results while carrying out the specificity assay, which in turn showed the higher specificity of the current *cm*PCR primers towards the genera *Bacillus* and *Pseudomonas*. The BLASTn results of the primer sequences selected also showed maximum homogeneity to the targeted genera when compared to others. ClustalW alignment of sequences of mPCR primers with the 16S rDNA sequence of *Bacillus*, *Pseudomonas* and other genera also showed the accuracy of primer combination (Table 3.4).

Table 3.4 Alignment of the primer sequences of BSF2, PAGSR and BK1R with 16S rDNA sequences of 32 strains belonging to *Pseudomonas*, *Bacillus* and other genera commonly found in the environment

Primer	BSF2		PAGSR		BK1R	
	10		10	20	10	
Sequence(5'-3')	TACGGGAGGC	AGCAGTRGG	TATAGGAAGG	AACACCAGTG	CATGCCGCGG	TGAATACG
Pseudomonas aeruginosa	....	....	....	....	A.-.T.A...	....
P.aeruginosa MBTDCMFRI Ps08	....	....	....	....	A.-.T.A...	....
Pseudomonas fluorescens	....	....	....	....	A.-.T.A...	....
Pseudomonas mendocina	....	....	....	....	A.-.T.A...	....
Pseudomonas putida	....	....	....	....	A.-.T.....	....
Pseudomonas stutzeri	....	....	....	....	A.-.T.....	....
Pseudomonas syringae	....	....	....	....	A.-.T.....	....
Bacillus amyloliquefaciens	....	....	G..GT.G...	....	....	....
Bacillus cereus	....	....	G...T.G...	....	....	....
Bacillus firmus	....	....	G..GT.G...	....	....	....
Bacillus licheniformis	....	....	G..GT.G...	....	....	....
Bacillus megaterium	....	....	G..GT.G...	....	....	....
Bacillus pumilus	....	....	G..GT.G...	....	....	....
Bacillus subtilis	....	....	G..GT.G...	....	....	....
B.subtilis MBTDCMFRI Ba37	....	....	G...T.G...	....	....	....
Aeromonas hydrophila	....	....	G..CT.G...	..T...G...	A...TT....	....
Alteromonas genovensis	....	....	...CT.G...	....T..A..	A..ATT....	....
Citrobacter freundii	....	....	G..CT.G...	..T...N...	A.....A...	....
Corynebacterium bovis	....	....	...CA.G...	......GA..	..C..T....	....
Enterobacter hormaechei	....	....	G..CT.G...	..T...G...	A.....A...	....
Enterococcus sp.	....	....	....T.G...	....	..C.......	....
Escherichia coli	....	....	G..CT.G...	..T...G...	A.....A...	....
Klebsiella oxytoca	....	....	G..CT.G...	..T...G...	A.....A...	....
Listeria monocytogenes	....	....	CG..AAGC.C	GCGCAGGCG.	......A...	....
Micrococcus luteus	....	....	...CA.G...	......GA..	..C..T....	....
Moraxella caprae	....	....	G..CT.G...	..T...GA..	A.-..T....	....
Pseudoalteromonas sp.	....	....	G..CT.....	..T...GA..	A...A.....	....
Serratia marcescens	....	....	G..CT.G...	..T...G...	A....TA...	....
Staphylococcus aureus	....	....	G...T.G...	....	.....TA...	....
Streptococcus bovis	....	....	....T.G...	......G...	..C.......	....
Vibrio harveyi	....	....	G..CT.....	..T...G...	A.....A...	....
Yersinia pestis	....	....	G..CT.G...	..T...G...	A....TA...	....

3.4.5 Application of *cm*PCR for Environmental Samples

A total of 711 bacterial isolates were subjected to *cm*PCR assay, of which 234 were *Bacillus* spp. and 63 were *Pseudomonas* spp. The details of the isolates and their sources were given in Table 3.5. Among the isolates obtained from culture collection, 74 isolates possessed antibacterial activity. Apart from this, 46 (9.7%) of 472 environmental isolates also exhibited antagonistic activity towards aquaculture pathogens. The majority of them belonged to the genera *Bacillus* (63%) followed by *Pseudomonas* (17.4%).

Table 3.5 Results of the colony multiplex PCR (*cm*PCR) and the antagonistic activity of the isolates obtained from the southwest coast of India.

Sampling site	Source	Sample	Number of isolates	Number of isolates detected positive in *cm* PCR		Number of antagonistic isolates detected by spot diffusion assay		
				Bacillus	*Pseudomonas*	*Bacillus*	*Pseudomonas*	Others
Andhakaranazhi	Ornamental fish hatchery	Water	37	0	1	0	0	0
		Fish	8	0	0	0	0	0
	Polluted canal	Sediment	36	18	1	4	0	0
Chettuva	Mangrove	Sediment	82	35	8	6	0	2
Edavanakkad	Canal	Sediment	20	1	2	0	1	0
Mandapam	Fish hatchery	Fish	14	5	1	0	0	0
Mangalavanam	Mangrove	Sediment	52	28	9	3	5	1
Mulavukad	Shrimp pond	Sediment	18	4	0	2	0	1
Njarakkal	Oyster hatchery	Water	21	0	0	0	0	1
		Oyster	44	1	6	0	0	0
Perandoor	Polluted canal	Sediment	37	12	0	4	0	1
Puthuvype	Mangrove	Sediment	22	4	0	2	0	1
	Canal	Sediment	17	5	0	1	0	0
	Refinery	Sediment	46	17	2	6	0	0
	Polluted canal	Water	18	3	2	1	2	2
			472	**133**	**32**	**29**	**8**	**9**
CMFRI	Microbial culture collection, MBTD	Culture collection	239	101	31	56	12	6
Total number of isolates screened			**711**	**234**	**63**	**85**	**20**	**15**

To confirm the consistency of detection, 160 isolates (amplified and non-amplified strains) were selected based on their antagonistic activity and *cm*PCR results. The results of the selected isolates were cross checked with their biochemical and 16S rDNA data. Upon comparison of the results of identification (biochemical methods and 16S rDNA sequencing), the specificity and reliability of *cm*PCR in the detection of the genera *Bacillus* and *Pseudomonas* were found to be 100% efficient. Among the 160 bacterial strains, 100 isolates which showed positive amplification in the *cm*PCR assay were *Bacillus* spp. (75 nos.) and *Pseudomonas* spp. (25 nos.). The rest 60 isolates which failed to amplify in *cm*PCR were identified as *Aeromonas*, *Vibrio*, *Alcaligenes*, *Enterobacter*, *Halomonas*, *Citrobacter*, *Enterococcus*, *Shewanella*, *Pseudoalteromonas*, *Escherichia*, *Arthrospira*, *Acinetobacter*, *Micrococcus*, *Alcanivorax*, *Pedobacter*, *Oceanimonas*, *Brevibacterium* and *Streptomyces* (Table 3.1a). Thus, the primer combinations developed for multiplex PCR assay were highly specific and sensitive by producing distinct bands for precise identification of the genera *Bacillus* and *Pseudomonas*.

While sequencing the *cm*PCR products of selected strains, the sequences exhibited similar identification results as 16S rDNA sequencing (Table 3.1a). This suggests that the products of *cm*PCR can also utilize for identification of *Bacillus* and *Pseudomonas* up to species level by sequencing when required. From the present study, 6 different isolates of *Pseudomonas* spp. and 14 different isolates of *Bacillus* spp. have been identified precisely. The accession numbers of the sequences submitted in GenBank were JF719759 to JF719808 (49 submissions) and KF317775 to KF317832 (57 submissions).

3.5 Discussion

A specific and sensitive DNA based identification method-*cm*PCR-for the rapid screening of antagonistically important genera *Bacillus* and *Pseudomonas* from environmental samples was developed in the present study. These two genera were reported to be ubiquitous and possess a wide spectrum of antagonistic activity towards various pathogens (Balcazar and Rojas-Luna 2007; Nithya and Pandian 2010; Sharma and Kaur 2010). The significance of these genera in the control of bacterial diseases in aquaculture systems was clearly depicted in our previous studies (Vijayan et al. 2006; Nair et al. 2012). The development of a consortium of antagonistic bacteria and their characterization offers a better alternative approach in developing novel microbial products for the control of bacterial diseases and to tackle the issue of antibiotic resistances, which requires screening of *en masse* of bacterial populations from diverse ecosystems. In this background, the present report on a novel molecular method is of important; to identify the isolates belonged to the promising genera of *Bacillus* and *Pseudomonas,* as an alternate to conventional diagnostic methods.

The current *cm*PCR assay employed the primer combinations targeting 16S rDNA region for simultaneous detection of *Bacillus* spp. and *Pseudomonas* spp. without noises from other genera. This region was widely accepted to infer phylogenetic relationships among bacteria and offers the benefit of robust databases and well-characterized phylogenetic primers (Sadeghi et al. 2012). Though 16S rDNA sequencing was widely used in bacterial taxonomy, its power to distinguish the species in certain genera was reported as poor (Janda and Abbott 2007). The present molecular screening tool involves a combination of colony PCR and multiplex PCR to identify both the genera in a rapid, specific, sensitive and cost effective mode. It demonstrates the use of purified bacterial colony to perform multiplex PCR

instead of isolated DNA to identify *Bacillus* spp. and *Pseudomonas* spp. This study also validated that the 'boiling water bath method' is an appropriate method for the preparation of crude bacterial DNA to be used in PCR reactions, for both *Bacillus* spp. and *Pseudomonas* spp. The assay described for the identification is much simpler and the time required to complete the whole process (DNA preparation, amplification and detection) was approximately two hours. Our result is in agreement with the report of Kwon et al. (2009) that the direct bacterial suspensions produce precise amplification and distinguishable PCR products.

For a successful multiplex PCR, a balance between primer combinations, magnesium chloride concentrations and annealing temperatures is important. These factors differed even with uniplex and multiplex systems which were also reported by Chen et al. (2012). The present study was successful in developing an efficient multiplex combinational primer mix using a single forward and two reverse primers providing high sensitivity and specificity in identifying the targeted genera *Bacillus* and *Pseudomonas*. In addition, the developed assay allowed the detection with less than 10 picogram and 50 picogram for genomic DNA and 1 x 10^1 and 7 x 10^2 CFU/µl for *Pseudomonas* spp. and *Bacillus* spp. respectively, which is comparable with results of Chiang et al. (2006) and Fan et al. (2008).

The reliability of bacterial identification by *cm*PCR assay was also checked and evaluated by comparing the results of 16S rDNA sequencing and biochemical methods. The results confirmed that the *cm*PCR assay developed in the present study is successful in the identification of the genera *Bacillus* and *Pseudomonas*. Moreover, the identification by sequencing the *cm*PCR product exactly matches with the results of 16S rDNA sequencing. Results observed with multiplex PCR and conventional methods were highly

consistent for all samples, indicating that the *cm*PCR is an efficient method for rapid screening of large number of samples. This clearly validates that the *cm*PCR protocol is a very useful and sensitive tool to obtain information on the composition and population dynamics of the specific genera from complex microbial communities. The combined application of multiplex PCR and crude DNA extraction as described here, will allow routine, high throughput analysis of environmental samples for the quick assessment of potential antagonistic bacteria (*Bacillus* spp. and *Pseudomonas* spp.).

When considering the extensive disease control or ecological studies, focusing on diversity of microbial interactions, simultaneous testing becomes more valuable and effective. The employment of this method may provide a new way to investigate the microbial populations in a wide range of environments in a short span of time. This will further help in finding distribution, abundance and screening of *Bacillus* spp. and *Pseudomonas* spp. from a large number of environmental sites and to explore its biotechnological potential.

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Chapter 4

CHARACTERIZATION OF THE *BACILLUS SUBTILIS* MBTDCMFRI Ba37 AND ITS BIOACTIVE COMPOUND

Contents

4.1 Abstract

Bacillus subtilis MBTDCMFRI Ba37 (*B. subtilis* Ba37) a marine bacterium isolated from the tropical estuarine habitats of Cochin, Kerala, India showed significant antibacterial activity against aquaculture pathogens. It showed resistance and growth under wide range of environmental conditions like temperature (20°C - 100°C), salinity (0 - 55 ppt) and pH (4 - 10). The highest antagonistic activity was detected in 3rd day culture, grown in modified *bacillus* medium with glycerol (1%) and glutamic acid (0.5%) as carbon and nitrogen source respectively, at 30°C, pH 7.0 and at 15 ppt saline conditions. In co-culture assay, *B. subtilis* Ba37 exhibited properties of inhibition on the growth of pathogenic *Vibrio anguillarum* O1. The *B. subtilis* Ba37 was found to be non-lethal to *Etroplus suratensis* juveniles in challenging experiments with oral route (challenged for 30 min at 10^8 CFU/mL) and by intramuscular

injection of ~5 g sub-adults (injected with 10^6 to 10^8 CFU/mL). The preliminary studies on the nature of the antibacterial action indicated that the antagonistic principle is stable even at 40% v/v dilution, up to 80°C and pH 6-9. Bioassay guided purification followed by the spectroscopic characterization of active fraction revealed that the compound 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [$8.7.0.0^{2,3}.0^{12,13}$] heptadecan-5-one, is responsible for its antibacterial activity. The results of this study indicated that *B. subtilis* Ba37 have beneficial antibacterial properties which could be useful in applying this microbe as a potential probiont, in aquaculture systems in the control bacterial infections.

4.2 Introduction

The bacterial infectious diseases are measured to be a foremost limiting factor leading to economic loss in the aquaculture industry, both in the hatchery and farm rearing sysytems (Aly et al. 2008; Vinoj et al. 2013). These bacterial infections can be partially controlled through the applications of antibiotics and drugs, however these are often hazardous to the environment and human health, due to serious issues such as development and transfer of antimicrobial resistance among pathogens (Balcazar and Rojas-Luna 2007; Motto et al. 2007; Hill 2009). This scenario emphasizes the need of alternative measures to be explored for a sustainable carryover of aquaculture. In this case the use of bio-control agents with anti-pathogenic activity has received wide acceptance (Lalloo et al. 2008; Vinoj et al. 2013; Priyaja et al. 2014). Antagonistic microbes are inhibitory to pathogenic bacteria by producing variety of bioactive metabolites (Cao et al. 2011; Sabate et al. 2013). Antagonistic bacteria used in aquaculture are mostly from the division Gammaproteobacteria (*Vibrio* spp., and *Pseudomonas* spp.) and Firmicutes (*Lactobacillus* spp., and *Bacillus* spp.) (Hill 2009; Nair et al. 2014).

Members of the genus *Bacillus* are common inhabitants of soil and capable to produce wide range of antimicrobial activities (Hong et al. 2005; Yilmaz et al. 2005). The application of *Bacillus* sp. as probiotic bacteria to control aquatic pathogens has been recognized by many earlier works (Hill et al. 2009; Touraki et al. 2012; Vinoj et al. 2013). *Bacillus* spp. were reported for its excellent nutrient source, as well as possess attributes to improve host digestion by producing vitamins, enzymes and other metabolites (Smoragiewicz et al. 1993; Bandyopadhyay et al. 2009). Furthermore, spore-forming ability of the genus *Bacillus* makes these bacteria to successfully colonize and survive extensive environments, and their heat stable and tolerance to high pressure properties makes the formulation and commercialization easier (Hong et al. 2005; Lalloo et al. 2008; Zhang et al. 2005; Cutting 2011). These characters have been taken onward the genus *Bacillus* to be recognized as potential antagonistic (beneficial) bacteria in aquaculture system.

In the exploration of bioactive natural products, microbes are the best sources of novel bioactive molecules and are often described as nature's greatest chemists (Subramani et al. 2012). Members of the genus *Bacillus* are considered as microbial factories, capable to produce vast array of active metabolites potentiated to inhibit wide range of pathogens (Ongena and Jacques 2007; Hammami et al. 2008). This genus presents a great variety of species that produce bacteriocins or bacteriocin-like substances (BLS), lipopeptides, non-peptide-based antibiotics etc. which are having potential applications (Bizani and Brandelli 2002; Baruzzi et al. 2011).

Among the various species of *Bacillus*, *B. subtilis* is a common candidate to be used as probiotic bacteria in aquaculture (Balcazar and Rojas-Luna 2007; Touraki et al. 2012). It is referred as an ideal biocontrol agents

used as alternative to control wide range of pathogens (Yang et al. 2009; Todorova et al. 2010; Kamgar et al. 2013). It was reported that 4-5% of its genome are dedicated to antibiotic synthesis with a capacity to produce more than two dozen structurally diverse antimicrobial compounds (Stein 2005; Fickers 2012). This versatile bacterium is considered as one of the important producers of metabolites including bacteriocin, subtilosin A, subtilin, bacilysin having wide spectrum of activities against pathogenic microorganisms (Sutyak et al. 2008; Todorova et al. 2010)**.** Characterization of these metabolites would help in understanding the detailed mechanisms of antibacterial activity of *Bacillus* species (Vaseeharan and Ramaswamy 2003).

B. subtilis Ba37 was isolated and selected as potential antagonistic bacteria because of its broad spectrum of antimicrobial activity against aquaculture pathogens (Nair et al. 2012). The properties of the genus *Bacillus* motivated us for the further exploration of *B. subtilis* Ba37 to characterize and evaluate as a candidate probiotic bacteria in aquaculture systems and also to identify the biologically active molecule which in turn would help to expand drug discovery program against aquaculture pathogens.

4.3 Materials and Methods

4.3.1 Bacterial Strains

Bacterial strains isolated from the tropical estuarine habitats of Cochin possessing antibacterial activity against aquaculture pathogens were maintained in Microbial Culture Collection of Marine Biotechnology Division (MCC-MBTD), Central Marine Fisheries Research Institute (CMFRI). Based on the consistency and broad spectrum antibacterial activity, *B. subtilis* Ba37 isolated from sediment sample collected from Mangalavanam (mangrove

ecosystem) was selected for further characterization. A reference strain *B. subtilis* MTCC441 was also used for the comparison studies.

For antagonistic assay, the test organisms used were *Vibrio harveyi* 101, *V. anguillarum* O1, *V. alginolyticus* 101 (*courtesy* Central Institute of Brackishwater Aquaculture, Chennai), *Aeromonas hydrophila* (*courtesy* National Centre for Aquatic Animal Health, Cochin University of Science and Technology), *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (*courtesy* Microbial Type Culture Collection, Chandigarh, India). All bacterial isolates were preserved at -80^{o}C as glycerol stocks and were inoculated into freshly prepared broth before each assay to attain uniform growth.

4.3.2 Characterization of *B. subtilis* Ba37

The isolate was characterized based on its morphological, biochemical, physiological, enzyme activity and antibiotic susceptibility tests and compare those with reference strains. *B. subtilis* Ba37 and the reference strain (*B. subtilis* MTCC 441) were grown on nutrient agar (Himedia, India) supplemented with 1% NaCl kept at 37°C for 48 h and used for characterization studies. Phenotypic characterisation of *B. subtilis* was carried out using the methods described by Reva et al. (2001).

The catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and oxidase reagent (1% tetramethyl-p- phenylenediamine dihydrochloride) respectively. Reduction of nitrate, indole production from tryptophan, methyl red and Voges–Proskauer reactions were carried out by adding specific indicators and reagents. H_2S production and citrate utilization were determined on Kligler triple-sugar iron agar and Simmon citrate agar respectively. Fermentative growth was tested using Hugh-Leifson oxidative-fermentative medium. Acid production from various carbohydrates was tested

by addition of 1% particular carbohydrate (arabinose, glucose, galactose, inositol, melibiose, mannitol, mannose, raffinose, salicin, sorbitol, trehalose and xylose) (HiMedia, India) to bromocresol purple broth media which contains bromo cresol purple as indicator. Hydrolysis of casein, gelatin, cellulose, urea, starch, lecithin, oil of olive and Tween 20 were studied for exoenzymatic activity, similarly haemolytic activity was also carried out. Activity was detected after incubation at 30 ± 2°C for 24 h, as growth, or a zone around the colony with/without the addition of respective reagents. Antibiotic susceptibility tests were performed using commercially available antibiotics impregnated discs (HiMedia, India) as described by Bauer et al. (1996). Growth at physiological conditions were studied on nutrient agar plates by growing them in a wide range of temperatures (20 - 60°C) and different pH gradients 4 -10 (adjusted using 0.1N HCl and 0.1N NaOH), and various NaCl concentrations using a medium containing peptone, yeast extract supplemented with respective concentrations of salt (NaCl) at 0%, 2%, 5%, 10%, 15%, 20%, 25% and 30% (w/v) (Yeon et al. 2005).

4.3.3 Antibacterial Assay

The initial screening of antagonism of *B. subtilis* Ba37 was carried out by spot diffusion assay against the test pathogens as described by Nair et al. (2012). In brief, isolated bacteria were spotted over pre-swabbed plates with aquaculture pathogens *viz*., *Vibrio vulnificus* MTCC1145, *V. harveyi* 101, *V. anguillarum* O1, *V. parahaemolyticus* MTCC451 and *V. alginolyticus* 101. After incubation, the bacteria having notable antagonistic potential (zone of clearance of 10 mm or greater observed around isolates) were selected, identified and preserved for further use.

Antagonism assay of cell-free supernatant was done by well diffusion method and disc diffusion method. The isolate was inoculated in nutrient broth supplemented with 1% NaCl and incubated at 30^{o}C for 72 h on a shaker (100 rpm). The culture was centrifuged at 8000 rpm for 15 min and filtered through a 0.2 µm pore size filter (Pall Life science, USA). For disc diffusion assay, 40 µl of this cell free preparation was impregnated on 6 mm diameter sterile discs (HiMedia, India) and air dried. These discs were placed on Muller Hinton Agar plates (salinity 15ppt) (HiMedia, India) previously swabbed with the target pathogens. For well diffusion assay, 40 µl of cell-free supernatant was introduced into the wells on the Muller Hinton Agar medium which were previously swabbed with the test organisms. Then the plates were then incubated at 30°C for 24 h and the zone of inhibition around the discs and well was measured and recorded against target organisms. Antibacterial activity of the selected isolate was compared with commercialized antibiotics against tested pathogens.

4.3.4 Evaluation as Candidate Antagonistic Bacteria

4.3.4.1 Growth Curve

4.3.4.1.1 Preparation of Culture

B. subtilis Ba37 and *V. anguillarum* O1 were taken from glycerol stock at -80°C and were streaked into nutrient agar plates supplemented with 1% NaCl and incubated at 30°C for 24 h. Five mL of nutrient broth (15 ppt) was inoculated with a single colony from the plates and incubated overnight at 30°C on a rotary shaker (New Brunswick Scientific Co. Inc., NJ, USA) at 200 rpm for 20 - 24 h. 1% of overnight grown culture was inoculated to 100 mL nutrient broth (15 ppt). The flasks were incubated on a shaker for 48 h as mentioned above.

4.3.4.1.2 Determination of Generation Time and Growth Curve

One mL of samples (bacterial cultures) were taken at every half an hour interval from 0 h to 8 h for the determination of generation time and for growth curve (change in cell number against time) studies, samples were taken at different time intervals from 0 to 48 h. Growth was determined by measuring the OD at 600 nm using Eppendorf Biophotometer Plus, Germany and viable cell count by serial dilution followed by plating on nutrient agar with 1% NaCl. The determination of generation time of the cultures was carried out as per the methodology described by Stephenson (1994).

4.3.4.2 Co-culture Experiment

Vibrio anguillarum was selected for co-culture experiment as it is one of the major disease causing bacterial pathogen in aquaculture rearing systems of finfish and shellfish, inflicting economic loss in larviculture (Frans et al. 2011). The *in vitro* inhibitory properties of *B. subtilis* Ba37 against *V. anguillarum* O1 was carried out using co-culture assay. Co-culture shows the interaction between two organisms in terms of antagonism and competition for micronutrients (Gram et al. 1999). Cultures were pre-cultured separately in nutrient broth supplemented with 1% NaCl and incubated at 28±2°C on a shaker at 120 rpm overnight. The above cultures were inoculated in 100 mL nutrient broth (with 1% NaCl) to obtain an initial cell count of approximately 10^2, 10^3, 10^4 CFU/mL for *V. anguillarum* O1, whereas the initial levels of *B. subtilis* Ba37 were 10^4, 10^5, 10^6, $10^7$and 10^8 CFU/mL respectively. The cultures were incubated at 28 ± 2°C on a shaker (120 rpm), and all samples (1 mL) were withdrawn at 24 h intervals for 4 days to determine the cell count. Counts of the pathogen were monitored by withdrawing daily 1 mL samples that were serially diluted 10 fold and 100 µl aliquots of each were spread

plated on TCBS agar and nutrient agar (15 ppt) supplemented with 5 mM potassium chromate (K_2CrO_4) plates. The plates were incubated at 28 ± 2°C for 24 h and the colonies obtained on plates were counted and expressed as log10 CFU/mL. *B. subtilis* Ba37 cannot grow on TCBS agar while *V. anguillarum* O1 cannot grow in 5 mM K_2CrO_4 so they could be easily differentiated in these specific agars.

4.3.4.3 Cytotoxicity Assay

Cyprinus carpio (Koi carp) fibroblast cell lines were grown in Leibovitz's Medium (L-15) supplemented with 5% fetal calf serum (FCS). For cytotoxicity assays, cells were seeded at a density of 10^5 cells/well, with 1 mL of medium being added into 24 well plates and incubated at 28 ± 2°C for 5days. Cell free supernatant were obtained by centrifugation of 10 mL of 18 h culture broth of *B. subtilis* Ba37 and *V. anguillarum* 101 and filtered through a 0.2 µm syringe filter. Cells were infected with 100 µl of each cultured cell-free supernatants obtained by filtration through a 0.2 µm syringe filter (Palls life science, USA) as a control and *V. anguillarum* 101 as a pathogenic control. Fresh media were added into the wells and incubated further for 24 h. After incubation period (24 - 48 h), the plates were visualized through microscope for morphological changes of the cells

4.3.4.4 Pathogenicity Test/ Safety of the Isolated Antagonistic Bacteria

4.3.4.4.1 On juveniles/larvae of Etroplus suratensis

Pathogenicity of *B. subtilis* Ba37 was tested on the juveniles/larvae of *E. suratensis* collected from a commercial hatchery. They were maintained for 7 days in 10ppt seawater and fed with commercial feed. Water temperature was 27 ± 1°C during the period of experiment. Continuous aeration was provided by using air pump. Twenty hour old culture grown in Nutrient agar

with 1% NaCl at 28 ± 2°C was scraped with sterile loop and centrifuged, washed the pellets in saline and the pellet were resuspended in sterile 1X PBS. The cells were washed twice in PBS and resuspended in sterile PBS to OD_{600} of 1.0, corresponding to 5×10^8 CFU/mL. The cell count was determined by plating serial 10-fold dilution in Nutrient agar with 1% NaCl by spread plate method. Suitable number of cells were added to 1 litre beaker containing 500 mL filter (0.45 mm) sterilized 10 ppt sea water to obtain 10^6, 10^7 and 10^8 CFU/L. The control beaker did not have any bacterial inoculum. To each of the four beakers, 10 juveniles/larvae of *E. suratensis* were introduced and were monitored for any signs of disease or mortality up to 7 days. The experiment was carried out in triplicate.

4.3.4.4.2 On sub adult E. suratensis

Sixty sub-adults of *E. suratensis* (average body weight of 5 ± 0.5 g) were acclimatized in 10 ppt seawater for 7 days in the laboratory and fed with commercial feed. Then they were distributed into groups of 5 animals each in 30 L capacity fiberglass tanks containing 20 L of 10 ppt seawater and maintained at 28 ± 2°C for 5 days. Bacterial cultures were prepared using the same procedures as described earlier. The cell density was observed to be around 10^9 CFU/mL. Fishes were inoculated (intraperitoneal) with 0.4 mL of prepared bacterial suspension. Negative control groups were injected with 0.4mL sterile saline and positive control groups were injected with 0.4 mL of *V. anguillarum* O1. All groups of fishes were monitored for any signs of disease or mortality up to 10 days. The experiment was carried out in triplicate.

4.3.5 Nutrient Profiling

The proximate chemical composition analysis of newly formulated feed was determined using the standard procedures of AOAC (1990). Briefly,

moisture was determined by oven drying at 105°C to constant weight. Dried samples were used for determination of crude fat, crude protein, crude fibre, soluble carbohydrate, acid insoluble ash, and crude ash contents. All analyses were done in triplicates.

4.3.5.1 Moisture Determination

About 4-5 g of sample taken in an aluminium dish, covered and dried at 100°C to constant weight. Moisture content was calculated in %.

$$\text{Moisture content } \% = \frac{\text{Wt. of fresh sample} - \text{Wt. of dry sample}}{\text{Wt. of fresh sample}} \times 100$$

4.3.5.2 Crude Protein

Crude protein content was measured as total nitrogen content multiplied with 6.25 by Kjeldahl method

Weighed approximately 0.25g of dried sample noting the exact weight, 'W' g, into clean dry digestion tubes. Into each tube added approximately 1g of digestion mixture (potassium sulphate & copper sulphate, 9:1 by weight). Then 12 ml of con.H_2SO_4 was added into each tube and placed on the digester (Kjeltec) assembly and digested at 400°C for $1^1/_2$ h. Sample was then cooled down to room temperature.

Each sample was then placed on the distillation unit (Kjeltec) and the program was set with water-70 ml, alkali-70 ml, receiver-30 ml, tube drain and distillation was made with steam in the unit. The instrument estimates the crude protein on entering the weight of sample W as,

$$\% \text{ crude protein} = \frac{\text{Volume of } 0.1\text{N Hcl} \times 0.00014 \times 6.25 \times 100}{\text{W}}$$

4.3.5.3 Crude Fat

Weighed 2-3 g of dried sample into an extraction thimble (residue from dry matter was used) and placed into the soxhlet apparatus, (Soxtec fat analyzer). Placed a dry, pre-weighed and marked aluminium cup in position beneath, and added 60 ml petroleum ether and connected to condenser. The temperature was adjusted to reach 100°C and boiling cycle was done for 15 min, by dipping the thimbles in solvent. The thimbles were raised and rinsed with condensed ether in the rinsing cycle for 30 min. This was followed by 10 min of recovery cycle where pure unsaturated ether was collected back and recovered. The fat containing cups with residual ether was then dried in hot air at 100°C for 1 h and then cooled in desiccator and weighed. The crude fat was calculated as

$$\text{Crude fat \% of dry mass} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

4.3.5.4 Crude Ash

Weighed about 3 g powdered sample into a dry, pre-weighed porcelain dish and then ignited in a muffle furnace at 600°C for 3 h. Allowed to cool overnight desiccators and weighed.

Calculation:

$$\text{Crude ash } (\text{CA}) \text{ \%} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

4.3.5.5 Acid Insoluble Ash

The residue obtained from ash determination was boiled with 25 ml 5N HCl and filtered through ash-less paper and washed with hot water until acid free. The paper with residue was transferred to respective crucible and dried in

hot air oven. It was then ignited in the muffle furnace at 600°C for 3 h. Cooled overnight and taken the weight. Percentage of acid insoluble ash was calculated as

$$\text{Acid insoluble ash \%} = \frac{\text{Weight of AIA}}{\text{Weight of sample}} \times 100$$

4.3.5.6 Crude Fibre

The thimbles containing fat free extract from the forgoing estimation of crude fat were dried in hot air oven at 50°C for overnight. Approximately 0.8 g of fat free sample was weighed into gooch crucibles provided with fibretec extraction assembly. They were set on the assembly and two digestions, acid & alkali digestions in 1.25% H_2SO_4 and 1.25% NaOH were done one after the other for 30 min. Draining of acid and alkali and flushing of hot distilled water were done in between each digestion. The residue containing crucibles were removed, over dried at 60°C for overnight and weighed. They were ashed at 600°C for 3 h in muffle furnace overnight, cooled and weighed again. Then percentage of crude fibre was calculated as

$$\text{\% crude fibre} = \frac{\text{Weight of crude fibre}}{\text{Weight of fat free extract}} \times 100$$

4.3.5.7 Soluble carbohydrate (NFE)

Calculated as,

100 – (% crude protein + % crude fat + % crude fibre + % crude ash + % moisture)

4.3.6 Optimization of Production Medium

B. subtilis Ba37 was taken from glycerol stock at -80°C and streaked on nutrient agar (15 ppt) and a single colony was inoculated into 5 mL of nutrient

broth supplemented with 1% NaCl and incubated at 30°C on a rotary shaker overnight (New Brunswick Scientific Co. Inc., NJ, USA) at 200 rpm for 20-24 h. 1% of overnight grown culture (starter culture) was inoculated to 100 mL of various medium (with 1% NaCl) like nutrient broth, Zobell marine broth, Tryptone soya broth and slightly modified *Bacillus* media (Jamil et al. 2007) (g/L) containing 5.0 g glutamic acid; 10 mL glycerol; 0.5 g KH_2PO_4; 0.5 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.01 g $MnSO_4.H_2O$; 0.01 g $FeSO_4.7H_2O$; 0.01 g $CuSO_4.7H_2O$; 0.015 g $CaCl.2H_2O$ at pH 7. All the flasks were incubated at 30°C for a period of 7 days in duplicate. Samples for antagonistic activity were drawn every 24 h interval and 1 mL of the culture was processed for evaluating the antagonistic potential against aquaculture pathogens by the disc diffusion method as explained earlier. The cell counts were determined by serial dilution.

4.3.6.1 Optimization of Carbon and Nitrogen sources and Physiochemical Conditions

The optimal conditions for growth and production of antagonistic compound by *B. subtilis* Ba37 was assessed by growing the culture in modified synthetic media at various temperature (25, 30, 35 and 40°C), pH (6.0, 7.0, 8.0 and 9.0), salinity (5, 10, 15, 20, 25, 30 and 35 ppt), carbon (glycerol and glucose at varying concentrations from 0.5-1%) and nitrogen sources (alanine and glutamic acid at varying concentrations from 0.5-1%) for a period of 7 days in duplicate. Samples for cell count and antagonistic activity were drawn every 24 h. One milliliter of the culture was processed for evaluating the antagonistic potential against pathogens by the disc diffusion method as explained earlier.

4.3.7 Preliminary Characterization of Antagonistic Compound

Sensitivity of antagonistic compound to enzymes, pH and heat treatment was carried out using cell free culture supernatant of *B. subtilis* Ba37. Heat sensitivity of the inhibitory substance was tested by heating the cell-free supernatant of *B. subtilis* Ba37 in a water bath for 30 min at 50, 60, 80 and 100°C, and autoclaving for 15 min at 121°C. Each of the treated and untreated supernatants was tested for antagonistic activity against pathogens using the disc diffusion method as described earlier. The sensitivity of the antibacterial principle to lyzozyme and proteolytic enzymes like proteinase K and trypsin was tested using 1, 2 and 5 mg/mL concentration. After 2 h incubation at 37°C, enzymatic action was inactivated by keeping at boiling water bath for 5min. Antibacterial activity was tested against pathogens using the disc diffusion method with cell free supernatant as control. The pH stability was tested by adjusting the pH of the cell free supernatant to 2, 4, 6, 8 and 10 using 1 $molL^{-1}$ HCl and 1 $molL^{-1}$ NaOH, with the help of a pH meter, and incubating for 2 h. The pH was subsequently readjusted to 7.0 and the activity was tested against pathogens as described above. The control consisted of a cell free supernatant with its pH adjusted to 7.0. Effect of dilution of the cell free supernatant was detected by diluting the filtrate containing the potential antibacterial compound up to 10% (v / v) using sterile phosphate buffered saline (pH 7.4). The antagonistic activity against pathogens was tested by the disc diffusion method as described earlier.

4.3.8 Isolation of Bioactive Compounds

4.3.8.1 Preparation and Purification of Bioactive Compounds from *B. subtilis* Ba37

Six litres of 3 days old *B. subtilis* Ba37 culture in modified synthetic medium incubated at 30°C at 150 rpm was centrifuged at 8000 rpm for 15 min

at 4°C and the supernatant was filter sterilized (0.2 µm) and lyophilized to obtained 5 g of supernatant powder which was partitioned between EtOAc/MeOH (1:1 v/v) and reflux for 2.5 h and filtered through anhydrous sodium sulphate (Na_2SO_4). The filtrate thus obtained was evaporated (40°C) using rotary evaporator under vacuum to dryness at a temperature below 42 ± 2 °C to afford to get pale yellow extract (4.5 g). The crude fraction obtained from EtOAc/MeOH extract was purified using various chromatographic techniques based on their antagonistic potential. Each time the different fractions obtained were evaluated using TLC and similar fractions were pooled accordingly. Each time the antagonistic potential of the fractions were evaluated and further purifications were carried out based on the zone of inhibition. All reagents and chemical solvents used for products isolation were of analytical grade or higher.

4.3.8.2 Identification of Bioactive Compounds

The purified fraction ($Bs_{2.2.4.2}$) was subjected to FT-IR (Fourier Transform Infra Red) spectrometric analysis to trace the spectra of the compounds under KBr pellets were recorded in a Thermo Nicolet, Avatar 370. The scanning was conducted in to mid IR range, i.e., between 4000 - 400 cm^{-1}. The LC-MS experiments were carried out on Quattro Premier XE (Micromass), an electrospray ionization (ESI) - triple quadrupole mass spectrometer, which is attached to Acquity UPLC (Waters). For each run, 5 µL of samples were injected onto a C_{18} column (Acquity UPLC BEH, 1.7 µm, 2.1 mm × 50 mm; Waters). A linear gradient elution was done utilizing water (H_2O; solvent A) and methanol (MeOH; solvent B) (0 - 20 min.: 50% - 90% B; 20 - 23 min.: 90% B; 23 - 25 min.: 90% - 50% B) and the flow rate was maintained at 0.2 mL min^{-1}. Eluents from the chromatographic column pass through photo diode array (PDA) detector and then enter into the mass spectrometer. Ultraviolet (UV)

absorbance data in the range 210 - 400 nm were recorded by PDA detector. Mass spectrometric data were acquired in positive ion polarity of ESI and the *m/z* range of detection was set to 50 - 1500. The ESI source parameters were: capillary 3 kV; Cone 30 V; Extractor 5 V; Source Temperature 100°C; Desolvation Temperature 300°C; Desolvation Gas Flow 800 Litre/hour (L/h); Cone Gas Flow 50 L/h. All data acquisitions and processing were facilitated by MassLynx (Waters). Followed by nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in $CDCl_3$ as aprotic solvent at ambient temperature with tetramethylsilane (TMS) as the internal standard (δ 0 ppm) equipped with 5 mm probes. The number of attached protons for the ^{13}C NMR signals was determined from DEPT experiments. Standard pulse sequences were used for DEPT, ^{1}H–^{1}H COSY, two-dimensional NOESY, HSQC, and HMBC experiments. All solvents were of analytical, spectroscopic or chromatographic reagent grade, and were obtained from E-Merck (Darmstadt, Germany).

