IDENTIFICATION AND ANALYSIS OF ANTIMICROBIAL BIOSYNTHETIC GENES IN MARINE MICROBIAL SYMBIONTS

By

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(2014-09-116)

THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"Identification and analysis of antimicrobial biosynthetic genes in marine microbial symbionts"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or society.

Place: Vellayani
Date: 16/11/2019

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CERTIFICATE

Certified that this thesis entitled **"Identification and analysis of antimicrobial biosynthetic genes in marine microbial symbionts"** is a record of research work done independently by **Ms. AKHITHA MARY BENNY (2014-09-116)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Akhitha Mary Benny

DEDICATED TO MY BELOVED FAMILY

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LIST OF ABBREVATIONS

ADH	Arginine decarboxylase test
BHIA	Brain heart infusion Agar
BLAST	Basic Local Alignment Search Tool
bp	Base pair
^{0}C	Degree Celsius
cm	Centimetre
CFU	Colony forming unit
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide triphosphates
EDTA	Ethylenediamine tetra-acetic acid
EHT	Esculin hydrolysis test
Fig.	Figure
g	Gram
h	Hour
H2O2	Hydrogen peroxide
H2S	Hydrogen sulphide
IU	International units

KOH	Potassium hydroxide
L	Litre
LB	Luria Bertani
LF	Lactose fermenter
MCA	MacConkey agar
MSA	Mannitol salt agar
MgCl2	Magnesium chloride
MHA	Muller Hinton Agar
µg	Microgram
µl	Microliter
mg	Milligram
ml	Millilitre
mM	Millimolar
min	Minutes
M	Molar
N	Normality
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
NFW	Nuclease free water

NLF	Non lactose fermenter
nm	Nanometre
ODT	Ornithine decarboxylase test
%	Percent
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pH	Log hydrogen ion concentration
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
sec	Second(s)
SD	Standard Deviation
sp.	Species
Taq	*Thermus aquaticus*
TCA	Tri Chloroacetic Acid
TCBS	Thiosulfate citrate bile salts sucrose agar
Tris HCL	Tris Hydrochloric acid
U	Unit(s)
UV	UltraViolet
V	Volts
X Gal	5-bromo-4-chloro-3-indolyl-β-D galactopyranoside

INTRODUCTION

1.INTRODUCTION

Emergence and spread of multidrug resistant (MDR) strains and newer diseases necessitates the development of novel and more effective antimicrobial agents (Doshi *et al.,* 2011). Since the discovery of the last effective resort to combat antimicrobial resistance in 1970's, limited number of new classes of antibiotics has been introduced into the clinic over the past few decades (Ventola *et al.,* 2015). Others were either modifications of the existing antibiotics or from the same class. Therefore development of novel drugs with novel mechanism of action and novel property is extremely urgent. Land resources are extensively explored for several decades and are not very ideal for the development of effective drugs (Doshi *et al.,* 2011). Moreover, the same drugs are being re-discovered from terrestrial environment causing loss of time, money and work (Zotchev, 2012). Therefore, over the past few years scientists are shifting their focus to virtually unexplored sources like marine environment for discovery of novel antibacterial agents.

The extreme habitat in sea compels both micro and macro organisms to evolve new mechanisms of survival which includes production of defence compounds to rescue themselves from predation and to tolerate severe environmental conditions (Abad *et al.,* 2011). Thus, marine bacteria are now being explored as the potential resources for novel bioactive secondary metabolites including new antimicrobials (Saha *et al.,* 2005). The increasing awareness on microbial symbionts and their involvment in the biosynthesis of their host's metabolites or as the true source of many host metabolites (Fortman and Sherman 2005; Konig *et al.*, 2006) leads to the blooming research on microbial symbionts of marine organisms in the exploration of novel marine natural products (Schmidt, 2005; Salomon *et al.*, 2004).

The studies on the genetic machinery responsible for the production of various secondary metabolites have helped the scientific community for the successful prediction of novel metabolites from the corresponding gene

sequence data (Tambadou *et al.,* 2014). It was found that production of biologically active secondary metabolites by microorganisms requires specific gene clusters encoding multi-modular enzymes. Non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) are the two major classes of these multi-modular enzymes. It is reported that any variations in these two enzymatic modules may result in the production of various biologically active compounds (Hochmuth and Piel, 2009; Graca *et al.,* 2015). Thus cognizance of these biosynthetic gene clusters associated with antibacterial properties is vital in the search for novel antibacterials, in the modification of existing compounds for the development of more fruitful antibiotics (Kalaitzis *et al.,* 2009).

Marine organisms like sponges, algae, tunicates as well as their symbiotic microbes has been screened in the search for novel antibacterial compounds (Abad *et al.,* 2011), while marine crabs and bivalves remain unexplored. The molecular basis of marine microbial natural product biosynthesis was first established for the streptomycete antibiotic enterocin (Piel *et al.,* 2000) and the Cyanobacterial agent barbamide (Chang *et al.,* 2002) based on the targeted cloning and sequencing of their respective biosynthetic gene clusters. When Zhang *et al.* (2014) done the genome survey of an antagonistic strain OH11, a Chinese *Lysobacter enzymogenes*, it was found that both PKS and NRPS gene were involved in the antibacterial activity of its novel antibiotic WAP-8294A2. Recently, antimicrobial potential and presence of NRPS genes in microbes associated with various tissues of mud crab (*Scylla olivacea*) were analyzed by Zote *et al.* (2018). However, marine crab/bivalve symbionts have not been tested for the presence of any biosynthetic gene clusters till date, and insights into the BGCs machinery of microbes associated with these marine invertebrates are virtually empty.

Keeping in view of the above facts, the objectives of the present study was kept as follows with a final target for finding out new antimicrobials from unexplored marine resources:

- Isolation and identification of microbial symbionts of some unexplored marine invertebrates having activity against human and aquatic pathogens including some multidrug resistant (MDR) strains and identification and analysis of antimicrobial biosynthetic genes in these antagonistic bacteria.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Antimicrobial therapy using different antibiotics and their derivatives forms the backbone of modern medicine (Friedman *et al.,* 2016). Pre- antibiotic era, that witnessed millions of emergencies and deaths due to severe but curable diseases were abated with the introduction of antibiotic therapies. The period from the discovery of salvarsan in 1910s to the late 1980s is regarded as the golden era for antibiotic discovery (WHO, 2014). Application of antibiotics has successfully reduced the fatality rates of pneumonia (Bartlett *et al.,*1995), bacteraemia (Karchmer *et al.,*1991) and various types of severe wounds (Hirsch *et al.,* 2008).

2.1. ANTIMICROBIAL AGENTS-BACKBONE OF MODERN MEDICINE

Antimicrobial agents are broadly classified into two as those that can kill bacteria (bactericidal) and those that can inhibit the growth of bacteria (bacteriostatic) without killing microbes. The mode of action of these agents can be either by interfering the integrity of cell walls, integrity of cell membranes, synthesis of nucleic acid, synthesis of protein or by inactivating a crucial metabolic pathway in bacteria (Neu, 1992). The antibiotics are classified into different classes based on these mechanisms. The cell wall inhibitors include beta lactams (such as penicillin, cephalosporins and monobactams) and glycopeptides (include vancomycin and teicoplanin) (Spratt, 1980; McManus, 1997). The second class containing polymyxins and daptomycin affect bacterial cell membrane by altering membrane permeability and depolarisation respectively (Storm *et al.,* 1977, Carpenter and Chambers, 2004). Protein synthesis inhibitors include macrolides (eg: clindamycin), aminoglycosides, oxazolidinones chloramphenicol, tetracyclines and streptogramins (Neu, 1992; McManus, 1997). DNA synthesis inhibitors forming the forth class include quinolones and rifampicin by targeting DNA gyrases and DNA directed RNA polymerases respectively (Drlica and Zhao, 1997). Fifth class containing metabolic pathway

inhibitors include sulphonamides and trimethoprim which inhibits folic acid synthesis (Yao and Moellering, 2003; Pteri, 2006).

2.2. ANTIMICROBIAL RESISTANCE (AMR) -AN EMERGING GLOBAL THREAT

Increasing AMR is recognised as a global health emergency that needs to be urgently tackled. This public threat can lead to huge economically and clinically adverse outcomes including increased mortality and morbidity in patients, utilisation of resources, hospitalisation and the need for board spectrum empiric therapies (Friedman *et al.,* 2016). As per the present estimates it is forecasted that 10 million human deaths may occur only due to AMR pathogens by 2040 if the present trend continues (O'Neill, 2014).

2.2.1. AMR- Basic concepts

Antimicrobial resistance (AMR) is the mechanism by which bacteria acquire resistance to a class of drugs with antimicrobial potential. This resistance may be innate or acquired. Innate resistance means that some bacteria may be naturally resistant to a few classes of antimicrobials. Acquired resistance means bacteria that were previously susceptible to a group of drugs become resistant to the same group due to the continuous exposure. Acquired resistance in bacteria to antimicrobial agents is much more serious and recognised as one of the major threats to medicine in future (Tenover *et al.,* 2006).

Bacteria have long been in this planet and has thrived on multiple environmental conditions that gave them the ability to adapt and survive in their surroundings (Economou and Gousia, 2015). Emergence of acquired AMR to more widely used antimicrobials may be one such adaptation mechanism due to the selective pressure exerted by the drug by the unnecessarily wide spread use (Tenover *et al.,* 2006). They may be in the form of acquired genes that encode enzymes (such as beta lactamase), efflux pumps that prevent drugs from reaching their site of action or genes that can perform alternative ways to the pathways that are inhibited by these antimicrobials (Tenover *et al.,* 2006). The mechanism of

acquired resistance from other resistant microbes were identified either by vertical or horizontal gene transfer. Vertical gene transfer involves transfer throughout generations of the same species of bacteria and latter is by any of the genetic transfer processes like conjugation, transformation and transduction which occur between same or different bacterial species (McManus, 1997).Conjugation involve the exchange of mobile genetic elements or transposons via sex pilus in case of gram negative bacteria or by sex pheromones in gram positive microbes. Transformation require bacteriophage and transduction is uptake of DNA from the surrounding medium. All these methods of gene exchange mechanism in horizontal gene transfer are reported to contribute for the development of multidrug resistant (MDR) strains which are the microbes resistant to three or more classes of antimicrobial agents. Acquired resistance can also be developed by mutation in existing genes (McManus, 1997) leading to the modification of antimicrobial target protein or its binding site, extensive production of enzymes that render antimicrobials inactive and deactivating an extraneous protein channel required by the drug (McManus, 1997; Tenover *et al.,* 2006).

The overuse and misuse of antimicrobials has alarmingly increased the speed of resistance acquisition in bacteria. Some examples include; introduction of the first effective antibiotic sulphonamide was followed by the development of resistance against it in the late 1930 (Levy, 1982). Penicillin resistant *S. aureus* were isolated in early 1940s from hospitals, shortly after the advent of penicillin (Rammelkamp and Maxon, 1942; Barber, 1948). Streptomycin resistant *Mycobacterium tuberculosis* aroused during its first course of treatment (Crofton and Mitchison, 1948). The emergence of resistance in *Haemophilus influenzae* and *Neisseria gonorrhoeae*, against ampicillin was reported in 1970s itself (Degraaff *et al.,* 1976; Elwell *et al.,* 1977). Similarly, inadequate empiric antimicrobial therapy has resulted in the emergence of many resistant strains in various pathogens such as *S. aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa (*Ibrahim *et al.,* 2000; Kang *et al.,* 2005).

2.2.2. Multidrug resistant (MDR) and extensively drug resistant (XDR) strains

MDR bacteria are those strains that are resistant to at least one antimicrobial agent belonging to three or more antimicrobial classes. Bacterial strains resistant to at least one agent in all antimicrobial classes except one or two are referred to as extensively drug resistant (XDR) strains (Sweeney *et al.,* 2018). Major MDR strains with increased hospital and community threats are recognised in the species like *K. pneumoniae, S. aureus, E. coli, Enterococcus faecium, Acinetobacter baumanii, P. aeruginosa* and *M. tuberculosis* (Hiramatsu, 1998; Levy, 1998; Weinstein, 2001; Walsh and Amyes, 2004;). These are also termed as 'super bugs' due to the increased rate of deaths and casualities caused by them (Davies and Davies, 2010). MDR *M. tuberculosis* is recognised as a grave public health menace in both developed and developing countries. Extensively drug resistant (XDR) strains of *M. tuberculosis* have also been described recently (Shah *et al.,* 2007; Sotgiu *et al.,* 2009). Human immune compromising *P. aeruginosa* has emerged from being a wound pathogen to lethal nocosomial pathogen through acquiring resistance to β-lactams and aminoglycosides (Horrevorts *et al.,* 1990). Similarly, MDR in *Acinetobacter baumanii* are evolving spontaneously as a result of their efficient transformation rates (Peleg *et al.,* 2008). MDR strains of *Vibrio cholerae, Clostridium difficle, Shigella flexneri* and *Salmonella enteritidis* are also reported to pose sober threats in developing countries as a consequence of irresponsible use of antimicrobials (Lipp *et al.,* 2002; Kelly and LaMont, 2008; Vernaz *et al.,* 2009). *Escherichia coli* is also known to be resistant to a board spectrum of beta lactams now a days (Stamm and Norrby, 2001). They acquire resistance via plasmid mediated transfer of beta lactamase encoding gene like *TEM* or *SHV* (Rupp and Fey, 2003). Extended spectrum beta lactamases (ESBLs) producing strains of *E.coli* are now evolved due to the mutations in these genes and are now considered as public health emergency as they resist the action of third generation cephalosporins and monobactams. Fortunately, these were not resistant to cephamycins (Bradford,

2001). However, recently loss of a porin channel (OmpF) in outer membrane that has resulted in resistance against cefoxitin and cefotetan also in *E. coli* (Clarke *et al.,* 2003; Ananthan and Subha, 2005). *E. coli* resistant to fluoroquinolones were discovered in Asia (Wang *et al.,* 2001). Treatment with carbepenems were found to be effective but resistance acquired to these drugs as a result of acquisition of metallo -beta lactamases is of great concern (WHO, 2014). As resistance against fluoroquinolones was higher than that of third generation cephalosporins WHO (2014) forecasted that fluoroquinolones, one of the ultimate oral treatment options may become useless in near future. *Campylobacter* spp., exhibiting resistance to fluoroquinolones, macrolides such as erythromycin, tetracyclines, beta lactams, aminoglycosides such as gentamicin (Luber *et al.,* 2003; Gupta *et al.,* 2004) has become grave concern in developed nations (Padungton and Kaneene, 2003; Koluman and Dikici, 2014). Salmonella that exhibit multidrug resistance to ceftriaxone, nalidixic acid and other lactams (Foley and Lynne, 2008) are reported to have the ability to spread globally (Davis *et al.,* 2002; McDermott, 2006).

2.2.3. *S. aureus:* Most debated MDR pathogen

MDR *S. aureus* strains are now recognised as the most scandalous bacterial pathogen (Enright *et al.,* 2002). WHO has selected *S. aureus* with resistance to methicillin and other lactam antibiotics as the bacterium of international concern in 2014. Shortly after the acquisition of penicillin resistance in 1940s (Rammelcamp and Maxon, 1942), it contracted macrolide and tetracycline resistance in 1950s (Enright *et al.,* 2002). Even the introduction of methicillin in 1959 to treat Penicillin resistant *S. aureus* (PRSA) was in vane with the emergence of methicillin resistant *S. aureus* (MRSA) strains in hospitals (Jevons *et al.,* 1961). It is now increasingly being spread as a community acquired infection and is known to cause more than 50% nocosomial infections in developed economies as compared to methicillin susceptible *S. aureus* (MSSA). Methicillin resistant gene, *mecA* carried on mobile genetic element has been transferred to susceptible *S. aureus* strains from distant bacterial relatives which contributed to the development of resistant MRSA (Katayama and Hiramatsu,

2000; Hiramatsu *et al.,* 2001). They encode proteins which synthesise peptidoglycan layer with reduced susceptibility to lactam antibiotics (Lim and Strynadka, 2002). Soon in 1980s quinolones were used for MRSA treatments. Unfortunately Methicillin resistant strains emerged resistance with mutations in chromosomal *DNA gyrase* gene rendering the antibiotic ineffective (Hooper, 2002). The last resort used to treat MRSA, vancomycin (Kirst *et al.,* 1998) was also left useless due to the emergence of Vancomycin intermediate- resistant *S. aureus* (VISA) (Hiramatsu *et al.,* 1997; Smith *et al.,* 1999). Emergence of Vancomycin resistant *S. aureus* (VRSA) strains that received *van* operon through conjugation from *Entercoccus sp.* have been reported (CDC, 2002). Currently, Quinupristin- Dalfopristin, sterptogramin (Jones *et al.,* 1998; Meka and Gold, 2004) and linezolid, daptomycin (Tsiodras *et al.,* 2001) has been used for the treatment of resistant strains especially VISA and VRSA. Teixobactin, a novel antibiotic discovered nearly after 30 years of antibiotic void period can also be used against many super bug resistance including MRSA (Ferri *et al.,* 2017).

2.2.4. AMR threat in developing countries

The spread of resistant bacterial strains is wider and rapid in developing countries rather than that of industrialised nations (Okeke *et al.,* 2000; Zhang *et al.,* 2006) due to poor economic and financial conditions. Poorest of the poor countries are the most affected (Murray and Lopez, 1997). They are extremely susceptible as they have reduced access to safe and clean meals and often malnutrition render their immune system weak, increasing room for potentially dangerous infections. An example is the spread of XDR tuberculosis in South Africa affecting the immune compromised HIV patients (Singh *et al.,* 2007). Moreover, people of the developing countries may not be able to afford the costs of many advanced drugs, leading to suboptimal dose which may be inevitable for development of certain resistance development (Berkley *et al.*, 2005; Kariuki *et al.,* 2006). Inappropriate surveillance and lack of frequent antimicrobial susceptibility testing worsen the conditions in these countries (Okeke 2006; Petti *et al.*, 2006). Treatments of patients with series of antibiotics without proper

antibiotic susceptibility testing pose serious disease threats. Hoarding, where a patient fail to complete the prescribed course and purchases antibiotic without expert prescription are among the causes of resistance. Copious availability of cheap and low quality antibiotics have also contributed to the accelerated resistance scenario (Okeke, 2006). Flexible laws and standards in low income countries result in poor regulation of distribution and quality of drugs (Sosa *et al.,* 2010). In an overpopulated country like India the risk of antimicrobial resistance is much higher due to impoverished living conditions, antibiotic over use and unhealthy effluent systems. Multi drug resistant, metallo-β lactamase producing *Klebsiella pneumonia* was first identified in India (Yong *et al.,* 2009). When Lamba *et al.* (2017) analysed and quantified faecal coliforms, carbapenem - resistant enterobacteriaceae (CRE) and ESBL resistant bacteria in hospital effluents and drainages in New Delhi, they found out that the bacterial count was nine times higher in hospital outflows rather than in drainages thus concluding that hospital drainages can contribute to the spread of AMR genes. Continuous emergence of MDR strains of *V. cholerae*, *S. flexneri*, *Salmonella* sp., *M. tuberculosis* may be the consequences of the same.