4.4 Results

4.4.1 Characterization

Morphological, biochemical and physiological characteristics of *B. subtilis* Ba37 were found to be similar to reference strain *B. subtilis* MTCC441 (Table 4.1). *B. subtilis* Ba37 was aerobic, Gram-positive, rods shaped (size ~ 0.4-0.7 X 1.5-3.0 μm), motile, endospores was ellipsoidal in shape and observed at sub-terminal or central positions in swollen sporangia.

Colonies were smooth, slightly undulate, irregular, slightly raised, creamish white in color and opaque and large in size after 3 days growth at 30°C on nutrient agar with 1% NaCl. It didn't give positive result for KOH

test. Oxidase and catalase were produced, Voges - Proskauer test is positive, but H_2S nitrate reduction and indole is negative, citrate utilization negative, K/A for TSI utilization, Late α - hemolytic activity was present. Negative for gas production from D-glucose, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not hydrolysed. Acid is produced from D-glucose, glycerol and salicin and slow utilization of mannitol and trehalose while L-arabinose, xylose, galactose, mannose, inositol, mellibiose and raffinose were not utilized.

B. subtilis Ba37 can grow in varied physiological conditions. Isolates grow at temperature range of 20-100°C, salinity of 0-55 ppt and pH of 4-10. Isolates were sensitive to other antibiotics tested and resistant to bacitracin only. *B. subtilis* Ba37 were possessing exoenzymatics activities such gelatinase, esterase, lipase, protease, amylase, cellullase, urease and lecithinase. It does not possess phosphatase activity and detailed results were given in Table 4.1

Table 4.1 Charcterization of *B.subtilis* Ba37 and reference strain *B. subtilis* MTCC 441

Characteristics	**Reaction**	
	B. *subtilis* MBTDCMFRI *B. subtilis* Ba37	*B. subtilis* MTCC 441
Morphology		
Colony colour on nutrient agar (1%NaCl/ 50% SW)	Creamish white	Creamish white
Size	Large	Large
Form	Irregular	Irregular
Margin	Undulate	Undulate
Elevation	Flat	Flat
Cell Shape	Rod	Rod
Gram stain	G+	G+
3% KOH	-	-
Endospore formation	+	+
Pigmentation	-	-

Physiological properties		
Aerobic growth	+	+
Growth at temperature	20-55°C	20-55°C
Growth at pH	4-10	4-10
Growth in NaCl (%)	0-100	0-100
Biochemical Characteristics		
Catalase reaction	+	+
Oxidase reaction	+	+
Nitrate reduction	+	+
Indole production	-	-
Methyl red test	-	-
Voges -Proskauer test	+	+
H_2S production	-	-
Citrate utilization	+	+
Arginine dihydrolase	-	-
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Hydrolysis of		
Tween 20	20	-
Starch	28	23
Cellulose	25	18
Skim milk	11	-
Gelatin	43	50
Egg yolk emulsion	27	-
Urease Activity	+	+
Haemolytic activity	-	-
Phosphatase activity	-	-
Acid production		
Arabinose	-	
Galactose	-	
Glucose	+	+
Inositol	-	

Mannitol	+	+
Mannose	-	-
Melibiose	-	-
Raffinose	-	-
Salicin	+	+
Trehalose	+	+
Xylose	-	-
Antibiotics susceptibility Pattern		
Ampicilin (10mcg)	23	18
Bacitracin (10U)	10	8
Cefoperazone(75mcg)	30	27
Cefoxitin (30mcg)	27	27
Cefuroxime(30mcg)	26	25
Cephalothin (30mcg)	29	37
Chloramphenicol(30mcg)	29	28
Ciprofloxacin (5mcg)	28	36
Colistin (10mcg)	7	0
Erythromycin (15mcg)	29	0
Gentamicin (100mcg)	27	26
Kanamycin (5mcg)	19	18
Levofloxacin (5mcg)	31	29
Lomefloxacin (10mcg)	27	24
Nitrofuran (300mcg)	19	20
Norfloxacin (10mcg)	27	23
Pencillin (10U)	29	24
Streptomycin (10mcg)	16	20
Tetracycline (30mcg)	32	35

4.4.2 Inhibitory Spectrum

The culture and cell-free supernatants of the *B. subtilis* Ba37 shows inhibitory towards the pathogens tested. The antibacterial activity of culture and filtered supernatant of *B. subtilis* Ba37 exhibited greater inhibitory activity against

V. vulnificus MTCC1145, *V. harveyi* 101 and least against *A. hydrophila.* The zone of inhibition against the tested pathogen by spot diffusion, well diffusion and disk diffusion method were around 9-20 mm were given in Table 4.2. Inhibition zones (mm) exhibited by commercial antibiotics against the test pathogens are shown in Table 4.3. Thus, upon comparison of the antibacterial activity of *B. subtilis* Ba37 and commercialized antibiotics against tested pathogens showed that the inhibitory levels of the isolates is equivalent to commercial antibiotics like ampicillin (10 mcg), penicillin (10 U), bacitracin (10 U), erythromycin (15 mcg), kanamycin (30 mcg) and streptomycin (10 mcg).

Table 4.2 Antagonistic activity of the *B. subtilis* Ba37

Assay Method	Zone of Inhibition (mm)					
	A	B	C	D	E	F
Spot diffusion	20	10	10	18	20	17
Well diffusion	16	12	10	14	15	16
Disc diffusion	16	11	9	13	15	15

A - *Vibrio vulnificus* MTCC1145; B - *V. parahaemolyticus* MTCC 451; C- *Aeromonas hydrophila*; D - *V. alginolyticus* 101; E- *V. harveyi* 101 and F- *V. anguillarum* 01. The measurement indicates the inhibition zone (mm) formed around the isolates. The values may differ ± 1mm from the size mentioned

Table 4.3 Antibiogram of standard commercial antibiotics against test pathogens

	Zone of inhibition of antibiotics (mm)									
Test organisms	P	B	E	T	S	Cf	C	G	A	K
Vibrio vulnificus MTCC1145	33	R	32	33	16	40	34	27	30	R
*Vibrio parahaemolyticus*MTCC 451	R	R	15	20	16	20	25	19	R	20
Aeromonas hydrophila	R	R	15	25	16	23	21	24	R	20
Vibrio harveyi 101	R	R	13	21	11	18	23	15	R	13
Vibrio anguillarum 01	R	R	15	28	14	14	33	24	R	20
Vibrio alginolyticus 101	R	R	11	15	12	17	19	16	R	13

P - Penicillin (10U), B - Bacitracin (10U), E - Erythromycin (15mcg), T- Tetracyclin (30mcg), S - Streptomycin (10mcg), Cf - Ciproflaxacin (5mcg), C - Chloramphenicol (30mcg), G- Gentamycin (10mcg), A - Ampicillin (10mcg), K - Kanamycin (30mcg

4.4.3 Determination of Generation Time and Growth Curve

Growth curve of the isolates was illustrated in Fig 4.1. There was a small lag phase, after which the microorganisms entered into a rapid logarithmic phase for *V. anguillarum* O1 while for *B. subtilis* Ba37 there is a typical lag phase and the entered logarithmic phase. The generation time was calculated from the growth data at 3rd h and 0th h. Thus, *B. subtilis* MBTDCMFRI Ba37 and *V. anguillarum* O1 has been shown to have a generation time of about 59 & 49 min generation^{-1} respectively.

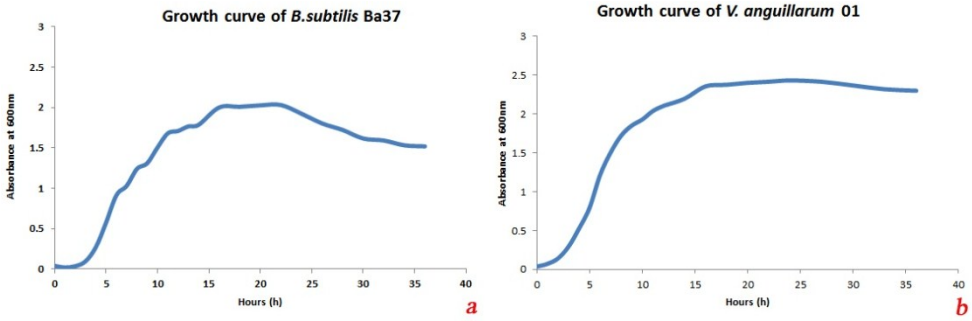

Fig 4.1 Growth curve of *B. subtilis* Ba37 and *V. anguillarum* 01

4.4.4 Co-culture Assay

The antagonism exhibited by *B. subtilis* Ba37 against *V. anguillarum* O1 was further confirmed by co-culture assay. The growth of pathogenic *V. anguillarum* O1 at different concentrations (10^2-10^4 CFU/mL) was inhibited by *B. subtilis* Ba37 (10^4-10^8 CFU/mL) (Fig 4.2). *B. subtilis* Ba37 inhibited *V. anguillarum* O1 in co-culture when the initial cell count of the probiont was even at 10^4 CFU/mL. Lower concentrations of *B. subtilis* Ba37 (10^4 CFU/mL) allowed initial growth of *V. anguillarum* 01 upon continued incubation but never allowed the cell densities of the pathogen to reach the level of control. High concentrations (10^6-10^8CFU/mL) of *B. subtilis* Ba37 allowed an initial increase of *V. anguillarum* 01 followed by a decrease in the total viable counts

(Fig 4.2). When the concentration of the probiotic increased, rate of inhibition also increased. After 96 h in co-culture, higher concentration of *B. subtilis* Ba37 inhibited complete growth of the *V. anguillarum* 01. Growth of the antagonistic bacteria was not affected by pathogen at any stage in the co-cultures. Results of co-culture experiment showed that the growth of *V. anguillarum* 01 was controlled when the concentration of *B. subtilis* Ba37 increased under *in vitro* conditions.

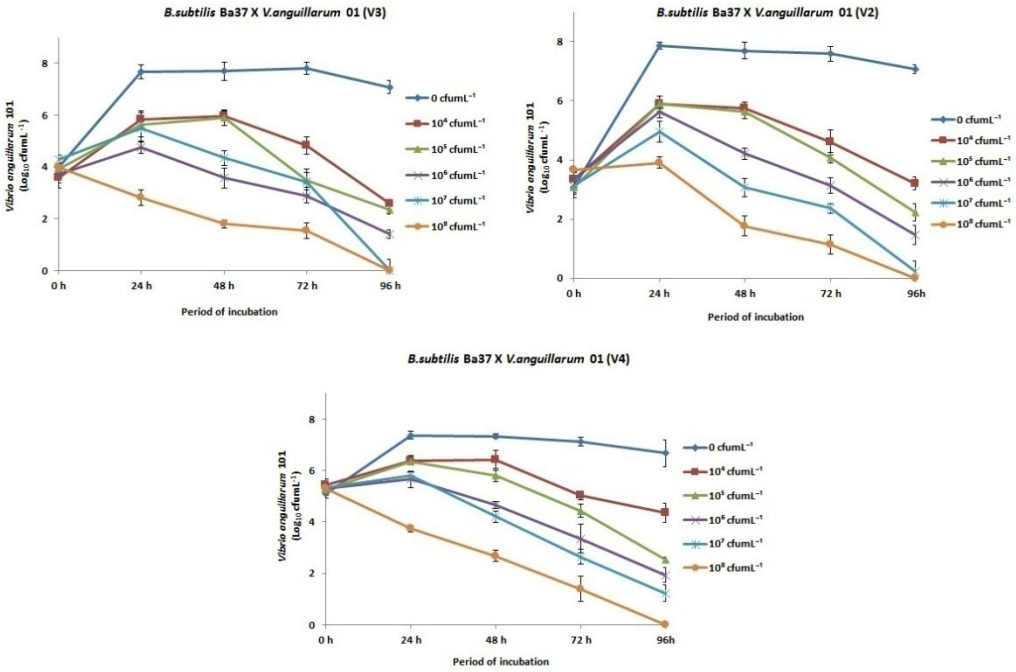

Fig 4.2 Growth of *V. anguillarum* 01 in co culture at increasing cell densities of *B. subtilis* Ba37

4.4.5 Cytotoxicity Assay

On microscopic observation, there is no morphological changes occurred in cells which were treated with filtered supernatant of *B. subtilis* Ba37 while the cells treated with supernatant of *V. anguillarum* 01 was ruptured completely. So the antagonistic bacterium was found not toxic to the koi carp cell lines but the pathogenic bacteria is highly toxic to the cell lines (Fig 4.3).

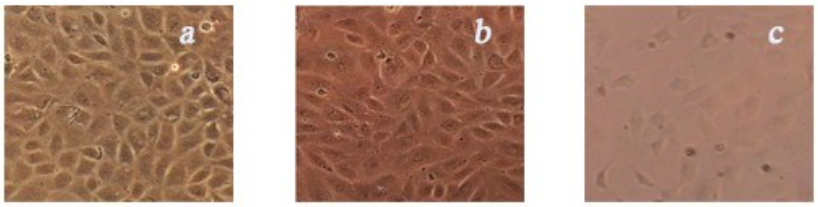

Fig 4.3 Cytotoxicity assay of koi carp cell lines with CFS of *B. subtilis* Ba37 and *V. anguillarum* 01
a - Control cell line, b - CFS of *B.subtilis* Ba37 treated cell line, c - CFS of *V.anguillarum 01* treated cell line

4.4.6 Safety of the Potential Antagonistic Bacteria (*in vivo*)

B. subtilis Ba37 was found to be harmless while carrying out the pathogenicity test. It did not cause any significant mortality after bath challenge on larvae of *E. suratensis*. The presence of *B. subtilis* Ba37 (10^6 CFU/mL) was found in larval rearing water for 4 days and subsequently decreased. It did not show any signs of disease or mortality after intra-peritoneal injection to sub adult *E. suratensis* and were therefore considered safe to be used in the fish. The bacterial analysis by the end of the experiment revealed the isolation of *B. subtilis* Ba37 from the corresponding fish group

4.4.7 Optimization of Media, Temperature, Salinity and pH for the Production of Antagonistic Compound

Optimization of culture media and its conditions highly influenced the production of antibacterial compound. Among different medium used, modified *Bacillus* media was found to be more effective in maximum production of antibacterial principle from *B. subtilis* Ba37 followed by TSB with 1% NaCl (Fig 4.4a). The best carbon and nitrogen source for optimum production was found to be glycerol (1%) and glutamic acid (0.5%) respectively. *B. subtilis* Ba37 can grow from 25 to 40°C, the optimum temperature for growth and the production of the antagonistic component was recorded at 30°C, with a maximum inhibitory zone of 12-21 mm on the 3rd day against tested pathogens (Fig 4.4b). Enhanced production was achieved at 15ppt salinity and maximum

antagonistic compound was produced after the 3rd day with a clearing zone of 13-21 mm. The inhibitory activity of the culture supernatants was less for higher and lower salinity of 35ppt and 5ppt respectively (Fig 4.4c). The pH does not influence much with inhibitory compound production. There was no significant variation in the growth of *B. subtilis* Ba37 or antagonistic activity with the tested pH range. However, the inhibitory activity was highest at pH 7.0 followed by pH 8.0 with 3 days of culture with a clearing zone of 12-20 mm (Fig 4.4d).

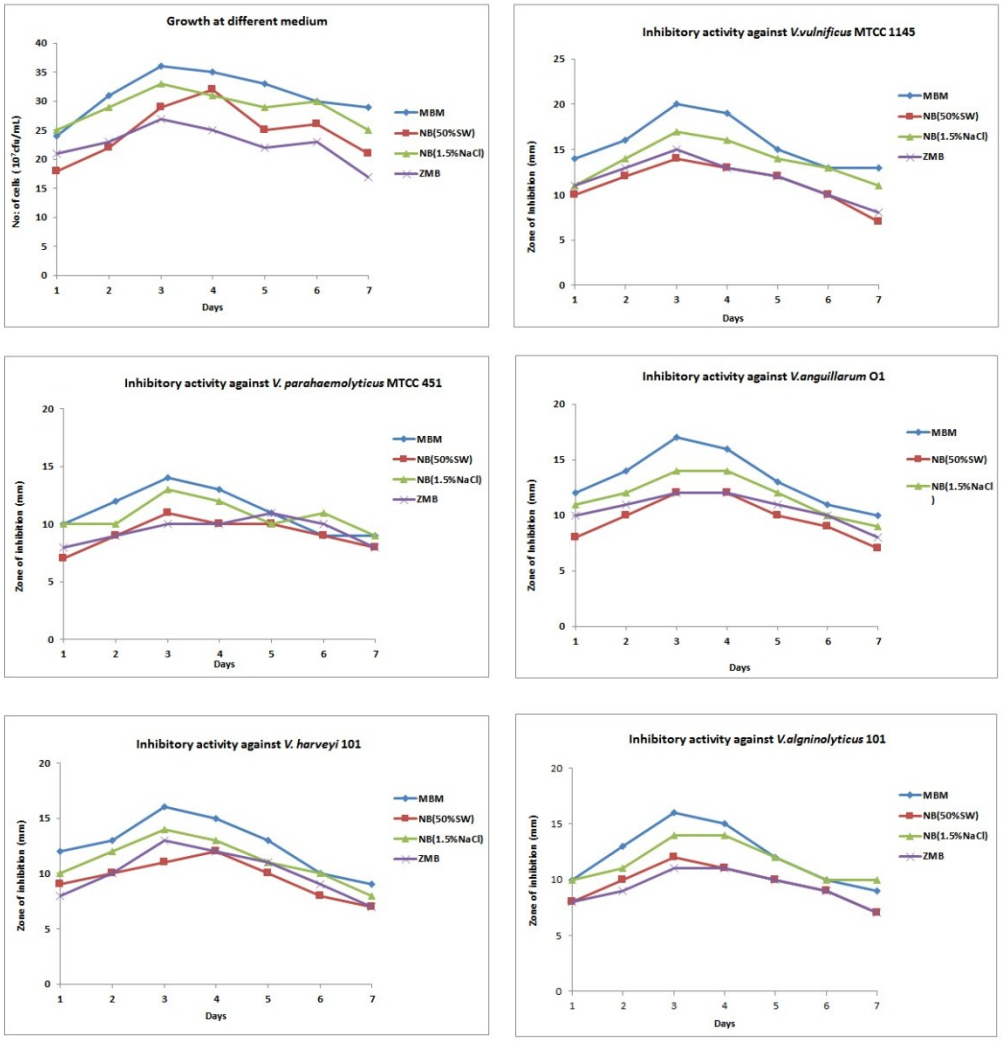

Fig 4.4a Effect of medium for the production of antibacterial metabolite in *B. subtilis* Ba37

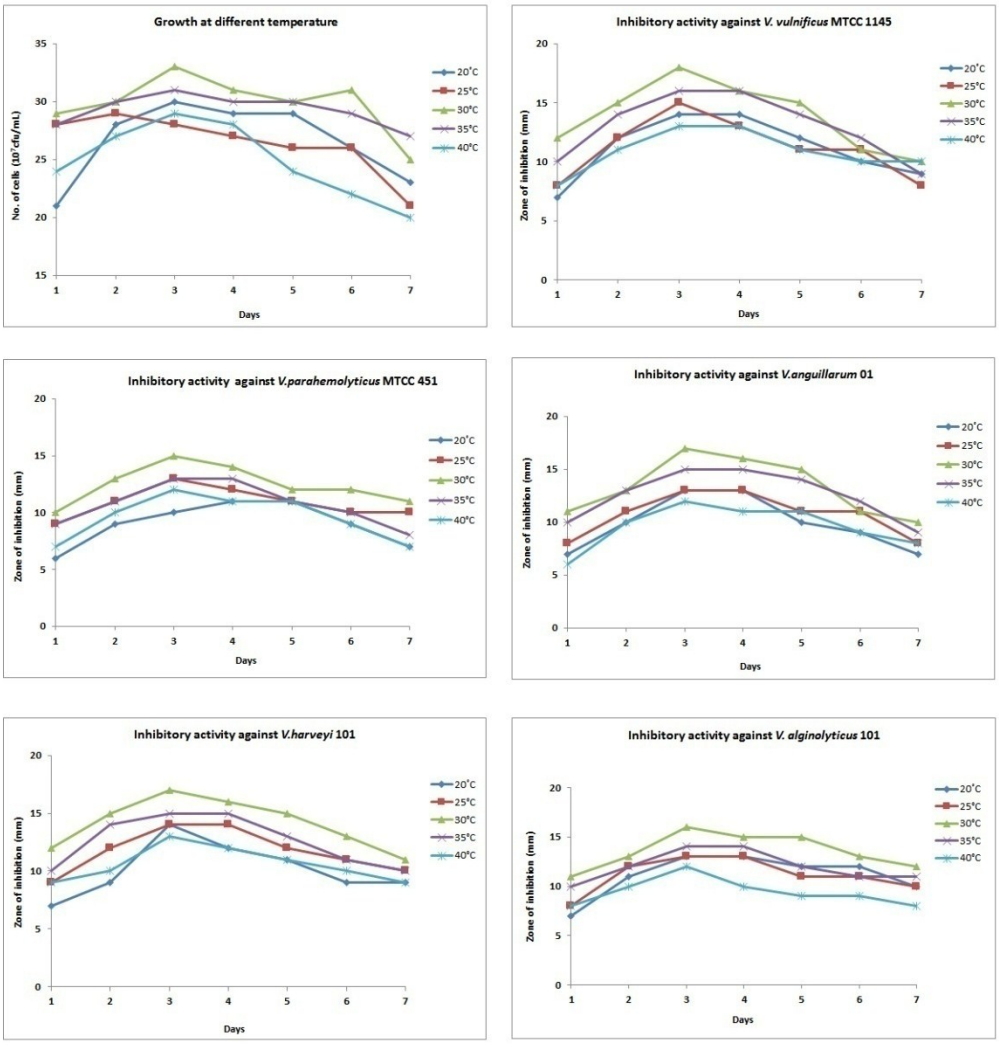

Fig 4.4b Effect of temperature for the production of antibacterial metabolite in *B. subtilis* Ba37

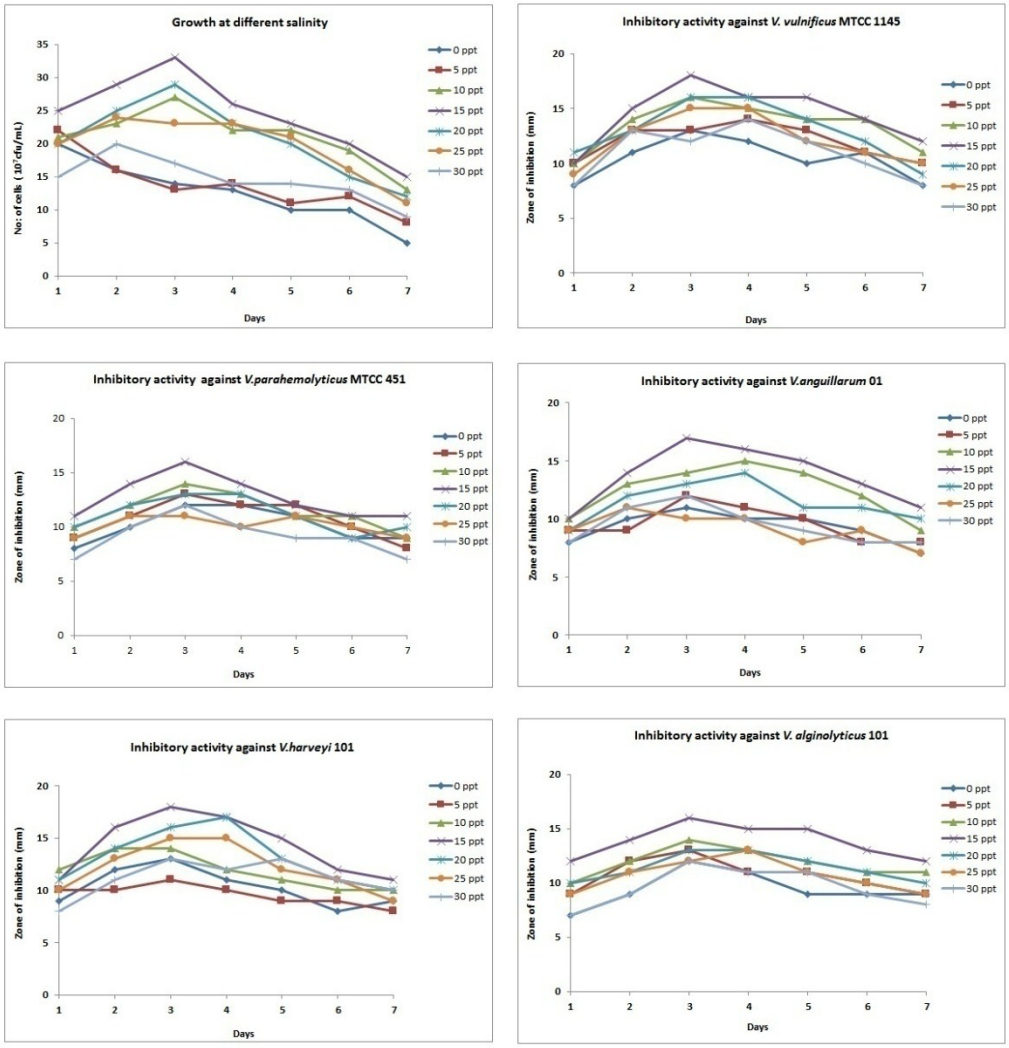

Fig 4.4c Effect of salinity for the production of antibacterial metabolite in *B. subtilis* Ba37

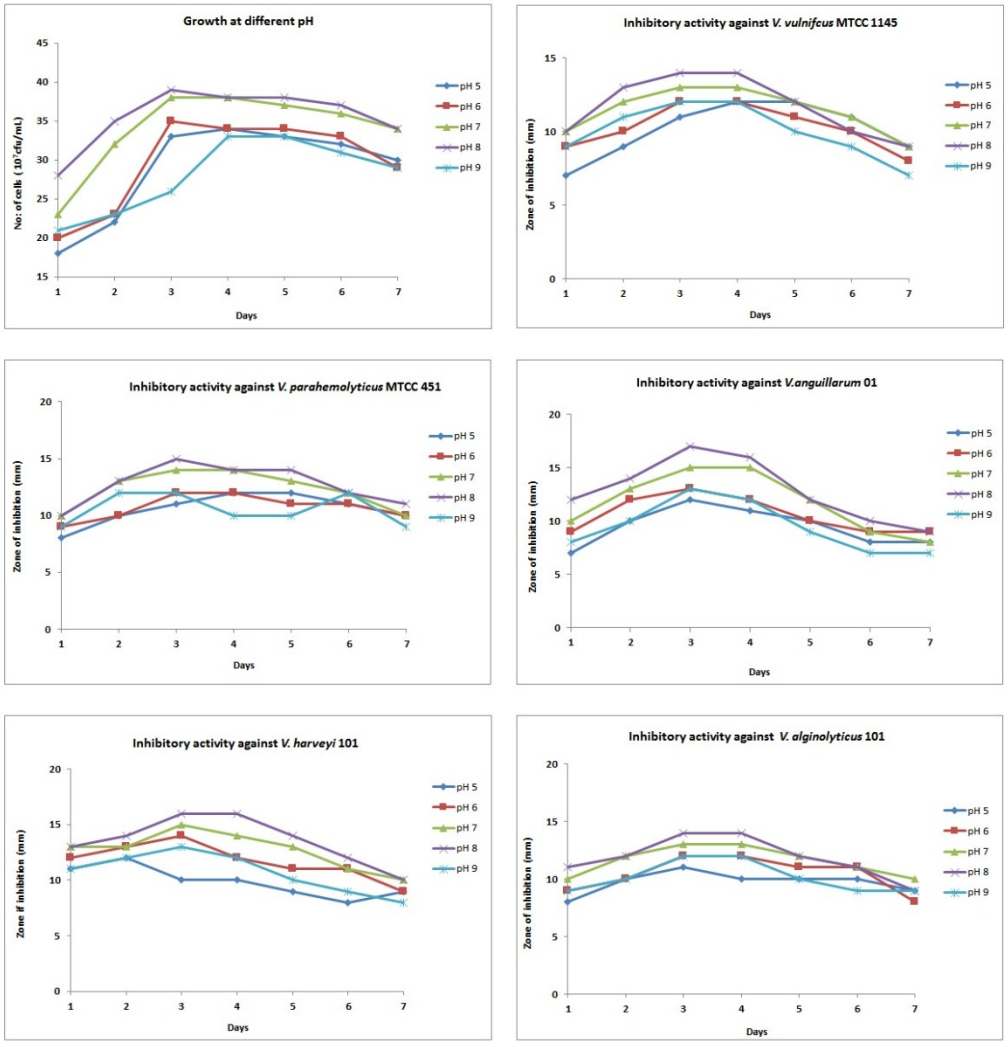

Fig 4.4d, Effect of pH for the production of antibacterial metabolite in *B. subtilis* Ba37

4.4.8 Nutritional Analysis

The main component of *B. subtilis* Ba37 was carbohydrate followed by protein and lipid. Proximate chemical composition of *B. subtilis* Ba37 is given in Table 4.4.

Table 4.4 Chemical composition analysis of *B. subtilis* Ba37 on dry matter basis

Sample name	% dry matter	% moisture	% crude protein	%crude fat	%crude ash	%crude fibre	% acid insoluble ash	% nitrogen free extract
B.subtilis Ba37	100	NIL	34.94	1.47	9.71	0.09	0.05	53.80

4.4.9 Preliminary Characterization of Antagonistic Principle

The inhibitory compound production of *B. subtilis* Ba37 was observed in the late phase of culture (about 40 h) in modified synthetic medium and activity reached maximum in the stationary phase and remained stable for two more days with small flux in activity. The filtered supernatant possessed inhibitory potential against pathogens even after dilution up to 60 times (40%) of the original concentration. The inhibitory zone produced by the diluted supernatant (40%) decreased to 8- 12 mm, compared to the control (12-16 mm). Study of the heat stability of the antagonistic principle showed that it was stable at temperatures up to 80°C and the activity gradually reduced as temperature increased. Activity was totally lost after incubation at 121°C. While in case of stability, at different pH, the antibacterial component was found to be stable at pH 6 - pH 9, but the activity was lesser at the extremes (pH >9 and pH <6) (Table 4.5). However antagonistic compound was not stable after enzymatic treatment.

Table 4.5 Preliminary characterization of the cell free supernatant of *B. subtilis* Ba37

Dilution	Zone of inhibition (mm)				
	A	B	C	D	E
10%	-	7	-	-	7
20%	7	7	8	9	7
30%	8	8	9	11	8
40%	9	8	9	11	8
50%	9	9	10	12	9
60%	11	10	10	12	10
70%	13	10	11	13	10
80%	13	11	12	13	10
90%	14	12	14	14	11
100%	15	13	16	15	12

pH	Zone of inhibition (mm)				
	A	B	C	D	E
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	9	8
6	11	10	13	12	11
7	13	12	13	14	12
8	12	12	14	15	12
9	9	9	8	13	8
10	-	7	-	8	-
11	-	-	-	-	-
12	-	-	-	-	-
Control	14	12	14	15	12

Temperature	Zone of inhibition (mm)				
	A	B	C	D	E
40°C	14	12	15	16	12
50°C	13	12	14	15	12
60°C	12	11	12	14	11
70°C	12	11	12	14	11
80°C	10	10	11	12	10
90°C	-	10	10	11	10
100°C	-	9	10	11	9
120°C	-		-	-	-
Control (RT)	15	12	15	16	12

A - *Vibrio harveyi* 101, B - *V. alginolyticus* 101, C - *V. anguillarum* 01, D - *V. vulnificus* MTCC1145, E - *V. parahaemolyticus* MTCC 451. The measurement indicates the inhibition zone (mm) formed around the isolates. The values may differ ± 1mm from the size mentioned.

4.4.10 Purification and Structural characterisation of 1-(1-Hydroxyethyl) -1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo 8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

The EtOAc/MeOH extract was chromatographed over silica column (60-120 mesh) with a stepwise gradient of solvents from CH_2Cl_2, CH_2Cl_2: MeOH and finally MeOH (100:0, 96:4, 90:10, 85:15, 80:20, 60:40, 20:80, 0:100). The fractions were collected and concentrated under vacuum and TLC was evaluated and fractions with same TLC profile were pooled together to furnish seven fractions (Bs_{1-7}). The column fractions obtained were evaluated for its antagonistic activity against aquaculture pathogens. The sub fraction Bs_2 (7.8% yield) which exhibited high antibacterial activity (zone size of 12-26 mm against various pathogens) was further purified using column chromatography with *n*- hexane, *n*- hexane:EtOAc (100:0, 97:3, 94:6, 91:9, 85:15, 75:25, 60:40, 50:50 and, 0:100) and then with EtOAc:MeOH (90:10, 80:20,0:100) to give another seven sub fractions. After concentrating and TLC evaluation, fractions with same TLC profile were pooled together to furnish seven sub fractions ($Bs_{2.1-7}$) and all fractions were checked for antagonistic activity. The sub fraction $Bs_{2.2}$ which exhibited high antagonistic potential (inhibition zone of 15- 24mm against various pathogens) was re-purified using preparative thin layer chromatography over Si gel GF_{254} (particle size 15 µm) (15% EtOAc: *n*- hexane) to obtain five sub fractions of which the two sub fraction $Bs_{2.2.4}$ and $Bs_{2.2.5}$ exhibited higher antibacterial potential were pooled ($Bs_{2.2.4}$) and further purified by preparative thin layer chromatography over Si gel GF_{254} (particle size 15 µm) (15% EtOAc: *n*- hexane) and were found to be single compound($Bs_{2.2.4.2}$) and active. Its R_f value is 0.68. The schematic representation of the purification pattern is given by (Fig 4.5).

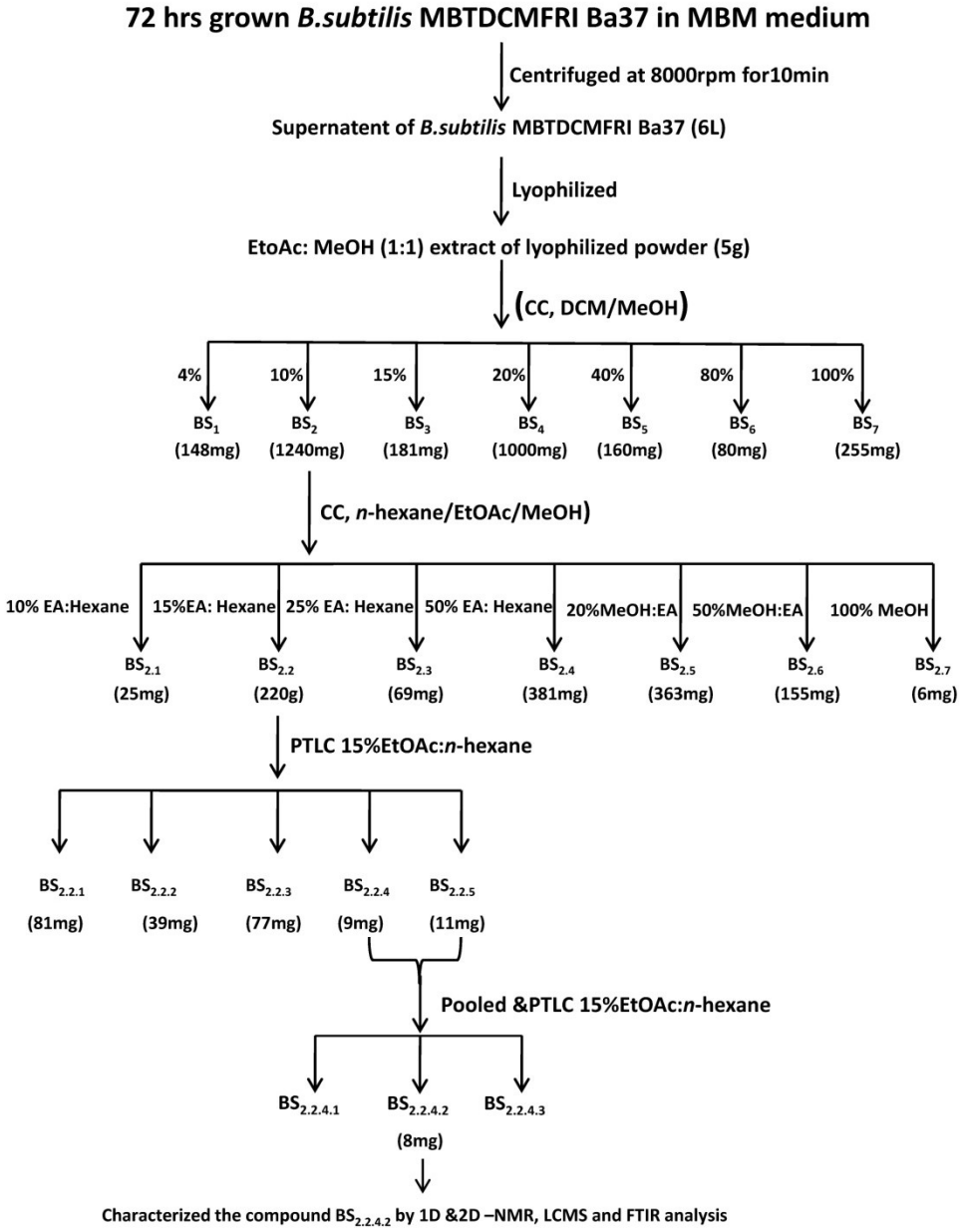

Fig 4.5 Schematic representation of the purification pattern of active compound from *B. subtilis* Ba37

1-(1-Hydroxyethyl)-1,7,10,12,13,15,17heptamethyl-16-oxatetracyclo [8.7.0 .$0^{2,3}$.$0^{12,13}$] heptadecan-5-one

Amorphous yellow solid; UV (MeOH) λmax (log ε): 247 nm (3.91); TLC (Si gel GF254 15 mm; $CHCl_3$/MeOH 10:90 v/ v) Rf: 0.55; LC (Column: Acquity UPLC BEH C18, 1.7 μm, 2.1 mm × 50 mm (Waters); gradient elution using H_2O (solvent A) and MeOH (solvent B) for 25 min : 50% B - 90% B in 20 min, hold at 90% B for 3 min and 90% B - 50% B in 2 min ; flow-rate: 0.2 mL/min ; volume of sample injection: 5 μl) Rt: 20.52 min. (Photo Diode Array (PDA) detection in the range, 200 nm - 400 nm); Elemental analysis found:C,72.42;H,7.13;N,3.67;O,16.78; IR(KBr, cm^{-1}) νmax 721.40cm^{-1} γr(C-H alkanes), 1034.84 cm^{-1} ν(C-N), 1168.90 cm^{-1} , 1313.57 cm^{-1}, 1377.22 cm^{-1} γr (C-H), 1464.02 cm^{-1} δ(C-H of alkanes), 1600.97 cm^{-1}, 1665.59 δ(N-H), 1741.78 cm^{-1} ν(C=O), 2728.40 cm^{-1}, 2852.81 cm^{-1}, 2924.18 cm^{-1}, 2956.01 cm^{-1} ν(C-H alkanes). 1H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$,125MHz, δ ppm) data, (Table 4.6) ; ESI MS m/z (rel. int. %): 803.68 ([2M+Na]+)(48), 413.44(4.5),391.45 ([M+H]+) (100), 279.33 (14), 181.18 (22), 167.16 (17).

Analysis of the IR spectra revealed a split peaks around 2900cm^{-1} and peaks around 3449 cm^{-1} and 1728 cm^{-1} (Fig 4. 6). The LC MS analysis of the compound exhibited a molecular ion peak (M+H peak) at m/z=391.45 and fragment peaks at m/z = 279.33, 181.18, and 167.16 (Fig 4.7). Peaks were also observed at m/z = 413, 429 and 803.68. The expected mass fragmentation pattern is represented by Fig 4.8. Analysis of the ^{13}C NMR spectrum (Table 4.6) with the DEPT-135 together showed one ketonic carbon (C-5, δ 211.36), three oxygenated CH groups (C-15, 17 and 24 with δ 71.31, 71.30 and 81.33

respectively), four CH_2 groups (C-4, C-6, C-11 and C-14 with δ 51.59, 51.57, 29.70, 29.36 respectively) and other 17 CH and CH_3 carbons together (δ values between 14-42) (Fig 4.10 & 4.11). The ^1H spectrum showed a quartet with a J value of 6.4 Hz appeared at a down field shift of δ 3.96 ppm of two hydrogen atoms (Fig 4.9). Similarly another quartet with a J value of 6.4Hz appeared at a down field shift of δ 3.80 ppm of one hydrogen atom. Consequently the CH_3 groups attached to CH groups were appeared as doublets around δ 1.2 ppm (C-18 & C-19) and δ 1.01 ppm (C-27). The structural correlation between hydrogen atoms bound to a particular carbon was further identified by ^1H-^1H COSY (Fig 4.12) The presence of CH groups attached to electronegative centers were further confirmed by the presence of ^{13}C signals appeared around δ 71.31, 71.30 and 81.33 ppm (Fig 4.10). The ^{13}C signals appeared around δ 211.36 ppm confirms the presence of a C=O group attached to the ring system (C-5). HSQC correlations observed between H-15 &17(δ 3.9 ppm) and C-15 &17 (δ 71.2 ppm) further confirms the proposed structure (Fig 4.13). Similarly HSQC correlation was also prominent between H-24 and C-24. The doublets formed at δ 1.01 and 1.2 with a J value of 6.4 Hz also support the present proposed structure that these CH_3 groups are only associated with a neighboring CH protons

Table 4.6. NMR spectroscopic data of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one in $CDCl_3$.[a]

Carbon no.	^{13}C NMR (DEPT)	H	δ^{1}H NMR (int., mult., *J* in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	34.24 (C)		…..		
2	42.81(CH)	H-2	1.00 (m, 1H)	H-7, H-3	C-23,C-10
3	41.38(CH)	H-3	1.77(m, 1H)	H-4,H-10	
4	51.59(CH_2)	H-4	3.6 (m, 2H)		
5	211.36(C)		------		
6	51.67(CH_2)	H-6	3.59(s, 2H)		C-24
7	22.69(CH)	H-7	1.54(m, 1H)	H-8	
8	41.29(CH)	H-8	3.57(m, 1H)	H-11,H-9	
9	40.91(CH)	H-9	3.52(m, 1H)	H-14	
10	22.93(CH)	H-10	1.18(m, 1H)	H-9	
11	29.70(CH_2)	H-11	1.20d(m, 2H)		
12	23.71(C)		……..		
13	23.73(C)		……		
14	29.36(CH_2)	H-14	1.20(m, 2H)		
15	71.31(CH)	H-15	3.99(q, *J*= 6.4 Hz, 2H)	H-18	
16	O		…….		
17	71.30(CH)	H-17	3.99(q, *J*= 6.4 Hz, 2H)	H-19	
18	22.69(CH_3)	H-18	1.21(d, *J*= 6.4 Hz, 3H)		
19	22.67(CH_3)	H-19	1.21(d, *J*= 6.4 Hz, 3H)		C-21,C-14
20	21.81(CH_3)	H-20	2.22(m, 3H)	H-12	C-11,C-18
21	21.79(CH_3)	H-21	2.22(m, 3H)	H-13	
22	21.59(CH_3)	H-22	1.19(m, 3H)	H-10	C-3,C-9
23	14.09(CH_3)	H-23	1.19(m, 3H)	H-7	C-25,C-8,C-11
24	81.33(CH)	H-24	3.79(q, *J*= 6.4 Hz, 1H)	H-27	
25	18.82(CH_3)	H-25	2.17(s, 3H)		
26	O (OH)		……..		
27	14.09(CH_3)	H-27	1.01(d, *J*= 6.4 Hz, 3H)		C-25, C-6
28	O		……		

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometer.

[b] Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY experiments

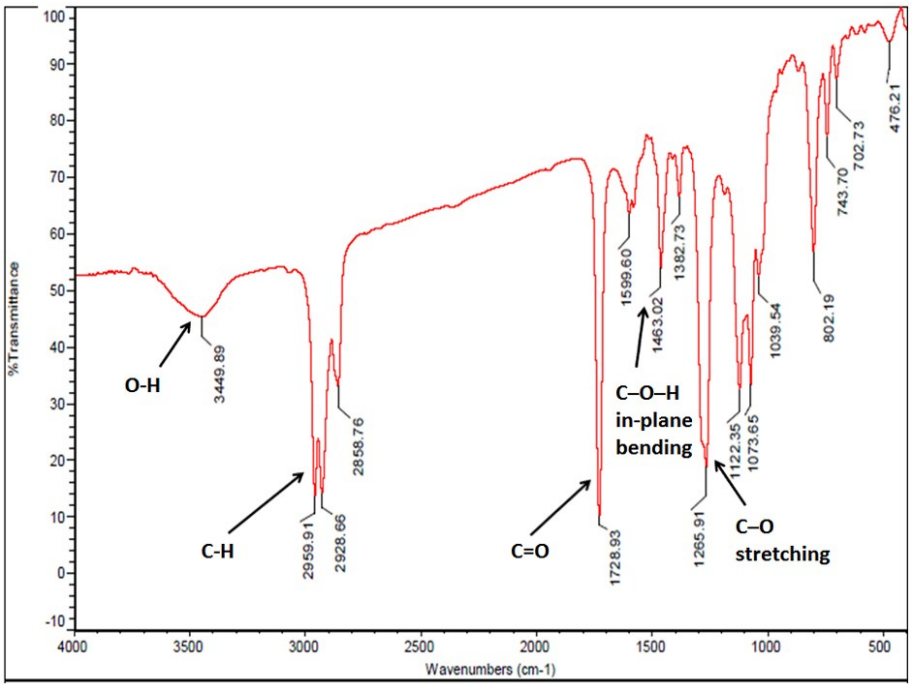

Fig 4.6 IR spectra of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

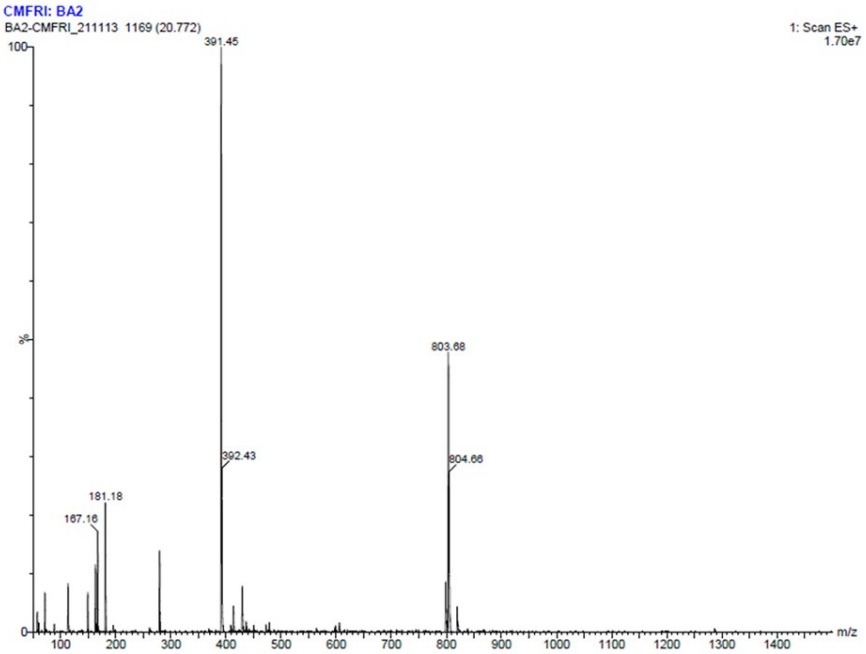

Fig 4.7 LC-MS spectra of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

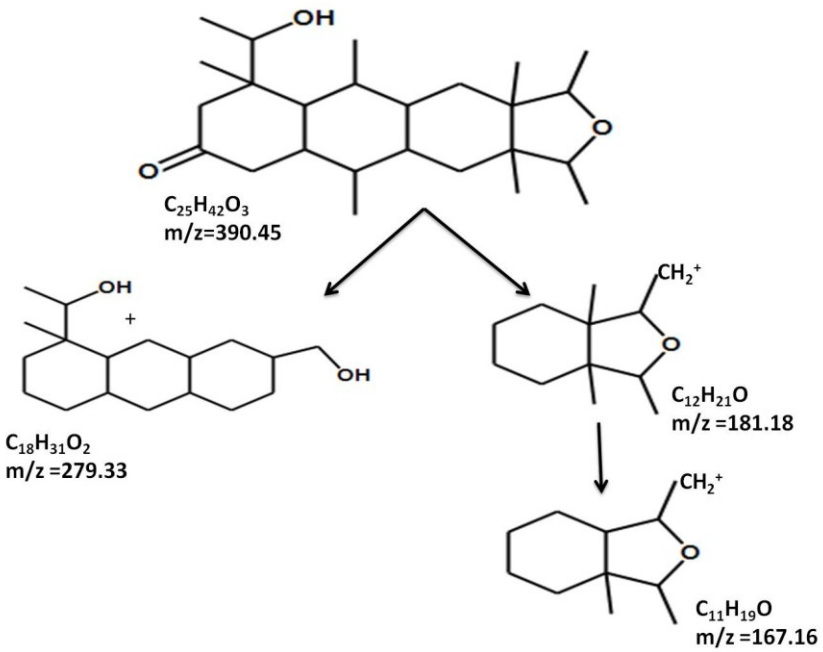

Fig 4.8 Mass fragmentation pattern of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo[8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

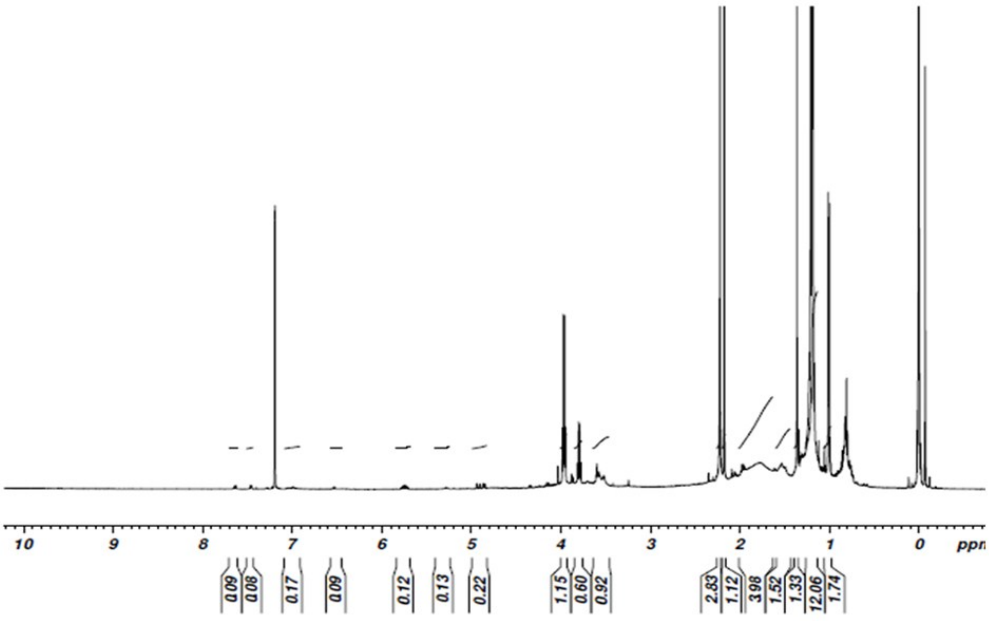

Fig 4.9 Proton NMR spectra of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.0^{2,3}.0^{12,13}]heptadecan-5-one

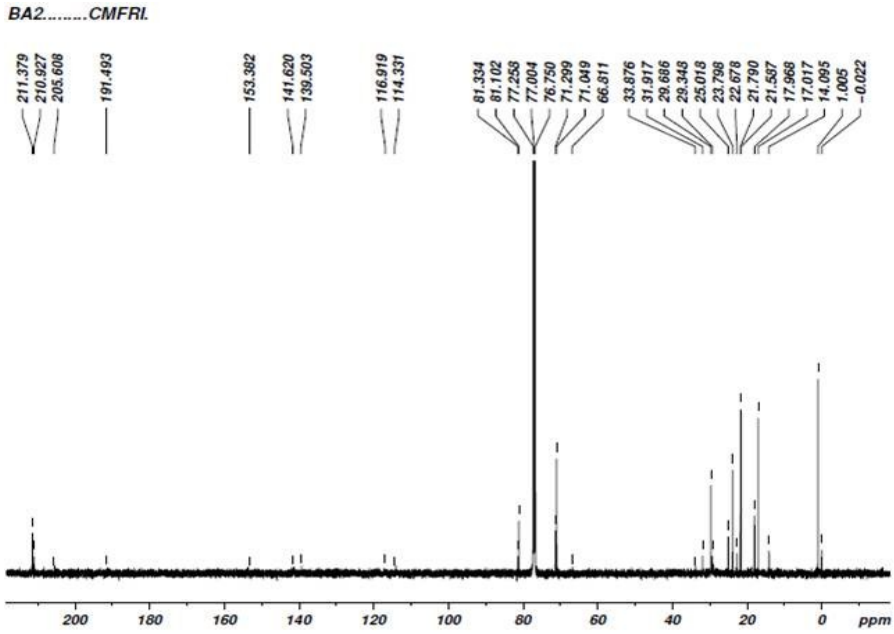

Fig 4.10 ^{13}C NMR spectrum of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.$0^{2,3}$.$0^{12,13}$]heptadecan-5-one

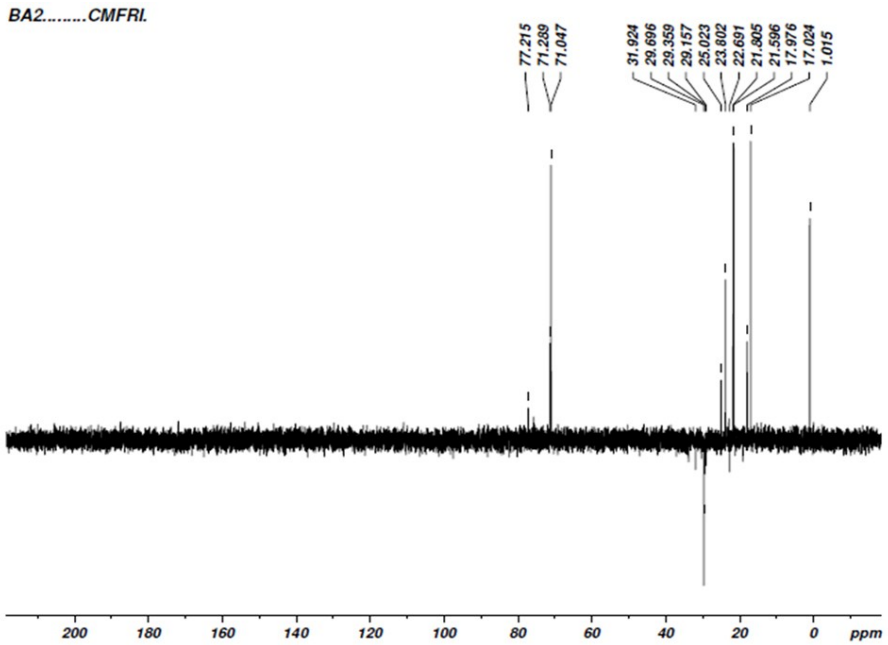

Fig 4.11 DEPT spectrum of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.$0^{2,3}$.$0^{12,13}$]heptadecan-5-one

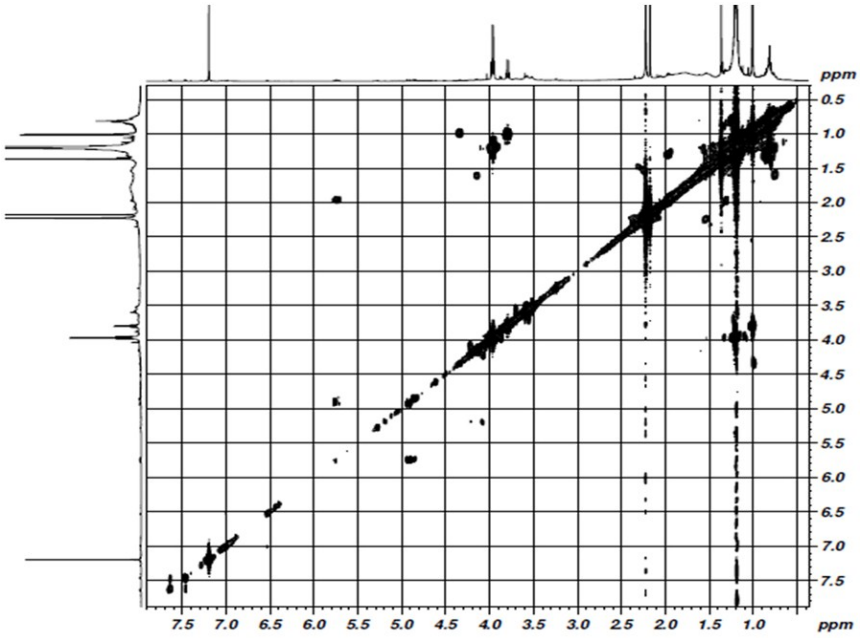

Fig 4.12 ^{1}H-^{1}H COSY spectrum of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.$0^{2,3}$.$0^{12,13}$]heptadecan-5-one

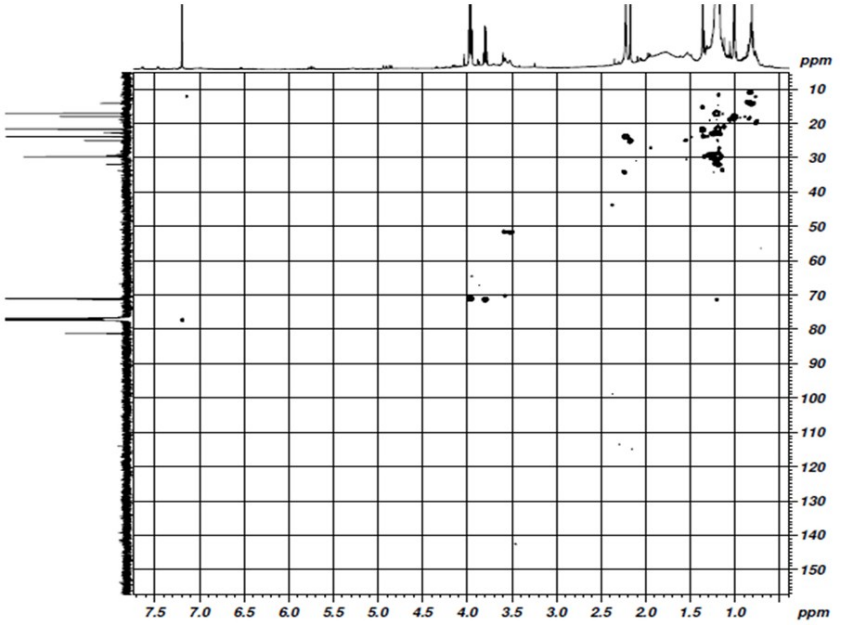

Fig 4.13 HSQC spectrum of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.$0^{2,3}$.$0^{12,13}$]heptadecan-5-one

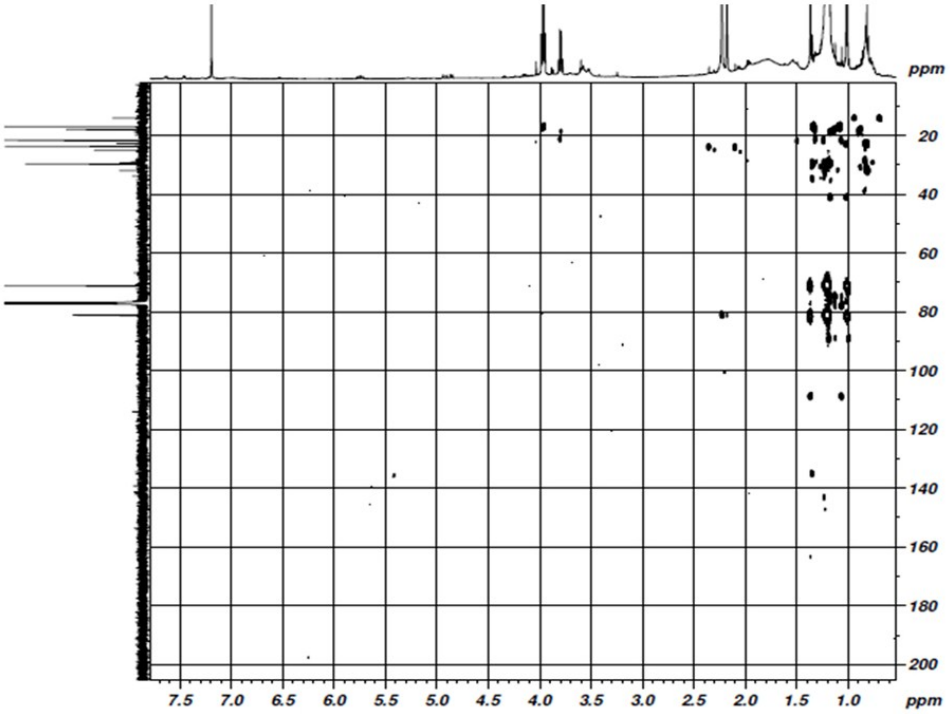

Fig 4.14 HMBC spectrum of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17 heptamethyl-16-oxatetracyclo [8.7.0.$0^{2,3}$.$0^{12,13}$]heptadecan-5-one

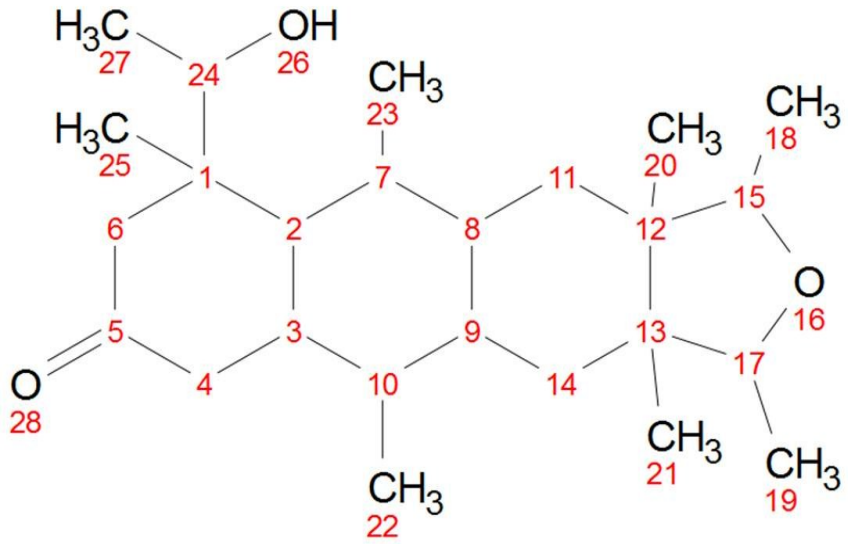

Fig 4.15 Struture of 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17heptamethyl-16-oxatetracyclo [8.7.0.$0^{2,3}$.$0^{12,13}$] heptadecan-5-one

4.5 Discussion

The present study illustrated a potential antagonistic bacterial strain, *B. subtilis* CMFRIMBTD Ba37, isolated from the sediments of mangrove ecosystem (Mangalavanam), a tropical estuarine habitat, Cochin. Further, the bioactive molecule behind the antibacterial activity was also characterized, which revealed the potential of this molecule as an alternative candidate for antibiotics, with broad spectrum of antibacterial activity against aquaculture pathogens.

B. subtilis is a ubiquitous bacterium and are relatively rich source of antimicrobial activity, since many species of this genus synthesize multitude of antimicrobial peptides (Lalloo et al. 2008) which inhibiting wide range of aquaculture pathogens (Vaseeharan and Ramaswamy 2003). They have been used as a promising antagonistic bacteria as well as a good bio-control agent in aquaculture (Balcazar and Rojas-Luna 2007; Kamgar et al. 2013) where the inhibitory effects of *Bacillus* might be due to the alteration of pH in the growth medium, outcompeting them for nutrients or mucosal space or the production of antagonistic compounds (Mahdhi et al. 2011; Nair et al. 2012; Keysami et al. 2012). Recently, Das et al. (2014) affirmed that outer membrane proteins of *B. subtilis* could be the best candidates for controlling the major bacterial infections in aquaculture sector as it have a broad spectrum of antimicrobial activity. Apart from this, *B. subtilis* has wide application against plant and animal pathogens (Nagorska et al. 2007, Thirabunyanon et al. 2012; Sabate et al. 2013; Moore et al. 2013). Safronova et al. 2012 stated that extreme genetic plasticity of *Bacillus* species endowed each strain with a unique spectrum of antagonistic activity.

The suitability and robustness of biological agents depends on its response to environmental conditions in which they must perform (Lalloo et al. 2008). Physiological conditions often affect functionality of biological agents and they must remain functional and effective over wide-ranging physiological conditions (Guetsky et al. 2002). *B. subtilis* Ba37 grown well under a wide range of temperature, salinity and pH which would help its better adaptation towards the host and the aquatic environment which helps to act as a good bio-control agent (Vine et al. 2006; Nair et al. 2012). Studies showed that *Bacillus* can grow within the intestinal tract and rather may exert a unique symbiotic relationship with their host and possibly be considered as temporary residents and hence there is also intense interest in using heat-stable *Bacillus* spores in aquaculture (Cutting 2011). It was reported that prospective probionts should not carry the genes for antibiotic resistance (Saarela et al. 2000) and gram positive probiotics minimize the chance of transfer of antibiotic resistant genes (Hill et al. 2009; Mahdhi et al. 2011). *B. subtilis* Ba37 were sensitive to twenty four antibiotics tested except three antibiotics tested showing that the isolate has only minimum number of antibiotic resistance genes whereas the reference strains also showed the similar antibiotic resistance pattern.

Besides, the isolate was able to produce several hydrolytic enzymes which could support/improve digestion, enhanced nutrient uptake by the host by degrading a variety of natural substrates and contribute to nutrient cycling (Geovanny et al. 2008; Keysami et al. 2012). Experiments conducted to study the the growth and survival of *Penaeus monodon* larvae after feeding with *B. subtilis* Ba37, showed improved survival and growth, either by supplementing the digestive system of the host with microbial enzymes or by enhanced nutrient digestion (Pai et al. 2010). In account of the higher nutritional levels,

with 35% crude protein content, the biomass of *B. subtilis* Ba37 would serve as an additional nutritional source to host. Thus it further increases the suitability of the bacteria to act as a nutrition supplement to the host animals. Spore forming capability of *Bacillus* spp. has provided excellent ecological advantages which allowed for long-term storage, resistance to toxic compounds, temperature extremes, desiccation and radiation; thus allowing for the relatively easy formulation and commercialization of *Bacillus*-based stable microbial products 'MPs' (Ongena and Jacques 2007; Hammami et al. 2008; Nemutanzhela et al. 2014). Being a *bacillus* sp, the *B. subtilis* Ba37 also possess similar character, which can be exploited to be incorporated as a feed additive in larval feeds in hatcheries.

The growth of the pathogen was successfully inhibited by antagonistic bacteria in co-culture, where the inhibitory activity increased with increasing biomass of the antagonist Similar results has been reported for coculture of antagonistic *B. subtilis* against pathogens (Vaseeharan and Ramasamy 2003; Touraki et al. 2012). Though the bacterium with a short lag period and short doubling time was reported to have an improved ability of out competing other bacteria (Vine et al. 2006), revealed that the *B. subtilis* possess higher production of antagonistic compound, which inhibits the growth of the pathogen. The rationale for using K_2CrO_4 medium for co-culture is based on the bio reduction potential of chromium by *Bacillus* spp. (Mangaiyarkarasi et al. 2011). Further, gram-positive microorganisms has the ability to accumulate higher amount of heavy metals than gram-negative microorganisms (Augusto da Costa and Duta 2001) and time than that of *V. anguillarum* but it inhibits the growth of *V. anguillarum*. This competitive advantage of *B. subtilis* Ba37 over *V. anguillarum* 101 is due to the production of antagonistic compound, which inhibits the growth of the pathogenic *Vibrios*. We have observed that *V.*

anguillarum cannot grow in nutrient agar supplemented with 5vmM potassium chromate whereas *Bacillus* can flourish in the same medium. As far our knowledge, this is the first report that depicted the use of 5 mM potassium chromate in nutrient agar be used as differential/specific medium for growth of *B. subtilis* while the same inhibits the growth of *V. anguillarum* 101. Therefore, this medium can be used for such studies. It could be demonstrated that *B. subtilis* Ba37 inhibits the growth of pathogens mainly by producing antagonistic compounds or by competitive exclusion, and eliminate it completely on long term incubation. And this antagonistic isolate has to be evaluated for its pathogenicity in order to be considered as safe to the host and which in turn can be applied to aquaculture systems (Verschuere et al. 2000; Vijayan et al. 2006). The administration of *B. subtilis* Ba37 did not cause any harmful effects, disease signs or mortality in sub adult/juvenile *E. suratensis* upon challenge even at a dose of $10^{7\text{-}8}$ CFU/mL cells through intramuscular and intraperitoneal routes. By this, *B. subtilis* Ba37 was found to be safe and hence could be used as candidate probiotic in aquaculture system.

The production of antimicrobial peptides by strains of *B. subtilis* is thought to be under complex genetic regulation and several antimicrobial peptides are produced by a single strain simultaneously or at least at different time intervals or growth conditions (Duitman et al. 1999). Modifications in culture conditions and nutrients may induce the production of different peptide antibiotics (Motta et al. 2007). Modified synthetic production medium optimized in this work has shown an increased production of antibacterial compound, probably because this medium satisfies all the necessary requirements for bioactive compound production than commercially available heterotropic rich medium (Jamil et al. 2007). The production pattern of the antagonistic component in synthetic media showed that production starts at

exponential phase and remained constant during the stationary phase indicating that this substance is a secondary metabolite (Jamil et al. 2007; Hammami et al. 2008).

In order to use *B. subtilis* Ba37 in aquaculture system or for its further application, the data on minimum inhibitory concentrations of cell free supernatants for inhibiting the growth of pathogen is vital (Vijayan et al. 2006). The cell free supernatant can be diluted up to 40% of its initial concentration to retain its antagonistic activity and activity was maintained at 80^{o}C and pH 6-9, indicating the inhibitory compound was relatively heat stable and possibility of their application in industrial level production of antagonistic compound. In addition, the cell free supernatant was sensitive to proteases tested, suggesting that a peptide moiety is associated with its activity (Lisboa et al. 2006).

Antibacterial substances produced by different bacteria seem to play an important role in the bacterial antagonism in aquatic ecosystems (Dopazo et al. 1988). Bacteriocins may play a defensive role to hinder the invasion of ecosystem of other strains or species into an occupied niche (Riley and Wertz 2002). One single *Bacillus* strain is often able to produce several types of molecules stable over a wide range of pH and temperature and partially resistant to enzyme treatments. *B. subtilis* produce various antimicrobial compounds which are usually protein or peptide-based compounds such as enzymes, bacteriocins and lipopeptides (Yoshida et al. 2001; Baruzzi et al. 2011). Given the diversity of bacteriocins produced in nature, they can be considered as an alternative to combat infections against specific pathogens (Neu 1992; Riley and Wertz 2002). Therefore, research for new products with antimicrobial activity is a very important field. The identification and chemical characterization of antagonistic compound produced by *Bacillus* spp., and

exploration of their potential use in the control of pathogens addresses this subject (Motto et al. 2007).

The bioactive compound obtained from *B. subtilis* Ba37 was isolated, purified and identified. The molecular formula of the bioactive compound was assigned to be $C_{25}H_{42}O_3$, based on their IR, MS, ID and 2D NMR spectral data analysis. In the IR spectra the split peaks around 2900 cm^{-1} confirms the presence of CH_3 groups. The peak around 3449 cm^{-1} is due to O-H stretching. The strong C=O stretching frequency appeared around 1728 cm^{-1} confirmed the fact that the ketonic group is attached to a six membered ring system. For five membered or lower ring system, due to higher ring strain the C=O stretching frequency will be 1745 cm^{-1} or higher (Clayden et al. 2012). The molecular ion peak (M+H peak) at m/z =391.45($C_{25}H_{42}O_3$+H) and fragments peaks at m/z = 279.33, 181.18, and 167.16 are due to fragments $C_{18}H_{31}O_3$, $C_{12}H_{21}O$ and $C_{11}H_{19}O$**.** The peak obtained at m/z =803.68 is due to 2M+Na. The M+Na M+K peaks at 413 and 429 also confirmed the molecular weight of the compound as 390.610**.** A quartet with a J value of 6.4Hz appeared at a down field shift of δ 3.96 ppm of two hydrogen atoms indicated the presence of two CH groups (C-15 & C-17) attached to an electronegative centre (oxygen atom) and to a CH_3 group. Similarly another quartet with a J value of 6.4Hz appeared at a down field shift of δ 3.80 ppm of one hydrogen atom indicated the presence of a CH group (C-24) attached to an electronegative centre (oxygen atom) and to a CH_3 group. The appearance of quartets further confirms the fact that both these CH groups are further attached to quaternary C atoms (C-1, C-12 & C-13) without any further H atoms to split the quartets formed. Consequently the CH_3 groups attached to CH groups were appeared as doublets around δ 1.2 ppm (C-18 & C-19) and δ 1.01 ppm(C-27).The presence of CH groups attached to electronegative centers were further

confirmed by the presence of ^{13}C signals appeared around δ 71.31, 71.30 and 81.33 ppm. The ^{13}C signals appeared around δ 211.36 ppm confirms the presence of a C=O group attached to the ring system (C-5). HSQC correlations observed between H-15 &17(δ 3.9 ppm) and C-15 &17 (δ 71.2 ppm) further confirms the proposed structure. Similarly HSQC correlation was also prominent between H-24 and C-24. The doublets formed at δ 1.01 and 1.2 with a J value of 6.4 Hz also support the present proposed structure that these CH_3 groups are only associated with a neighboring CH protons. H-24 (3.61 ppm) showed HSQC correlation with C-24 (81.33 ppm) indicating that they are directly connected and moreover a down field appearance of the proton and carbon signals indicate the presence of an electronegative group (OH) attached directly with C-24. Proton at H-4 (3.6 ppm) position exhibited HSQC correlation with C-4 (51.59). Similarly correlation between H-6 and C-6 were confirmed by their HSQC correlation signals appeared at 3.58 ppm and 51.59 ppm. Proton at H-2 exhibited HMBC correlation with C-23 and C-10. H-27 exhibited HMBC correlations with C-25 and C-6, whereas H-6 showed a correlation with C-24 (Fig 4.14). The ^{13}C NMR spectrum of the purified compound in combination with DEPT experiments indicated the occurrence of 25 carbon atoms in the molecule. The relative positions of the different carbon atoms and corresponding H atoms were further assigned from the ^{1}H–^{1}H COSY, HSQC, HMBC, and NOESY spectra to confirm the established skeleton (Fig 4.15).

The present study demonstrates that the *B. subtilis* MTDCMFRI Ba37 possess unique properties such as extensive antibacterial property towards wide spectrum of aquaculture bacterial pathogens, tolerance to a wide range of physico-chemical conditions of aquaculture systems, suppress the *Vibrio* population on co-culture assay through an antagonistic mode of action. The

strain also proved to be safe when tested in fish cell lines and to fish as such. These results prove the suitability of the strain as an ideal *Vibrio* antagonistic strain to use in aquatic rearing systems. Further we identified a new compound with antibacterial property from the strain against aquaculture pathogens, but further specific and detailed study of the novel compounds is required to develop novel antimicrobial therapeutics ideal for aquaculture in near future.

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Chapter 5

CHARACTERIZATION OF THE *PSEUDOMONAS AERUGINOSA* MBTDCMFRI Ps04 AND ITS BIOACTIVE COMPOUNDS

Contents

5.1 Abstract

Significant antibacterial activity against aquaculture pathogens was exhibited by the marine bacterium, *Pseudomonas aeruginosa* MBTDCMFRI Ps04 (*P. aeruginosa* Ps04) isolated from Marine Science Jetty of tropical estuarine habitats of Cochin, Kerala, India. It showed a wide range of environmental tolerance including temperature (20 - 100ºC), salinity (0 - 55 ppt) and pH (4 - 10). Co-culture assay revealed antagonistic properties of *P. aeruginosa* Ps04 and showed inhibitory action on the aquatic bacterial pathogen *Vibrio harveyi* 101. Besides, Ps04 was found to be harmless to the juvenile stage of *E. suratensis* when challenged at 10^8 CFU/mL and by intramuscular injection into ~5 g sub-adults shrimp at a concentration range from 10^6 - 10^8 CFU/mL. The antagonistic activity peaked after 3rd day culture, grown in modified synthetic medium with glycerol (1%) and alanine (1%) as carbon and nitrogen source respectively, at 30ºC, pH 7.0 and at 15 ppt saline conditions. The preliminary studies also revealed that the antagonistic property is stable even at 40% v/v dilution, up to 120ºC and pH 6 - 9. Bioassay guided

purification followed by the spectroscopic characterization of active fraction revealed that the compound 4-Hydroxy-11-methylpentacyclo [11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9),17-penten-14-one, is responsible for its antibacterial activity. The results of this study indicated that the candidate species could be employed as a potential probiont in aquaculture systems and also to act as a rich source of novel bioactive secondary metabolites with pharmaceutical applications.