2.2.5. AMR in animal sectors

Emergence of AMR in livestock and food animals has made them potential reservoirs of resistance transmission. Antibiotics are widely used in animals and poultry as growth enhancers and for abating livestock infections by prophylaxis and metaphylaxis (Marshall and Levy, 2011). The sub-inhibitory levels of antibiotics administered for growth promotion increasingly contribute to the development of resistance (USFDA, 2014). Therefore, use of antibiotics as growth promoters in animal sectors has been banned in some developed countries like European Union. Antibiotics ranging from penicillin to third generation cephalosporin are being extensively used in cattle (McEwan and Fedorka-Cray, 2002). *Staphylococcus*, *Enterococcus* and ESBL producing *enterobacteriacea* are the major bacteria that emerged resistance due to the abuse of antibiotic in animal sectors. They are transported to humans through food chain, contact with people

involved in maintenance or by environment contaminated with animal wastes ((Levy *et al.,* 1976; Peterson *et al.,* 2002; Soonthornchaikul and Garelick, 2009; Shah *et al.,* 2012). There are chances that these resistant strains may modify human intestinal flora rendering them more perilous to humans (Smet *et al.,* 2011). Consuming contaminated or raw milk is one of the serious reasons for contracting MDR food borne bacteria in humans. Extensive antibiotic uses in aquaculture and fisheries along with feeds have added to the development of MDR strains (Cabello *et al.,* 2013). Phenicols, quinolones and tetracyclines are increasingly being used in salmon farming. The connections established between human and marine pathogen resistomes by gene transfer through R plasmids, integrons and transposons can add to accelerated resistance. Antibiotic use in marine niches have also led to their leaching out to sediments thus contributing resistance in more than half of microbial flora. The elevated concentrations of tetracycline and oxolinic in sediments have exerted selective pressure on *Edwarsiella* and *Aeromonas* spp. (Bengtsson-Palme and Larsson, 2016).

2.3. SOLUTIONS TO TACKLE ANTIMICROBIAL RESISTANCE

From being a "wonder drug" antimicrobials are racing towards becoming a "perilous drug" because of resistant bacterial threat. Outsmarting the long term survivors of this earth (bacteria) seem impossible, but the rapid rise of resistance in them can be controlled. Plethoras of methods are being advised for the same. These include measures to control disease spread, judicious and careful use of existing drugs, development of novel drugs and global regulatory systems. Global regulation that may be legally binding or non- binding is one approach for antibiotic consumption control. Legally binding regulations like treaties put forth by multilateral organisations such as World Health Organization (WHO), Food and agricultural organization (FAO), World Organisation for Animal Health (OIE) have quick, sturdy and harmonizing effect on this public health threat (Padiyara *et al.,* 2018). These are more efficient as they keep the countries involved in it responsible for implementation of the same. Though not effective as treaties, non-binding mechanisms like political proclamation, decisions and

recommendations by intergovernmental organizations plays an important role in preserving antimicrobial effectiveness. Of the total antibiotic use, human and animal consumptions account for 50% each and around 60% of its use in both is questionable (Wise *et al.,* 1998). Promoting judicious use and preventing fraudulent use is therefore necessary. The prudent use of antibiotics decelerate rise of resistance and provide time for the development of alternatives for existing moribund drugs (Hoffman and Outterson, 2015). Preventive measures like vaccination, disease control, cohorting and access to safe and clean food and water can contribute to conservation (Clift *et al.,* 2015). Cohorting was done in Australian hospitals were severely ill patients with MRSA were moved to isolation wards that reduced the spread of MDR Staphylococcal strains (Turnidge *et al.,* 1989; Pearman and Grubb, 1993). Fast and efficient methods for disease diagnostic and antibiotic susceptibility testing saves time and allow medical practitioners to choose appropriate drugs accordingly (Levy and Marshall, 2004).

Continuous development of antibacterial drugs are inevitable as pathogenic microorganisms may gradually develop resistance against existing drugs. The aforementioned strategies can slow down the rate of resistance but cannot treat emerging resistant antibacterial threat. This can only be solved by innovative development of novel therapeutic or non- therapeutic agents that can function against resistant mechanism or target new site of action (Levy and Marshall, 2004). Several drugs are under clinical trials in pharmaceutical companies (Table 1). Since 1960s sufficient development of novel antibiotics were not made. The numbers of companies in this quest are declining due to high costs, time consumption and failure of drugs in clinical trials (Spellberg *et al.,* 2004). Antimicrobial peptides (AMPs) may contribute to the development of novel antibiotics. For example, Plazomicin belonging to aminoglycosides were developed to treat various Gram negative strains including MRSA (Livermore *et al.,* 2011).

Other than therapeutic agents, non-therapeutics can also be used for treatment of bacterial infections. Nanoparticles, which are able to penetrate the

cell wall of bacteria, are found to be efficient. A study conducted revealed the potential of Zinc oxide to cure Bacillus infection (Azam *et al.,* 2012). Quorom sensing (QS) in *P. aeruginosa* can be used as a target by antimicrobial compounds (Que *et al.,* 2011). Combination therapies involving lytic phages are promising as they destroy the pathogenic bacteria more precisely compared to antibiotics (Zhang *et al.,* 2012). Antibiotics in combination with antisense therapy can be used to alter pathogenic gene expression rendering them useless. They are promising as they do not create any selection pressure on pathogens and do not give rise to resistance. These are oligonucleotides that target either transcription or translation. Beta lactam antibiotics are co administered with antisense agents like tazobactam, sulbactam to restore their effectiveness towards beta lactamase producing strains (Woodford and Wareham, 2009). In animals AMR can be tackled by the use of prebiotics, probiotics and synbiotics that improves the intestinal flora and do not provide room for pathogens (Callaway *et al.,* 2008; Gaggia *et al.,* 2010). Use of antagonistic bacterium against pathogens is effective in combating resistance as former release enzymes degrading nucleic acid and proteins. *Bdellovibrio* spp. predate on both MDR and non- MDR *Pseudomonas* and *Klebsiella* strains (Kadouri *et al.,* 2013; Lambert and Sockett, 2013; Allen *et al.,* 2014).

Table 1. Antimicrobial compounds in current clinical pipeline (Boucher *et al.,* 2009)

Antimicrobial compound	Classification	Pathogen	Company	Associated infection
TD-1792	Vanco-Cephalosporin	Gram positive (G+) bacteria	Theravance	Skin infection
Ceftobiprole	Cephalosporin	G+ bacteria	Peninsula Pharmaceuticals	Skin infection
Telavancin	Lipoglycopeptide	G+ bacteria	Theravance	Pneumonia and skin infection
Dalbavancin	Lipoglycopeptide	G+ bacteria	Pfizer	Skin infection
Iclaprim	Diaminopyrimidine	G+ bacteria	Arpida	Pneumonia and skin infection

RX-1741	Oxazolidinone	G+ bacteria	Rib-X	Pneumonia and skin infection
Doripenem	Carbapenem	*P. aeruginosa*	Johnson and Johnson	intra-abdominal infection and complicated urinary tract infection
Tomopenem	Carbapenem	*P. aeruginosa* and MRSA	Daiichi Sankyo	Pneumonia and skin infection
PTK-0796	Aminomethylcycline	MRSA, VRE, and some resistant G- pathogens, including *A. baumannii*	Paratek Pharmaceuticals	Skin infections
ME 1036	Carbapenem	MRSA and VRE, ESBL-producing *E. coli, K. pneumonia*	Forest laboratories	Pneumonia and skin infection
PZ-601	Carbapenem	MRSA, P. aeruginosa, *A. baumannii*	Protez, Dainippon Sumitomo	Skin infection
Sulopenem	Orally active penem	G- pathogens including ESBL-producing Enterobacteriacea, G+ pathogens, and anaerobes	Pfizer	Pneumonia
BAL 30376	Combination of monobactam, oxapenem and beta lactamase inhibitor	*Acinetobacter* species, non-fermenting bacilli (*P. aeruginosa* and *S. maltophilia*	Basilea	Peritonitis and sepsis in murine
ME1071	Metallo-beta-lactamase inhibitor	*A. baumannii* (*P. aeruginosa*)	Meiji Seika Kaisha	Nosocomial infections

2.4. MARINE MICROBES AS A RESOURCE FOR ANTIMICROBIAL AGENT

As the emergence of AMR strains and newer diseases needs to be addressed urgently, antimicrobial potential of marine habitat should be exploited. Terrestrial environment has been extensively exploited for novel drug discovery so that the focus has now shifted to least explored or underexplored sources. Marine ecosystem with its vastness, diverse life forms and extreme habitats form one such source that needs attention and have been in focus recently (Abad *et al.,* 2011). They encompass diverse forms of life that have the ability to produce manifold metabolites with pharmaceutical potential and biomedical importance (Haygood *et al.,* 1999 and Hentschel *et al.,* 2001). This is because they are exposed to harsh and competitive environmental conditions with varying degrees of salinity, temperature, pressure, pathogens and predators. As a result they produce compounds to thrive these environments, to protect themselves from predators and to survive infections (Wenzel and Muller, 2005). Sponges, algae, molluscs, bivalves are marine organisms which are recognised as having vast potential for the production of bioactives (Abad *et al.,* 2011). Different classes of compounds like polyketides, non-ribosomal and ribosomal peptides, terpenes with antimicrobial activity are reported to be produced by them (Hughes and Fenical, 2010; Smith *et al.,* 2010). Studies have shown that these compounds which were attributed to be produced from marine organisms are actually of microbial origin (Fotrman and Sherman, 2005; Konig *et al.,* 2006 and Zhang *et al.,* 2009). Thus, symbiotic microbes of marine organisms play significant role in their host's defence system and are novel leads for drug development. The advent of marine microbe into drug development began with the discovery of Cephalosporin C from *Cephalosporium* sp. isolated from Sardinian coast (Pandey, 2019). Most of the antimicrobials being discovered from marine microbes are mainly from bacteria (*Actinomycetes)* and fungi (Cragg and Newman, 2007; Butler and Buss, 2006). The first antibiotic from marine bacteria was developed in mid-1990's (Ocio *et al.,* 2009). Many of the bacteria belonging to genus *Actinomycetes,*

Pseudomonas, Bacillus, Pseudoalteromonas were identified for their activities against pathogens (Mc Envoy, 1993; Lucas-Elio *et al.,* 2005; Charyulu *et al.,* 2009; Darabpour *et al.,* 2012; Tawiah *et al.,* 2012). These bacteria may be either symbionts or free living. Symbiotic bacteria associated with various marine invertebrates were recently known to be precious sources of antimicrobials.

Sponges from marine ecosystem were the research hotspot in antimicrobial search for the past few decades. These sessile marine filter feeders harbour a wide range of organisms both intracellularly and extracellularly with diverse behavioural habits. They occupy almost half of the sponge biomass (Wilkinson, 1987).These symbionts that survive sponge digestive system are involved in sponge defence mechanism with tremendous capacity for the production novel metabolites (Wilkinson, 1987; Bultel-Ponce *et al.,* 1999; Schmidt *et al.,* 2000). Sponges belonging to Class *Desmospongiae* are the major contributors to symbionts derived bioactives of medicinal value (Thomas *et al.,* 2010).

Marine bacteria associated with marine invertebrates other than sponges were also found to be beneficial for novel drug quest. Soft coral *Alcyonium digitatum* was studied for its antimicrobial potential against *Staphylococcus* sp., *Bacillus* sp. and *E. coli* where, around 50% of the total symbionts were found to exhibit activity (Pham *et al.,* 2016). Gram positive pathogenic strains were shown to be susceptible to symbiotic bacteria associated with Bryozoans obtained from Baltic and Mediterranean Sea (Heindl *et al.,* 2010). Wiese *et al.* (2018) discovered Bacicyclin, a cyclic hexapeptide from *Bacillus* sp. associated with marine mollusc *Mytilus edulis* which exhibited antibacterial activity against clinically relevant pathogens like *Enterococcus faecalis* and *Staphylococcus aureus.* The lists of the important antimicrobial compounds derived from various marine bacterial sources are given in Table 2.

Table 2. Major antimicrobial compounds derived from marine bacterial sources

Compound	Activity	*Marine host*	Bacteria	Reference
Not identified	Antimicrobial	*Homophymia* sp	*Pseudomonas* sp.	Bultel- Ponce *et al.* (1999)
Not identified	Antimicrobial	*Xestospongia* sp.	*Micrococcus luteus*	Bultel- Ponce *et al.* (1998)
Andrimid	Anti-*Bacillus*	*Hyatella* sp.	*Vibrio* sp.	Oclarit *et al.* (1994)
Polybrominated biphenyl ethers	Antimicrobial	*Dysidea herbacea*	*Oscillatoria Spongeliae*	Floweres *et al.* (1998)
Onegramide	Antifungal cyclic peptide	Not known	Filamentous heterotrophic bacteria	Bewley *et al.* (1996)
Quinolones	Antimicrobial	*Suberea creba*	Pseudomonad	Debitus *et al.* (1998)
Quinolones and phosphatidyl Glyceride	Antimicrobial	*Homophymia* sp.	*Pseudomonas* sp	Bultel- Ponce *et al.* (1999)
Not identified	Antibacterial activity against *Pseudomonas* sp. and *S. aureus*	Mediterranean Sea at Murcia coast	*Marinomonas mediterranea*	Lucas-Elio *et al.*, 2005
Not identified	Antimicrobial	Iran	*Pseudoalteromonas piscicida*	Tawiah *et al.* (2012)
Not identified	Antimicrobial	Ghana	*Pseudomonas aeruginosa*	Darabpour *et al.* (2012)
2,2',3-tribromo-biphenyl-4,4'-dicarboxylic acid	Antibacterial activity against MR-*Staphylococcus*	-	*Pseudoalteromonas phenolica*	Isnansetyo and Kamei (2003)
Not identified	Antibacterial	-	*Pseudoalteromonas luteoviolacea*	Radjasa *et al.* (2007)
Not identified	Antibacterial	Tunicates.	*Pseudoalteromonas*	Holmstrom

			sp.	and Kjelleberg (1999)
Phenolic compounds such as 4,4,6-tribromo-2,2'-biphenol ,CMMED 290	Against MRSA	-	*Pseudoalteromonas* sp	Feher *et al.* (2010)
Bromophenyl compounds	Antibacterial	-	*Pseudoalteromonas haloplanktis*	Hayashida-Soiza *et al.* (2008)
Not identified	Antimicrobial	-	*Streptomyces* sp	El Gendy *et al.* (2008)
Ammonificins A and B	Antimicrobial	Marine hydrothermal vent	*Thermovibrio ammonifican*	Andrianasolo *et al.* (2009)
Cyclic maribasins A and B, lipopeptides	Antifungal activity against phytopathogens	Suaeda salsa on the Bohai coastline of China	*Bacillus marinus* B-9987	Zhang *et al.* (2010)
Two antifungal lipopeptides	Antifungal against 5 plant pathogens	-	*B.amyloliquefaciens* SH-B10	Chen *et al.* (2010)
Tauramamide	*Enterococcus* sp.	Papua New Guinea	*Brevibacillus laterosporus* PNG276	Desjardine *et al.* (2007)
Unnarmicine A and C antibacterial depsipeptides	Against *Pseudovibrio.*	-	*Photobacterium* MBIC06485	Oku *et al.* (2008)
Cyclic hybrid polyketide-peptide antibiotics miuraenamides A and B	Antimicrobial	Japan	*Paraliomyxa miuraensis*	Ojika *et al.* (2008)

Ariakemicins A and B -linear hybrid polyketide-nonribosomal peptide antiobiotics	G+ bacteria	-	*Rapidithrix*	Oku *et al.* (2008)
Not identified	Antimicrobial	*Erylus deficiens*	*Cellulomonas* and *Proteus* sp.	Graca *et al.* (2015)
Symplostatin 1 (currently in phase II clinical trials.)	Antimicrobial	Marine mollusk *Dolabella auricularia*	Blue-green alga *Symploca hydnoides*	Pandey (2019)
Cyclic peptide theopalauamide and macrolide swinholide.	Antifungal	Sponge *Theonella swinhoei* Philippines	Filamentous bacteria	Bewley *et al.* (1996); Bewley and Faulkner (1998)
Zafrin (4b-methyl-5,6,7,8–tetrahydro-1 (4b-H) – phenanthrenone)	Anti MRSA	Abdomen of fish at Balochistan Coast, Pakistan	*Pseudomonas stutzeri* (CMG 1030)	Uzair *et al.* (2008)
Mayamycin 53	Antibacterial activity against different human pathogens	Sponge *Halichondria* associated	*Streptomyces* sp.	Schneemann *et al.* (2010)
Kocurin - member of the thiazolyl peptide family of antibiotics	Anti-MRSA	Marine sponge	*Kocuria* and *Micrococcus*	Palomo *et al.* (2013)
Norharman (Beta-carboline alkaloid)	Against *S. aureus*, *A. tumefaciens* and *B. subtilis*	Sponge *Hymeniacidon perleve*	*Pseudoalteromonas piscicida*	Zheng *et al.* (2005)
Antibiotics	-	*Halichondria*	*Bacillus cereus*	Suzumura *et*

		japonica		*al.* (2003)
Cyclic decapeptide (loloatin B)	Against VRE, MRSA	Marine worm- Papua New Guinea	*Bacillus* species	Javed *et al.* (2011)

2.5. MICROBIAL BIOSYNTHETIC GENES ENCODING ANTIBACTERIAL AGENTS

The major drawback of marine antibacterial sources, which open up new windows towards discovery of novel antibiotics, is that they cannot be cultured or preserved efficiently compared to terrestrial antibacterial sources (Jensen *et al.,* 2005). The antibacterials produced by them may be insufficient for large scale commercial production, may have scarce expression under laboratory culture conditions or loss their viability during preservation (Unson *et al.,* 1994 and Lee *et al.,* 2001). Therefore alternative method for the isolation of these antibacterial compounds should be identified after the identification of resources. Molecular approaches serves as potential tools to overcome this challenge. Identification and analysis of biosynthetic gene clusters necessary for the production of these compounds and their expression in suitable culturable host is an ideal strategy that is beneficial for mass production of antibacterial metabolites. Moreover detailed insilico and invitro analysis of biosynthetic gene clusters can contribute to the development of modified compounds with unique and beneficial properties which is extremely useful in the field of novel drug development (Kalaitzis *et al.,* 2009).

Biosynthetic gene clusters (BGC's) are a group of closely related non-homologues genes that are involved in the synthesis of a secondary metabolite. They are self- contained arrays that include genes involved in pathways for the synthesis of the basic structure of different classes of compounds and tailoring enzymes that modify the skeletal metabolite structure (Osbourn, 2010). In-vivo, they are expressed under the influence of various abiotic, biotic or developmental factors (Hoffmeister and Keller, 2007; Osbourn and Field, 2009).They form

super-clusters of different metabolite producing genes and are overlapped resulting in more complex bio-actives (Trefzer *et al.,* 2002; Fischbach, 2009). Within the genome they are located in close proximity to the variable end regions of chromosomes (Challis and Hopwood, 2003). In eukaryotes like fungi, they may undergo chromosomal rearrangements like insertion, deletion, inversion and recombination including other modifications (Farman, 2007). Clustering offer much advantage to organisms as genes can be efficiently co-regulated and transferred between and within species (Wenzl *et al.,* 2005; Ridley *et al.,* 2008; Osbourn, 2010). Cluster organisation and the biosynthetic pathway is quiet complex with all of them displaying substrate flexibility and thus producing congeners of bio-actives (Zotchev, 2012). BGCs are frequently grouped based on their products that include ribosomally synthesised and post translationally modified peptides (RiPPs), non-ribosomal peptides (NRPs), terpenes and saccharides (Xiong *et al.,* 2013; Naughton *et al.,* 2017*)*.