5.2 Introduction

Aquaculture is considered as the viable option in the production of valuable protein for the growing population across the world. The aquafarming sector is also is one among the fastest growing production sectors in the world and 90% of the global production to this sector is contributed by Asian countries (Sahu et al. 2008; Aly et al. 2008). However, aquaculture industry is very adversely affected by the pathogenic bacteria and related disease and mortalities which consecutively affect the profitability (Aly et al. 2008; Vinoj et. al 2013). Among the pathogens, *Vibrio* is one of the most important pathogen causing a high mortality in aquaculture (Austin and Zhang 2006). Pathogenic outbreaks could be controlled to an extent by maintaining the water quality, with the aid of some safe chemicals and antibiotics. Use of antibiotics in aquaculture, is a reason for more serious issue of antibiotic residues and to human health, and can even lead to the development of antimicrobial resistance (Balcazar et al. 2006; Motto et al. 2007; Hill 2009). In this regard, there is an imperative need to find alternatives to antibiotics, where a bacterium with antagonistic properties is a choice. Use of probiotics appear to be an eco friendly alternative and is gaining importance in controlling potential pathogens (Sahu et al. 2008; Defer et al. 2009). Bacteria producing inhibitory substances are thought to play a major role as a barrier against the proliferation of pathogens and in some cases by enhancing the host

resistance by synthesizing wide range of bioactive metabolites with varied properties (Cao et al. 2011; Sabate et al. 2013).

In the exploration of novel antagonistic metabolites, it is wise to investigate untapped coastal and marine environment which is a rich source of extremely potent compounds including those with interesting antagonist properties (Schulz et al. 2002). This is because the microbes in the coastal and marine ecosystem is exposed and adapted to stressful habitat and produces a range of structurally complex natural products (Zhang et al. 2005; Debbab et al. 2010). Among these, marine microorganisms have emerged as a good source for the isolation of natural products and also as a successful source of drugs (Harvey 2000). Marine bacteria produce compounds like antibiotics, variety of enzymes, organic acids, bacteriocins, hydrogen peroxide etc. which can function as probiotic in nature (Verschuere et al. 2000). Production of bacteriocins is considered as the main reason for the pathogenic activity of bacteria. It may serve as an anti-competitor compound enabling an invasion of a strain or species in an established microbial community (Lenski and Riley 2002) or act as communication molecules in bacterial consortia like biofilms (Gillor 2008). Antagonistic bacteria used in aquaculture commonly belong to the division of Gammaproteobacteria (*Vibrio* spp., *Pseudomonas* spp., etc.) and Firmicutes (*Lactobacillus* spp., *Bacillus* spp., etc.) (Hill 2009; Nair et al. 2014).

Pseudomonas spp. is the common inhabitants of soil, aquatic environment and is commonly associated with gills, skin and intestinal tract of live fish (Cahill 1990; Raaijmakers et al 1997; Otta et al. 1999). Interestingly, *Pseudomonas* spp. are the most extensively studied group of biocontrol bacteria because they have many traits that make them effective biocontrol agents of plant and animal pathogens (Vijayan et al 2006; Bakker et al. 2007; Weller 2007; Hofte and Altier 2010). Among the various species of *Pseudomonas, P. aeruginosa* have been used widely in agriculture as microbial control agents,

due to their high abundance, growth rate and low environmental sensitivity (Raaijmakers et al. 1997; Weller 2007). Moreover they are reported to produce a wide range of antagonistic secondary metabolites. Meanwhile, Torrento and Torres (1996) have reported *P. aeruginosa*, which showed inhibitory activity against *Vibrio harveyi*, the causative agent of luminescent vibriosis in *Penaeus monodon*. Later, it was reported that *Pseudomonas* I-2 antagonized shrimp pathogenic *Vibrio harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. vulnificus* and *Photobacterium damselae* (Chythanya et al. 2002). Preetha et al. 2007 isolated a phenazine antibiotic compound from *Pseudomonas* strains 102 and 103 with potent antimicrobial activity. Studies by Vijayan et al. 2006 indicated that the organism *Pseudomonas* sp. PS 102 could be employed as a potential microbial product in shellfish and finfish aquaculture systems for management and control of bacterial infections. Our previous study had isolated a marine bacterium, *P. aeruginosa* Ps04 which inhibited the growth of *Vibrio* spp. under *in vitro* conditions through inhibitory compounds. It can be selected as potential antagonistic bacteria because of the broad spectrum of antimicrobial activity against aquaculture pathogens and other beneficial potentials (Nair et al. 2012).

The antagonistic mechanism of these marine organisms derived secondary metabolities can be better understood by their activity guided by purification and structural characterization. An elaborate study of the nature of the antagonistic compounds produced by *P. aeruginosa* Ps04 is required to understand about their detailed mode of action**.** Following these leads, in this research work, a study on the characterization and evaluation of the active metabolites of *P. aeruginosa* Ps04 to act as candidate antagonistic bacteria has been taken up. The study also envisage to identify the novel, biologically active organic molecules which in turn will help to find a novel aquaculture-grade chemical which could be used as an alternative for antibiotics in the control of bacterial disease in aquaculture

5.3 Materials and Methods

5.3.1 Bacterial Strains

Bacterial strains isolated from the tropical estuarine habitats of Cochin possessing antibacterial activity against aquaculture pathogens were maintained in Microbial Culture Collection of Marine Biotechnology Division (MCC-MBTD), Central Marine Fisheries Research Institute (CMFRI). Based on the consistency and broad spectrum of antibacterial activity, *P. aeruginosa* Ps04 isolated from subsurface water sample collected from Marine science jetty, Foreshore Road, Kochi was selected for further characterization and a reference strain *P.aeruginosa* MTCC1688 was also used for comparison studies.

The test organisms used for antagonistic assay were *Vibrio harveyi* 101, *V. anguillarum* O1, *V. alginolyticus* 101 (*courtesy* Central Institute of Brackishwater Aquaculture, Chennai), *Aeromonas hydrophila* (*courtesy* National Centre for Aquatic Animal Health, Cochin University of Science and Technology) *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (*courtesy* Microbial Type Culture Collection, Chandigarh, India). All bacterial isolates were preserved at -80ºC as glycerol stocks and were inoculated into freshly prepared broth before each assay to attain uniform growth.

5.3.2 Characterization of *P.aeruginosa* MBTDCMFRI Ps04

The colony was taken from glycerol stocks and purified, and further characterized based on its morphological, biochemical, physiological, enzymatic activity and antibiotic susceptibility tests by comparing with reference strains. *P. aeruginosa* Ps04 and the reference strain (*P. aeruginosa* MTCC 1688) were grown on Nutrient Agar with 50% SW (Himedia, India) kept at 37ºC for 48 h were used for characterization studies. Phenotypic characterisation of *P. aeruginosa* was carried out using the methods described by Krieg and Holt 1984. The biochemical tests, antibiotic susceptibility assay,

exoenzyme activity and growth at different physiological conditions of the isolates were carried out as described by section 4.3.2.

5.3.3 Antibacterial Assay

The initial screening of antagonism of *P. aeruginosa* Ps04 was carried out by spot diffusion assay against the test pathogens as described by Nair et al. (2012). In brief, isolated bacteria were spotted over pre-swabbed plates with aquaculture pathogens viz., *V. vulnificus* MTCC1145, *V. harveyi* 101, *V. anguillarum* O1, *V. parahaemolyticus* MTCC451 and *V. alginolyticus* 101. After incubation, the bacteria having notable antagonistic potential (zone of clearance of 10 mm or greater observed around isolates) were selected, identified and preserved for further use.

Antagonism assay of cell-free supernatant was done by well diffusion method and disc diffusion method. The isolate was inoculated in nutrient broth supplemented with 50% seawater and incubated at 30ºC for 72 h on a shaker (100 rpm). Antagonism assay by well diffusion and disc diffusion method were done as described by section 4.3.3.

5.3.4 Evaluation as candidate antagonistic bacteria

5.3.4.1 Growth Curve

5.3.4.1.1 Preparation of Culture

P. aeruginosa Ps04 and *V. harveyi* 101 were taken from glycerol stock at -80ºC and were streaked into nutrient agar plates supplemented with 50% SW and incubated at 30ºC for 24 h. 5 mL of nutrient broth (50% SW) was inoculated with a single colony from the plates and incubated at 30ºC on a rotary shaker overnight (New Brunswick Scientific Co. Inc., NJ, USA) at 200 rpm for 20 - 24 h. 1% of overnight grown culture was inoculated to 100 mL

nutrient broth (50% SW). The flasks were incubated on a shaker for 48 h as mentioned above.

5.3.4.1.2 Determination of Generation time and Growth Curve

Growth and generation time of *P. aeruginosa* Ps04 and *V. harveyi* 101 were determined as described by section 4.3.4.1.2

5.3.4.2 Co-culture Experiment

V. harveyi was selected for coculture experiment as it has been known to be a key shell fish pathogen mainly affecting *Penaeus* spp. (Janakiram et al. 2014). The *in vitro* inhibitory properties of *P. aeruginosa* Ps04 against *V. harveyi* 101 was carried out using co-culture assay. Cultures were pre-cultured separately in nutrient broth supplemented with 50% SW and incubated at 28 ± 2ºC on a shaker at 120 rpm overnight. The above cultures were inoculated in 100 mL nutrient broth (with 50% SW) to obtain an initial cell count of approximately 10^{-2}, 10^{-3}, 10^{-4} CFU/mL for *V. harveyi* 101, whereas the initial levels of *P. aeruginosa* Ps04 were 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} CFU/mL respectively. The cultures were incubated at 28 ± 2ºC on a shaker (120 rpm), and all samples (1mL) were withdrawn at 24 h intervals for 4 days to determine the cell count. Counts of the pathogen were monitored by withdrawing daily 1 mL samples that were serially diluted 10-fold and 100 µl aliquots of each were spread on nutrient agar with 50% SW and 1 mL of diluted samples were mixed with 4 mL of Hugh leifson medium. The plates and tubes were incubated at 28 ± 2ºC for 24 h and the colonies obtained on plates and tubes were counted and expressed as log10 CFU/mL. *V. harveyi* 101 grow well on Hugh leifson medium and produce fermentation of medium while *P. aeruginosa* Ps04 produce greenish blue pigmentation on nutrient agar with 50% SW which would be easy to differentiate the isolates on these specific agars.

5.3.4.3 Cytotoxicity Assay

Cell free supernatant were obtained by centrifugation of 10 mL of 18 h culture broth of *P. aeruginosa* Ps04 and *V. harveyi* 101 and filtered through a 0.2 µm syringe filter. Cells were infected with 100 µl of each cultured cell-free supernatants obtained by filtration through a 0.2 µm syringe filter (Palls life science, USA) as a control and *V. harveyi* 101 as a pathogenic control. The cytotoxicity assay was carried out as described by section 4.3.4.3

5.3.4.4 Pathogenicity Test/ Safety of the Isolated Antagonistic Bacteria

5.3.4.4.1 On juveniles of *Etroplus suratensis*

Pathogenicity of *P. aeruginosa* Ps04 was tested on the juveniles/ larvae of *E. suratensis* collected from a commercial hatchery. They were maintained for 7 days in 10 ppt seawater and fed with commercial feed. The experiment was done as described by section 4.3.4.4.1. The experiment was carried out in triplicate. The cell count was determined by plating serial 10-fold dilution in Nutrient agar with 50% SW by spread plate method.

5.3.4.4.2 On sub adult *E. suratensis*

Sixty sub-adults of *E. suratensis* (average body weight of 5 ± 0.5 g) were acclimatized in 10 ppt seawater for 7 days in the laboratory and fed with commercial feed. Then they were distributed into groups of 5 animals each in 30 L capacity fiberglass tanks containing 20 L of 10 ppt seawater and maintained at 28 ± 20ºC for 5 days. The experiment was carried out in triplicate. The experiment was carried out as explained by section 4.3.4.4.2. Negative control groups were injected with 0.4 mL sterile saline and positive control groups were injected with 0.4 mL of *V. harveyi* 101.

5.3.5 Nutrient Profiling

The proximate chemical composition analyses of *P. aeruginosa* Ps04 was determined using the standard procedures of AOAC (1990). Briefly, moisture was determined by oven drying at 105ºC to constant weight. Dried samples were used for determination of crude fat, crude protein, soluble carbohydrate, crude fibre, acid insoluble ash and crude ash contents as explained in section 4.3.5. All analyses were done in triplicates.

5.3.6 Optimization of Production Medium

P. aeruginosa Ps04 was taken from glycerol stock at ¯80ºC and streaked on nutrient agar (15 ppt) and a single colony was inoculated into 5 mL of nutrient broth supplemented with 50% SW and incubated at 30ºC on a rotary shaker overnight (New Brunswick Scientific Co. Inc., NJ, USA) at 200 rpm for 20 - 24 h. 1% of overnight grown culture (starter culture) was inoculated to 100 mL of various medium (with 50% SW) like nutrient broth, Zobell marine broth, Tryptone soya broth and Glycerol alanine medium (Saha et al. 2008) (g/L) containing 10.0g DL - Alanine; 20mL glycerol; 0.139 g K_2HPO_4; 4.06 g $MgCl_2.6H_2O$; 14.2 g Na_2SO_4; 0.01 g Ferric citrate at pH 7 ± 0.2. All the flasks were incubated at 30°C for a period of 7 days in duplicates. Samples for antagonistic activity were drawn after every 24 h interval and 1 mL of the culture was processed for evaluating the antagonistic potential against aquaculture pathogens by the disc diffusion method as explained earlier. The cell counts were determined by serial dilution.

5.3.6.1 Optimization of Carbon and Nitrogen sources and Physiochemical Conditions

The optimal conditions for growth and production of antagonistic compound by *P. aeruginosa* Ps04 was assessed by growing the culture in

modified synthetic media at various temperature (25, 30, 35 and 40ºC), pH (6.0, 7.0, 8.0 and 9.0), salinity (5, 10, 15, 20, 25, 30 and 35 ppt), carbon sources (glycerol and glucose at varying concentrations from 0.5 - 1%) and nitrogen sources (alanine and glutamic acid at varying concentrations from 0.5 - 1%) for a period of 7 days in duplicates. Samples for cell count and antagonistic activity were drawn every 24 h. One milliliter of the culture was processed for evaluating the antagonistic potential against pathogens by the disc diffusion method as explained earlier.

5.3.7 Preliminary Characterization of Antagonistic Compound

Sensitivity of antagonistic compound to enzymes, pH and heat treatment was carried out using cell free culture supernatant of *P. aeruginosa* Ps04. The preliminary characterization of antagonistic metabolites was carried out as described by section 4.3.7.

5.3.8 Isolation of Bioactive Compounds

5.3.8.1 Preparation and Purification of Bioactive Compounds from *P. aeruginosa* Ps04

Ten litres of 3 days old *P. aeruginosa* Ps04 culture in glycerol-alanine medium incubated at 30ºC in 150 rpm was centrifuged at 8000 rpm for 15 min at 4ºC and the supernatant was filter sterilized (0.2 µm) and lyophilized to obtained 10 g of supernatant powder which was partitioned between EtOAc/MeOH (1:1, v/v) and reflux for 2.5 h and filtered through anhydrous sodium sulphate (Na_2SO_4). The filtrate thus obtained was evaporated (40ºC) using rotary evaporator under vacuum to dryness at a temperature below 42 ± 2ºC to obtain a dark pinkish extract (4.5 g). The crude fraction obtained from EtOAc/MeOH extract was purified using various chromatographic techniques based on their antagonistic potential. Each time the different fractions obtained were evaluated

using TLC and similar fractions were pooled accordingly. In all cases the antagonistic potential of the fractions were evaluated and further purifications were carried out based on the zone of inhibition. All reagents and chemical solvents used for products isolation were of analytical grade or higher.

5.3.8.2 Identification of Bio active Compounds

The purified fraction ($PA_{3.3.6}$) was subjected to FT-IR (Fourier Transform Infra-Red) spectrometric analysis to trace the spectra of the compounds under KBr pellets and was recorded in a Thermo Nicolet, Avatar 370. The scanning was conducted in to mid IR range, i.e., between 4000 - 400cm^{-1}. The LC-MS experiments were carried out on Quattro Premier XE (Micromass), an electrospray ionization (ESI) - triple quadrupole mass spectrometer, which is attached to Acquity UPLC (Waters). For each run, 5 µL of samples were injected onto a C_{18} column (Acquity UPLC BEH, 1.7 µm, 2.1 mm × 50 mm; Waters). A linear gradient elution was done utilizing water (H_2O; solvent A) and methanol (MeOH; solvent B) (0 - 20 min: 50% - 90% B; 20 - 23 min: 90% B; 23 - 25 min: 90% - 50% B) and the flow rate was maintained at 0.2 mL min^{-1}. Eluents from the chromatographic column pass through photo diode array (PDA) detector and then enter into the mass spectrometer. Ultraviolet (UV) absorbance data in the range 210 - 400 nm were recorded by PDA detector. Mass spectrometric data were acquired in positive ion polarity of ESI and the *m/z* range of detection was set to 50 - 1500. The ESI source parameters were: capillary 3 kV; Cone 30 V; Extractor 5 V; Source Temperature 100ºC; Desolvation Temperature 300ºC; Desolvation Gas Flow 800 Litre/hour (L/h); Cone Gas Flow 50 L/h. All data acquisitions and processing were facilitated by MassLynx (Waters). Followed by nuclear magnetic resonance (^1H and ^{13}CNMR) spectra were recorded on a Bruker AVANCE III 500 MHz (AV 500) DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) in $CDCl_3$ as aprotic solvent at ambient temperature with tetramethylsilane (TMS) as the internal standard (δ 0 ppm) equipped with 5 mm probes. The number of attached protons for the ^{13}C NMR signals was determined from DEPT

experiments. Standard pulse sequences were used for DEPT, ^{1}H-^{1}H COSY, two-dimensional NOESY, HSQC, and HMBC experiments. All solvents were of analytical, spectroscopic or chromatographic reagent grade, and were obtained from E-Merck (Darmstadt, Germany).

5.4 Results

5.4.1 Characterization

Morphological, biochemical and physiological characteristics of *P. aeruginosa* Ps04 were found to be similar to the reference strain *P. aeruginosa* MTCC 1688 (Table 5.1). *P. aeruginosa* Ps04 was Gram negative, small rod shaped (size $\simeq$ 0.5 - 0.8 µm X 1.5 - 3.0 µm), motile and facultative anaerobic. Colonies were smooth, slightly undulate, regular, raised, opaque, medium and blue green in colour after 2-3 days growth at 30ºC on nutrient agar 50% SW. It didn't give positive result for KOH test. Oxidase and catalase were produced, Voges - Proskauer test is positive, but H_2S, nitrate reduction and indole is negative, citrate utilization positive, K/A for TSI utilization, Late α - hemolytic activity was present. Negative for gas production from D-glucose, lysine decarboxylase and ornithine decarboxylase are not hydrolysed. Positive for arginine dihydrolase. Sugars were not utilized and there is no acid production.

Table 5.1 Characterization of *P. aeruginosa* Ps04 and reference strain *P. aeruginosa* MTCC 1688

Characteristics	Reaction	
	P.aeruginosa MBTDCMFRI Ps04	*P.aeruginosa* MTCC 1688
Morphology		
Colony colour on nutrient agar (50% SW)	Greenish blue colour	Greenish yellow
Size	Medium	Medium
Form	Irregular	Irregular
Margin	Undulate	Undulate
Elevation	Umbonate	Umbonate
Cell Shape	Small rod	Small rod
Gram stain	G-	G-
3% KOH	+	+
Endospore formation	-	-
Pigmentation	Greenish blue	Greenish yellow

Physiological properties		
Aerobic growth	+	+
Growth at temperature	20-40°C	20-40°C
Growth at pH	4-10	4-10
Growth in NaCl (%)	0-50	0.50
Biochemical Characteristics		
Catalase reaction	+	+
Oxidase reaction	+	+
Nitrate reduction	-	-
Indole production	-	-
Methyl red test	-	-
Voges -Proskauer test	-	-
H_2S production	-	-
Citrate utilization	+	+
Arginine dihydrolase	+	+
Lysine decarboxylase	-	-
Ornithine decarboxylase	-	-
Hydrolysis of		
Tween 20	16	19
Starch	-	-
Cellulose	-	-
Skim milk	9	12
Gelatin	42	41
Egg yolk emulsion	24	25
Urease Activity	+	+
Haemolytic activity	Late α	Late α
Phosphatase activity	+	+
Acid production		
Arabinose	-	-
Galactose	-	-
Glucose	-	-
Inositol	-	-
Mannitol	-	-
Mannose	-	-
Melibiose	-	-
Raffinose	-	-
Salicin	-	-
Trehalose	-	-
Xylose	-	-
Antibiotics susceptibility pattern		
Ampicilin (10mcg)	0	0
Bacitracin (10U)	8	0

Cefoperazone(75mcg)	27	19
Cefoxitin (30mcg)	0	0
Cefuroxime(30mcg)	0	0
Cephalothin (30mcg)	0	0
Chloramphenicol(30mcg)	12	11
Ciprofloxacin (5mcg)	32	30
Colistin (10mcg)	14	11
Erythromycin (15mcg)	26	0
Gentamicin (100mcg)	23	16
Kanamycin (5mcg)	0	0
Levofloxacin (5mcg)	30	25
Lomefloxacin (10mcg)	23	18
Nitrofuran (300mcg)	0	0
Norfloxacin (10mcg)	30	26
Pencillin (10U)	0	0
Streptomycin (10mcg)	16	8
Tetracycline (30mcg)	11	10

P. aeruginosa Ps04 can grow in varied physiological conditions. Isolates grow at temperature range of 20 - 40^{o}C, salinity of 0 - 50 ppt and pH of 4 - 10. Isolates were resistant to seven antibiotics tested and resistant to rest. *P.aeruginosa* Ps04 were possessing exoenzymatic activities such as gelatinase, lipase, protease, phosphatase, urease and lecithinase. It does not possess cellullase and amylase activity and detailed results are given in Table 5.1.

5.4.2 Inhibitory Spectrum

The culture and cell-free supernatants of the *P. aeruginosa* Ps04 shows inhibition towards the pathogens tested. The antibacterial activity of culture and filtered supernatant of *P. aeruginosa* Ps04 exhibited greater inhibitory activity against *V. vulnificus* MTCC 1145, *V. harveyi* 101 and least against *A .hydrophilla.* The zone of inhibition against the tested pathogen by spot diffusion, well diffusion and disk diffusion method were around 9 - 20 mm were given in Table 5. 2. Inhibition zones (mm) exhibited by commercial antibiotics against the test pathogens are shown in Table 4.3. Thus, upon comparison of the antibacterial activity of *P. aeruginosa* Ps04 and commercialized antibiotics against tested pathogens showed that the inhibitory levels of the isolates is

comparatively higher than commercial antibiotics like ampicillin (10 mcg), penicillin (10 U), bacitracin (10 U), erythromycin (15 mcg), kanamycin (30 mcg) and streptomycin (10 mcg)

Table 5.2 Antagonistic activity of the *P. aeruginosa* Ps04

Assay Method	Zone of Inhibition (mm)					
	A	B	C	D	E	F
Spot diffusion	22	15	21	14	19	22
Well diffusion	21	16	21	14	17	21
Disc diffusion	20	15	19	16	19	22

A - *Vibrio vulnificus* MTCC1145; B - *Vibrio parahaemolyticus* MTCC 451; C- *Aeromonas hydrophila*; D - *Vibrio alginolyticus* 101; E- *Vibrio harveyi* 101 and F- *Vibrio anguillarum* A1. The measurement indicates the inhibition zone (mm) formed around the isolates. The values may differ ±1 mm from the size mentioned

5.4.3 Determination of Generation Time and Growth Curve

Growth curve of the isolates is illustrated in Fig. 5.1. There was a small lag phase, after which *P. aeruginosa* Ps04 and *V. harveyi* 101 entered into a rapid logarithmic phase. The generation time was calculated from the growth data at 3rd h and 0th h. *P. aeruginosa* Ps04 and *V. harveyi* 101 has been shown to have a generation time of about 51.8 & 49.3 min generation^{-1} respectively

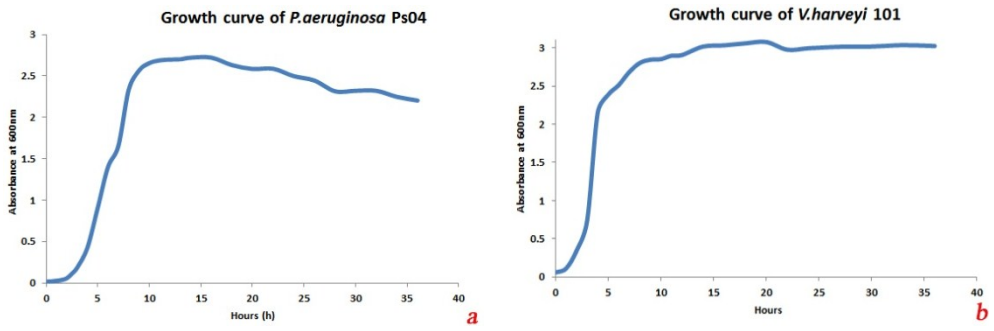

Fig 5.1 Growth curve of *P. aeruginosa* Ps04 and *V. harveyi* 101

5.4.4 Co-culture Assay

The antagonism exhibited by *P. aeruginosa* Ps04 against *V. harveyi* 101 was further confirmed by co-culture assay. The growth of pathogenic *V. harveyi* 101 at different concentrations (10^2 - 10^4 CFU/mL) was inhibited by *P.*

aeruginosa Ps04 (10^4 - 10^8 CFU/mL). *P. aeruginosa* Ps04 inhibited *V. harveyi* 101 in co-culture when the initial cell count of the probiont was even at 10^4 CFU/mL. Lower concentrations of *P. aeruginosa* Ps04 (10^4 CFU/mL) allowed initial growth of *V. harveyi* 101 upon continued incubation but never allowed the cell densities of the pathogen to reach the level of control. High concentrations (10^6 - 10^8 CFU/mL) of *P. aeruginosa* Ps04 allowed an initial increase of *V. harveyi* 101 followed by a decrease in the total viable counts (Fig. 5.2). When the concentration of the probiotic increased, rate of inhibition also increased. After 96 h in co-culture, higher concentration of *P. aeruginosa* Ps04 inhibited complete growth of the *V. harveyi* 101. Growth of the antagonistic bacteria was not affected by pathogen at any stage in the co-cultures. Results of co-culture experiment showed that under in vitro conditions when the concentration of *P. aeruginosa* Ps04 increases, the growth of *V. harveyi* 101 was controlled.

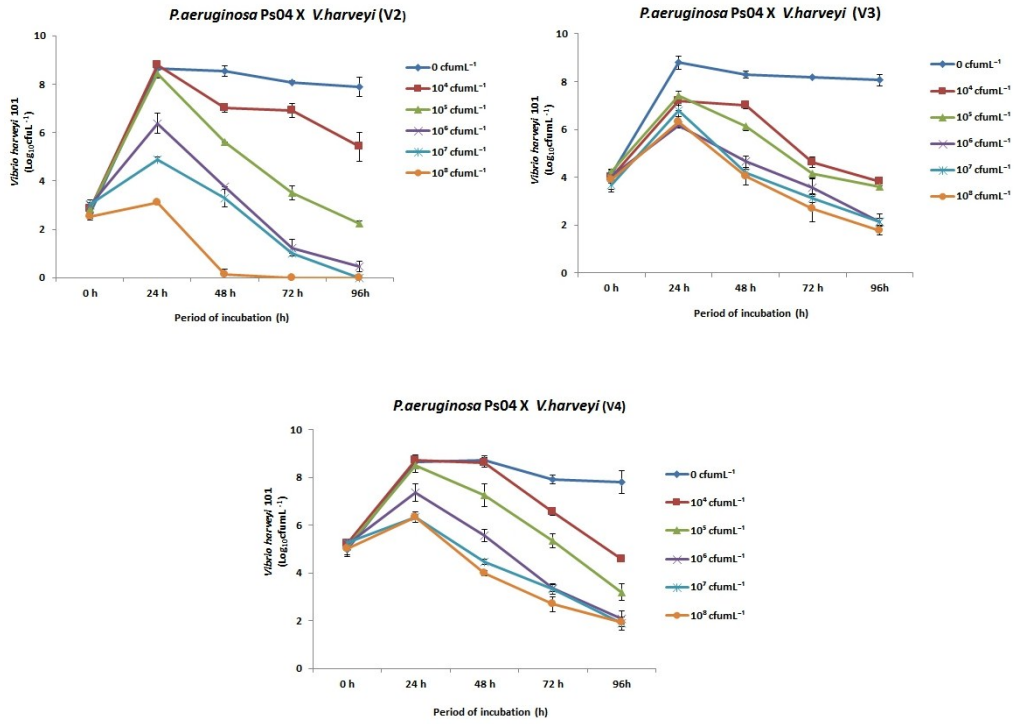

Fig 5.2 Growth of *V. harveyi* 101 in co culture at increasing cell densities of *P. aeruginosa* Ps04

5.4.5 Cytotoxicity Assay

On microscopic observation, there were no morphological changes observed in cells which were treated with filtered supernatant of *P. aeruginosa* Ps04 while the cells treated with supernatant of *V. harveyi* 101 were ruptured completely. So the antagonistic bacterium was found not toxic to the koi carp cell lines but the pathogenic bacteria was highly toxic to the cell lines (Fig 5.3).

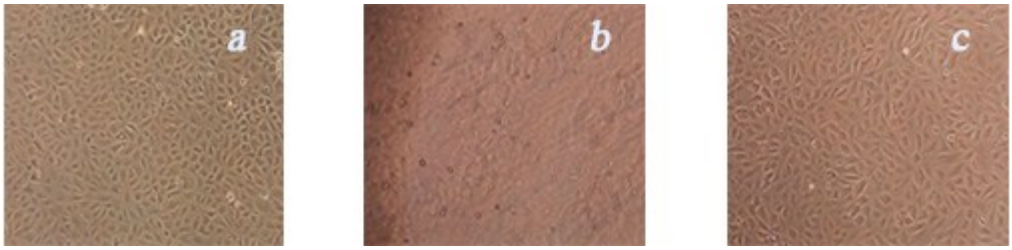

Fig 5.3 Cytotoxicity assay of koi carp cell lines with CFS of *P. aeruginosa* Ps04 and *V. harveyi* 101
a - Control cell line, b - CFS of *V. harveyi* 101 treated cell line, c - CFS of *P. aeruginosa* Ps04 treated cell line

5.4.6 Safety of the Potential Antagonistic Bacteria (*in vivo*)

P. aeruginosa Ps04 was found to be harmless while carrying out the pathogenicity test**.** It did not cause any significant mortality after bath challenge on larvae of *E. suratensis*. The presence of *P. aeruginosa* Ps04 (10^6 CFU/mL) was found in larval rearing water for 4 days and subsequently decreased. It did not show any signs of disease or mortality after intraperitoneal injection to sub adult fishes and were therefore considered safe to be used in the fish. The bacterial analysis by the end of the experiment revealed the isolation of *P. aeruginosa* Ps04 from the corresponding fish groups.

5.4.7 Optimization of Media, Temperature, Salinity and pH for the Production of Antagonistic Compound

Optimization of culture media and its conditions highly influenced the production of antibacterial compound. Among different medium used, glycerol-alanine media was found to be more effective in maximum production of antibacterial principle from *P. aeruginosa* Ps04 followed by nutrient broth with 50% SW (Fig 5.4a). The best carbon and nitrogen source for optimum production was found to be glycerol (1%) and alanine (1%) respectively. *P. aeruginosa* Ps04 can grow from 25 to 40ºC, the optimum temperature for growth and the production of the antagonistic component was recorded at 30ºC, with a maximum inhibitory zone of 12 - 21 mm on the 3rd day against tested pathogens (Fig 5.4b). Enhanced production was achieved at 20 ppt salinity and maximum antagonistic compound was produced after the 3rd day with a clearing zone of 13 - 21 mm. The inhibitory activity of the culture supernatants was less for higher and lower salinity of 35 ppt and 5 ppt respectively (Fig 5.4c). The pH does not influence much with inhibitory compound production. There was no significant variation in the growth of *P. aeruginosa* Ps04 or antagonistic activity with the tested pH range. However, the inhibitory activity was highest at pH 7.0 followed by pH 8.0 with 3 days of culture with a clearing zone of 12 - 20 mm (Fig 5.4d).

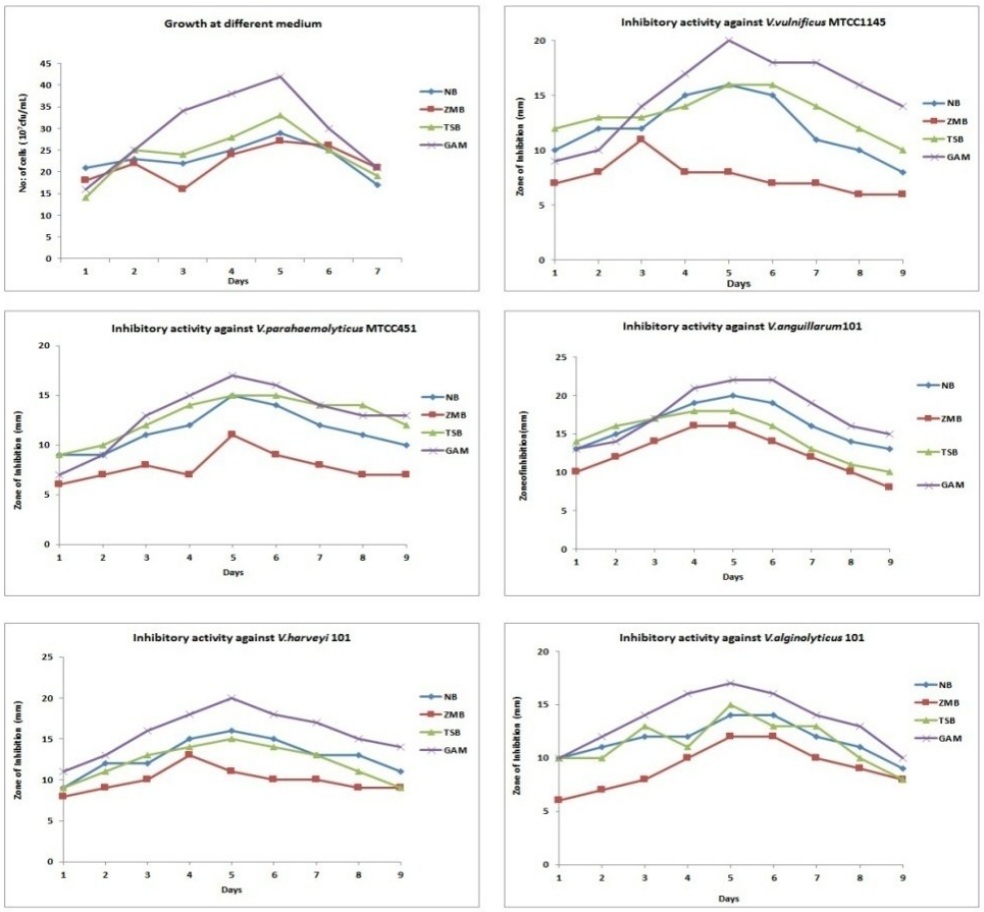

Fig 5.4a Effect of medium for the production of antibacterial metabolite in *B. subtilis* Ba37

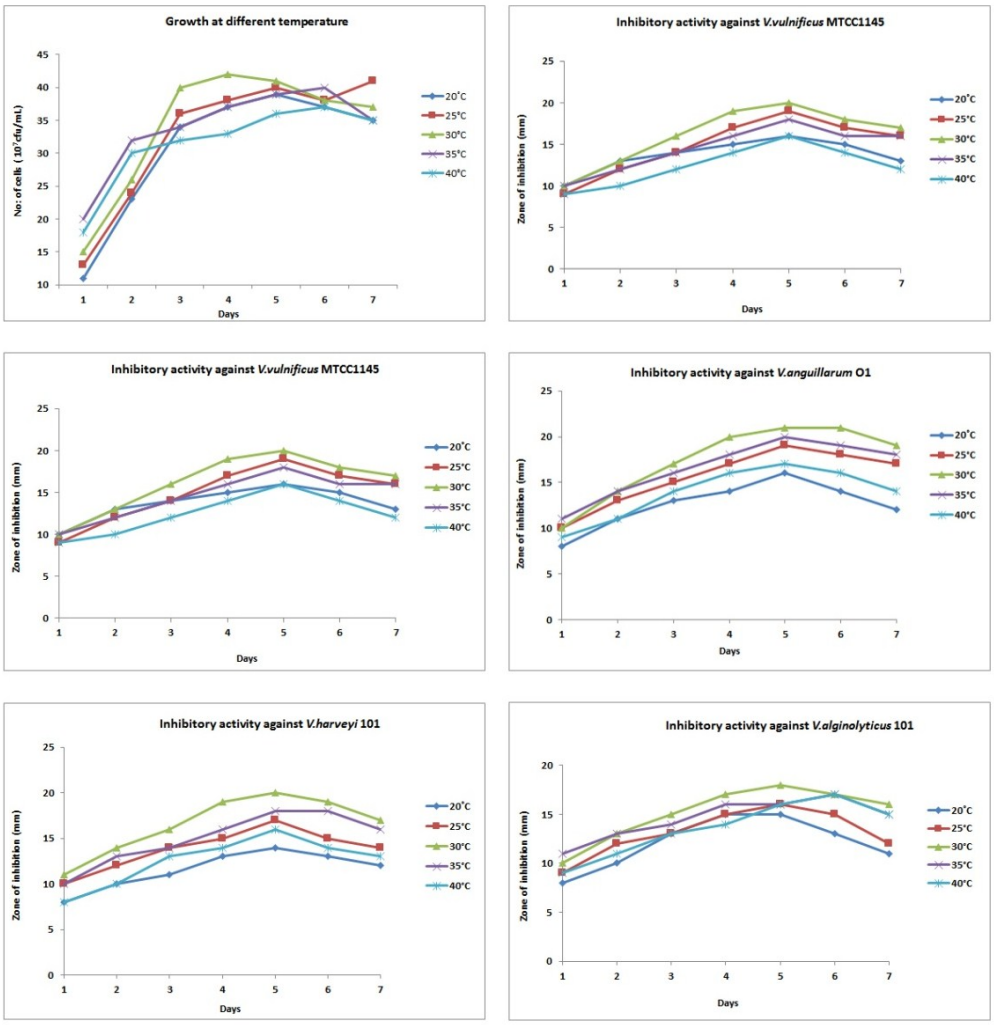

Fig 5.4b Effect of temperature for the production of antibacterial metabolite in *B. subtilis* Ba37

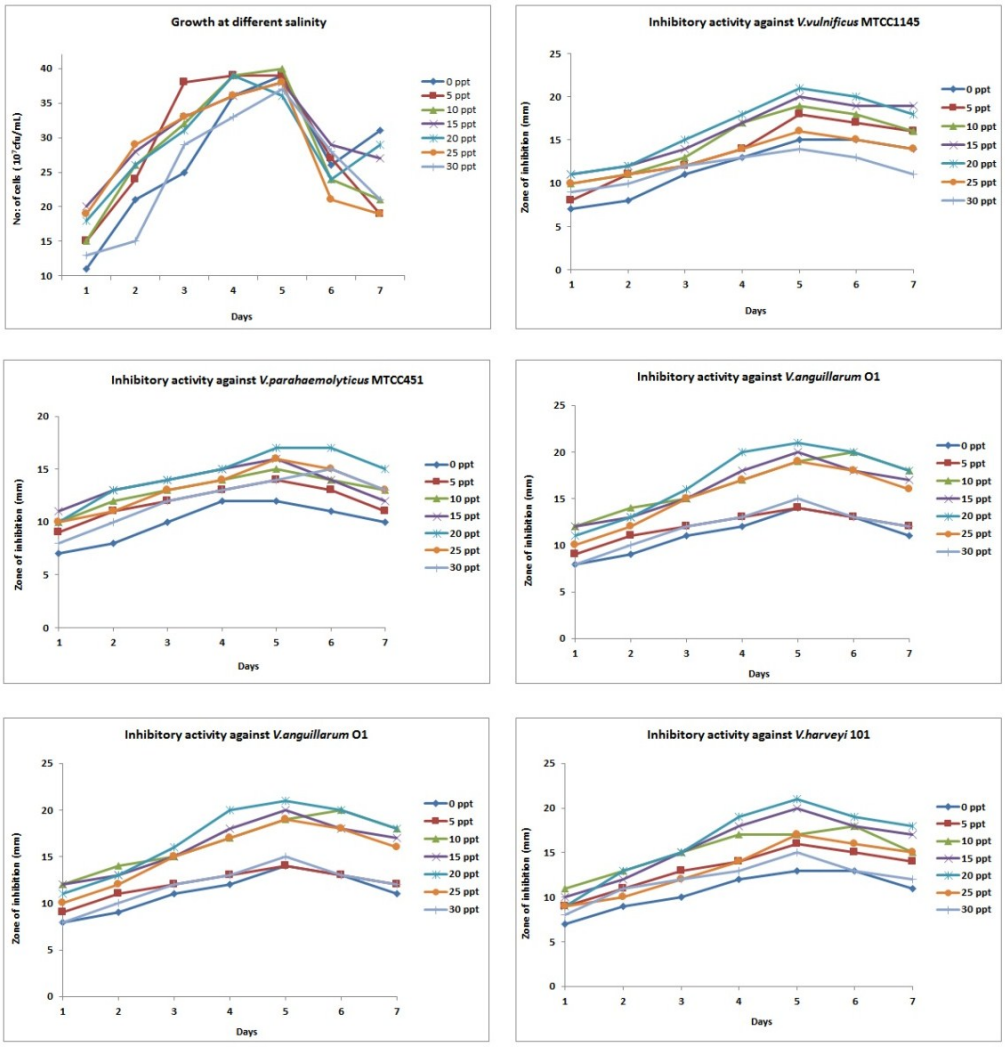

Fig 5.4c Effect of salinity for the production of antibacterial metabolite in *B. subtilis* Ba37

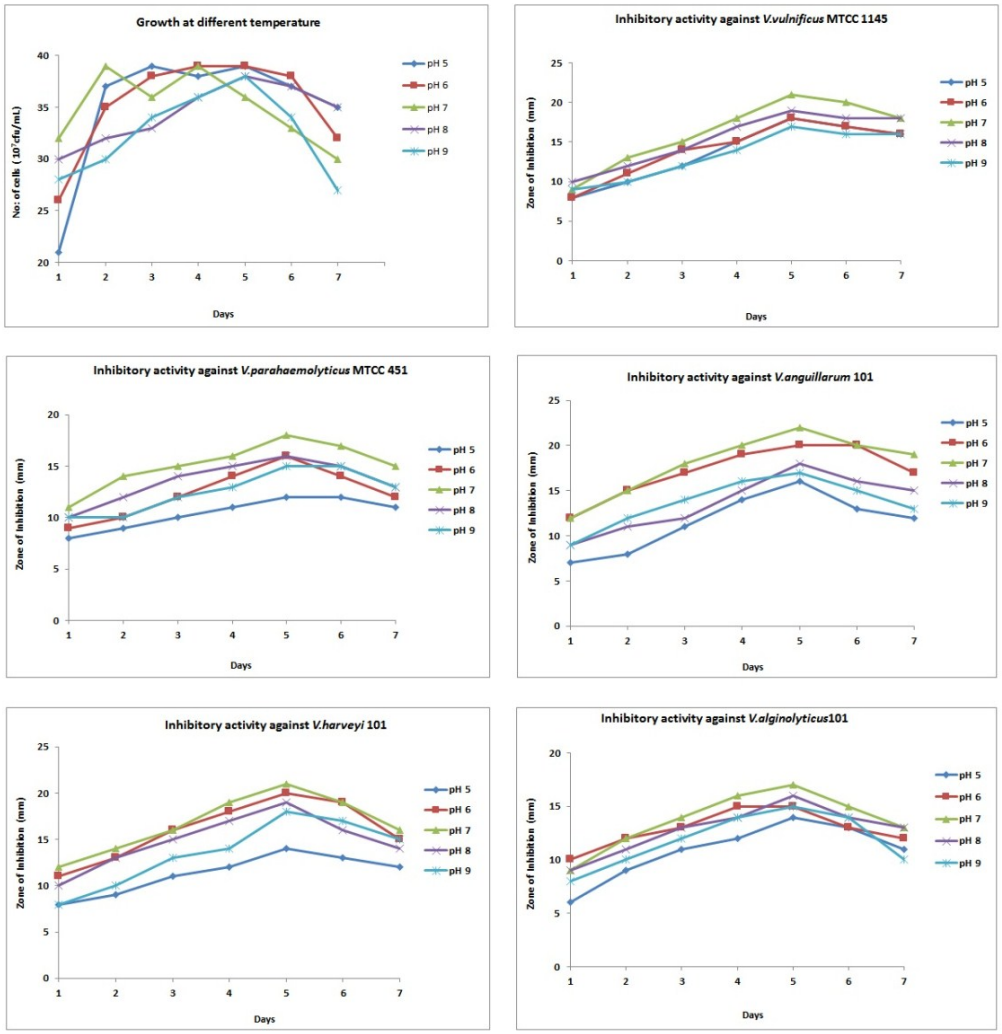

Fig 5.4d Effect of pH for the production of antibacterial metabolite in *P. aeruginosa* Ps04

5.4.8 Nutritional Analysis

The main component of *P. aeruginosa* Ps04 was carbohydrate followed by protein and lipid. Proximate chemical composition of *P. aeruginosa* Ps04 is given in Table 5.3.

Table 5.3 Chemical composition analysis of *P. aeruginosa* Ps04 on dry matter basis

Sample name	% dry matter	% moisture	% crude protein	%crude fat	%crude ash	%crude fibre	% acid insoluble ash	% nitrogen free extract
P. aeruginosa Ps04	100	NIL	51.66	4.44	11.84	0.35	0.19	31.71

5.4.9 Preliminary Characterization of Antagonistic Principle

The inhibitory compound production of *P. aeruginosa* Ps04 was observed in the late phase of culture (about 40 h) in modified synthetic medium. The activity reached maximum in the stationary phase and remained stable for two more days with small flux in activity. The filtered supernatant possessed inhibitory potential against pathogens even after dilution upto 80 times (20%) of the original concentration. The inhibitory zone produced by the diluted supernatant (20%) decreased to 9 - 13 mm, compared to the control (16 - 23 mm). Study of the heat stability of the antagonistic principle showed that it was stable at temperatures up to 120ºC and the activity gradually reduced at increasing temperature. In case of stability at different pH, the antibacterial component was found to be stable at pH 2 - pH 12. However antagonistic compound was stable after treatment of proteinase K, trypsin and lyzozyme at varying concentrations (Table 5.4).

Table 5.4. Preliminary characterization of the cell free supernatant of *P. aeruginosa* Ps04

Dilution	Zone of inhibition (mm)				
	A	B	C	D	E
10%	-	-	7	7	-
20%	-	-	10	9	-
30%	13	-	12	12	9
40%	13	10	13	13	10
50%	14	11	15	16	12
60%	14	13	17	18	13
70%	15	13	18	18	13
80%	18	13	20	21	14
90%	19	14	22	22	15
100%	21	16	23	22	15

pH	Zone of inhibition (mm)				
	A	B	C	D	E
2	20	10	20	18	12
3	21	11	20	18	12
4	21	13	21	20	12
5	20	13	22	21	13
6	21	14	22	21	13
7	22	15	23	22	14
8	23	16	24	22	15
9	21	14	23	21	14
10	20	14	22	22	13
11	21	15	22	21	11
12	20	12	21	20	12
Control	22	15	24	22	14

Temparature	Zone of inhibition (mm)				
	A	B	C	D	E
40°C	21	15	20	19	19
50°C	21	15	19	19	19
60°C	20	15	19	19	18
70°C	20	14	19	18	17
80°C	20	15	19	18	17
90°C	19	14	19	18	17
100°C	19	14	18	17	16
120°C	18	13	17	17	16
Control (RT)	21	15	20	19	19

Enzyme	Zone of inhibition (mm)				
	A	B	C	D	E
Lyzozyme (1mg/ml)	21	18	21	20	17
Lyzozyme (2mg/ml)	20	17	21	19	17
Lyzozyme (5mg/ml)	21	14	20	18	16
Trypsin (1mg/ml)	21	17	22	19	16
Trypsin (2mg/ml)	22	18	21	19	17
Trypsin (5mg/ml)	21	17	22	17	15
Proteinase K(1mg/ml)	18	17	19	18	14
Proteinase K(2mg/ml)	18	16	19	17	13
Proteinase K(5mg/ml)	16	16	18	16	12
Control	22	18	22	20	18

5.4.10 Purification and Structural Characterization of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9), 17-penten-14-one

The EtOAc/MeOH extract was chromatographed over silica column (60 - 120 mesh) with a stepwise gradient of solvents from CH_2Cl_2, CH_2Cl_2: MeOH and finally MeOH (100%, 50%, 30% and 100%). The fractions were collected and concentrated under vacuum and TLC was evaluated and fractions with same TLC profile were pooled together to furnish four fractions (PA_{1-4}). The sub fraction PA_3 and PA_4 which exhibited high antibacterial activity (zone size of 16 - 28 mm against various pathogens) was pooled together and purified using column chromatography with, *n*- hexane, EtOAc: *n*- hexane, CH_2Cl_2:MeOH, MeOH (100:0, 50:50, 75:25) and then with CH_2Cl_2 :MeOH (10:90, 30:70, 0:100) to give another six sub fractions. After concentrating, all fractions were checked for antagonistic activity and TLC evaluation. The sub fraction $PA_{3.3}$ exhibited more antagonistic activity (zone size of 14 - 23 mm) against various pathogens. The sub fraction $PA_{3.3}$ was re-purified using preparative thin layer chromatography over Si gel GF_{254} (particle size 15 μm) (0.5% MeOH:CH_2Cl_2) to obtain six sub fractions of which the sub fraction $PA_{3.3.6}$ exhibited higher antibacterial potential and was found to be pure. This was selected for further analysis. Its R_f value is 0.37. The schematic representation of the purification pattern is given (Fig 5.5). All reagents and chemical solvents used for product isolation were of analytical grade or higher.

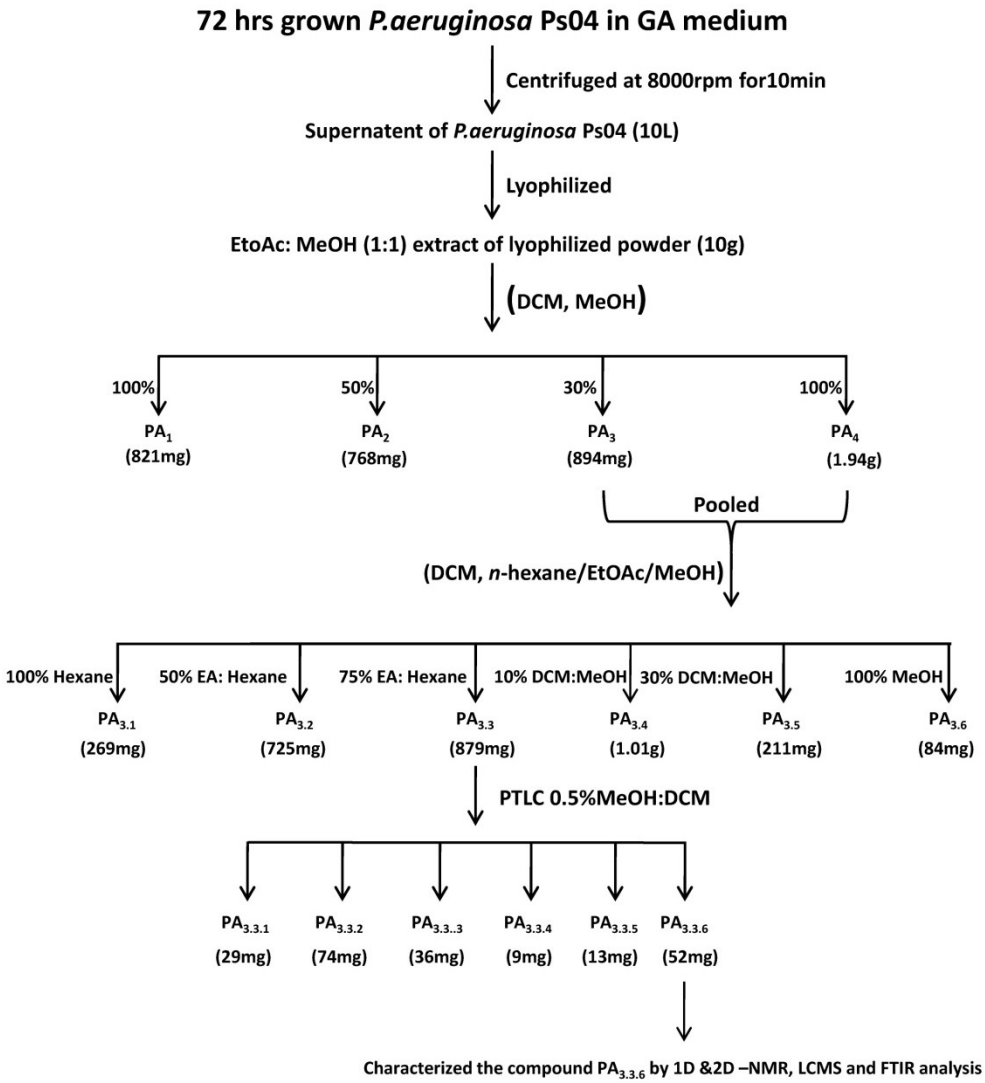

Fig 5.5 Schematic representation of the purification pattern of active compound from *P. aeruginosa* Ps04.

4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}]henicosa-1,3,5,8(9), 17-penten-14-one

Amorphous brownish solid; UV (MeOH) λ_{max} (log ε): 273 nm; TLC (Si gel GF_{254} 15 mm; $CHCl_3$/MeOH 10:90, v/ v) R_f: 0.37; LC (Column: Acquity UPLC BEH C18, 1.7 µm, 2.1 mm × 50 mm (Waters); gradient elution using H_2O

(solvent A) and MeOH (solvent B) for 25 min: 50% B - 90% B in 20 min, hold at 90% B for 3 min and 90% B - 50% B in 2 min; flow-rate: 0.2 mL/min; volume of sample injection: 5 µl), Elemental analysis found: C,82.46; H,7.55;O,9.99; IR (KBr, cm^{-1}). ν_{max} 762.18cm^{-1} γ_r (C-H alkanes), 1510.88 cm^{-1} and 1596.11 cm^{-1} ν(C-C aromatic ring), 1726.24 cm^{-1} ν(C=O), 2928.88 cm^{-1} ν (C-H alkanes), 3396.01 cm^{-1} ν (C-H alkanes). ^{1}H NMR ($CDCl_3$, 500 MHz, δ ppm) and ^{13}C NMR ($CDCl_3$, 125MHz, δ ppm) data (Table 5.5); ESI MS m/z (rel. int. %): 350.58 (M+Na) (100%), 321.76 ([M+H]) (9.28%), 313.89 (11.4%), 280.07 (12%), 137.48 (25.29%), 124.53 (24.29%), 105.25 (18.75%).

The IR spectra of the compound showed major bands at 3396.01cm^{-1}, 2928.88 cm^{-1}, 1726.24 cm^{-1}, 1510.88 cm^{-1}, 1726cm^{-1} and 1046.43 cm^{-1} (Fig 5. 6). The LC MS analysis of the compound exhibited a base peak at m/z = 359.46 (100%) The molecular ion peak was observed at 321.76 ([M+H]) (9.28%) Other major fragment peaks at m/z = 321.76, 313.89 (11.4%), 280.07 (12%), 137.48 (25.29%), 124.53 (24.29%), 105.25 (18.75%) (Fig 5. 7). The detailed fragmentation pattern was given by (Fig 5. 8). Proton NMR analysis of the compound revealed three sets of peaks between δ 7 - 8, a singlet at δ 6.2 and different sets of multiplets between δ 0.8 - 4.8 (Fig 5. 9). Evaluation of the ^{13}C spectra of the compound contained 22 C signals ranging from δ 14 - 171 (Fig 5. 10). The seven CH_2 signals between δ 20-70 were confirmed by evaluating the DEPT 135 spectrum (Fig 5. 11). The ^{1}H -^{1}H and ^{1}H-^{13}C correlations were evaluated using ^{1}H -^{1}H COSY (Fig 5. 12), HSQC (Fig 5. 13), and HMBC (Fig 5. 14) spectrums.

Table 5.5 NMR spectroscopic data of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one in $CDCl_3$.[a]

Carbon No.	^{13}C NMR (DEPT)	H	δ^1H NMR (int., mult., *J* in Hz)[b]	^{1}H-^{1}H COSY	HMBC (^{1}H-^{13}C)
1	123.77 (CH)	H1	7.53 (m, 1H)	H-6	C-2, C-6
2	140.01 (C)				
3	117.84 (C)				
4	171.12 (C)				
5	108.33 (CH)	H5	7.32(m, 1H)	H-6	C-4, C-6
6	131.93 (CH)	H6	8.2 (t, J = 7.5 Hz, 1H)		
7	29.17 (CH_2)	H7	3.60 (s, 2H)		C-2, C-8
8	171.46 (C)				
9	124.69 (C)				
10	28.96 (CH_2)	H10	3.73 (m, 2H)		C-3, C-9
11	28.79 (C)				
12	68.1 (CH)	H12	2.51 (m, 1H)	H-18	C-18, C-11
13	65.24 (CH_2)	H13	2.55 (d, *J*=17.5Hz, 2H)	H-12	C-12, C-14
14	178.94 (C)				
15	65.38 (CH_2)	H15	1.26 (s, 2H)	H-16	C-11, C-16
16	20.8 (CH)	H16	2.08 (m, 1H)	H-19	C-17
17	154.51 (C)				
18	125.51 (CH)	H18	4.81 (m, 1H)		C-17
19	31.77 (CH_2)	H19	0.86 (m, 2H)	H-20	C-16, C-20
20	22.63 (CH_2)	H20	1.23 (m, 2H)		C-21
21	31.64 (CH_2)	H21	2.01(m, 2H)	H-20	C-17
22	14.04 (CH_3)	H22	1.17 (s, 3H)		C-11
23		OH	6.2 (s, 1H)		

[a] NMR spectra recorded using Bruker AVANCE III 500 MHz (AV 500) spectrometer.

[b] Values in ppm, multiplicity and coupling constants (*J*¼ Hz) are indicated in parentheses. Assignments were made with the aid of the ^{1}H–^{1}H COSY, HSQC and HMBC experiments

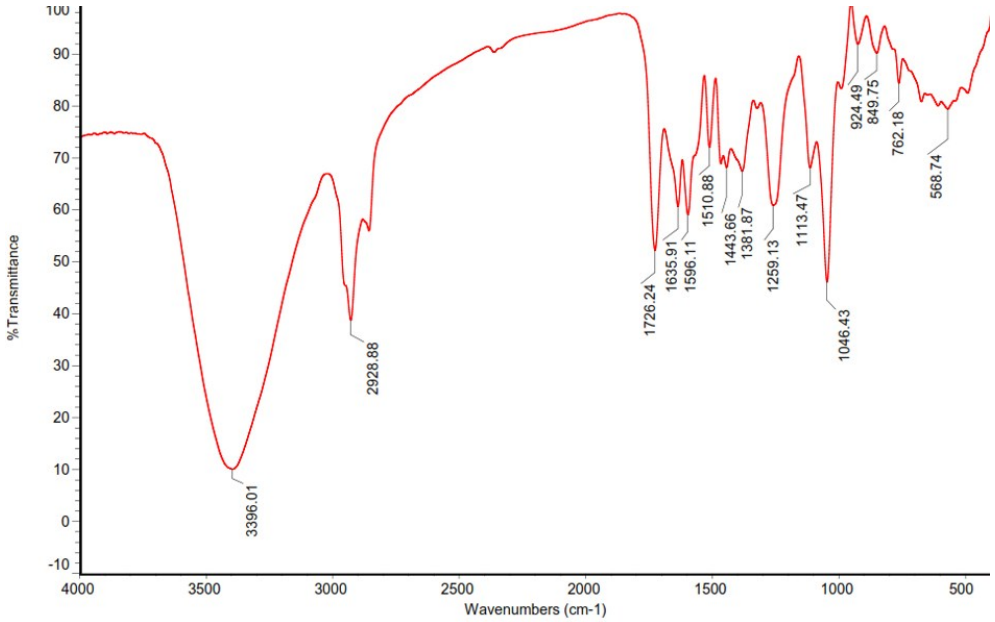

Fig 5.6 IR spectra of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9), 17-penten-14-one

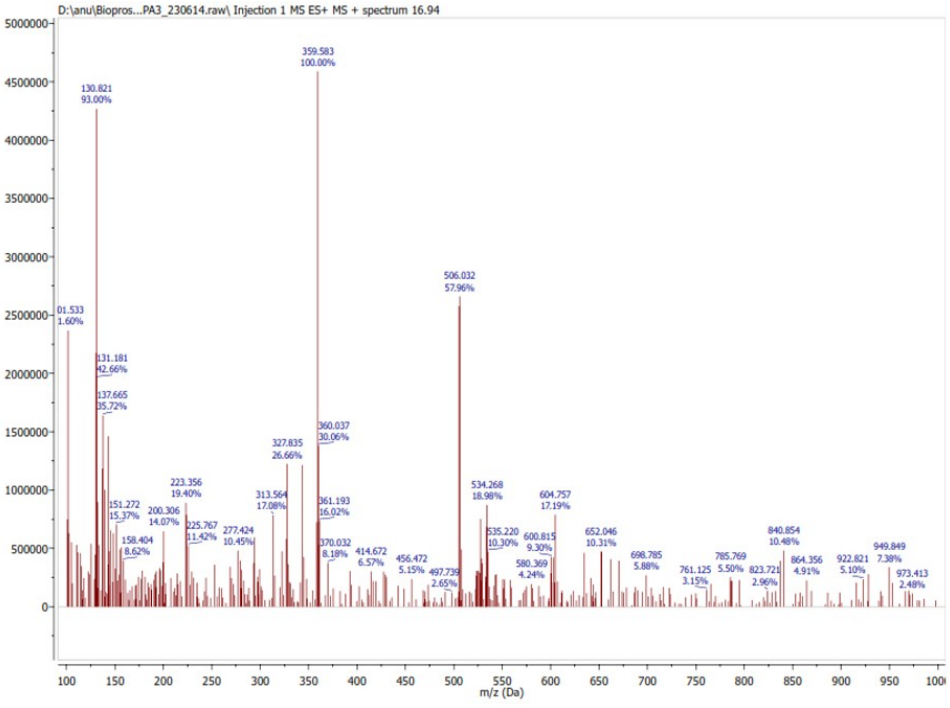

Fig 5.7 LC-MS spectra of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

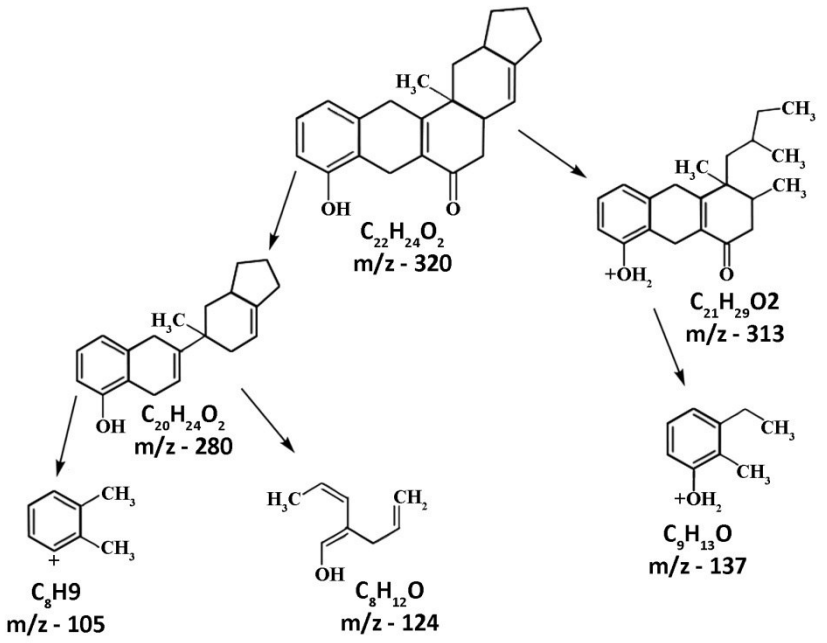

Fig 5.8 Mass fragmentation pattern of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

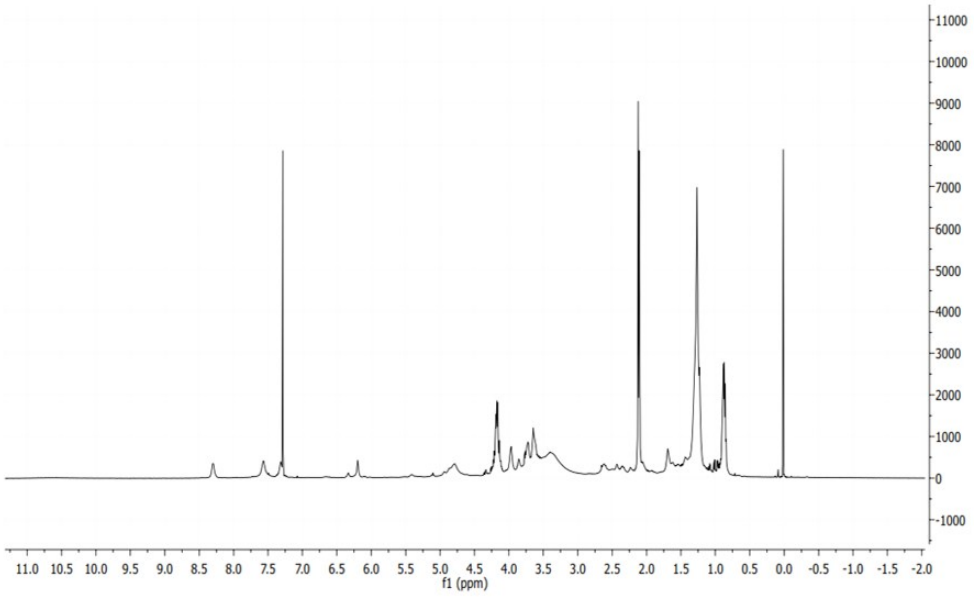

Fig 5.9 Proton NMR spectra of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

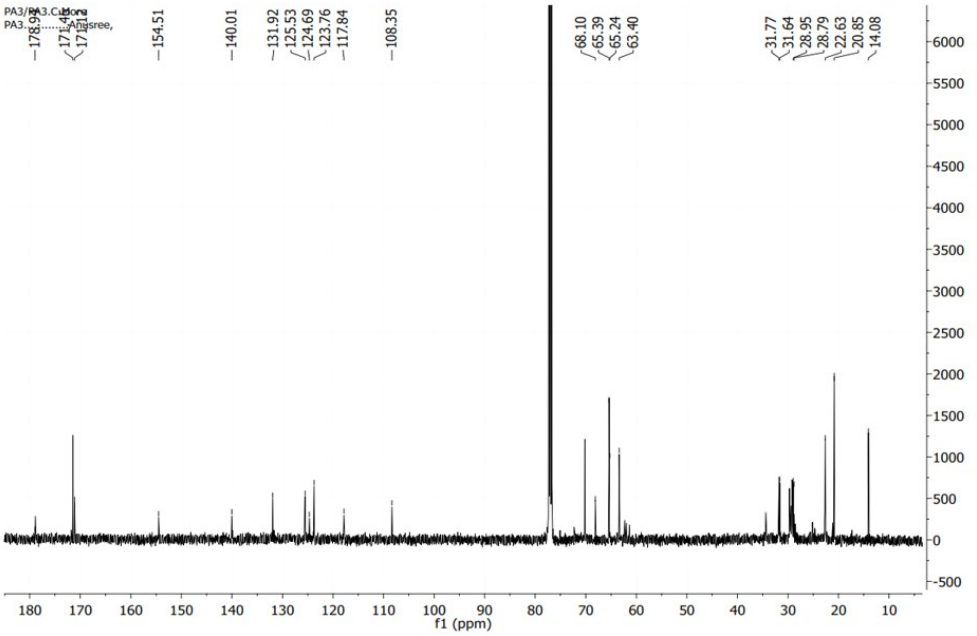

Fig 5.10 ^{13}C NMR spectrum of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

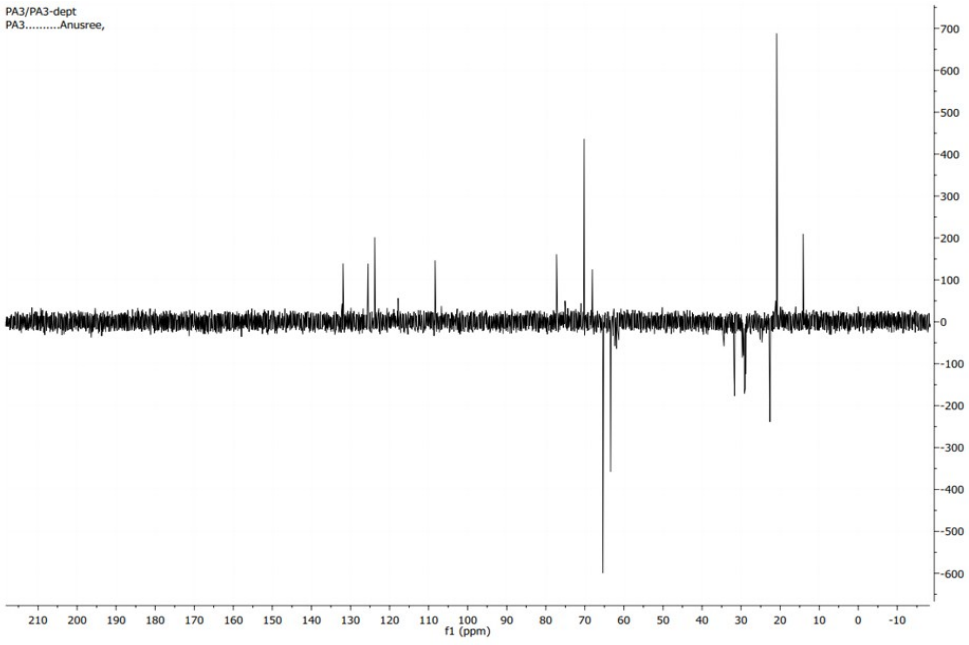

Fig 5.11 DEPT spectrum of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

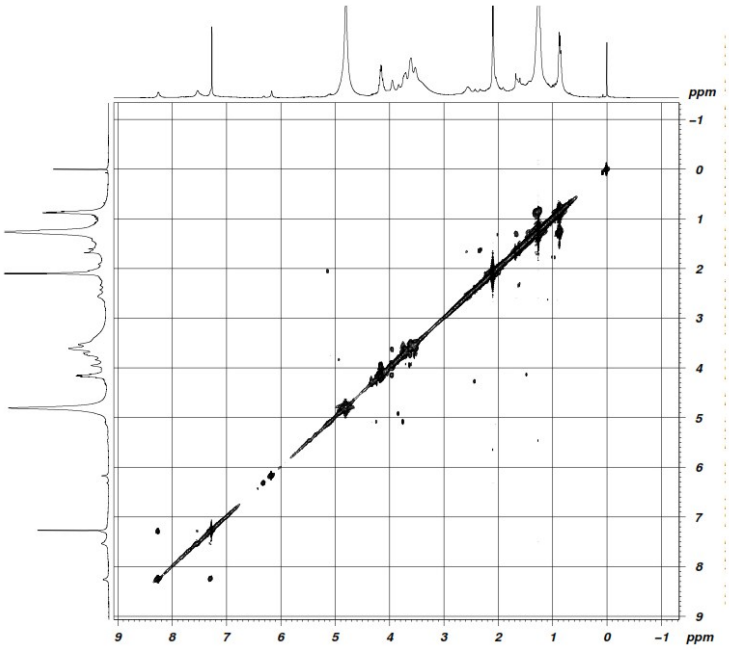

Fig 5.12 ^{1}H-^{1}H COSY spectrum of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

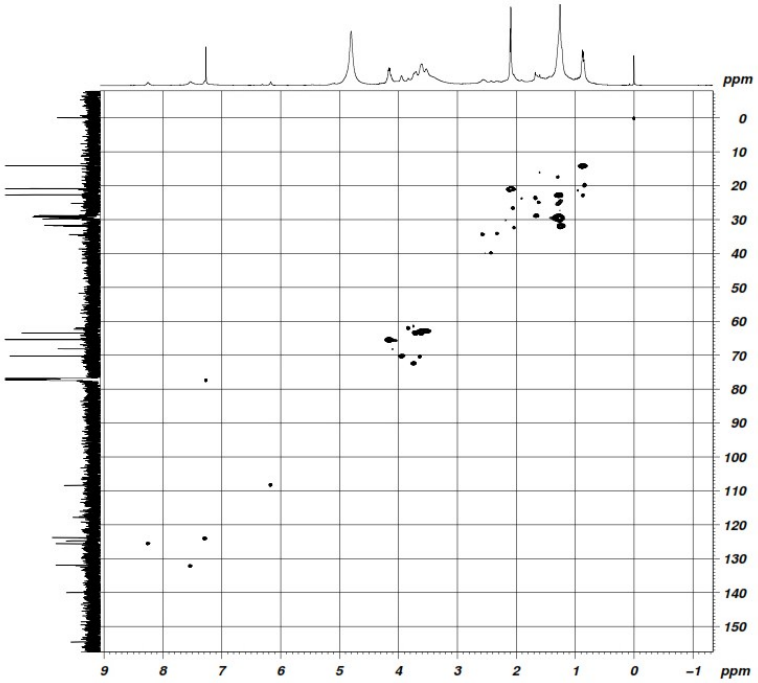

Fig 5.13 HSQC spectrum of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

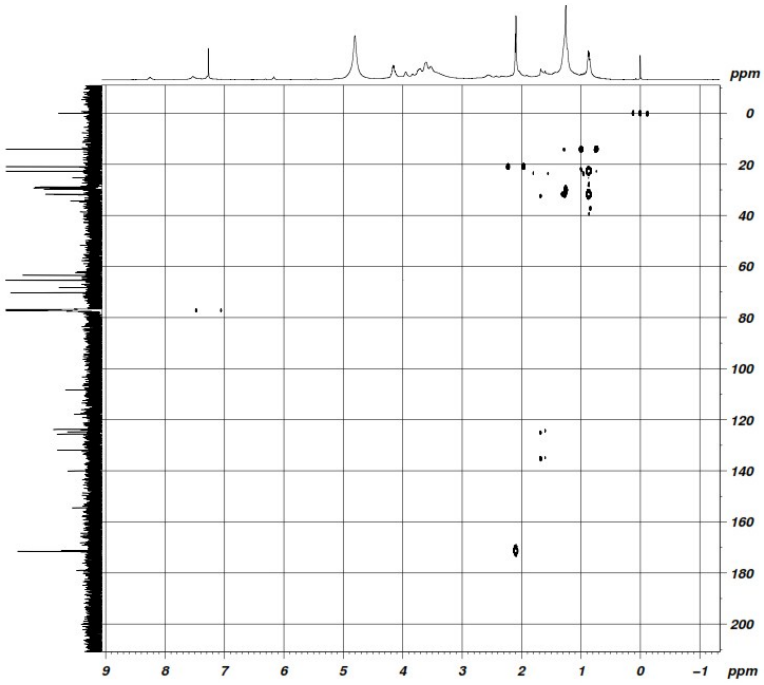

Fig 5.14 HMBC spectrum of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9),17-penten-14-one

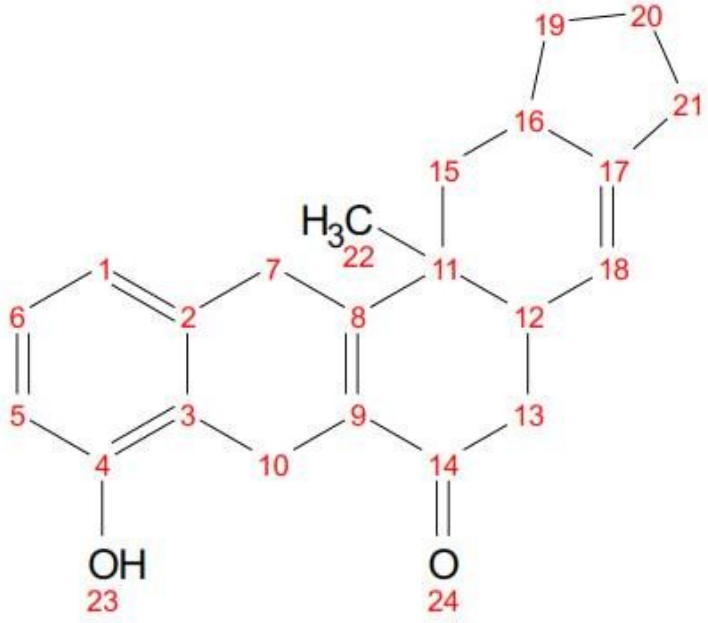

Fig 5.15 Structure of 4-Hydroxy-11-methylpentacyclo[11.8.0.0^{2,3}.0^{11,12}.0^{16,17}] henicosa-1,3,5,8(9), 17-penten-14-one

5.5 Discussion

The present study illustrated that a potential antagonistic bacterial strain, *P. aeruginosa* Ps04 isolated from the subsurface waters from tropical estuarine habitats of Cochin was evaluated as a promising antagonistic native bacteria. The study revealed information on the structural aspects of metabolite, which was also identified and characterized for the bioactive molecule behind the antagonistic activity which can be a lead for the drug discovery program. This strain used in the study possessed broad spectrum of antibacterial activity against aquaculture pathogens. This is in line with the observation that marine flora of antagonistic bacteria could be considered as an emerging unexplored resource to yield secondary metabolites leading to drug and feed application (Kasanah & Hamann 2004). *P. aeruginosa* belongs to the same class with versatile metabolic potential and great ecological and clinical significance (Raaijmakers et al. 1997; Nair et al. 2015). They are well recognized with metabolites having inhibitive properties against pathogenic *Vibrios* and hence can be explored its potential to be used in aquaculture as a antagonistic organism (Nair et al. 2012; Priyaja et al. 2014).