Among the marine microbes, Actinomycetes are the most widely studied group for secondary metabolite producing BGCs (Watve *et al.,* 2001). Among them, Streptomyces are recognized as the most potent producer of antibiotics (Challis and Hopwood, 2003). Since the first cloning and heterologous expression of actinorhodin biosynthetic gene cluster from Streptomyces coelicolor in S. parvulus (Malpartida and Hopwood, 1984) the molecular approaches for the mining of antibiotic BGC from Actinomycetes began. The synthesis of actinorhodin is driven by polyketide synthase pathway using acetyl units as substrates (Gorst-Allman *et al.,* 1981). Super hosts like M512 mutant of S. coelicolor was developed to stably express antibiotic BGCs (Baltz, 2010). Non ribosomal peptide synthetase (NRPS), polyketide synthase (PKS) and hybrid NRPS -PKS are the most common BGCs responsible for bioactive production in them (Ayuso-Sacido and Genilloud, 2005*)*.They may be a few to 100 kb in size in case of Streptomyces (Bentley *et al.,* 2002 and Ohnishi *et al.,* 2008). These gene clusters are responsible for the formation of more than 50% of bio-actives discovered so far (Baltz, 2014). They encode enzyme with a set of functional

domains called modules and require a phosphopantetheinyl transferase (PPTase) for the activation of these enzymes. As in terrestrial microbes, investigation on BGC from marine microbes has been done (Table 3). NRPS and PKS clusters are recognised as responsible for the synthesis of many classes of bio-actives from marine bacteria especially Gram positive marine actinomycetes (Mizuno *et al.*, 2013).

2.5.1. Non ribosomal peptide synthetases (NRPS) gene clusters

Non ribosomal peptide synthetases (NRPS) gene clusters are the most studied compared to PKS and hybrids (Donadio *et al.*, 2007). Non ribosomal peptides are formed independent of ribosomal synthesis. Non proteinogenic amino acid or hydroxyl acids are used as substrates in this pathway and the synthesis is carried out on a protein template (Finking and Marahiel, 2004; Seiber and Marahiel, 2005). These assembly lines include loading, elongation and termination modules with multiple domains. Adenylation (A), Peptidyl Carrier Protein (PCP), thiolation (T) and Condensation (C) domains are present in all elongation modules. Loading module lack C domain and termination module include an extra thioesterase (TE) domain (Royer *et al.*, 2013). These domains are inevitable for the selection and oligomerisation of the amino acid unit. Methylation (M), epimerisation (E) and reduction (R) domains carry out additional specialised functions thus modifying the so formed product (Donadio *et al.*, 2007).

2.5.2. Polyketide synthase (PKS) biosynthetic gene clusters

Polyketide synthase (PKS) biosynthetic gene clusters encode enzymes necessary for the production of polyketides (Dewick, 2002). They are functionally similar to NRPS biosynthetic pathway and contain modules with multiple functions that involve the utilisation of acetate or propionate units. Each module except loading module contains three domains which perform vital function necessary for the formation of corresponding compound. They are acyltransferase (AT) domain, ketosynthase (KS) domain and acyl carrier protein (ACP) domain (Ayuso-Sacido and Genilloud, 2005). Loading module lacks KS domain and termination module involve an additional TE domain for the release of the final

product. The coenzyme A bound starter attaches to ACP via thioester bond formation catalysed by AT domain. The AT domain is responsible for the selection of suitable substrate for the corresponding pathway. The ACP bound starter then attaches via cysteine residue to KS domain those catalyses the elongation of the attached unit by decarboxylative condensation of the same with the polyketide intermediate of the preceding module. This pathway catalyse the formation of compounds via claisen condensation (Donadio *et al.*, 2007). The substrate selection for each PKS pathway differ according to the difference in the sequence motif of the AT domain. Thus, diverse and complex polyketides are produced depending on both the substrate being utilised and modifications incorporated by extra domains such as enoylreductase (ER), dehydratase (DH) and ketoreductase (KR) domains (Olano *et al.*, 2009). Type 1 and type 2 PKS clusters have been reported in Streptomyces. Type 1 PKS clusters are again classified to modular and iterative types where multiple functions are carried out by different modules and single modules respectively. Modular PKS clusters are the most identified as potent producers of many important bioactives (Ayuso-Sacido and Genilloud, 2005; Olano *et al.*, 2009).

Table 3. Biosynthetic gene clusters associated with marine microbes

Compound	Activity	Gene/BGC	Bacteria	Source	Reference
Tirandamycin	Antibacterial	*PKS 1* and *NRPS*	*Streptomyces* sp.	South China Sea	Mo *et al.* (2011)
Abyssomicin C-Polycyclic Antibiotic	Antibacterial (including MRSA) Inhibitor of PABA/Tetrahydrofolate Biosynthesis	*PKS*	*Verrucosispora* sp.	Sea sediment from Japan	Bister *et al.* (2004)
TP1161-Thiopeptide	Antibacterial	*PKS 1, PKS 2 and NRPS*	*Nocardiopsis sp.*	Sediments of Trondheimfjord	Engelhardt *et al.* (2010)
Unidentified	Antibacterial	Hybrid	*Pseudovibrio*	*Haliclona*	Kennedy

		PKS and *NRPS*		*simulans*	*et al.* (2009)
Enterocin	Bacteriostatic	*PKS 2*	*Streptomyces maritimus*	Not known	Piel *et al.* (2000)
Griseorhodin A	Antibiotic	*PKS*	*Streptomyces*	Aplidium lenticulum	Li and Piel (2002)
2Allyloxypheno l (*Terpenoid)*	Antimicrobial	Not known	*Streptomyces*	Bay of Bengal, India	Arumuga m *et al.* (2009)
Unidentified-related to Bleomycin	Antitumor and antibiotic	Hybrid *NRPS-PKS*	*Alteromonas macleodii*	Water from the Adriatic Sea	Mizuno *et al.* (2013)
Alteramide A	Antimicrobial	Alkaloid	*Alteromonas* sp.	*Halichondri a okadai*	Shigemori *et al.* (1992)
Thiomarinal	Antimicrobial	Hybrid *NRPS-PKS*	*Pseudoalteromo nas* sp.	Not known	Murphy *et al.* (2011); Fukuda *et al.* (2011)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 EQUIPMENTS

Cooling micro centrifuge (Remi, India and Hermle, Germany),Thermal cycler (Proflex, India and ABI Veriti 96 well thermal cycler, Thermofischer scientific), pH meter (Elico Ltd., India), Vortex mixer (Spinix, India), Laminar Air flow system (Labline, India), Class II Biological Safety Cabinet (ESCO), UV trans illuminator (Gelstan, India), Orbital shaker cum incubator (Labline, India), Microwave oven (Samsung, India),Water bath (Labline) Incubator (Kemi, India and Labline, India), Electronic weighing balance (Shimadzu, India), Hot airoven (Kemi, India), Autoclave(Labline, India), Refrigerator(Samsung, Whirlpool, India) Ultra low temperature freezer -80°C (New Brunswick Scientific, India), Microscope (Leica micro system, India), spectrophotometer (Multiskan go, Thermofisher scientific) and bio photometer (Eppendorf) were used in the present study.

3.1.2 GLASS WARE AND PLASTIC WARE

Test tubes, petridishes, conical flasks, beakers, glass rods etc. used in this study were procured from reputed firms such as Borosil (India). They were thoroughly washed and sterilized as per standard procedures. Plastic wares including micro centrifuge tubes, micropipette tips and centrifuge tubes were sterilized and used.

3.1.3 CHEMICALS AND REAGENTS

Chemicals and reagents used have been mentioned either at appropriate places or listed in the Appendix. Chemicals were from reputed firms such as Sigmaaldrich, Himedia (India), and other reputed firms. The compositions of media are enclosed in Appendix I.

LPS

3.1.4 BACTERIAL ISOLATES USED IN THE STUDY

A total of 12 pathogenic bacterial strains including 6 human and 6 aquatic pathogens were used as indicator strains for the antibacterial assay. Details of these strains were given in Table 4.

Table 4. Pathogens used in the study

Sl no.	Pathogens	Isolate ID	Source
Aquatic pathogens			
1	*Vibrio harveyi*	CMFRI/VHa-03	Marine microbial culture collection, ICAR-CMFRI, Kochi
2	*Vibrio vulnificus*	CMFRI/VV-01	Marine microbial culture collection, ICAR-CMFRI, Kochi
3	*Vibrio anguillarum*	CMFRI/VAn-1	ICAR- Central Institute of Brackish water Aquaculture (CIBA), Chennai
4	*Vibrio parahaemolyticus*	CMFRI/VP-08	Marine microbial culture collection, ICAR-CMFRI, Kochi
5	*Vibrio alginolyticus*	CMFRI/VAl-01	ICAR-CIBA, Chennai
6	*Photobacterium damselae*	CMFRI/PhD-05	Marine microbial culture collection, ICAR-CMFRI, Kochi
Human pathogens			
7	*Salmonella* Typhimurium	MTCC 3224	Microbial Type Culture Collection (MTCC), Chandigarh, India
8	*Shigella flexneri*	MTCC 1357	MTCC, Chandigarh, India
9	*Staphylococcus aureus*	MTCC 96	MTCC, Chandigarh, India
10	*Escherichia coli*	MTCC 25922	MTCC, Chandigarh, India
Multidrug resistant (MDR) pathogens			
11	Methicillin Resistant *S. aureus* (MRSA)	MTCC 1430	MTCC, Chandigarh, India
12	Beta lactamase producing *E. coli*	MTCC 35218	MTCC, Chandigarh, India

3.1.5 PRIMERS USED IN THE STUDY

Various primers used in the present study are shown in Table 5.

Table 5. Primers used in the study

SL. No.	Primers	Sequence (5'-3')	Reference
1	NP1F	GAGTTTGATCCTGGCTCA	Nair *et al.*, 2012
2	NP1R	ACGGCTACCTTGTTACGACTT	Nair *et al.*, 2012
3	KSI 1f	GCIATGGAYCCICCARCARMGIVT	Schirmer *et al.*, 2005
4	KSI 2r	GTICCIGTICCRTGISCYTCIAC	Schirmer *et al.*, 2005
5	MTF	CCNCGDATYTTNACYTG	Neilan *et al.*, 1999
6	MTR	GCNGGYGGYGCNTAYGTNCC	Neilan *et al.*, 1999

3.2. METHODS

3.2.1 SAMPLES COLLECTION AND PROCESSING

Four commercially significant marine crab species namely, *Charybdis feriatus* (Linnaeus, 1758); *Portunus pelagicus* (Linnaeus, 1758); *Portunus sanguinolentus* (Herbst, 1783) and *Scylla olivacea* (Herbst, 1796) were collected using trawl nets. The animals were retained in sterile polythene bags containing sea water. The bags were then placed in thermocol boxes containing ice packs and brought to laboratory within 1 hour after collection. A total of 9 apparently healthy marine crabs from each species were selected, anaesthetized on ice. External surfaces especially, base of periopods were disinfected with 70% ethanol before collection of hemolymph, to prevent contamination with surface bacteria (Colwell *et al.*, 1975) and hemolymph was aseptically withdrawn.