The physiological and environmental conditions were reported to be affecting the functionality and specificity of bio control agents (Guetsky et al. 2002; Lalloo et al. 2008). Our results showed that *P. aeruginosa* Ps04 grows well under a wide range of temperature, salinity and pH thereby supporting its better functioning as a bio control agent in aquaculture system. Even though *P. aeruginosa* Ps04 showed resistance to a few antibiotics, this variation could be due to various factors viz., the low permeability of its outer-membrane, the constitutive expression of various efflux pumps or may be related to the large size and the versatility of its genome (Hancock 1998 ; Vaisvila et al. 2001). Similarly such multiple resistances have already been reported for *P.*

aeruginosa isolated from open ocean by Khan et al. (2007). This may decline the chance of transfer of antibiotic resistant genes to other organisms. The ability of *P. aeruginosa* Ps04 to generate a number of hydrolytic enzymes can help in nutrient cycling, improving host's nutrient uptake and digestion (Geovanny et al. 2008; Keysami et al. 2012). In account of the higher protein levels, the biomass of *P. aeruginosa* Ps04 would serve as a good nutritional source to host and also would serve as single cell protein (Vijayan et al. 2006). Thus it further increases the suitability of the bacteria to act as a nutrition supplement to the host animals, added to the bacterial inhibiting property.

The antagonism and challenge for nutrients between the probiont and pathogen can be better understood by co-culture assays (Pai et al. 2010). The growth of the *V. harveyi* 101 was successfully inhibited by *P. aeruginosa* Ps04 in co-culture mediated by the killing of the pathogen. Further it demonstrated that the inhibitory activity increased with increasing density of the antagonist. Similar results have been reported for coculture of antagonistic *Pseudomonas* MCCB 102 and MCCB 103 against *V. harveyi* MCCB111 (Pai et al. 2010). In our study it was observed that even though the *P. aeruginosa* Ps04 possess slightly higher generation time than *V. harveyi* 101, it inhibits the pathogen completely. This is contradictory to the observation reported by Vine et al. (2006), which suggested a higher antagonistic potential for a bacteria having a shorter lag period and doubling time. This could be due to the production of antagonistic compound or competitive exclusion. The challenge experiments conducted in juvenile *E. suratensis* with *P. aeruginosa* Ps04 even at a dose of 10^{7-8} CFU/mL cells showed no mortality or harmful effects. These results clearly showed that the administration of *P. aeruginosa* Ps04 is found to be harmless but it is better to use in the inactivated form as microbial product in the aquaculture rearing system.

The composition of a fermentation medium has always been a vital component in the optimization of growth conditions, because it affects productivity and overall cost of the production process (Lee 2005). Media formulation and optimization of physiochemical conditions are key considerations in development of bioprocesses that can produce affordable aquaculture biological agents (Irianto and Austin 2003; Preetha et al. 2007). The type of carbon source and the carbon to nitrogen ratio plays an important role in microbial growth (Gandhi et al. 1997) and the variations in culture conditions and nutrients may stimulate the production of different metabolites (Motta et al. 2007). The production of secondary metabolites by the bacteria is influenced by the unique ecological characteristics of their environment and is thought to be under complex genetic regulation (Isnansetyo and Kamel 2009). Several antimicrobial peptides are produced by a single strain simultaneously or at least at different time intervals or growth conditions (Duitman et al. 1999).

The optimization of the mass production medium is vital as it affects the growth conditions, production of secondary metabolites etc. of the bacteria (Lee 2005; Preetha et al. 2007; Isnansetyo and Kamel 2009). Glycerol-alanine medium used in this study showed an increased production of antibacterial compound. It was observed that the production starts at exponential phase and remained constant during the stationary phase. This indicated the efficiency of the selected medium in terms of its nutritional requirements and its potential to produce secondary metabolites (Jamil et al. 2007; Hammami et al. 2008; Saha et al. 2008; Nair et al. 2015). Vijayan et al. 2006 reported the importance of minimum inhibitory concentrations of bacterial cell free supernatants for inhibiting the growth of pathogens in aqua culture. In our study it was observed that the cell free supernatant diluted even to 20% of its initial concentration could retain its antagonistic activity. More over the activity was

maintained at 120ºC and pH 2 - 12 and was not sensitive to the tested enzymes. This further indicated that the inhibitory compound was relatively heat stable and can be used in industrial level production of antagonistic compound.

Bacteriocins may play a defensive role to hinder the invasion of ecosystem of other strains or species into an occupied niche (Riley and Wertz 2002). Antibacterial substances produced by different bacteria seem to play an important role in the bacterial antagonism in aquatic ecosystems (Dopazo et al. 1988). Marine *Pseudomonas* spp. produced various novel and diverse bioactive substances which possess pyroles, pseudopeptide pyrrolidinedione, phloroglucinol, phenazine, benzaldehyde, quinoline, quinolone, phenanthren, phthalate, andrimid, moiramides, zafrin and bushrin as chemical structures (Isnansetyo and Kamel 2009). Major bioactive compounds obtained from marine *P. aeruginosa* were diketopiperazines, phenazine alkaloids such as pyocyanin and pyorubrin (Jayatilake et al. 1996 and Saha et al. 2008). Bacteriocins are considered as an alternative to combat infections against specific pathogens (Riley and Wertz 2002) because they mediate inhibition of other bacteria due to their unusual redox properties, which resulted in an enhanced production of hydroxide (OH^-) and super oxide (O_2^-) radicals, resulting in oxidative damage, DNA damage and lipid peroxidation (Pai et al. 2010).Therefore, research for new products with antimicrobial activity gained importance. The identification and chemical characterization of antagonistic compound produced by *Pseudomonas spp.* and exploration of their potential use in the control of pathogens addresses this subject. The IR band at 1046 cm^{-1} is due to δ OH deformation mode (Frost et al. 2007). The peak at 1510.88 cm^{-1} is due to C-C stretches in aromatic ring. The strong peak at 1726.88 cm^{-1} indicate the presence of C=O functional group. A broad band around 3396 cm^{-1} confirms

the presence of –OH functional group in the compound. The C-H stretching vibrations are observed around 2928 cm^{-1}. The molecular formula of the compound is confirmed to be $C_{22}H_{24}O_2$ (m/z 320). The base peak was M+K peak (359.583, 100%). M+H peak was observed at 321.763(9.28%). M+Na peak was also observed at 343.063 (11.89%). Other major peaks were obtained at 313 (11.4%), 280 (12%), 124 (24%), 137 (25%) and at 105 (18.75%). The mass splitting pattern of the compound is given by (Fig 5. 8). From the mass spectral data it was clear that the molecular mass of the compound was 320 and IR data confirmed the presence of OH functional group. Proton NMR signals obtained between δ 7-8 revealed the presence of an aromatic ring with in the compound. After evaluating the 2D spectra the corresponding C atoms were assigned as C1 to C6 and as the part of aromatic six membered ring (Fig 5.15). Evaluation of the ^{13}C spectra in combination with DEPT 135 revealed that the compound contained 22 C atoms of which 8 were quaternary, 7 were CH_2 and the rest as CH or CH_3. The peak at quaternary C signal obtained at δ 171 confirmed the presence of C=O functional group which causes maximum de-shielding to carbon atom. Similar is the result in case of C atom that attached with an electronegative OH functional group which appeared at δ 171. More over a singlet peak obtained at δ 6.2 in proton MNR was assigned due to H atom of OH group. From these points it can be concluded that the molecule contain 22 C atoms and two O atoms and 24 H atoms. Further out of these two O atoms one is a hydroxyl functional group and another one is a ketonic moiety. The C signals revealed that there were 10 un-saturated carbon atoms of which 6 were quaternary and 4 were CH groups. The absence of C signals after 100 in DEPT revealed that The CH_2 groups present in the compound were not from any unsaturated C atom. All the DEPT signals were observed between δ 21 - 66. Out of the eight quaternary C atoms all but except one exhibited C signals between δ

108 - 171 which confirms the fact that all of them are the part of an unsaturated frame work. The quaternary C signal obtained at δ 28.7 is identified as normal saturated C atom. The position CH_3, C=O and OH functional groups were assigned with the help of both 1D and 2D spectral data. Carefully evaluating the spectral data the structure of compound was assigned as 4-Hydroxy-11-methylpentacyclo[11.8.0.$0^{2,3}$.$0^{11,12}$.$0^{16,17}$]henicosa-1,3,5,8(9),17-penten-14-one (Fig 5.15).

The present study demonstrates that *P. aeruginosa* MBTDCMFRI Ps04 possess unique properties such as extensive antibacterial property towards wide spectrum of aquaculture bacterial pathogens, tolerance to a wide range of physico-chemical conditions of aquaculture systems, suppress the *Vibrio* population on coculture assay through an antagonistic mode of action. Further, we identified a new compound with antibacterial property from the candidate strain, and using the primary data generated on this compound, the possibility of tracking the exact molecular component, responsible for the antagonistic property is a possibility. Once this is done, using the synthetic chemistry route, synthesizing an aquaculture grade chemical is also realistic possibility, which would open up new avenues in antimicrobial therapeutics in aquaculture

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Chapter 6

DEVELOPMENT OF MICROBIAL PRODUCT (MP) AND THEIR APPLICATION IN AQUACULTURE

Contents

6.1 Abstract

Applications of antagonistic microorganisms in aquatic feed formulations are widely accepted, and have been used as dietary supplement for many years. This chapter deals with the preparation of a functional micro feed for using in early larviculture, by incorporating an antagonistic bacterial isolate from the present study, which details are already depicted in Chapter 5. This *Pseudomonas* based microbial product (PMP), can contribute the improvement of health and survivability during the larval rearing cycle. *P. aeruginosa* MBTDCMFRI Ps04 was selected for the development of MP, due to its broad spectrum of antibacterial activity, higher stability and higher nutritional value, where the protein content is in the range of 45 - 55%. Optimal conditions for pilot scale production of antagonistic compound using nutrient broth supplemented with 50% SW were achieved at aeration 0.5 LPM (litres per minute), agitation 100 rpm, inoculum size of 4%, pH - 7.5 ± 0.2 , 31ºC at 96 h in New Brunswick BioFlo/ CelliGen 115 Fermentor. A minimum inhibitory concentration of PMP (781 µg/ml) was found to inhibit 10^5 CFU/ml of *V. harveyi.* This product was found to be non - toxic to fish cell lines up to 0.2%.

The inactivated microbial biomass was spray dried and blended with the nutritional components to make a complete formulated feed essential for finfish and shellfish larvae. The homogenous blend of raw material was then pulverized and dried. The filaments were then grounded and granulated with fillers and binders to obtain the desired micro size. The granulated feeds were separated according to mesh size as 60 - 80 µm, 100 - 120 µm and 200 - 220 µm. The developed feeds were checked for acceptability in various larval stages (zoea, mysis and post larvae) of Indian white shrimp, *Fenneropenaeus indicus (F. indicus)* larvae in wet laboratory. Growth and stage changes of the PMP feed larvae were compared with commercially available feed (Micro elite - Taiwan) were used as control. The rates of feeding were adjusted depending upon the changes in larval density and variability in consumption. The animals showed good acceptance and better survival rate with the indigenously developed feed, when compared with the commercial one. Co-feeding at the earlier stages proved to be more effective [(5 times feed + 2 times algae)/ day] especially in zoea and mysis stages than 7 times feed/ day. Microfeed developed using an antagonistic isolate of P. *aeruginosa*, when used as a larval feed in shrimp is found to have a key effect on larval survivability and growth, enhancing productivity in the hatchery phase, proving the potential of the microfeed in larval culture systems of aquaculture species.

6.2 Introduction

Aquaculture is one of the growing food production sector which is on a steep path of development and growth in recent years including India. But the intensification related issues has brought stress to the rearing environment and disease to the cultured species, affecting the productivity and profitability (Cruz et al. 2012; FAO 2012). The United Nations 'Food and Agriculture Organization' (FAO 2012) reported the disease outbreak due to disease

causing pathogens such as virus, bacteria and fungi in aquaculture had an annual loss of US$3 billion (Defoirdt et al. 2007). Mortalities due to pathogenic bacteria and their disease management have become a greater challenge to the industry (Gomez-Gil et al. 2000; Balcazar and Rojas - Luna 2007). Conventionally, antibiotics and chemotherapeutics are employed to control bacterial diseases in aquaculture. However, the excessive use of drugs will alter the natural microbial population and will damage the environment and in turn raise the issues of multidrug resistance among pathogens. The repeated uses of antibiotics not only makes the treatment ineffective, but also creates food safety issues and pose threat to the human health and the environment in the form of residues (Defoirdt et al. 2007; Cruz et al. 2012). The use of biocontrol agents emerged as a sustainable ecofriendly option to prevent and control diseases with a potential to replace antibiotics/drugs used in aquaculture (Defoirdt et al. 2007; Boonthai et al. 2011). Thus, the use of beneficial bacteria with antagonistic properties will have a significant role in controlling the bacterial disease with additional benefits of nutrition, toxic waste degradation and water quality.

The use of bacteria either in live or dead form was found to be effective in controlling or reducing bacterial infections due to the production of inhibitory compounds (Vaseeharan and Ramasamy 2003; Vijayan et al. 2006; Pai et al. 2010; Preetha et al. 2010), increasing growth and survival of the host by supplementing various essential enzymes/nutrients (Vine et al. 2006; Kesarcodi - Watson et al. 2008; Boonthai et al. 2011) and may also act as immune modulators (Irianto and Austin 2003; Taoka et al. 2006; Pai et al. 2010). Probiotic bacteria also work by competitive exclusion in which bacteria/microbial products decrease the pathogenic bacterial population by competing for nutrients and adhesion sites on the hosts (Balcazar et al. 2007).

The diets ought to provide sufficient proportions of essential nutrients and energy which are considered as primary and leading criterion to meet the requirement of host animal. The developed feed should be easily accepted by the host animal and should have fewer adverse environmental impacts (Kaushik 2000). Based on the species and stages of life cycle of aquaculture animals, the formulation, technology and inherent restrictions in feed development diverges. Additives such as potential antioxidants, enzymes, probiotics as well as emulsifying, binding or preserving agents were also added to improve the efficiency of feed (Rico et al. 2013). In aquaculture industry, the development of functional feed is considered as a new opportunity, where diets are formulated with dietary ingredients which are beneficial to the growth and health of aqua cultured animals, which can enhance the immune system and improve physiological benefits, than conventional feeds (Ibrahem 2015; Soto et al 2015). Moreover it is gaining importance due to its ease in application and absence of side effects (Irianto and Austin 2003; Salinas 2006). Previous studies have clearly emphasized the advantages of genus *Pseudomonas* as they are ubiquitous, tolerated to a wide range of abiotic conditions, various enzyme production and consistent antibacterial activity which helps in controlling bacterial diseases in aquaculture (Vijayan et al. 2006; Nair et al. 2012; Vinoj et al. 2013; Nair et al. 2015).

The use of microorganisms with antagonistic properties, in the form of formulated aqua feed as a carrier, is comparatively easy, effective and well accepted. Present study aims to develop a formulated larval feed incorporating the potential antagonistic strain *P. aeruginosa* Ps04 described in the Chapter 5. The present work also attempted to standardize the process of larval feed formulation, evaluation using *F. indicus* larvae along with a commercial larval feed as control.

6.3 Materials and Methods

6.3.1 Optimization of Fermentation Technology for Pilot Scale Production of Antimicrobial Metabolite

P. aeruginosa Ps04 was taken from glycerol stock at -80^{o}C and streaked on nutrient agar (15 ppt) and from this a single colony was inoculated into 5 ml of nutrient broth supplemented with 50% SW and incubated at 30^{o}C on a rotary shaker (New Brunswick Scientific Co. Inc., NJ, USA) at 200 rpm for 20 - 24 h. Optimal concentration of grown culture (starter culture) for pilot scale production using fermenter was assessed by inoculating the cultures at various concentrations (0.5, 1, 2, 3, 4, 5 and 10%) in 2 L nutrient broth supplemented with 50% SW at the optimized conditions viz, at 30°C, pH 7.0 and at 15 ppt saline conditions for 1 - 7 days. Later on all the said conditions were optimized for a scale up volume of 10 L also. Agitation speed and aeration was optimized by varying the agitation speed (25, 50, 100, 200 and 250 rpm) and rate of aeration (0.1, 0.5 and 1 LPM). Samples for antagonistic activity were drawn every 24 h interval and 1ml of the culture was processed for evaluating the antagonistic potential as explained in chapter 4 (Section 4.3.3).

6.3.2 Inactivation of Culture Broth

The bacterial biomass in the scaled up fermented broth was inactivated by heat and formalin treatment, in separate experiments. Heat treatment was carried out at various temperatures (40, 50, 60, 70, 80, 90, 100, 120 and 160 ºC) for a period of 15-30 min. Chemical treatment using formalin was carried out at varying concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1, 2 and 5%) for 15 - 30 min. The activity of both treated culture broths were assayed against aquaculture pathogens by disc diffusion method. The viability of the treated

cells was checked by plating the inactivated cells on nutrient agar (Himedia, India) with 50% seawater.

6.3.3 Spray Drying Process

The fermented broth was diluted in sterile water at 4% v/v. The scaled up cultured broth was spray dried [Advanced drying systems (SD - 50 kg h^{-1})]. The inactivated bacterial preparation along with additives (micelle) was pumped into the spray drier through a screw pump at a pressure of 3 bars. The micelle was oozed out through a nozzle into the spray drier chamber set at 200ºC and vacuum of -10 mm Hg. The sprayed micelle evaporates to form the dry part which is then sucked out of the chamber. The outlet temperature was set to 95ºC (Table 6.1).

Table 6.1 Conditions of spray drying process

Operating conditions	
% dry matter	4%
pH	4.50
Gas (L/min)	Air
Spraying pressure(bar)	3 bar
Air at the inlet (°C)	205 °C
Hot air pressure (mbar)	2 bar
Air at the outlet (°C)	95 °C
Humidity of the air at the outlet (%)	< 1%
Humidity of the powder	< 2%
Powder yield (kg/h)	2 kg h^{-1}

6.3.4 Optimization of Minimum Inhibitory Concentration of the Crude Fermented Spray Dried Product

The antimicrobial activities of the samples were evaluated using the method followed by Ericsson and Sherris (1971) with suitable modifications to determine the minimum inhibitory concentration (MIC). The crude fermented

spray dried product (Mp) was dissolved in autoclaved distilled water for preparing dilutions of varying concentrations of compound (12.5, 50, 100, 200, 400, 800, 1600, 3200, 6400, 12800, 25600 and 50000 µg /mL) by double dilution method. The tubes containing tested samples and controls were inoculated with test pathogens (*V. harveyi* 101, *V. alginolyticus* 101 and *V. anguillarum* 01) approximately at a concentration of 10^5 CFU/mL and incubated for 20 - 48 h at 28 ± 2ºC. The growth of the viable cells was visually monitored using tetrazolium salt (2 mg/mL). Control tests were also conducted using sterile water and nutrient broth in order to identify the growth of pathogens in water and the selected medium respectively.

6.3.5 Cytotoxicity Assay

Cytotoxicity assay was carried out on *Cyprinus carpio* (Koi carp) fibroblast cell lines. Cell lines were grown in Leibovitz's Medium (L - 15) supplemented with 5% fetal calf serum (FCS) and the cytotoxicity assay was carried out using standard protocol (Freshney 2006) and incubated at 28 ± 2 °C for 5 days. Cells were treated with varying concentrations of spray dried product (50 µg/mL - 5 mg/mL) as described earlier. The cell free supernatants of *V. harveyi* 101 obtained through filtration by 0.2 µm syringe filter (Palls life science, USA) is used as a pathogenic control, followed by incubation at 28 ± 2°C for 24 h. The growth media was removed and the cells were trypsinized and the plates were visualized through microscope for morphological changes in the cells periodically.

6.3.6 Formulation of Antagonistic Larval Feed

Feed ingredients available in and around Cochin were used to formulate the experimental diets and the compositions of ingredients were shown in Table 6.2. The key protein sources are fish meal (dried unsalted

anchovies), shrimp meal (dried *Parapenaeopsis stylifera*), clam meal (*Villorita cyprinoides*) and soy flour. The proximate composition of the feed ingredients was determined prior to the experiment. All the ingredients were mixed well and blended with fish oil, vitamin and mineral mixtures and fortified with certain additives making it nutritionally complete, along with microbial product (0.2% of spray dried crude fermented product).

Table 6.2 Ingredient composition and chemical composition of *Vibrio* antagonistic larval feed

Ingredients	per 1000g
Fish meal	200
Shrimp Meal	200
Clam Meal	200
Soy flours	300
Fish oil	50
Vitamin C	5
Mineral mixture	28
Vitamin mixture	3
Mixed carotenoids	4
Spirulina	4
Antioxidant	1
Antifungal	1
Lecithin	2
Microbial product	2
Chemical composition (% on dry matter basis)	
Crude protein	55.60±0.92
Crude fat	14.72±2.28
Soluble carbohydrate (NFE)	14.67±1.41
Crude ash	10.65±1.05
Crude fibre	3.30±0.51
Acid insoluble ash	1.07±0.78

Values are the mean of three observations

Then the homogenous blend of raw material was extruded in an extruder (Basic Technologies Pvt. Ltd., Kolkata) with a time-temperature combination of 10 sec and 80ºC, to obtain 2 mm diameter long cylindrical filament pellets, followed by drying the filaments for 24 h at constant temperature (60 ± 2ºC). The dried filaments were then crumbled and then submitted to grinder to crush the filaments. It is then sieved through 100 µm mesh to obtain uniform particle size and further granulated using 0.2% binders into corresponding size. The feeds were coloured coded with different natural oil resins (green, yellow and red) to differentiate the different micron sizes. The granulated feed were then stored in airtight containers for subsequent nutritional analyses and feeding. The schematic representation of preparation of newly formulated *Vibrio* antagonistic larval feed using *P. aeruginosa* MBTDCMFRI Ps04 is given in Fig 6.1.

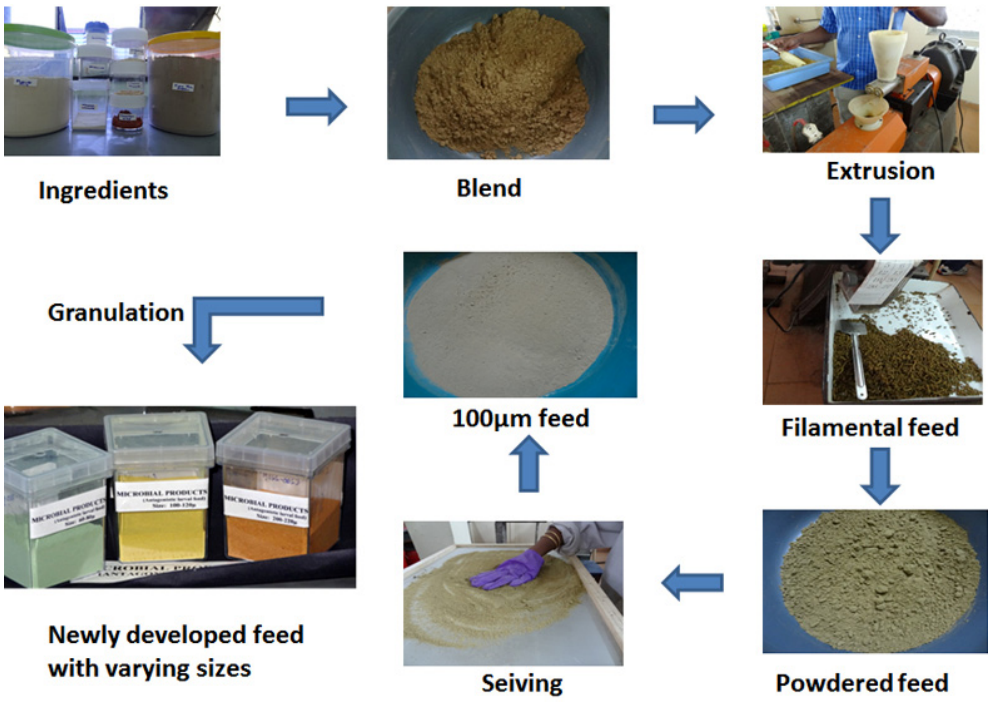

Fig 6.1 Schematic representation of preparation of newly formulated *Vibrio* antagonistic larval feed

6.3.7 Nutritional Analysis of Micro Feed

The proximate chemical composition analysis of newly formulated feed was determined using the standard procedures (AOAC 1990). Briefly, moisture was determined by oven drying at 105ºC to constant weight. Dried samples were used for determination of crude fat, crude protein, soluble carbohydrate, crude fibre, acid insoluble ash and crude ash contents as explained in chapter 4 (Section 4.3.5). All analyses were done in triplicates.

6.3.8 Acceptability of Micro Feed Developed

The survival and growth of shrimp larvae was observed for 25 days, fed with the newly developed *Vibrio* antagonistic larval feed in comparison with a commercially available larval feed

Feeding:

A feeding experiment was conducted for 25 days with zoea to post larval stage of the *F. indicus* (Indian white shrimp) in the wet laboratory of Department of Fisheries, Matsyafed hatchery, Azhikode (Fig 6.2). *F. indicus* at a density of 100 larvae/L have been distributed in 50 L glass tank with 30 L of seawater (salinity 34 ppt). The larvae (zoea stage) have been divided into four lots and the larval survival and growth have been observed from 1 - 25 days after hatching. The first group of four lots has exclusively been fed using the newly formulated antagonistic feed. The second group has been fed with commercially available feed (Microelite - Taiwan). The third group has been fed with newly formulated feed along with microalgae. The fourth group has been fed with commercially available feed (Microelite - Taiwan) with microalgae. All experiments were carried out in triplicate. Feeding pattern was given in Table 6.3. Phytoplankton cultures of *Chaetoceros* sp. maintained at the Matsyafed hatchery was used as live feed in few experimental tanks. The

rearing conditions have been standardized in glass tanks, the water temperature being maintained at 28.5 ± 0.5ºC with 28 to 34 ppt salinity depending upon the stages of larvae and pH 7.8 ± 0.2.

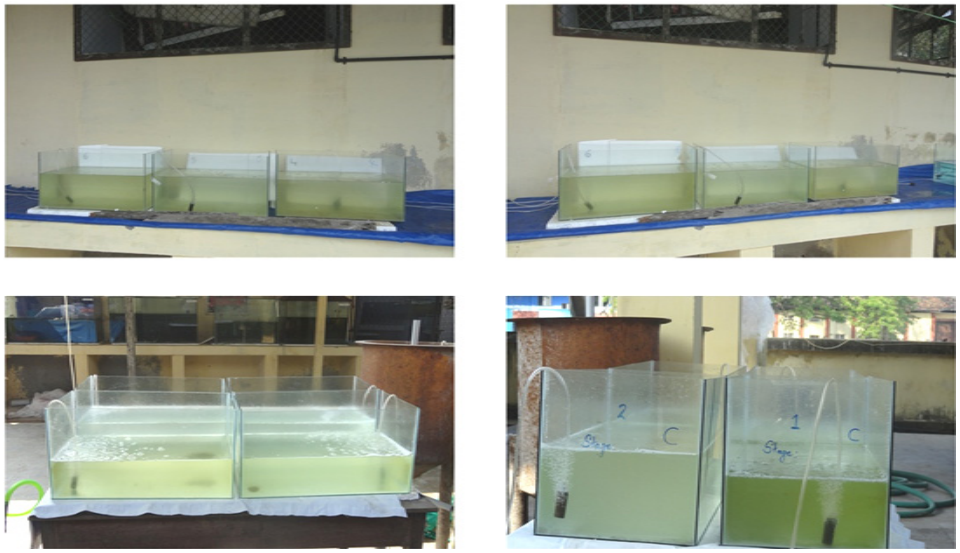

Fig 6.2 Experimental set up for the feeding trial of newly developed larval feed at Regional Shrimp hatchery, Azhikode

Table 6.3 Feeding pattern at different stages of *F. indicus* larvae

Stage	Acceptable Feed size
Zoea 1 - 3	Microalgae, Feed 3 (50- 60 µm)
Mysis 1 - 3	Feed 3 (50- 60 µm), Feed 2 (100- 120 µm)
Post larvae 1 - 20	Feed 2 (100- 120 µm), Feed 1 (200-220 µm)

After acclimatization and conditioning of the experimental animals for 5 h, shrimp were fed at a rate of 7 times per day with an interval of 3 h manually. The formulated feed and the commercial feed were daily fed at the rate of 0.5 g/day per lakh larvae. At the beginning of the feeding cycle, for zoea stage the larval feed size was 60 - 80 µm was used, which was progressively increased to 0.8 g/day per lakh larvae. For feeding the mysis, the larval feed size of 60 - 80 and 100 - 120 µm was used. For the post larval stage

(PL1 - PL5) 1.3 g/day per lakh larvae feed of particle size is 100 - 120 µm was used. The post larval stage (PL6 - PL15) was fed at the rate of 1.7 g/day per lakh larvae (larval feed size is 200 - 220 µm). In the tanks with co feeding regime (dietary feed + microalgae), the dry feed was given 5 times and microalgae were provided two times per day. Every day, the excess feed and fecal output accumulated are removed.

6.4 Results

6.4.1 Optimization of Fermentation Technology for Pilot Scale Production of Antimicrobial Metabolite

The conditions were optimized for mass production of broth in New Brunswick BioFlo/CelliGen 115 fermentor (Eppendorf, Germany). The best concentration of starter culture for optimum production was found to be 4%. Agitation speed was optimized as 100 rpm whereas aeration as 0.5 LPM for 4 days in nutrient broth supplemented with 50% SW (Fig 6.3). After the pilot scale production the consistency of antibacterial property was also evaluated (Table 6.4).

Fig 6.3 Optimization of pilot scale production of *P.aeruginosa* Ps04 using New Brunswick BioFlo/ CelliGen 115 fermentor

Legends: (a) Fermenter vessel (2 L) connected to the automated system for the pilot scale production of biomass, (b) Culture in log phase of growth (10 L) in fermenter vessel

Table 6.4 Spectrum of inhibitory activity of scaled up cultured broth using New Brunswick BioFlo/CelliGen 115 fermentor

Volume of medium	Zone of inhibition (mm) on different days						
	1	2	3	4	5	6	7
2L	11	15	17	20	20	18	15
10L	10	14	17	21	20	19	16

The values may differ ± 1 mm from the size mentioned

6.4.2 Inactivation and Spray Drying of Broth

It was observed that the heat treatment and chemical treatment inhibits the survival of bacteria and even single bacteria were not grown after treatment at 60°C and 0.4 % of 40% formalin. The antibacterial potential of metabolite was persistent even after treating at 160°C for 30 min and even at 5% of 40% formalin (Table 6.5). A small difference in the zone of inhibition (~2 - 4mm) was observed with the increase in temperature and concentration of formalin. The heat inactivation step was followed by spray drying [Advanced drying systems (SD - 50 kg h^{-1})] to yield a crude fermented spray dried product (2%) with bioactive properties (microbial product - Mp) (Fig 6.4).

Table 6.5 The inhibitory activity and bacterial growth of scaled up cultured broth using disc diffusion against *V. harveyi* 101 after inactivation step

Heat treatment (°C)										
Temperature	**40**	**50**	**60**	**70**	**80**	**90**	**100**	**120**	**160**	**Control (RT)**
Zone of inhibition (mm)	20	20	19	19	19	18	18	17	16	20
CFU/mL	3×10^8	1×10^8	0	0	0	0	0	0	0	22×10^8
Chemical treatment										
40% Formalin (%)	**0.1**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**	**2.0**	**5.0**	**Control**	
Zone of inhibition (mm)	18	17	16	16	16	15	15	14	18	
CFU/mL	120	80	0	0	0	0	0	0	15×10^8	

The inhibition zone (mm) values may differ $\pm$ 1mm from the size mentioned

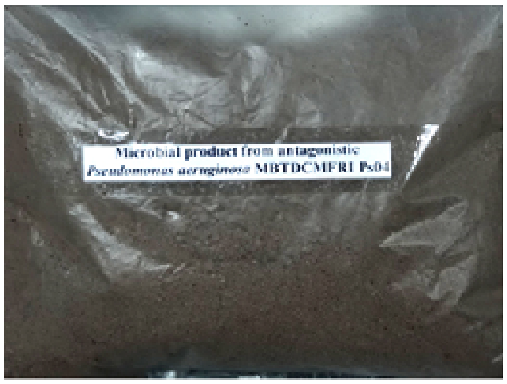

Fig 6.4 Crude fermented powder (Microbial product-Mp) after spray drying of scaled up cultured broth

6.4.3 Minimum Inhibitory Concentration and Cytotoxicity of the Crude Fermented Product

Different concentrations of microbial product in the range (12.5-50000 µg /mL) were tested for identifying the minimum inhibitory concentration (MIC) and the results were given in Table 6.6. A variation was observed in the MIC results of the tested microorganisms (*V. harveyi* 101, *V. alginolyticus* 101 and *V. anguillarum* O1) and the growths were completely inhibited at the concentration of 782 - 3125 µg/mL of microbial product (Fig 6.5).

Table 6.6 Minimum inhibitory concentration of the microbial product

Pathogens	*V. harveyi* 101	*V.alginolyticus* 101	*V.anguillarum* 01
MIC value(µg/ml)	782	3125	3125

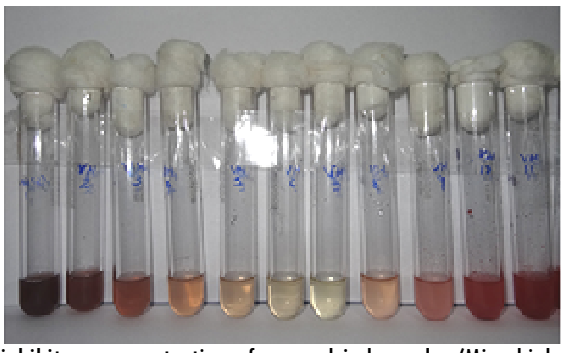

Fig 6.5 Minimum inhibitory concentration of spray dried powder (Microbial product-Mp) against *V.harveyi* 101

The effect of crude fermented product on the growth of fish fibroblast cell line was examined. Monolayer cultures of the cells were exposed to various concentrations of spray dried powder and the morphological changes in the cells were evaluated periodically for 48 h by exposing under microscope (Fig 6.6). The crude fermented product was found to be non - toxic to fish cell lines up to 0.2%. Beyond that it started disrupting the cells slowly and further increase in the concentration of powder led to the lysis of the cell gradually. A high toxicity was observed with pathogenic control with its small concentration of cell free supernatant.

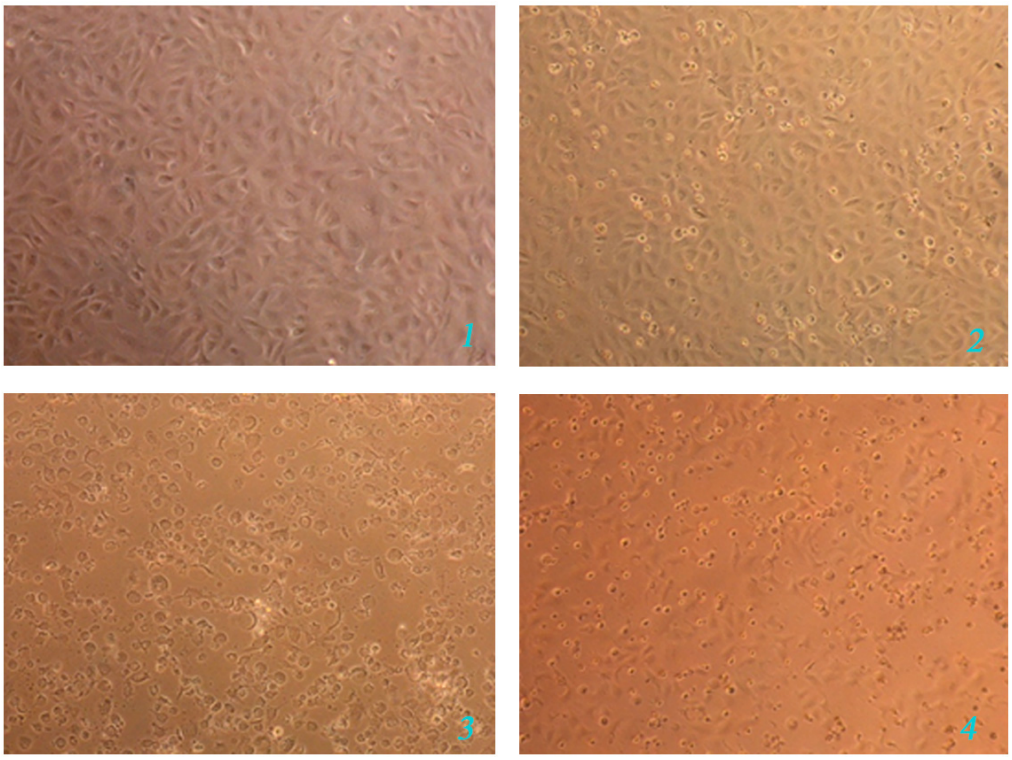

Fig 6.6 Effect of different concentration of microbial product in fish fibroblast cell lines

Legends : Concentration of Mp's - (1) - 2 mg/ml , (2) - 3 mg/ml, (3) - 5 mg/ml, (4) - 10 mg/ml

6.4.4 Nutritional Evaluation and Feeding of the Newly Developed Feed

The granules obtained after granulation technology were passed through successive sieves to separate the granules in various fractions, with a size ranging between 200 - 220 µm (red colour), 100 - 120 µm (yellow colour) and 60 - 80 µm (green colour), were named as Feed 1, Feed 2 and Feed 3 respectively (Fig 6.7). The result of proximate analysis of feed on dry basis is tabulated on Table 6.2. There was only slight variation in protein, lipid and carbohydrates level in all the tested feeds. The nutrient profile of newly developed micro feed revealed that it contained of 55.60±0.92% of crude protein, 14.72±2.28% of crude fat, 14.67±1.41% of Soluble carbohydrate (NFE), 10.65±1.05% of crude ash, 3.30±0.51% of crude fibre and 1.07±0.78% of crude silicates.

Fig 6.7 Newly developed *Vibrio* antagonistic larval feed with various micron sizes

During the initial phase of the study (on zoea and mysis stage), the growth and survivability of the larvae of *F. indicus* did not vary much between the experimental and control tanks. At the end of the experiment the growth

diverge between experimental and control tanks. It was observed that experimental feeds (Feed 1, Feed 2 and Feed3) produced high survivability and growth of larvae (Fig 6.8). There was a higher percentage of mortality in zoea and mysis stage compared to post larval stage. The larvae fed with newly formulated feed from the present work had a higher growth rate than fed with the commercial feed used as control. Also the novel antagonistic feed was found to be more effective in enhancing the health benefits of the cultured animals than commercial feed used (Table 6.7 & Fig 6.9). Moreover the conversion/completion of larval stages was also fast when fed with newly granulated feed. Further, our result showed that if the newly formulated feed was used along with the selected microalgae (co feeding) up to mysis stage, it further enhances the health benefits of the cultured animals at the initial phase to a great extent The results clearly showed that the newly formulated antagonistic micro feed can advantageously replace the live feed in the larval nutrition of shrimp and the new formulated feed also helps in the higher survival rate of larvae, compared to commercial feed. The present study opens up new vistas in the development of indigenous and novel larval feed with added antagonistic benefits for shrimp hatchery rearing, in the place of costly imported feeds.

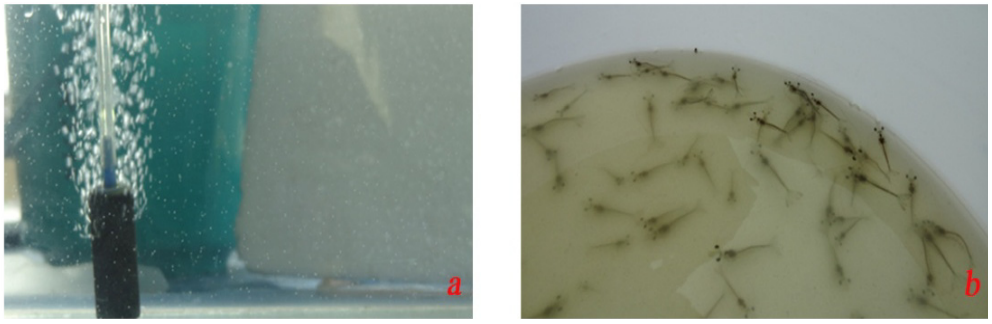

Fig 6.8 Different stages of *F.indicus* larvae before and after the feeding trial with the newly developed feed
Legend: (a) Naupli stage for the intiation of feeding trail (b) Post larval stage (PL20) after finishing the feeding trial

Table 6.7 Percentage of survival of *F. indicus* larvae

Stage Conversion	Survival rate (%)			
	New feed	New feed + algae	Commercial feed	Commercial feed+ algae
Zoea - Mysis	66	72	60	68
Mysis - Post larvae 1	83	89	77	84
Post larvae 2 - Post larvae 20	89	94	80	87

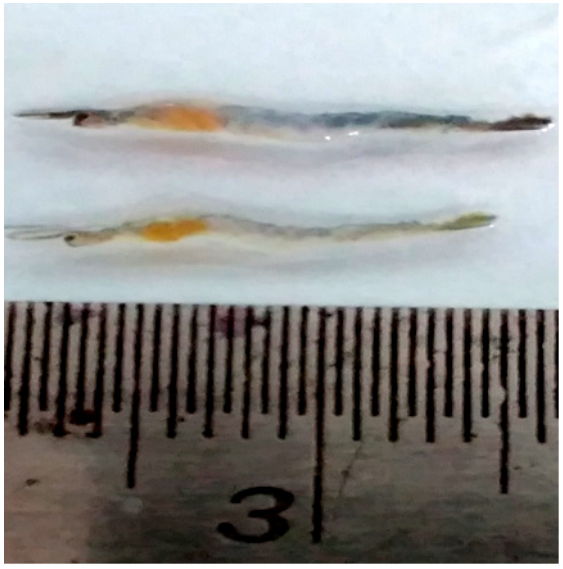

Fig 6.9 Effect of feed on *F.indicus*
Top - animals fed with newly deveoped feed, Bottom: animals fed with commercial diet

6.5 Discussion

The present work exemplified the development of an appropriate bioprocess technology for mass production of the already identified potential antagonistic bacterial strain *P. aeruginosa* Ps04. Further, this work lead to the formulation of a novel functional feed incorporating a microbial product with proven antibacterial properties from the bacterial isolate.

The culture conditions such as pH, temperature and salinity have important roles to play in the biomass production and antagonistic activity as it responded more or less uniformly to the combination of these three factors

(Vijayan et al. 2006). It has been observed that for improving the efficacy of the bioprocess for the commercial production, optimization of physiochemical factors is a pre requisite as majority of the probiotics/ antagonistic bacteria used in aquaculture system have been isolated from natural environment (Jayaprakash et a1. 2005). Considering the biomass and antagonistic activity against aquaculture pathogens, the culture conditions of the *P. aeruginosa* Ps04 was already standardized as 20 ppt salinity, pH 7 ± 0.2 & and temperature 30 ± 2°C. A proportional increase on biomass and antibacterial activity was observed with fermenter conditions maintained at 100 rpm with 4% starter concentration and 0.5 LPM aeration.

Inactivation of bacteria can be achieved by heat, pressure and chemical agents (formaldehyde and sodium hypochlorite). However the chemical agents were found to be toxic or irritating to skin and mucus membrane (Baker et al. 2000) of the host. In our study also, temperature inactivation was found to be more efficient method (by heating above 60ºC for 15 - 30 min) in the inactivation of the tested bacterial strains. This is in agreement with the study of Villamiel and Jong (2000) who reported the reduction in growth of *Pseudomonas fluorescens* when they were inactivated at a temperature higher than 58ºC. Though inhibition of bacterial growth and maintaining the antagonistic potential was sustained after inactivation by both chemical treatment and heat treatment, we preferred to go for inactivation of *P. aeruginosa* Ps04 by heat treatment at 120ºC for 15 min, as it may not produce any toxic side effects.

The microbial product completely inhibited the growth and multiplication of tested bacterial pathogens. The variation in the MIC values against different tested pathogens may be strain dependent factor (Yousef and Danial 2012). Tetrazolium salts were commonly used for the detection of

bacterial growth by colour change in the broth micro dilution method (Johnson 1985), which used in the present study was proven useful in the determination of pathogenic susceptibility. Cytotoxicity assay results of the microbial product proved that it is non - toxic to fish cell lines up to 0.2% while cell free supernatant of pathogenic control was highly toxic to the monolayer even at small concentrations. From the above results we can suggest that the spray dried crude fermented powder (Microbial product- Mp) at 0.2% can be incorporated as an active ingredient in formulated functional feed for aquaculture animals.

The energy requirements of aquatic animals are made available mainly by nutritional resources like protein, carbohydrate and lipid, among which protein sources are considered to be the most important feed components for higher growth rate and survivability (Johnston et al. 2003; Martinez-Cordova et al. 2003). In the newly formulated feed, the protein concentration was above 50% in all three feeds, and protein levels are considered as an important criterion in the larval feed. The newly formulated larval feed blend can be considered as an improved formulation, as it produced comparatively higher growth and larger sized larvae than those fed with the commercial feed. Stage conversion was also slightly faster in larvae fed with newly formulated feed. Almost similar growth and survival rate of larvae was observed in all treatments fed with experimental diets. Larvae with better survival percentages and growth rate suggested that the diet supplemented was sufficient to satisfy the essential macronutrient requirements required for the growing shrimp larval stages (Jones et al. 1997; Pedroza-Islas et al. 2004). The differences in the nutritional composition and suitability of the diet will affect the larval survival which is the driver of the economic viability of the hatcheries, and the availability of health seed is vital for the successful grow out phase (Vijayagopal et al. 2012). The survivability rate was observed to be less in zoea and mysis stages compared to

post larval stage, which need to be addressed further in the future studies. Co feeding with experimental diet and microalgae showed comparatively higher survivability of larvae than that fed with experimental feed alone, suggests the importance of live feed along with artificial feed in the early stages of larval cycle to complete its nutritional balance.

The present study illustrated the optimization of bioprocess technology for pilot scale production of active metabolites from *P. aeruginosa* MBTDCMFRI Ps04 which possess wide spectrum of antibacterial activity against aquaculture bacterial pathogens. The formulation of *Vibrio* antagonistic larval feed was developed using microbial product (Mp) obtained as spray dried form of fermented cultured broth. The acceptance of newly formulated feed by *F. indicus* larvae and their better survivability showed that it can also be grown effectively on formulated larval feed like live feed. Higher survival and growth rates of larvae have been achieved by supplementing the newly developed formulation in comparison with the commercial one. It further showed that substitution of live feed with formulated larval diet will be sufficient enough to enhance the growth, survival and development of the tested *F. indicus* larvae. The newly developed larval micro feed was tested and proved its suitability as a larval micro feed using *F. indicus* as a model, and can also serve as an import substitute. The formulated microfeed can also be used as a replacement for live microalgal feed in the hatchery rearing of aquaculture species, of both shellfish and finfishes.

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CONCLUSION AND SUMMARY

Disease outbreaks due to pathogenic bacteria especially in larval rearing systems have presented as a major challenge. This adversely affects the development of aquaculture which is the fastest growing food production sector in the world. *Vibrio* is considered as the major causative agent causing mass mortalities in shrimp larval rearing system. Conventional therapeutic methods to control bacterial diseases are the use of chemicals, antibiotics, vaccination etc. But later on it resulted in multiple antibiotic resistance in pathogens and horizontal transmission of plasmids from aquaculture pathogens to those of humans. Apart from this, the chemical residues which are left behind, adversely affect the environment and public health. Vaccination or immunization for the control of bacterial diseases is not recommended due to non-specific immune response in shellfish and individual vaccination to the huge population is not feasible. Thus the use of live bacteria or heat killed microbial product (MP) with antagonistic properties emerged as an alternative approach to address the problem and subsequently became a standard practice in aquaculture. Because of the ease in application and lack of side effects the use of antagonistic bacteria such as *Bacillus*, *Pseudomonas*, *Alteromonas* and *Flavobacterium* are gaining importance in aquaculture industry to control bacterial diseases.

The tropical estuarine habitat of Cochin, located along the southwest coast of India, is one of the most biologically productive regions with assorted ecological systems.The present research work deals with a detailed exploration of the diversity of culturable bacteria with antagonistic properties from various ecological niches of Cochin estuary for developing strategies to control bacterial

diseases in aquaculture. Rapid screening and identification of bacterial populations by conventional methods is laborious, cost-intensive, and time-consuming. In order to develop a faster screening system, a colony multiplex PCR (cmPCR) method was developed and evaluated for the rapid detection of major antagonistic genera *Bacillus* and *Pseudomonas*. Later on two potent antagonistic strain *Bacillus subtilis* MBTDCMFRI Ba37 and *Pseudomonas aeruginosa* MBTDCMFRI Ps04 have been evaluated as potential biocontrol agent. The bioactive compounds extracted from these species were found to be useful as a drug of choice against pathogens. Further the potentials of the microbial products as a supplement to feed were also explored to use as an alternative eco-friendly approach for the control of bacterial diseases in aquaculture.

The present work has been divided into following:

- Diversity and characterization of antagonistic bacteria
- Molecular approach for the rapid detection of *Bacillus* and *Pseudomonas* genera using colony multiplex PCR
- Characterization of the *Bacillus subtilis* MBTDCMFRI Ba37 and its bioactive compound
- Characterization of the *Pseudomonas aeruginosa* MBTDCMFRI Ps04 and its bioactive compounds
- Development of microbial products and evaluating their efficacy as aquaculture supplement

Salient Finding of the Present Investigations

- From eight locations of Cochin estuary during the collection period (June 2009 - May 2010), 4,870 bacteria were isolated, out of which

2,960 isolates were from sediment samples and 1,910 were from sub-surface water samples

- Among the 4,870 isolates screened, approximately 1 % (48 isolates) showed significant antibacterial activity against six common aquaculture pathogens belonging to the genera *Aeromonas* and *Vibrio*.
- The antagonistic bacteria were identified by biochemical and16S rRNA gene sequence homology and they belonged to the genera *Bacillus* (81 %) and *Pseudomonas* (19 %).
- The antagonistic isolates were also efficient in producing different varieties of enzymes, tolerate and grow under a wide range of environmental conditions which would help them in the better adaptation towards the host and the aquatic environment aiding their function as biocontrol agent.
- A colony multiplex PCR (cmPCR) method was developed and evaluated for the rapid detection of genus *Bacillus* and *Pseudomonas*. This technique amplifies the partial 16s rRNA gene of *Bacillus* and *Pseudomonas* with a product size of ~1,100 and ~375 bp, respectively, using single forward (BSF2) and two reverse primers (PAGSR and BK1R)
- This molecular screening tool developed helps in the rapid and specific identification of the genera *Bacillus* and *Pseudomonas* simultaneously. Thus, the newly developed molecular approach provides a quick, sensitive, and potential screening tool to detect novel, antagonistically important *Bacillus* and *Pseudomonas* genera for their use in aquaculture.

- *B. subtilis* Ba37 (10^4 - 10^8 CFU/ml) *controls the grow*th of *V. anguillarum* O1 (10^2 - 10^4 CFU/ml) in co-culture assay.
- The cell free supernatant of *B. subtilis* Ba37 was found to be non-toxic to the koi carp fibroblast cell lines but the *V. anguillarum* O1 is highly toxic to the cell lines
- *B. subtilis* Ba37 was found to be non-lethal to *E. suratensis* juveniles when challenged at 10^8 CFU/ml and on sub-adults at a concentration of 10^6 CFU/ml to 10^8 CFU/ml.
- A synthetic medium (modified *Bacillus* medium) has been optimized for *Bacillus* spp. which helps in the increased production of bioactive compounds and biomass.
- Bioassay guided purification followed by the spectroscopic characterization of active fraction revealed that the compound 1-(1-Hydroxyethyl)-1,7,10,12,13,15,17heptamethyl-16-oxatetracyclo 8.7.0.$0^{2,3}$.$0^{12,13}$]heptadecan-5-one is responsible for its antibacterial activity in *B. subtilis* Ba37
- In co-culture experiment, *P. aeruginosa* Ps04 (10^4 - 10^8 CFU/ml) controls the growth of *V. harveyi* 101 (10^2 - 10^4 CFU/ml).
- The cell free supernatant of *P. aeruginosa* Ps04 was found not toxic to the koi carp fibroblast cell lines but the *V. harveyi* 101 is highly toxic to the cell lines
- *P.aeruginosa* Ps04 was found to be non-lethal to *E. suratensis* juveniles when challenged at 10^8 CFU/ml and on sub-adults at a concentration of 10^6 CFU ml^{-1} to 10^8 CFU/ml.

- A synthetic medium (glycerol alanine medium) has been optimized for *Pseudomonas* spp. which helps in the increased production of bioactive compounds and biomass.
- Bioassay guided purification followed by the spectroscopic characterization of active fraction revealed that the compound 4-Hydroxy-11-methylpentacyclo[11.8.0.$0^{2,3}$.$0^{11,12}$.$0^{16,17}$]henicosa-1,3,5,8(9),17-penten-14-one is responsible for its antibacterial activity in *P. aeruginosa* Ps04
- Optimal conditions for pilot scale production of antagonistic compound (2 L & 10 L) using the nutrient broth supplemented with 50% SW were achieved at aeration 0.5 LPM, agitation 100 rpm, inoculum size of 4%, pH - 7.5 ± 0.2 , 31 °C at 96 h in New Brunswick BioFlo/ CelliGen 115 Fermentor.
- The fermented cultured broth was inactivated according to standard protocol by heating at 120 °C for 15 minutes as the bacteria is an opportunistic pathogen to aquaculture systems followed by spray drying yield a crude fermented product (2%) with bioactive properties (microbial product - Mp)
- The growths of pathogens (*V. harveyi* 101, *V. alginolyticus* 101 and *V. anguillarum* O1) at 10^5 CFU/mL were completely inhibited by 782 - 3125 µg/mL of Mp**.**
- The crude fermented product was found to be non - toxic to fish cell lines up to 0.2%.
- The new formulated blend contains 200 g anchovy meal, 200 g shrimp meal, 200 g clam meal, 300 g soya flour, 50 g fish oil, 5 g vitamin C,

28 g mineral mixture, 3g vitamin mixture, 4 g mixed carotenoids, 4 g spirulina, 1 g antioxidant, 1 g antifungal, 2 g lecithin and 2 g microbial product with bioactive properties per kg.

- The feed mix was extruded and powdered into various micron sizes of 60 - 80 µm, 100 - 120 µm and 200 - 220 µm using granulation technology wherein said suspending aids are fillers in an amount of at least 2% by weight of micro feed
- The nutrient profile of newly developed micro feed revealed that it contained of 55.60 ± 0.92% of crude protein, 14.72 ± 2.28% of crude fat, 14.67 ± 1.41% of soluble carbohydrate (NFE), 10.65 ± 1.05% of crude ash, 3.30 ± 0.51% of crude fibre and 1.07 ± 0.78% of crude silicates.
- The newly formulated feed is found to be more effective in enhancing the health benefits of the aquacultured animals in the larval rearing systems than commercial feed used.
- The newly formulated feed is used along with the selected microalgae (co feeding) thus enhancing the health benefits of the cultured animals at the initial phase to a great extent.
- The invented formulated feed increases the survival rate of larvae compared to commercial feed. The results clearly showed that the newly formulated micro feed can advantageously replaces the live feed in the larval stage.

Conclusion

The use of the indigenous probiotic isolates generated in this study with potential antibacterial activity have proven as an effective alternative,

substituting commercial antibiotics in aquaculture. This would open up new horizons for the development and practice of ecofriendly strategies which can be readily adapted in aquaculture industry. The study stands as the first of its kind and emerged with a novel molecular tool, which is quick, sensitive and consistently reliable to screen important genera *Bacillus* and *Pseudomonas* for their use in aquaculture. The same methodologies standardized in this study could also serve as a molecular taxonomic tool to understand the distribution of these genera from wide ecological niches and their exploitation for diverse biotechnological applications. The bacteria with beneficial characteristics could be useful as a candidate potential probiont in aquaculture systems to manage and control bacterial infections We have further characterized the biologically active molecule through various spectroscopic methods from the antagonistic bacteria which in turn helps to expand drug discovery program against aquaculture pathogens. Utilizing the baseline data and products evolved a novel *Vibrio* antagonistic larval feed with nutritional ingredients supplemented by microbial product (Mp) was developed for use in aquaculture and readily substitutes the antibiotics to a large extent. The present study stands as the first one of its kind involved in screening the indigenous habitat for want of potential probiotic and readily utilized its potential in the form of a feed to be used in aquaculture. Such approaches would definitely initiate the process of screening more of natural environments and thereby making the aquaculture more ecofriendly and sustainable which is an important step towards food security.

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REFERENCES

Altinok I (2011) Multiplex PCR assay for detection of four major bacterial pathogens causing rainbow trout disease. Dis Aquat Organ 93(3):199-206

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI- BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17):3389-3402

Aly SM, Abd-El-Rahman AM, John G, Mohamed MF (2008) Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. Aquaculture 277:1–6

Association of Official Analytical Chemist (1990) Official methods of analysis of the association of official analytical chemists (W. Worwitz ed.) 18th Ed. Washington D.C.

Augusto da Costa AC, Duta FP (2001) Bioaccumulation of copper, zinc, cadmium and lead by *Bacillus* sp., *Bacillus cereus*, *Bacillus sphaericus* and *Bacillus subtilis*. Brazil J Microbiol 32:1-5

Austin B, Zhang XH (2006) *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett. Appl. Microbiol 43:119–124

Baker JR, Wright DC, Hayes MM, Hamouda T and Brisker J (2000) Methods of inactivating bacteria including bacterial spores .U S patent No. 6015832

Bakker PAHM, Pieterse CMJ, Van Loon LC (2007) Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology 97:239–243

Balcazar JL, De Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Mu´zquiz JL (2006) The role of probiotics in aquaculture. Vet Microbiol 114:173–186

Balcazar JL, Rojas-Luna T (2007) Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). Curr Microbiol 55(5):409-412

Bandyopadhyay P, Mohapatra PKD (2009) Effect of a probiotic bacterium *Bacillus circulans* PB7 in the formulated diets: on growth, nutritional quality and immunity of *Catla catla* (Ham.). Fish Physiol Biochem 35:467–478

Barker G (2000) Novel methods to reduce disease in aquaculture. Fish Vet J 5:66-71

Baruzzi F, Quintieri L, Morea M and Caputo L (2011) Antimicrobial compounds produced by *Bacillus Spp*. and applications in food science against microbial pathogens: communicating current research and technological advance. A Méndez Vilas (E d.) 1102-1111

Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45(4): 493–496

Berdy J (2005) Bioactive microbial metabolites. J Antibiot (Tokyo) 58(1):1-26

Bizani D and Brandelli A (2002) Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. Strain 8 A. J Appl Microbiol 93:512–519

Bobbie RJ, White DC (1980) Characterization of benthic microbial community structure by high-resolution gas chromatography of fatty acid methyl esters. Appl Environ Microbiol 39(6):1212-1222

Boonthai T, Vuthiphandchai V, Nimrat S (2011) Probiotic bacteria effects on growth and bacterial composition of black tiger shrimp (*Penaeus monodon*). Aquacult Nutr 17:634-644

Boyd CE (1985) Chemical budgets for channel catfish ponds. Trans Am Fish Soc 114:291-298

Cahill MM (1990) Bacterial flora of fishes: A review Microbial Ecology 19:21-41

Cao H, He S, Wei R, Diong M, Lu L (2011) *Bacillus amyloliquefaciens* G1: A Potential antagonistic bacterium against Eel-pathogenic *Aeromonas hydrophila*. Evid Based Complement Alternat Med. doi: 10.1155/ 2011/824104.

Chamberlain JS, Chamberlain JR (1994) Optimization of multiplex PCRs. In: The polymerase chain reaction (Mullis KB, Ferre F, Gibbs RA [eds]). Birkhauser, Boston

Chen J, Tanq J, Liu J, Cai Z, Bai X (2012) Development and evaluation of a multiplex PCR for simultaneous detection of five food borne pathogens. J Appl Microbiol 112(4):823–830

Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z (2014) Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front Microbiol 13:5:217. doi: 10.3389/fmicb.2014.0021

Chiang YC, Yang CY, Li C, Ho YC, Lin CK, Tsen HY (2006) Identification of *Bacillus* spp., *Escherichia coli*, *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp. with 16S ribosomal DNA-based oligonucleotide array hybridization. Int J Food Microbiol 107(2):131-137

Chythanya R, Karnasagar I, Karunasagar I (2002) Inhibition of shrimp pathogenic *vibrios* by a marine *Pseudomonas* I-2 strain. Aquaculture 208(1–2):1–10

Clayden J, Geeves N, Warren S (2012) Organic Chemistry 2nd edition, ISBN: 978-0199270293. Oxford University Press

Cruz PM, Ibanez AL, Monroy Hermosillo OA, Ramirez Saad HC, Hermosillo OAM, Mart P, Ib AL, Ram HC (2012) Use of probiotics in aquaculture. ISRN Microbiol doi:10.5402/2012/916845

Cunningham CO (2002) Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture 206:19-55

Cutting S M (2011) *Bacillus* probiotics. Food Microbiol 28:214-220

Das BK, Neha Nidhi NRG, Roy P, Muduli AK, Swain P, Mishra SS and Jayasankar P (2014) Antagonistic activity of cellular components of *Bacillus subtilis* AN11 against bacterial pathogens. Int J Curr Microbiol App Sci 3(5):795-809

Debbab A, Aly AH, Lin WH, Proksc P (2010) Bioactive compounds from marine bacteria and fungi. Microb Biotechnol 3(5):544–563

Defer D, Bourgougnon N, Fleury Y (2009). Screening for antibacterial and antiviral activities in three bivalve and two gastropod marine molluscs. Aquaculture 293:1–7

Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P (2007) Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends Biotechnol 25:472–479

Dopazo CP, Lemos ML, Lodeiros C, Bolinches J, Barja JL and Toranzo E (1988) Inhibitory activity of antibiotic producing marine bacteria against fish pathogens. J Appl Bacteriol 65:97-101

Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ulrich C, Stein T, Leenders F, Vater J (1999) The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: A multifunctional hybrid between a peptide synthetase, an amino transferase and a fatty acid synthetase. Proc Natl Acad Sci USA 96:13294–13299

Ericsson HM and Sherris JC (1971) Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol Microbiol Scand Sect B 217(Suppl.):1-90

Fan H, Wu Q, Kou X (2008) Co-detection of five species of water-borne bacteria by multiplex PCR. Life Science Journal 5(4):47-54

FAO (2010) Statistics and information service of the Fisheries and Aquaculture Department. Fishery and Aquaculture Statistics - 2008. FAO yearbook

FAO (2012) The State of World Fisheries and Aquaculture 2012. Rome, Italy

Felten A, Barreau C, Bixet C, Lagrange PH, Philippon A (1999) *Lactobacillus* species identification, H_2O_2 production, and antibiotic resistance and correlation with human clinical status. J Clin Microbiol 37(3):729-733

Fickers P (2012) Antibiotic Compounds from *Bacillus*: Why are they so Amazing? Am J Biochem Biotech 8(1):40-46

Frans I, Michiels CW, Bossier P, Willems KA, Lievens B, Rediers H (2011) *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. J Fish Dis 34(9):643-61

Frost RL, Wain DL, Martens WN, Reddy BJ (2007) Vibrational spectroscopy of selected minerals of the rosasite group. Spectrochimic Acta Part A: Mol Biomol Spectrosc 66(4-5): 1068-1074

Freshney RI (2006) Culture of Animal Cells: A manual of basic technique. (Fifth Ed.) New Jersey: Wiley Liss, Inc

Gandhi M, Sangwan V, Kapoor KK, Dilbaghi N (1997) Composting of household wastes with and without earthworms. Environ Ecol 15:432–434

Garbeva P, Veen JA, Elsas JD (2004) Assessment of the diversity, and antagonism towards *Rhizoctonia solani* AG3, of *Pseudomonas* species in soil from different agricultural regimes. FEMS Microbiol Ecol 47(1):51-64

Geovanny D Gomez, Balcazar JL (2008) A review on the interactions between gut microbiota and innate immunity of fish. FEMS Immunol Med Microbiol 52:145–154

Gillor O, Etzion A, Riley MA (2008) The dual role of bacteriocins as anti- and probiotics. Appl Microbiol Biotechnol 81(4):591-606

Gomez-Gil B, Roque A, Turnbull JF (2000) The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191:259–270

Gram L, Melchiorsen J, Spanggaard B, Huber I & Nielsen TF (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Appl Environ Microbiol 65:969-973

Guetsky R, Shtienberg Y, Elad Y, Fischer E, Dinoor A (2002) Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. Phytopathology 92:976–985

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment and editor program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98

Hammami I, Rhouma A, Jaouadi B, Rebai A, Nesme X (2008) Optimization and biochemical characterization of a bacteriocin from a newly isolated *Bacillus subtilis* strain 14B for biocontrol of *Agrobacterium* spp. strains. Lett Appl Microbiol 48(2):253-260

Hancock RE (1998) Resistance mechanisms in *Pseudomonas aeruginosa* and other non fermentative gram-negative bacteria. Clin Infect Dis 27(suppl 1):S93–S99

Harvey A (2000) Strategies for discovering drugs from previously unexplored natural products. Drug Discov Tod 5(7) 254-.

Heuer OE, Kruse H, Grave K, Cillignon P, Karunasagar I, Angulo FJ (2009) Human health consequences of use of antimicrobial agents in aquaculture. Clin Infect Dis 49(8):1248-1253

Hill JE, Baiano JCF and Barnes AC (2009) Isolation of a novel strain of *Bacillus pumilus* from *penaeid* shrimp that is inhibitory against marine pathogens. J Fish Disease 32:1007–1016

Hofmann MA, Brian DA (1991) Sequencing PCR DNA amplified directly from a bacterial colony. Biotechniques 11(1):30-31

Hofte M, Altier N (2010) Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. Res Micro 161:464–471

Hong HA, Duc LH, Cutting SM (2005) The use of bacterial spore formers as probiotics. FEMS Micro Rev 29(2005):813–835

Ibrahem M D (2015) Evolution of probiotics in aquatic world: Potential effects, the current status in Egypt and recent prospective. J Adv Res (2015) 6:765–791

Irianto A, Austin B (2003) Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 26(1):59–62

Irianto A, Robertson PAW, Austin B (2003) Short communication Oral administration of formalin-inactivated cells of *Aeromonas hydrophila* A3-51 controls infection by atypical *A. salmonicida* in goldfish, *Carassius auratus* (L.). J Fish Dis 26(2):117–120

Irie T, Watarai S, Iwasaki T, Kodama H (2005) Protection against experimental *Aeromonas salmonicida* infection in carp by oral immunization with bacterial antigen entrapped liposomes. Fish Shellfish Immunol 18:235-242

Isnansetyo A, Kamei Y (2009) Bioactive substances produced by marine isolates of *Pseudomonas* sp. J Ind Microbiol Biotechnol 36:1239–1248

Jamil B, Hasan F, Hameed A, Ahmed S (2007) Isolation of *Bacillus subtilis* MH-4 from soil and its potential of polypeptidic antibiotic production. Pak J Pharm Sci (2007) 20(1):26-31

Janakiram P, Veerendra kumar M, Jayasree L, Sivaprasad B (2014) Probiotic activity of *Pseudomonas aeruginosa* (PIC-4) isolated from Visakhapatnam coast, Bay of Bengal, India, against *Vibrio harveyi* in *Penaeus monodon.* Int Fish Aquat Stud 1(6):199-207

Janda JM, Abbott SL (2007) 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. J Clin Microbiol 45(9):2761–2764

Jayaprakash NS, Pai SS, Anas A, Preetha R, Philip R, Singh ISB (2005) A marine *Micrococcus* MCCB 1 04 antagonistic to *vibrios* in prawn larval rearing systems. Dis Aquat Org 68:39-45

Jayatilake GS, Thornton MP, Leonard AC, Grimwade JE, BakerBJ (1996) Metabolites from an antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. J Nat Prod 59:293–296

Johnson TL, Forbes BA, O'Connor-Scarlet M, Machinski A and McClatchey KD (1985) Rapid method of MIC determinations utilizing tetrazolium reduction. Am J Clin Pathol 83:374–378

Johnston DJ, Calvert KA, Crear BJ and Carter CG (2003) Dietary carbohydrate/lipid ratio and nutritional condition in juvenile southern rock lobster, *Jasus edwardsii*. Aquaculture 220: 667-682

Jones DA, Yule AB & Holland DL (1997) Larval Nutrition. In: Crustacean Nutrition (D'Abramo LR, Conklin DE & Akiyama DM eds), World Aquaculture Society, Louisiana, USA pp 353–382

Jose J, Giridhar R, Anas A, Bharathi PAL, Nair S (2011) Heavy metal pollution exerts reduction/adaptation in the diversity and enzyme expression profile of heterotrophic bacteria in Cochin estuary, India. Environ Pollut 159(10):2775-2780

Kamgar M, Pourgholam R, Ghiasi M, Ghane M (2013) Studies on *Bacillus subtilis* as Potential Probiotics, on the Biochemical Parameters of Rainbow trout, *Oncorhynchus mykiss* (Walbaum) to Challenge. Inf Adv Stud Biol 5(1):37-50

Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A (2008) A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Curr Microbiol 57(5):503-507

Kasanah N, Hamann MT (2004) Development of antibiotics and the future of marine microoganisms to stem the tide of antibiotic resistance. Curr Opin Investig Drugs 5(8):827-37

Kaushik SJ (2000) Feed formulation, diet development and feed technology CIHEAM - Options Mediterraneennes 43-51

Kennedy J, Baker P, Piper C, Cotter PD, Walsh M, Mooij MJ, Bourke MB, Rea MC, O'Connor PM, Ross RP, Hill C, O'Gara F, Marchesi JR, Dobson ADW (2009) Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. Mar Biotechnol 11(3):384-396

Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L (2008) Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. Aquaculture 274(1):1–14

Keysami MA, Mohammadpour M, Saad CR (2012) Probiotic activity of *Bacillus subtilis* in juvenile freshwater prawn, *Macrobrachium rosenbergii* (de Man) at different methods of administration to the feed. Aquacult Int 20:499–511

Khan NH, Ishii Y, Kimata-Kino N, Esaki H, Nishino T, Nishimura M, Kogure K (2007) Isolation of *Pseudomonas aeruginosa* from Open Ocean and comparison with freshwater, clinical, and animal isolates. Microb Ecol 53(2):173-86

Kong P, Richardson PA, Hong C (2005) Direct colony PCR-SSCP for detection of multiple pythiaceous oomycetes in environmental samples. J Microbiol Methods 61(1):25-32

Krieg NR, Holt JG (1984) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore

Kwon GH, Lee HA, Park JY, Kim JS, Lim J, Park CS, Kwon DY, Kim YS, Kim JH (2009) Development of a RAPD-PCR method for identification of *Bacillus* sp. isolated from Cheonggukjang. Int J Food Microbiol 129:282-287

Kwon HS, Yang EH, Lee SH, Yeon SW, Kang BH, Kim TY (2005) Rapid identification of potentially probiotic *Bifidobacterium* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol Lett 250:55-62

Lalloo R (2010) Development of a bioprocess for the production of an aquaculture biological agent. University of Stellenbosch

Lalloo R, Maharajh D, Görgens J, Gardiner N (2008) Functionality of a *Bacillus cereus* biological agent in response to physiological variables encountered in aquaculture. Appl Microbiol Biotechnol 79:111–118

Lalloo R, Maharajh D, Gorgens J, Gardiner N (2010) A downstream process for production of a viable and stable *Bacillus cereus* aquaculture biological agent. Appl Microbiol Biotechnol 86:499–508

Lambert PA (2002) Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. J R Soc Med 95(Suppl. 41):22–26

Lauzon HL, Gudmundsdottir S, Pedersen MH, Budde BB, Gudmundsdottir BK (2008) Isolation of putative probionts from cod rearing environment. Vet Microbiol 132:328–39

Lee KB (2005) A media design program for lactic acid production coupled with extraction by electrodialysis. Bioresour Technol 96:1505-1510

Lenski RE, Riley MA (2002) Chemical warfare from an ecological perspective. Proc Natl Acad Sci USA 99:556–558

Liao PB, Mayo RD (1974) Intensified fish culture combining water re-conditioning with pollution abatement. Aquaculture 3:61-85

Lisboa MP, Bonatto D, Bizani D, Henriques JA, Brandelli A (2006) Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquifaciens* isolated from the Brazilian Atlantic forest. Int Microbiol 9:111–118

Logan NA, Berkeley RCW (1984) Identification of *Bacillus* strains using the API system. J Gen Microbiol 130(7):1871-1882

Long RA, Azam F (2001) Antagonistic interactions among marine pelagic bacteria. Appl Environ Microbiol 67(11):4975-4983

Maeda Y, Shinohara H, Kiba A, Ohnishi K, Furuya N, Kawamura Y, Ezaki T, Vandamme P, Tsushima S, Hikichi Y (2006) Phylogenetic study and multiplex PCR-based detection of *Burkholderia plantarii*, *Burkholderia glumae* and *Burkholderia gladioli* using gyrB and rpoD sequences. Int J Syst Evol Microbiol 56(Pt 5):1031-1038

Mahdhi A, Kamoun F, Bakhrouf A (2011) Inhibitory activity and adhesive ability of potential probiotic *Bacillus* species to confer protection for *Artemia* gnotobiotic culture against pathogenic *Vibrio* spp. Turk J Vet Anim Sci 35(4): 227-233

Mangaiyarkarasi MSM, Vincent S, Janarthanan S, Rao TS, Tata BVR (2011) Bioreduction of Cr(VI) by alkaliphilic *Bacillus subtilis* and interaction of the membrane groups. Saud of Biol Sci 18:157–16

Mann CJ, Wetzel RG (1995) Dissolved organic carbon and its utilization in a riverine wetland ecosystem. Biogeochemistry 31:99-120

Martinez-Cordova LR, Campana-Torres A and Porchas-Cornejo MA (2003) Dietary protein level and natural food management in the culture of blue (*Litopenaeus stylirostris*) and white shrimp (*Litopenaeus vannamei*) in microscosms. Aquacult. Nutr 9:155-160

Menon NN, Balchand AN, Menon NR (2000) Hydrobiology of the Cochin backwater system – a review. Hydrobiologia 430(1-3):149-183

Mohapatra S, Chakraborty T, Kumar V, DeBoeck G, Mohanta KN (2013) Aquaculture and stress management: a review of probiotic intervention. J Anim Physiol Anim Nutr 97:405–430

Moore T, Globa L, Barbaree J, Vodyanoy V, Sorokulova I (2013) Antagonistic activity of *Bacillus* bacteria against food-borne pathogens. J Prob Health 1:110 doi: 10.4172/2329-8901.1000110

Motta AS, Cannavan FS, Tsai SM, Brandelli A (2007) Characterization of a broad range antibacterial substance from a new *Bacillus* species isolated from Amazon basin. Arch Microbiol 188(4):367-75

Nagorska K, Bikowski M, Obuchowski M (2007) Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochimica Polonica 54:495–508

Nair AV, Joseph N, Krishna K, Sneha KG, Tom N, Jangid K, Nair S (2015) A comparative study of coastal and clinical isolates of *Pseudomonas aeruginosa*. Brazil J Microbiol 46(3):725-734

Nair AV, Pradeep MA, Vijayan KK (2014) Molecular approach for the rapid detection of *Bacillus* and *Pseudomonas* genera-dominant antagonistic groups—from diverse ecological niches using colony multiplex PCR. J Ind Microbiol Biotechnol. doi 10.1007/s10295-014-1441-4

Nair AV, Vijayan KK, Chakraborty K, Leo Antony M (2012) Diversity and characterization of antagonistic bacteria from tropical estuarine habitats of Cochin, India for fish health management. World J Microbiol Biotechnol 28(7):2581–2592

Nemutanzhela ME, Roets Y, Gardiner N, Lalloo R (2014) The use and benefits of *bacillus* based biological agents in aquaculture. Intech Publishing: Rijeka, Croatia ISBN 978-953-51-1224-2

Neu HC (1992) The crisis in antibiotic resistance. Science 257:1064–1073

Nithya C, Pandian SK (2010) Isolation of heterotrophic bacteria from Palk Bay sediments showing heavy metal tolerance and antibiotic production. Microbiol Res 165(7):578- 593

Olive DM, Bean P (1999) Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol 37(6):1661-1669

Ongena M and Jacques P (2007) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Tren Microbiol 16(3):115-125

Ortega-Morales BO, Ortega-Morales FN, Lara-Reyna J, De la Rosa-García SC, Martínez-Hernandez A, Montero MJ (2009) Antagonism of *Bacillus* spp. isolated from marine biofilms against terrestrial phytopathogenic fungi. Mar Biotechnol 11(3):375-383

Otta SK, Karunasager I, Karunasagar I (1999) Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon*. J Aqua Trop 14:309-318

Ozdemir Z (2009) Development of a multiplex PCR assay for the simultaneous detection of *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato* and *Xanthomonas axonopodis* pv. *vesicatoria* using pure cultures. J Plant Pathol 91(2):495-497

Pai SS, Anas A, Jayaprakash NS, Priyaja P, Sreelakshmi B, Preetha R, Philip R, Mohandas A, Singh ISB (2010) *Penaeus monodon* larvae can be protected from *Vibrio harveyi* infection by pre - emptive treatment of a rearing system with antagonistic or non - antagonistic bacterial probiotics. Aquacult Res 41:847–860

Pedroza-Islas R, Gallardo P, Vernon-Carter EJ, Garci A-Galano T, Rosas C, Pascual C and Gaxiola G (2004) Growth, survival, quality and digestive enzyme activities of larval shrimp fed microencapsulated, mixed and live diets. Aquacult Nut 10:167-173

Peduzzi P, Herndl GJ (1992) Zooplankton activity fueling the microbial loop: differential growth response of bacteria from oligotrophic and eutrophic waters. Limnol Oceanogr 37:1087-1092

Penesyan A, Marshall-Jones Z, Holmstrom C, Kjelleberg S, EganS (2009) Antimicrobial activity observed among cultured marine epiphytic bacteria reflects their potential as a source of new drugs. FEMS Microbiol Ecol 69:113–124.