Similarly, a total of 48 apparently healthy Asian green lipped mussel, *Perna viridis* (Linnaeus, 1758) were collected manually from Cochin coast, Kerala, India and used for the present study. The animals were retained in sterile polythene bags containing sea water and the bags were then placed in thermocol

boxes containing ice packs and brought to laboratory within 1 hour after collection. Animals were randomly divided into 8 groups. The collected animals were washed with sterile marine water and wiped with alcohol to remove non-adherent bacteria. The haemolymph was then withdrawn using sterile 22 gauge syringe and haemolymph collected from animals of each group were pooled. The shells were opened and different tissues including gill, gut, muscle and mantle was dissected out aseptically using sterile knife and forceps. The corresponding tissues from six mussels were pooled together and were transferred to a sterile 1.5 ml microcentrifuge tube. Weight of each tissue pool was taken and the homogenized using sterile normal saline.

3.2.2. ESTIMATION OF DENSITY OF CULTIVABLE MICROBIAL SYMBIONTS

The pooled haemolymph (0.1 ml) was diluted with 900 µl of sterile normal saline. Pooled tissue samples weighing 0.1 g was homogenized in a blender with 1000 µl of sterile normal saline using mortar and pestle. 100 µL of each tissue homogenate were diluted in 900 µL of sterile normal saline. These were then serially diluted using sterile normal saline. Hundred microliter of each dilution was transferred to sterile ZMA (Zobell Marine Agar), TCBS (Thiosulphate Citrate Bile Salt Sugar) agar and BHIA (Brain Heart Infusion Agar) plates and uniformly spread using sterile L rods. Each dilution was plated in duplicates. The plates were incubated at 30°C for 24-48 h. After incubation, the plates with 30-300 colonies were chosen for counting and the total viable count was expressed as the number of colony forming units (CFU) per ml and g of haemolymph and tissue respectively (Hovda *et al.*, 2007).

3.2.3. ISOLATION OF MICROBIAL SYMBIONTS

Morphologically unique bacteria from the various culture plates were picked up and were checked for purity by sub-culturing. These were then aseptically transferred to sterile nutrient slants such as ZMA and BHIA slants and kept at 4^0C and sub-culturing was done periodically to preserve the viability of the isolates.

These were used for further characterization. At the same time glycerol stock of pure culture of the isolates were also made and stored at -80^{0}C.

3.2.4. SCREENING FOR ANTAGONISTIC ACTIVITY AGAINST PATHOGENS

In-vitro antibacterial assay was carried out using spot on lawn culture method (Rojo-Bezares *et al.,* 2007) against indicator pathogens. Briefly, bacterial suspension in log phase of growth was swabbed onto Muller Hinton Agar. Afterwards, the pure culture of each isolate was spotted onto these agar plates. The plates were incubated at 30^{0}C and 37^{0}C overnight for aquatic and human pathogens respectively. The inhibition zone diameter were measured and recorded with the help of a scale. Versatility of each isolates and number of positive isolates against each pathogen were also noted down. Scoring of positive isolates based on their zone of inhibition diameter was also done (Nandi *et al.,* 2017) in which score 4, 3, 2, 1 and 0 were assigned to very high (> 15 mm), high (10-14 mm), moderate (5-9 mm), Low (1-4 mm) and no inhibition activities respectively.

3.2.5. IDENTIFICATION OF BACTERIA HAVING ANTAGONISTIC POTENTIAL

The positive isolates showing antagonistic potential against any of tested pathogens were identified by polyphasic taxonomic approach (a series of morphological, cultural, biochemical and molecular tests).

3.2.5.1. Morphological characterization

3.2.5.1.1. Gram staining

Bacterial smear was prepared from the colony and was heat fixed. The slide was flooded with crystal violet reagent for 1 min and the smear was gently washed by direct stream of tap water for 1 min again the slide was flooded with Grams iodine solution for 1 min. The slide was washed and decolorized with 95% ethanol for few seconds, then flooded with water and then counter stain safranin

was added, allowed to react for 1 min. The slide was washed further, dried and observed under microscope with 100 X magnification using oil immersion.

3.2.5.1.2. KOH string test

A drop of 3% KOH was placed onto a glass slide. Using a loop a visible amount of fresh bacteria from a well isolated colony was removed and transferred into KOH. This was then mixed continuously on the glass slide for a maximum of 1 min and by slowly lifting the loop, formation of a string was observed.

3.2.5.2 Cultural characterization

The bacterial isolates were sub-cultured on various media such as ZMA, TCBS, BHIA, MacConket agar (MCA) and Luria Bertani (LB) Broth and cultural characteristics were noted down.

3.2.5.3. Biochemical characterization

3.2.5.3.1. Preliminary biochemical characterization

A series of biochemical tests were done for the preliminary clustering of isolates and details of the same were given below.

a. Catalase test: A drop of hydrogen peroxide (3%) was placed on a microscopic slide. Using loop, test organism was placed on the slide and effervescence was observed.

b. Oxidase test: Oxidase test was carried out by touching and spreading a well isolated colony on the oxidase disc and the formation of a violet color was observed after 5-10 sec.

c. Indole test: Pure bacterial cultures were grown in sterile tryptophan broth for 24-48 hours. After incubation, few drops of Kovac's reagent were layered onto the culture broth. A red ring formation was observed on the addition of Kovac's reagent.

d. Methyl Red test: Isolate was inoculated into MR-VP broth (Himedia) with sterile loop. The tube was incubated at 28°C for 2 days. After incubation five drops of pH indicator methyl red was added to this tube and color change was observed.

e. Voges-Proskauer test: Isolate was inoculated into MR-VP broth with sterile loop. The tube was incubated at 28°C for 2 days. An aliquot of 0.6 ml of 5% alpha naphthol is added followed by 0.2 ml of 40% KOH and the tube was shaken gently to expose the medium to atmospheric oxygen. The tube was allowed to remain undisturbed for 30 to 45 min.

f. Citrate test: The isolate was inoculated into Simmons citrate agar and incubated at 28°C for 24-48 hour. The color change was observed.

g. Oxidative Fermentative test (OF): Two tubes of OF test medium were inoculated with the test organism using a straight loop by stabbing "half way to the bottom" of the tube. One tube of each pair is covered with 1 cm layer of sterile mineral oil or liquid paraffin (it creates anaerobic condition in the tube by preventing diffusion of oxygen), leaving the other tube open to the air. Both tubes were incubated at 28°C for 48 hours.

After the preliminary characterization by the above methods the isolates having similar morphological, cultural and biochemical characteristics were clubbed together to one group and representative one isolate from each group were then characterized by 16S *rRNA* gene sequencing.

3.2.5.4. Molecular characterization by *16S rRNA* gene sequencing

3.2.5.4.1 Extraction of genomic DNA from bacteria

A well isolated colony was inoculated in 5 ml of LB broth and kept overnight in a shaker incubator. After incubation the cells were harvested by centrifugation at 10,000 rpm for 10 min. The pellet was re-suspended in 570 µl TEG buffer with lyzozyme and vortexed well. 30 µl of 10% SDS and 3 µL of proteinase K was added and mixed well. Sample was then incubated at 60°C for 1

h. 100 µL of 5M NaCl and 80 µL of CTAB were added and incubated at 65^0C for 15 min. After incubation, equal volume of phenol: chloroform: isoamyl alcohol in the ratio 25:24:1 was added and centrifuged at 12,000 rpm for 20 min. This step was repeated twice. The aqueous phase was collected in another micro centrifuge tube without disturbing the inter phase and lower phase. To the supernatant equal volume of chloroform: isoamyl alcohol in the ratio 24:1 was added and centrifuged at 12,000 rpm for 20 min. Then $1/10^{th}$ volume of 3 M sodium acetate followed by 80% of isopropanol was added and incubated at -20°C for 20 min so that the DNA got precipitated. This was subjected to centrifugation at 10,000 rpm for 20 min. The supernatant was discarded and the pellet was re suspended in 1mL of 70% ethanol and centrifuged at 10,000 rpm for 15 min. Supernatant was discarded and the pellet was air dried for 30 min. DNA samples were then dissolved in 30 µl of DNA dissolving buffer (TE buffer) and stored at 4°C. Further purity of DNA was analyzed by Agarose gel electrophoresis.

3.2.5.4.2. Analysis of genomic DNA by agarose gel electrophoresis

5 µl of DNA with 1 µl loading dye (6X) (Takara) was loaded on agarose gel. The agarose gel (0.7%) was run at constant voltage of 100 V and current of 45 A till the bromophenol blue has reached the extreme opposite side of the wells with ladder of gene ruler (Thermo scientific). Viewed the gel on UV transilluminator with the safety shield and photograph was taken.

3.2.5.4.3. PCR amplification of 16SrRNA gene

The method of *16S* rRNA gene amplification was carried out using universal prokaryotic primers; NP1F 5'GAGTTTGATCCTGGCTCA-3' and NP1R 5'-ACGGCTACCTTGTTACGACTT- 3' (Nair *et al.*, 2012). Each polymerase chain reaction (PCR) mixture consisted of 1 µl of template DNA, 2.5 µl of 1X Biolab *Taq* buffer, 0.5 µl of dNTP mix, 0.5 µl of Forward and Reverse Primer and 1.25 U of Biolab *Taq* DNA polymerase. The PCR programme of each sample included initial denaturation at (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 30 sec), annealing (58°C for 1 min) and extension (72°C

for 1.30 min). Final extension was carried out at (72°C for 5 min). The PCR products were then characterized by submarine gel electrophoresis (1% agarose gel). 1 Kb DNA ladder (Fermentas) was used as molecular weight marker. After electrophoresis the gel was visualized in Gel documentation system.