Perry L, Heard P, Kane M, Kim H, Savikhin S, Dominguez W, Applegate B (2007) Application of multiplex polymerase chain reaction to the detection of pathogens in food. J Rapid Methods Autom Microbiol 15(2):176–198

Preetha R, Jose S, Prathapan S, Vijayan KK, Jayaprakash NS, Philip R, Singh ISB (2010) An inhibitory compound produced by *Pseudomonas* with effectiveness on *Vibrio harveyi*. Aquacult Res 41:1452–1461

Preetha R, Jayaprakash NS, Philip R & Singh ISB (2007) Optimization of carbon and nitrogen sources and growth factors for the production of an aquaculture probiotic (*Pseudomonas* MCCB 103) using response surface methodology. J Appl Microbiol 102:1043-1051

Pridgeon JW, Klesius PH (2012) Major bacterial diseases in aquaculture and their vaccine development. CAB Rev Perspect Agric Vet Sci Nutr Nat Resour 7 doi:10.1079/PAVSNNR20127048

Priest FG, Goodfellow M, Shute LA, Berkeley RCW (1987) *Bacillus amyloliquefaciens* sp. nov nom rev Int J Syst Bacteriol 37(1):69-71

Prieur G, Mevel G, Nicolas JL, Plusquellec A, Vigneulle M (1990) Interactions between bivalve molluscs and bacteria in the marine environment. Oceanogr Mar Biol Annu Rev 28:227-352

Priyaja P, Jayesh P, Neil Scolastin Correya, B Sreelakshmi, NS Sudheer, Rosamma Philip and IS Bright Singh (2014) Antagonistic effect of *Pseudomonas aeruginosa* isolates from various ecological niches on *Vibrio* species pathogenic to crustaceans. J Coast Lif Med 2(1):76-84

Raaijmakers IM, David MW, Thomasshow LS (1997) Frequency of antibiotic-producing *Pseudomonas* spp. in natural environment. Appl Environ MicrobioI 63:881-887

Reva ON, Sorokulova IB, Smirnov VV (2001) Simplified technique for identification of the aerobic spore-forming bacteria by phenotype. Int J Syst Evol Microbiol 51:1361–1371

Rico A, Phu TM, Satapornvanit K, Min J, Shahabuddin AM, Henriksson PJG, Murray FJ, Little DC, Dalsgaard A, Van den Brink PJ (2013) Use of veterinary medicines, feed additives and probiotics in four major internationally traded aquaculture species farmed in Asia. Aquaculture 412–413 231–243

Riley MA, Wertz JE (2002) Bacteriocins: evolution, ecology and application. Ann Rev Microbiol 56:117-137

Saarela M, Mogensen G, Fonden R, Matto J, Mattila-Sandholm T (2000) Probiotic bacteria: safety, functional and technological properties. J Biotechnol 84(3):197–215

Sabate DC Audisio CM (2013) Inhibitory activity of surfactin, produced by different *Bacillus subtilis* subsp. *subtilis* strains, against *Listeria monocytogenes* sensitive and bacteriocin-resistant strains. Microbiol Res 168:125–129

Sadeghi A, Mortazavi SA, Bahrami AR, Sadeghi B (2012) Design of multiplex PCR for simultaneous detection of rope-forming *Bacillus* strains in Iranian bread dough. J Sci Food Agric 92(13):2652-2656

Safronova LA, Zelena LB, Klochko VV, Reva ON (2012) Does the applicability of *Bacillus* strains in probiotics rely upon their taxonomy? Can J Microbiol 58(2):212- 219

Saha S, Thavasi R, Jayalakshmi S (2008) Phenazine pigments from *Pseudomonas aeruginosa* and their application as antibacterial agent and food colourants. Res J Microbial 3:122–128

Sahoo K, Dhal NK (2009) Potential microbial diversity in mangrove ecosystem: A review. Indian J Mar Sci 38(2):249-256

Sahu MK, Swarnakumar NS, Sivakumar K, Thangaradjou T, Kannan L (2008) Probiotics in aquaculture: importance and future perspectives. Indian J Microbiol 48:299–308

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406-425

Salinas I, Diaz-Rosales P, Cuesta A, Meseguer M, Chabrillo M, Morinigo MA, Esteban MA (2006) Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata L.*). Vet Immun and Immunopath : 279–286

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Sanchez-Porro C, Martin S, Mellado E, Ventosa A (2003) Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. J Appl Microbiol 94(2):295-300

Saulnier D, Haffner P, Goarant C, Levy P, Ansquer D (2000) Experimental infection models for shrimp vibriosis studies: a review. Aquaculture 191:133-144

Schulz B, Boyle C, Draeger S, Rommert A, Krohn K (2002) Endophytic fungi: a source of novel biologically active secondary metabolites. J Mycological Soc 106:996-1004

Schulze AD, Alabi AO, Tattersall-Sheldrake AR, Miller KM (2006) Bacterial diversity in a marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains. Aquaculture 256:50–73

Serrano PH (2005) Responsible use of antibiotics in aquaculture. FAO Fisheries Technical Paper. No. 469. Rome, FAO p 97

Sharma S, Kaur M (2010) Antimicrobial activities of rhizobacterial strains of *Pseudomonas* and *Bacillus* strains isolated from rhizosphere soil of carnation (*Dianthus caryophyllus* cv. Sunrise). Indian J Microbiol 50:229-232

Shen WY, Fu LL, Li WF, Zhu YR (2010) Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). Aquac Res 41:1691–1698

Sirisha E, Rajasekar N, Narasu ML (2010) Isolation and optimization of lipase producing bacteria from oil contaminated soils. Adv Biol Res 4(5):249-252

Smoragiewicz W, Bielecka M, Babuchowski A, Boutard A, Dubeau H (1993) Les probiotiques. Can J Microbiol 39:1089–1095

Soto JO, Paniagua-Michel JJ, Lopez L, Ochoa L (2015) Functional Feeds in Aquaculture, Part J Industrial Applications 1304-1317

Spilker T, Coenye T, Vandamme P, LiPuma JJ (2004) PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. J Clin Microbiol 42(5): 2074-2079

Srinivas K, Revichandran C, Maheswaran PA, Mohammed Ashraf TT, Murukesh N (2003) Propagation of tides in the Cochin estuarine system, south west coast of India. Indian Mar Sci 32:14–24

Stackebrandt E, Goebel BM (1994) Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 44(4):846-849

Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56:845-857

Stephenson FH (1994) Calculations for molecular biology and biotechnology - a guide to mathematics in the laboratory. USA: Academic Press. Cell growth pp 42–75

Subasinghe R (2009) Disease control in aquaculture and the responsible use of veterinary drugs and vaccines: The issues, prospects and challenges. The use of veterinary drugs and vaccines in Mediterranean aquaculture Options Mediterraneennes. A/no 86

Subramani R, Aalbersberg W (2012) Marine actinomycetes: An ongoing source of novel bioactive metabolites. Microbiol Res 167:571-580

Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML (2008) Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. J Appl Microbiol 104:1067–1074

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731-2739

Tanaka T, F Rassoulzadegan (2004) Vertical and seasonal variations of bacterial abundance and production in the mesopelagic layer of the NW Mediterranean Sea: bottom-up and top-down controls. Deep Sea Res (I) 51:531-544

Taoka Y, Maeda H, Jo JY, Kim SM, Park SI, Yoshikawa T, Sakata T (2006) Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. Fish Sci 72:755–766

Thirabunyanon M, Thongwittaya N (2012) Protection activity of a novel probiotic strain of *Bacillus subtilis* against *Salmonella enteritidis* infection. Res Vet Sci 93:74–81

Thompson J, Gregory S, Plummer S, Shields RJ, Rowley AF (2010) An in vitro and in vivo assessment of the potential of *Vibrio* spp. as probiotics for the Pacific white shrimp, *Litopenaeus vannamei*. J Appl Microbiol 109:1177–1187

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673-4680

Todorova S, Kozhuharova L (2010) Characteristics and antimicrobial activity of *Bacillus subtilis*. World J Microbiol Biotechnol 26(7):1207-16

Toranzo AE, Romalde JL, Magarinos B, Barja JL (2009) Present and future of aquaculture vaccines against fish bacterial diseases. Options Mediterraneennes 86:155-176

Torrento AE, Torres JL (1996) *In vitro* inhibition of *Vibrio harveyi* by *Pseudomonas* species isolated from aquatic environment. UPV J Nat Sci 1:130-138

Touraki M, Karamanlidou G, Karavida P, Chrysi K (2012) Evaluation of the probiotics *Bacillus subtilis* and *Lactobacillus plantarum* bioencapsulated in *Artemia nauplii* against vibriosis in European sea bass larvae (*Dicentrarchus labrax* L.). World J Microbiol Biotechnol 28(6):2425-33

Vaerewijck MJ, De Vos P, Lebbe L, Scheldeman P, Hoste B, Heyndrickx M (2001) Occurrence of *Bacillus sporothermodurans* and other aerobic spore-forming species in feed concentrate for dairy cattle. J Appl Microbiol 91(6):1074-1084

Van Zeijl CM, van de Kamp EH, Punt PJ, Selten GC, Hauer B, van Gorcom RF, van den Hondel CA (1998) An improved colony-PCR method for filamentous fungi for amplification of PCR-fragments of several kilobases. J Biotechnol 59(3):221-224

Vaisvila, R., Morgan, R. D., Posfai, J. & Raleigh, E. A. (2001). Discovery and distribution of super-integrons among pseudomonads. Mol Microbiol 42, 587–601

Vaseeharan B, Ramasamy P (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett Appl Microbiol 36(2):83–87

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671

Vijayagopal P, Chakraborty K, Iyyapparajanarasimapallavan G, Anil MK, Ignatius B, Correya NS and Vijayan KK (2012) Development of live feed enrichment product for marine fish larviculture. Indian J Fish 59(2):121-125

Vijayan KK, Singh ISB, Jayaprakash NS, Alavandi SV, Pai SS, Preetha R, Rajan JJS, SantiagoTC (2006) A brackishwater isolate of *Pseudomonas* PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. Aquaculture 251:192-200

Villamiel M and Jong P (2000) Inactivation of *Pseudomonas fluorescens* and *Streptococcus thermophilus* in Trypticase Soy Broth and total bacteria in milk by continuous-flow ultrasonic treatment and conventional heating. J Food Eng 45:171–179

Vine NG, Leukes WD, Kaiser H (2006) Probiotics in marine larviculture. FEMS Microbiol Rev 30(3):404–427

Vinoj G, Vaseeharan B, Jayaseelan BD, Rajakumaran P, Ravi C (2013) Inhibitory effects of *Bacillus licheniformis* (DAB1) and *Pseudomonas aeruginosa* (DAP1) against *Vibrio parahaemolyticus* isolated from *Fenneropenaeus indicus.* Aquac Int. 21:1121-1135

Vynne NG, Mansson M, Nielsen KF, Gram L (2011) Bioactivity, chemical profiling, and 16S rRNA-based phylogeny of *Pseudoalteromonas* strains collected on a global research cruise. Mar Biotechnol 13(6):1062-1073

Wan M, Rosenberg JN, Faruq J, Betenbaugh MJ, Xia J (2011) An improved colony PCR procedure for genetic screening of *Chlorella* and related microalgae. Biotechnol Lett 33(8):1615-1619

Wang GX, Liu YT, Li FY, Gao HT, Lei Y, Liu XL (2010) Immunostimulatory activities of simplex DR-834 to carp (*Cyprinus carpio*). Fish Shellfish Immunol 29:378–387

Wang Y-B, Li J-R, Lin J (2008) Probiotics in aquaculture: challenges and outlook. Aquaculture 281(1-4):1-4

Ward AC (1992) Rapid analysis of yeast transformants using colony-PCR. Biotechniques 13(3):350

Wattiau P, Renard ME, Ledent P, Debois V, Blackman G, Agathos SN (2001) A PCR test to identify *Bacillus subtilis* and closely related species and its application to the monitoring of wastewater biotreatment. Appl Microbiol Biotechnol 56(5-6):816-819

Weller DM (2007) *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology 97:250–256

Wiese J, Thiel V, Nagel K, Staufenberger T, Imhoff JF (2009) Diversity of antibiotic-active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. Mar Biotechnol 11(2):287-300

Wu XY, Walker MJ, Hornitzky M, Chin J (2006) Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. J Microbiol Met 64:107-119

Wulff EG, Mguni CM, Mansfeld-Giese K, Fels J, Lubeck M, Hockenhull J (2002) Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris*. Plant Pathol 51:574-584

Xie F, Zhu T, Zhang F, Zhou K, Zhao Y, Li Z (2013) Using *Bacillus amyloliquefaciens* for remediation of aquaculture water. Springerplus 2:119 doi:10.1186/2193-1801-2-119

Yang D, Wang B, Wang J, Chen Y, Zhou M (2009) Activity and efficacy of *Bacillus subtilis* strain NJ-18 against rice sheath blight and sclerotinia stem rot of rape. Biological Control 51:61–65

Yeon SH, Jeong WJ, Park JS (2005) The diversity of culturable organotrophic bacteria from local solar salterns. J Microbiol 43(1):1-10

Yilmaz M, Soran H, Beyatli Y (2005) Determination of poly-β-hydroxybutyrate (PHB) production by some *Bacillus* spp. World J Microbiol Biotechnol 21:565–566 doi:10.1007/s11274-004-3274-1

Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A (2001) Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 Isolated from mulberry leaves. Phytopathology 91(2):181-187

Yoshida T, Hayashi KI Ohmoto H (2002) Dissolution of iron hydroxides by marine bacterial siderophore. Chem Geol 184:1-9

Yousef JM and Danial EN (2012) *In vitro* antibacterial activity and minimum inhibitory concentration of zinc oxide and nano-particle zinc oxide against pathogenic strains. J Health Sci 2(4):38-42

Zhang L (2005) Integrated approaches for discovering novel drugs from microbial natural products. In: Zhang L, Demain A editors. Natural products: drug discovery and therapeutics medicines. Totowa NJ Human Press p 33–56

Zheng L, Han X, Chen H, Lin W, Yan X (2005) Marine bacteria associated with marine macroorganisms: the potential antimicrobial resources. Ann Microbiol 55(2):119-124

Zhou Q, Li K, Jun X, Bo L (2009) Role and functions of beneficial microorganisms in sustainable aquaculture. Bioresour Technol 100:3780–3786

******

PUBLICATIONS

Nair AV, Vijayan KK, Chakraborty K, Leo Antony M (2012) Diversity and characterization of antagonistic bacteria from tropical estuarine habitats of Cochin, India for fish health management. World J Microbiol Biotechnol 28(7):2581–2592.

Nair A V, Pradeep M A, Vijayan K K (2014) Molecular approach for the rapid detection of *Bacillus* and *Pseudomonas* genera—dominant antagonistic groups—from diverse ecological niches using colony multiplex PCR. J Ind Microbiol Biotechnol DOI 10.1007/s10295-014-1441-4.

Nair AV, Joseph N, Krishna K, Sneha KG, Tom N, Jangid K, Nair S (2015) A comparative study of coastal and clinical isolates of *Pseudomonas aeruginosa*. Brazilian Journal of Microbiology 46, 3, 725-734

Patent

"A PROCESS AND FORMULATION TO PREPARE *VIBRIO* ANTAGONISTIC MICRO FEED FOR FINFISH AND SHELLFISH LARVAE USING MARINE BACTERIA AND A PRODUCT THEREOF" (Delhi Patent Office - Application No: 201611004807, dated: 11.02.2016)

Inventors: Anusree Velappan Nair, Leo Antony Maria Louis, Pananghat Vijayagopal, Koyadan Kizhakedath Vijayan

Paper presented

1. **Nair A.V**., Ghosh S., Chakraborty K., Chakraborty R.D., and Vijayan K.K. (2011). I**solation and identification of potential probionts having antimicrobial activity from south west and south east coast of India.** Conference paper presented in Asian Pacific Aquaculture 2011, January 17-20, 2011, Le Méridien Resort and Convention Center, Kochi, India, pp. 358.

2. **Nair A.V**, Antony M L and Vijayan K K (2014) ***In vitro*** **and** ***in vivo*** **screening of the antagonistic bacteria** ***Bacillus subtilis*** **MBTDCMFRI Ba37 and** ***Pseudomonas aeruginosa*** **MBTDCMFRI Ps04 to use as biological disease control agents in aquaculture**, Conference paper presented in MECOZ 2, December 2-5, 2014, Dream Hotel , Kochi, India, pp. 235

Genbank Submissions

Number of Submissions: 106

Accession No: JF719759 - JF719808, KF317775 - KF317832

Sequence description: Partial 16S rRNA gene

Sources: Sediment and water

Microbial Taxon:

Bacillus, Pseudomonas, Vibrio, Alcaligenes, Enterobacter, Halomonas, Citrobacter, Enterococcus, Shewanella, Pseudoalteromonas, Escherichia, Arthrospira, Acinetobacter, Micrococcus, Alcanivorax, Pedobacter, Oceanimonas, Brevibacterium and *Streptomyces* spp

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RESEARCH PAPERS PUBLISHED

World J Microbiol Biotechnol (2012) 28:2581–2592
DOI 10.1007/s11274-012-1067-5

ORIGINAL PAPER

Diversity and characterization of antagonistic bacteria from tropical estuarine habitats of Cochin, India for fish health management

Anusree V. Nair · K. K. Vijayan · Kajal Chakraborty · M. Leo Antony

Received: 21 January 2012 / Accepted: 20 April 2012 / Published online: 13 May 2012

Abstract Mortalities due to pathogenic bacteria are a major problem in aquaculture, especially in larval rearing systems. Use of antibiotics to overcome this problem is not an option any more due to the increasing antibiotic resistance among pathogens. The present study aims to understand the diversity of bacteria with antagonistic properties in the tropical estuarine habitats of Cochin, located along the southwest coast of India, and to use them as an alternative to antibiotics in aquaculture. Among the 4,870 isolates screened, approximately 1 % showed significant antibacterial activity against six common aquaculture pathogens belonging to the genera *Aeromonas* and *Vibrio*. The antagonistic bacteria were identified as *Bacillus* (81 %) and *Pseudomonas* (19 %) using biochemical and 16S rRNA gene sequence homology. The isolates showing stable and higher levels of antibacterial activity were subjected to enzymatic expression profile, antibiotic resistance pattern and abiotic stress tolerance assays. As a result, five *Pseudomonas* spp. and four *Bacillus* spp., were identified as promising antagonistic isolates that could be exploited as probionts or microbial products (MP's), to control bacterial diseases in aquaculture rearing systems.

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Keywords Antibacterial diversity · Aquaculture · Bacterial antagonism · Fish health management · Tropical estuarine habitats

Introduction

Aquaculture production has grown steadily over the past decade, contributing 46 % of the global fish supply (FAO 2010). However, diseases are a major limiting factor with larval mortalities due to pathogenic *Vibrio* and *Aeromonas* infections being one of the chief causes (Balcazar and Rojas-Luna 2007). Good husbandry techniques and the usage of chemical additives, disinfectants, antimicrobials and vaccines are primarily practiced to control bacterial infections in aquaculture (Wang et al. 2008). However, the increased use of antibiotics in aquaculture systems leads to complications such as increased stress among aquatic animals, development of drug resistance among fish and human pathogens subsequently via transfer of genes from drug resistant microbes (Wang et al. 2008; Heuer et al. 2009). Apart from this, the residues which are left behind adversely affect the environment and public health (Defoirdt et al. 2007). Vaccination or immunization for the control of bacterial diseases is of limited practicality in shellfish due to non-specific immune responses (Vine et al. 2006; Defoirdt et al. 2007). Individual vaccination in finfish is infeasible under intensive culture environments (Toranzo et al. 2009). In this backdrop, use of live bacteria or in the form of heat-killed microbial products (MP's) with antagonistic properties emerged as an alternate strategy to control bacterial disease problems (Vine et al. 2006; Kesarcodi-Watson et al. 2008). Probiotics reduce bacterial infections by producing inhibitory compounds (Vijayan et al. 2006; Vaseeharan and Ramasamy 2003; Preetha et al.

J Ind Microbiol Biotechnol
DOI 10.1007/s10295-014-1441-4

BIOTECHNOLOGY METHODS

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Molecular approach for the rapid detection of *Bacillus* and *Pseudomonas* genera—dominant antagonistic groups—from diverse ecological niches using colony multiplex PCR

Anusree V. Nair · M. A. Pradeep · K. K. Vijayan

Received: 31 October 2013 / Accepted: 20 March 2014

Abstract *Bacillus* and *Pseudomonas* are the dominant groups of bacteria known for their antagonistic potential against many plant and animal pathogens. Presently, exploration of these genera with antagonistic property for disease management of aquaculture system is gaining more importance to overcome the use of antibiotics and related resistance issues. Rapid screening and identification of these genera from diverse bacterial populations by conventional methods is laborious, cost-intensive, and time-consuming. To overcome these limiting factors, in the present study, a colony multiplex PCR (*cm*PCR) method was developed and evaluated for the rapid detection of *Bacillus* and *Pseudomonas*. The technique amplifies the partial 16S rRNA gene of *Bacillus* and *Pseudomonas* with a product size of ~1,100 and ~375 bp, respectively, using single forward (BSF2) and two reverse primers (PAGSR and BK1R). Reliability of the *cm*PCR method was confirmed by screening 472 isolates obtained from ten different eco-stations, of which 133 isolates belonged to *Bacillus* and 32 to *Pseudomonas*. The *cm*PCR method also helped to identify six different *Pseudomonas* spp. and 14 different *Bacillus* spp. from environmental samples. Of the total 472 isolates studied, 46 showed antagonistic activity, among which 63 % were *Bacillus* and 17.4 % were *Pseudomonas*. Thus, the newly developed molecular approach provides a quick, sensitive, and potential screening tool to detect novel, antagonistically important *Bacillus* and *Pseudomonas* genera for their use in aquaculture. Further, it can also act as a taxonomic tool to understand the distribution of these genera from wide ecological niches and their exploitation for diverse biotechnological applications.

Keywords *Bacillus* · Bacterial identification · Colony multiplex PCR · *Pseudomonas* · Taxonomical tool

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Introduction

Aquaculture is the fastest-growing food production sector in the world and accounts for approximately 47 % of the world's total fish food supply [9]. Disease outbreaks due to pathogenic bacteria have presented a major challenge, which adversely affects the development of aquaculture-based fish production [12]. The therapeutic options available in the control of bacterial diseases are the use of approved antibiotics, chemicals, and vaccination, but these applications are limited due to the development of antibiotic resistance and lack of consistency. Nowadays, the use of antagonistic bacteria such as *Bacillus*, *Pseudomonas*, *Alteromonas*, and *Flavobacterium* are also gaining importance in aquaculture industry to control bacterial diseases because of its ease in application and absence of side effects [3, 35, 40]. Our previous studies have clearly emphasized the importance of the genera *Bacillus* and *Pseudomonas*, with antagonistic activity and its application in aquaculture systems [22, 34]. These genera were also reported for its potential antimicrobial activity against many plant and human pathogens [23, 29].

Generally, the identification of bacteria by conventional methods is time-consuming, invariably mono-specific, as well as laborious, especially when screening a large number of field samples [6]. The methods like fatty acid methyl ester (FAME) profiling [4, 33] and the API system-based identification have been shown to be more useful than

Published online: 22 April 2014

Brazilian Journal of Microbiology 46, 3, 725-734 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246320140502

Research Paper

A comparative study of coastal and clinical isolates of *Pseudomonas aeruginosa*

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Submitted: June 12, 2014; Approved: December 19, 2014.

Abstract

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium having a versatile metabolic potential and great ecological and clinical significance. The geographical distribution of *P. aeruginosa* has revealed the existence of an unbiased genetic arrangement in terrestrial isolates. In contrast, there are very few reports about *P. aeruginosa* strains from marine environments. The present work was aimed at studying the distribution of *P. aeruginosa* in coastal waters along the Indian Peninsula and understanding the environmental influence on genotypic, metabolic and phenotypic characteristics by comparing marine and clinical isolates. Of the 785 marine isolates obtained on selective media, only 32 (~4.1%) were identified as *P. aeruginosa*, based on their fatty acid methyl ester profiles. A low Euclidian distance value (< 2.5) obtained from chemotaxonomic analysis suggested that all the environmental (coastal and marine) isolates originated from a single species. While UPGMA analyses of AP-PCR and phenotypic profiles separated the environmental and clinical isolates, fatty acid biotyping showed overlapping between most clinical and environmental isolates. Our study revealed the genetic diversity among different environmental isolates of *P. aeruginosa.* While biogeographical separation was not evident based solely on phenotypic and metabolic typing, genomic and metatranscriptomic studies are more likely to show differences between these isolates. Thus, newer and more insightful methods are required to understand the ecological distribution of this complex group of bacteria.

Key words: *Pseudomonas aeruginosa*, biogeography, FAME profiling, arbitrarily primed PCR, environmental distribution.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative γ-proteobacterium having a large and flexible genome (> 6.2 Mb) with a high number of protein-coding genes (5,570 ORFs) matching simpler eukaryotes (Stover *et al.*, 2000). Its influential adaptive capacity and metabolic potential allow this bacterium to live in very dissimilar environments (Römling *et al.*, 1994; Finnan *et al.*, 2004; LaBaer *et al.*, 2004; Pirnay *et al.*, 2005; Shen *et al.*, 2006; Khan *et al.*, 2008). The distribution of this species suggests a net-like population structure with equivalent genotypes and functionally similar characters elicited by both clinical and environmental strains without any specialisation (Römling *et al.*, 1994; Alonso *et al.*, 1999; Kiewitz and Tummler, 2000; Pirnay *et al.*, 2005). The species' indigenous adaptation in marine environments was a matter of debate until its presence was first reported by Kimata *et al.* (2004). Recent isolation of *P. aeruginosa* strains from an open-ocean site has shown that they possess a unique genotype. Thus, the geographical origin of the strains is reflected in their phylogeny (Khan *et al.*, 2007; Khan *et al.*, 2008; Nonaka *et al.*, 2010).

Although *P. aeruginosa* is an extensively studied organism, the bacterium in its natural reservoirs has a lot to

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ISOLATION AND IDENTIFICATION OF POTENTIAL PROBIONTS HAVING ANTIMICROBIAL ACTIVITY FROM SOUTH WEST AND SOUTH EAST COAST OF INDIA

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Marine bacteria are important and unexplored resources for novel microbial products. Compared with terrestrial organisms, the secondary metabolites produced by micro organisms are more novel and unique structures owing to the complex living circumstance and diversity of species The present work aims to isolate marine bacteria from sediment and seawater in terms of the capabilities of producing secondary metabolite with antimicrobial activity and to identify the potential isolates using morphological, biochemical and molecular methods.

In the present work marine bacteria were isolated from sediment and seawater samples collected from different ecosystems from South West and South East coast of India. The samples were processed by standard microbiological methods. Spread plate method was used for recording the heterotrophic bacterial counts (cfu/ml/mg) using Zobell's marine agar and Nutrient agar. Colonies were randomly picked to check the antimicrobial activity by spot inoculation and disc diffusion methods using Muller Hinton Agar. Antagonism was tested against 9 aquaculturally important pathogenic strains viz *V. harveyi, V. alginolyticus*, *V. parahaemolyticus, V. vulnificus* and *A.hydrophilla*. Colonies giving prominent zone of activity were selected and identified on the basis of morphological and biochemical characteristics. Genomic DNA was extracted from the potential isolates.The bacteria with wide antimicrobial spectrum were identified to the species level by PCR amplification of the 16S rRNA gene using the universal bacterial primer. The amplified product was purified and sequenced. Homology of the obtained sequence was compared to the sequence available in GenBank nucleotide database of NCBI using BLAST. Among 3270 isolates, total of 30 isolates (1%) were found to have prominent zone of (>10 mm) antimicrobial activity against *V.vulnificus*, *V.harveyi*, *Aeromonas hydrophilla*, *V.alginolyticus* and *V.parahemolyticus*. Majority of the potential isolates were deduced to be *Bacillus sp*(75%) followed by *Pseudomonas sp* (25%) (Table1 & Figure 1). Antimicrobial spectra of different bacteria were found to be different and this could be related to the survival competent superiority. The potential microbes could be used for marine bioprospecting.

Ref code	Identification	Sampling site	Zone of inhibition (mm) against different pathogens					
			V.vulnificus 1145	*V.vulnificus 1146*	*V.parahemolyticus US*	*A. hydrophilla*	*V.alginolyticus 101*	*V.harveyi 101*
P101	*P.aeruginosa*	Vypin	21	30	10	25	16	20
P104	*P.aeruginosa*	Jetty	19	23	10	23	12	17
P107	*P.aeruginosa*	Kakkinadaa	22	26	12	26	13	20
B097	*B.subtilis*	Vypin	20	27	14	24	15	14
B098	*B.subtilis*	Vypin	20	22	16	19	13	15
B101	*B.subtilis*	Njarakkal	18	27	16	20	15	17

TABLE 1. Antimicrobial activiy of *Bacillus sp* and *Pseudomonas sp.* against aquaculturally important pathogen

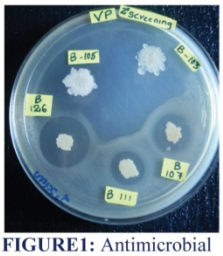

FIGURE1: Antimicrobial activity of bacterial isolates against *V. parahemolyticus* using Muller Hinton Agar

MECOS 2 | MBTO 06

In vitro and *In vivo* screening of the antagonistic bacteria *Bacillus subtilis* MBTD CMFRI Ba37 and *Pseudomonas aeruginosa* MBTDCMFRI Ps04 for use as biological disease control agents in aquaculture

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Aquaculture is one of the fastest growing food production sectors and supplies nearly fifty percent of the world fish food production. Disease outbreaks due to pathogenic bacteria have become a major disease problem in aquaculture rearing systems. The use of antibiotics, synthetic drugs, disinfectant like compounds and chemicals in aquatic environments are widely criticized for their negative effect on environment and also on the consumer. Hence, the need of the hour is to develop an eco-friendly approach to tackle the bacterial disease issues in aquaculture. In recent years, the use of probiotics as biological control seems to be a potential alternative for antibiotics in aquaculture. In this perspective, the identification and introduction of bacteria with antagonistic activity against pathogens into aquaculture system has proven to be a more effective disease control strategy. Our previous studies reported that the genus *Bacillus* and *Pseudomonas* were the common groups that possess significant antagonistic activity against aquaculture pathogens.

The present study evaluated the *In vitro* and *In vivo* antagonistic effect of the two potential antagonistic bacteria - *Bacillus subtilis* MBTDCMFRI Ba37 and *Pseudomonas aeruginosa* MBTDCMFRI Ps04 for use in aquaculture as preventive measures against Vibrio infections. These two bacteria were isolated from tropical estuarine habitats of Cochin and screened for antagonistic activity against aquaculture pathogens. The cultured and filtered supernatant of *B. subtilis* Ba37 and *P. aeruginosa* Ps04 exhibited higher inhibitory activity against *Vibrio vulnificus* MTCC1145, *V. anguillarum* 01, *V. harveyi* 101 and least activity against *Aeromonas hydrophila*. The zone of inhibition against the tested pathogen by disk diffusion method was around 9- 22 mm (Fig 1). The *Candida*te bacteria evaluated for inhibitory potential by co-culturing assay, has revealed that *B. subtilis* Ba37 and *P. aeruginosa* Ps04 were inhibitory to *V. anguillarum* 01 and *V. harveyi* 101 respectively Fig.1. Antagonistic activity of *B. subtilis* MBTD CMFRI Ba37 and *P. aeruginosa* MBTDCMFRI Ps04 by disc diffusion method. A - *Vibrio vulnificus* MTCC1145; B - Vibrio parahaemolyticus MTCC 451; C- Aeromonas hydrophila; D - Vibrio alginolyticus 101; E- *Vibrio harveyi* 101 and F- *Vibrio anguillarum* A1. The measurement indicates the inhibition zone (mm) formed around the isolates. The values may differ ± 1mm from the size mentioned Fig .2. Co-culture assay of *P. aeruginosa* MBTDCMFRI Ps04 against *V. harveyi* 101. Tubes showed the results of co-culture assay- *P. aeruginosa* MBTDCMFRI Ps04 inhibits the growth of *V. harveyi* 101. Yellow colour indicates the growth of *V. harveyi* 101 whereas green indicates the inhibition. even at 104 CFU/ml concentrations (Fig. 2). On evaluating the pathogenicity of antagonistic strains, *B. subtilis* MBTDCMFRI Ba37 and *P. aeruginosa* MBTDCMFRI Ps04 were found to be safe and did not cause any infections by intramuscular injection into ~5 g of sub-adults of *Etroplus suratensis* at concentration range from 106 to 109 CFU ml^{-1}.The results of this study illustrated that *B. subtilis* Ba37 and *P. aeruginosa* Ps04 were found to be

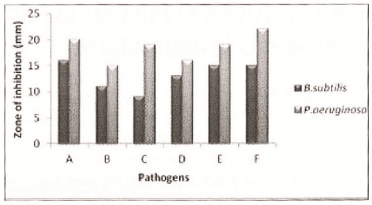

Fig.1. Antagonistic activity of *B. subtilis* MBTD CMFRI Ba37 and *P. aeruginosa* MBTDCMFRI Ps04 by disc diffusion method. A - *Vibrio vulnificus* MTCC1145; B - *Vibrio parahaemolyticus* MTCC 451; C- *Aeromonas hydrophila*; D - *Vibrio alginolyticus* 101; E- *Vibrio harveyi* 101 and F- *Vibrio anguillarum* A1. The measurement indicates the inhibition zone (mm) formed around the isolates. The values may differ ± 1mm from the size mentioned

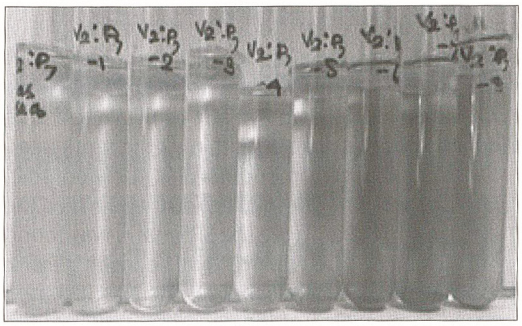

Fig .2. Co-culture assay of *P. aeruginosa* MBTDCMFRI Ps04 against *V. harveyi* 101. Tubes showed the results of co-culture assay- *P. aeruginosa* MBTDCMFRI Ps04 inhibits the growth of *V. harveyi* 101. Yellow colour indicates the growth of *V. harveyi* 101 whereas green indicates the inhibition.

promising *Candida*tes that could be employed as potential antagonistic bacteria in aquaculture systems for the management and control of bacterial infections. Further studies on these bacteria will allow their utilization as live or inactivated biological disease control agents in aquaculture to control bacterial diseases.

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