3.2.5.4.4. Sequence Analysis

The PCR products were sequenced at Agrigenome, Kochi. The obtained sequences were then subjected to BLAST search (NCBI) and the genus of the bacteria were identified. Further species/ sub-species level was confirmed by different biochemical tests based on the genus identified. Finally, the sequences were submitted in NCBI and got assigned with Accession numbers.

3.2.5.5. Identification of antagonistic bacteria up to species level

3.2.5.5.1. Characterization of Bacillus up to species level

a. Sugar Fermentation Test: Aseptically inoculated pure culture of the test organism to labelled bromocresol purple broth (Himedia) to which various sugar discs (Himedia) (Mannitol, mannose, sucrose, trehalose, starch, glycogen) were added. Incubated the tubes at 18-24 hours at 28°C.
b. Citrate test: The isolate was inoculated into Simmons citrate agar and incubated at 28°C for 24-48 h.
c. Antibiotic sensitivity test: Pure culture in Luria Bertani Broth was swabbed aseptically to Mueller Hinton Agar (MHA) plate and antibiotic disc (penicillin) was fixed. The plate was incubated at 28°C for 24 h.
d. Arginine Decarboxylase Test: An inoculum from a pure culture is transferred aseptically to a sterile tube of Arginine Decarboxylase broth (Himedia). The inoculated tube is incubated at 28°C for 24-48 h. After inoculation over layered the tubes with 2-3 ml mineral oil.
e. Motility: The isolate was inoculated into Mannitol motility agar and incubated at 28°C for 24-48 h.
f. Colony morphology: The isolate was streaked in BHIA plate and incubated at 28°C for 24-48 h. Colony characters were noted.

g. Test for gelatinase: An inoculum from a pure culture was spotted aseptically to gelatin embedded agar plates containing 1% of NaCl and inoculated plate was incubated at 28°C for 24 h.
h. Growth at 45^0C: An inoculum from a pure culture was transferred aseptically to Luria Bertani Broth containing 1% of NaCl and inoculated tube was incubated at 45^0C for 24 h.

The combined analysis of the results of these biochemical tests and the results of NCBI-BLAST Analysis was done in order to precisely identify the species.

3.2.5.5.2. Characterization of Pseudomonas up to species level

The various tests included

a. Arginine Decarboxylase Test: An inoculum from the pure culture was transferred aseptically to a sterile tube of Arginine Decarboxylase broth (Himedia). The inoculated tube was incubated at 28°C for 24 h. After inoculation over layered the tubes with 2-3 ml mineral oil.
b. Growth at different w/v concentration of NaCl: An inoculum from a pure culture was transferred aseptically to peptone broth containing w/v concentration of NaCl (8%) and inoculated tube was incubated at 28°C for 24 h.
c. Growth at different temperature: An inoculum from a pure culture was transferred aseptically to Luria Bertani Broth containing 1% of NaCl and inoculated tube was incubated at (4°C, 42°C and 46°C) for 28°C 24 h.
d. Sugar Fermentation Test: Aseptically inoculated pure culture of the test organism to labelled bromocresol purple broth (Himedia) to which Mannitol disc was added. Incubated the tube at 18-24 h at 28°C.
e. Citrate test: The isolate was inoculated into Simmons citrate agar and incubated at 28°C for 24-48 h.

The combined analysis of the results of these biochemical tests and the results of NCBI-BLAST Analysis was done in order to precisely identify the species.

3.2.5.5.3. Characterization of Shewanella up to species level

The various tests included

a. Ornithine Decarboxylase Test: An inoculum from a pure culture was transferred aseptically to a sterile tube of ornithine decarboxylase broth (Himedia). The inoculated tube was incubated at 28°C for 24 h. After inoculation over layered the tubes with 2-3 ml mineral oil.
b. Urease test: Streak the surface of a urea agar slant (Himedia) with a portion of a well-isolated colony and incubate the tube at 28°C in ambient air for 24 h.
c. Sugar Fermentation Test: Aseptically inoculated pure culture of the test organism to each labelled bromocresol purple broth (Himedia) to which various sugar discs (Himedia) (sucrose, mannose, ribose, glucose, fructose, salicin, mannose, and sorbitol) were added. Incubated the tubes at 18-24 h at 28°C.
d. Triple Sugar Iron Test: An inoculum from a pure culture was stabbed aseptically to a sterile tube of TSI (Himedia). The inoculated tube was incubated at 28°C for 24 h.
e. Growth at different w/v concentration of NaCl: An inoculum from a pure culture was spotted in to nutrient agar plates containing different w/v concentration of NaCl (6% and 10%) and plates was incubated at 28°C for 24 h.
f. Growth at different temperature: An inoculum from a pure culture was transferred aseptically to LB Broth containing 1% of NaCl and inoculated tube was incubated at temperatures (4°C and 45°C) for 24 h.
g. Antibiotic sensitivity test: Pure culture in LB Broth was swabbed aseptically to Mueller Hinton Agar (MHA) plate and different antibiotic discs (vancomycin, penicillin, ampicillin, and carbenicillin) were fixed. The plate was incubated at 28°C for 24 h.

The combined analysis of the results of these biochemical tests and the results of NCBI-BLAST Analysis was done in order to precisely identify the species.

3.2.5.5.4. Characterization of Staphylococcus up to species level

The various tests included

a. Sugar Fermentation Test: Aseptically inoculated pure culture of the test organism to labelled bromocresol purple broth (Himedia) to which various sugar discs (Himedia) (sucrose, Mannitol, trehalose, mannose, ribose and salicin) were added. Incubated the tubes at 18-24 h at 28°C.
b. Catalase test: A drop of hydrogen peroxide (3%) was placed on a microscopic slide. Using loop, test organism is placed on that and effervescence was observed.
c. Antibiotic sensitivity test: Pure culture in Luria Bertani Broth was swabbed aseptically to MHA plate and antibiotic disc (novobiocin) was fixed. The plate was incubated at 28°C for 24 h.
d. Urease test: Streaked the surface of a urea agar slant (Himedia) with a portion of a well-isolated colony and incubated the tube at 28°C in ambient air for 24 h.
e. Nitrate reduction test: An inoculum from the pure culture was transferred aseptically to nitrate broth and inoculated tube was incubated at 28°C for 24 h. At the end of incubation add one drop full of sulfanilic acid and one drop full of α-naphthylamine and observed for the red color formation for the positive results. A small amount of zinc powder was added for the confirmation of positive results.
f. Ornithine Decarboxylase Test: An inoculum from the pure culture was transferred aseptically to a sterile tube of ornithine decarboxylase broth (Himedia). The inoculated tube was incubated at 28°C for 24 h. After inoculation over layered the tubes with 2-3 ml mineral oil.

g. Mannitol salt agar: An inoculum from a pure culture was transferred aseptically to Mannitol salt agar containing 1 % of NaCl and inoculated plate was incubated at 28°C for 24 h.

The combined analysis of the results of these biochemical tests and the results of NCBI-BLAST Analysis was done in order to precisely identify the species.

3.2.5.5.5. Characterization of Vibrio up to species level

A practical set of biochemical keys designed for the routine identification of *Vibrio* sp. developed by Noguerola and Blanch (2007) was primarily used for the characterization of Vibrios up to the species level. The procedures for these tests were as follows:

a. Ornithine Decarboxylase Test: An inoculum from a pure culture was transferred aseptically to a sterile tube of ornithine decarboxylase broth (Himedia). The inoculated tube was incubated at 28°C for 24 h. After inoculation over layered the tubes with 2-3 ml mineral oil.
b. Lysine Decarboxylase Test: An inoculum from a pure culture was transferred aseptically to a sterile tube of Lysine Decarboxylase broth (Himedia). The inoculated tube was incubated at 28°C for 24 h. After inoculation over layered the tubes with 2-3 ml mineral oil
c. Arginine Decarboxylase Test: An inoculum from a pure culture is transferred aseptically to a sterile tube of Arginine Decarboxylase broth (Himedia). The inoculated tube is incubated at 28°C for 24 h. After inoculation over layered the tubes with 2-3 ml mineral oil.
d. Esculin hydrolysis test: An inoculum from a pure culture was transferred aseptically to esculin hydrolysis test broth (Qadri *et al.*, 1980) and inoculated tube was incubated at 28°C for 24 h.
e. Urease test: Streak the surface of a urea agar slant (Himedia) with a portion of a well-isolated colony and incubate the tube at 28°C in ambient air for 24 h.

f. Sugar Fermentation Test: Aseptically inoculated pure culture of the test organism to each labelled bromocresol purple broth (Himedia) to which various sugar discs (Himedia) (sucrose, arabinose, salicin, inositol, Mannitol, trehalose, raffinose, mannose, maltose, lactose, glycogen, ribose, rhamnose, and sorbitol) were added. Incubated the tubes at 18-24 h at 28°C.

g. Growth at different w/v concentration of NaCl: An inoculum from a pure culture was transferred aseptically to peptone broth containing different w/v concentration of NaCl (0%, 6%, 8%, 10% and 12%) and inoculated tube was incubated at 28°C for 24 h.

h. Nitrate reduction test: An inoculum from a pure culture was transferred aseptically to nitrate broth and inoculated tube was incubated at 28°C for 24 h. At the end of incubation add one drop full of sulfanilic acid and one drop full of α-naphthylamine and observed for the red color formation for the positive results. A small amount of zinc powder is added for the confirmation of positive results.

i. Vibrio 0129 Differential Disc (10 mcg and 150 mcg): With a sterile swab, a lawn culture of the test organism was made onto Muller-Hinton agar (Himedia). Aseptically placed both Vibrio 0129 differential discs (10 mcg and 150 mcg) on the swabbed plates. Incubate at 28°C for 24 h. Observed for zone of inhibition.

j. Testing for enzymatic activity: An inoculum from a pure culture was spotted aseptically to different substrate embedded agar plates (test for protease, amylase, caseinase, gelatinase) containing 1 % of NaCl and inoculated plate was incubated at 28°C for 24 h.

k. Growth at different temperature: An inoculum from a pure culture was transferred aseptically to Luria Bertani Broth containing 1% of NaCl and inoculated tube was incubated at different temperature (4°C and 45°C) for 24 h.

The combined analysis of the results of these biochemical tests and the results of NCBI-BLAST Analysis was done in order to precisely identify the species.

3.2.5.5.6. Characterization of Photobacterium up to species level

The various tests included:

a. Growth on TCBS: The pure cultures of the bacterial samples were streaked onto the TCBS agar plates and the color of the colonies were checked after 24 h of incubation.
b. Motility Test: The inoculation needle was used to stab the soft Oxidative fermentation (OF) medium with the culture and the tubes were incubated at 30℃ for 24 h.
c. Nitrate Reduction Test: An inoculum from a pure culture was transferred aseptically to nitrate broth and inoculated tube was incubated at 28°C for 24 h. At the end of incubation add one drop full of sulfanilic acid and one drop full of α-naphthylamine and observed for the red color formation for the positive results. A small amount of zinc powder is added for the confirmation of positive results.
d. Urease Test: The urea agar base was sterilized and added with sterile 40% urea. Slants were prepared and the surface of the agar slant was streaked with the test organism. The cap is left loose and the tubes are incubated at 30°C in ambient air for 18 to 24 h. The color of the slant was noted after incubation.
e. Sugar Fermentation Test: The Bromcresol purple broth was sterilized and inoculated with the pure culture of the bacterial samples. After inoculation, the discs of sucrose, arabinose, salicin, inositol, Mannitol, trehalose, raffinose, mannose, maltose, lactose, glycogen, ribose, rhamnose, and sorbitol were added. The tubes were observed after 24 h of incubation at 30℃ and the results were noted.

The combined analysis of the results of these biochemical tests and the results of NCBI-BLAST Analysis was done in order to precisely identify the species.

3.2.5.5.7. Characterization of Pantoea sp. up to species level

The various tests included:

a. Indole test: Pure bacterial cultures were grown in sterile tryptophan broth for 24-48 h. After incubation, few drops of Kovac's reagent were layered onto the culture broth. A red ring formation was observed on the addition of Kovac's reagent.

b. Voges Proskauer test: Isolate was inoculated into MR-VP broth (Himedia) with sterile loop. The tube was incubated at 28°C for 2 days. An aliquot of 0.6 ml of 5% alpha naphthol is added followed by 0.2 ml of 40% KOH and the tube was shaken gently to expose the medium to atmospheric oxygen. The tube was allowed to remain undisturbed for 30 to 45 min.

c. Siderophore and hydrocyanic acid (HCN) production test (Schwyn and Neilands, 1987): The isolate was inoculated on Chrome Azurol-S (CAS) agar and was incubated at 30^0C for 18- 24 h. After incubation, the diameter of inhibition zone was measured. The qualitative method of Bakker and Schipper, 1987 are employed in HCN production test. The change in the color of the filter paper previously dipped in 2% sodium carbonate prepared in 0.05% picric acid, from yellow to dark brown was rated visually depending on the intensity of the colour change.

d. Sugar Fermentation Test: The Bromcresol purple broth was sterilised and inoculated with the pure culture of the bacterial samples. After inoculation, the discs of inositol and melibiose were added. The tubes were observed after 24 h of incubation at 30^0C and the results were noted.

e. Arginine dihydrolase test: The isolates were stabbed on to arginine dihydrolase broth and were incubated at 30^0C for 24 h. The colour change to purple was noted.

i. Gelatin hydrolysis test: An inoculum from a pure culture was spotted aseptically to gelatin embedded agar plates containing 1% of NaCl and inoculated plate was incubated at 28°C for 24 h.

3.2.6. PHYLOGENETIC ANALYSIS OF ANTAGONISTIC ISOLATES

The overlapping 770 bp size segment of *16SrRNA* gene in the isolates of each species was used for phylogenetic study. The sequences were aligned using ClustalW and neighbor-joining (NJ) tree was constructed by MEGA version 10 using Tamura Nei model (Kumar *et al.,* 2018). The confidence in the NJ tree was estimated by 1000 bootstrap replicates. Separate phylogram was made for crab and *P. viridis* antagonistic isolates.

3.2.7. SCREENING FOR BIOSYNTHETIC GENES IN ANTAGONISTIC BACTERIA

3.2.7.1. Extraction of genomic DNA

3.2.7.1.1. Extraction of genomic DNA from Gram positive bacteria

Extraction of genomic DNA was done using HiPurA Bacterial genomic DNA purification kit (Himedia) according to manufacturer's instructions. Briefly, 5 mL of an overnight bacterial culture was pelleted in a 2 mL capped centrifuge tube (Tarsons) by centrifuging for 10 min at 13,000 rpm at room temperature (15-25^{0}C). The culture media was discarded and the pellet was re suspended thoroughly in 200 µL of lysozyme solution and was incubated for 30 min at 37^{0}C. 20 µL of proteinase K solution (20 mg/mL) was added to the sample. 20 µL of RNase A solution was added, mixed and incubated for 5 min at room temperature (15- 25^{0}C).Then 200 µL of lysis solution was added, vortexed thoroughly for few sec and incubated at 55^{0}C for 10 min. 200 µL of ethanol (95-100%) was added to the lysate and mixed thoroughly by vortexing for few sec. Lysate was then transferred to HiElute Miniprep Spin Column and centrifuged at 10,000 rpm for 5 min at room temperature (15-25^{0}C). The flow –through liquid was discarded and the spin column was placed in same 2 mL collection tube. 500 µL of Prewash

solution was added to the column and centrifuged at 10,000 rpm for 2 min at room temperature (15- 25^{0}C). The flow through liquid was discarded and 200 µL of absolute ethanol was then added and centrifuged at 10,000 rpm for 2 min. 500µL of diluted Wash Solution was added to the column and centrifuged for 5 min at 13, 000 rpm at room temperature (15-25^{0}C).The flow through was discarded and spin for additional 2 min to dry the column. Column was kept at 70^{0}C for 10 min to remove residual ethanol. The column was transferred to fresh uncapped collection tube. 75 µL of elution buffer was added without spilling to the sides and incubated at room temperature (15- 25^{0}C) for 5 min. This was then centrifuged at 10,000 rpm for 5 min at room temperature (15-25^{0}C) to elute DNA. This was repeated two times more with 50 µL and 25 µL of elution buffer and the eluate containing pure genomic DNA was stored at -20^{0}C for future use.

3.2.7.1.2. Extraction of genomic DNA from Gram negative bacteria

Extraction of genomic DNA was done using HiPurA Bacterial genomic DNA purification kit (Himedia) according to manufacturer's instructions. 5 mL of an overnight bacterial culture was pelleted in a 2 mL capped centrifuge tube from eppendrof by centrifuging for 10 min at 13,000 rpm at room temperature (15-25^{0}C). The culture media was discarded and the pellet was re-suspended thoroughly in 570 µL of TEG buffer with lysozyme (5 mg/ mL) and vortexed well. 30 µl of 10% SDS and 3 µL of proteinase K was added and mixed well. Sample was then incubated at 60°C for 1 h. 100 µL of 5M NaCl and 80 µL of CTAB were added and incubated at 65^{0}C for 15 min. 600 µL of ethanol (95-100%) was added to the lysate and mixed thoroughly by vortexing for few sec. Lysate was then transferred to HiElute Miniprep Spin Column and centrifuged at 10,000 rpm for 5 min at room temperature (15-25^{0}C). The flow-through liquid was discarded and the spin column was placed in same 2 mL collection tube. 500 µL of Prewash solution was added to the column and centrifuged at 10,000 rpm for 2 min at room temperature (15- 25^{0}C). The flow through liquid was discarded and 200 µL of absolute ethanol was then added and centrifuged at 10,000 rpm for 2 min. 500 µL of diluted Wash Solution was added to the column and centrifuged

for 5 minutes at 13, 000 rpm at room temperature (15-25^{0}C).The flow through was discarded and spin for additional 2 min to dry the column. Column was kept at 70^{0}C for 10 min to remove residual ethanol. The column was transferred to fresh uncapped collection tube. 75 µL of elution buffer (ET) was added without spilling to the sides and incubated at room temperature (15- 25^{0}C) for 5 min. This was then centrifuged at 10,000 rpm for 5 min at room temperature (15-25^{0}C) to elute DNA. This was repeated two times more with 50 µL and 25 µL of elution buffer and the eluate containing pure genomic DNA was stored at -20^{0}C for future use.

3.2.7.1.3 Analysis of genomic DNA by Agarose gel electrophoresis

Genomic DNA was analyzed as explained in section 3.2.5.4.2.

3.2.7.2. Amplification of biosynthetic genes

The presence of PKS 1 and NRPS biosynthetic genes were screened in the antagonistic bacterial genome using reported primers (Table 5). PCR conditions and mixture was optimized using standard protocol (Sambrook and Russell, 2006). The optimized PCR mixture consisted of 1 µl of template DNA, 2.5 µl of 1X Takara *Taq* buffer, 0.5 µl of dNTP mix, 0.5 µl of Forward and Reverse Primer and 1.25 U of Takara *Taq* DNA polymerase. In case of PCR using MTS primers, an extra 1.5 µL $MgCl_2$ was added to the reaction mix for successful yield of the amplicon. The optimized PCR programme included initial denaturation at (95°C for 5 min) followed by 35 cycles of denaturation (95°C for 1 min), annealing (55°C for 1 min for PKS-1 and MTS primers and 60°C for 1 min for PKS-II primers) and extension (72°C for 1.30 min). Final extension was carried out at (72°C for 10 min).

3.2.7.3 Analysis of amplicons by Agarose gel electrophoresis

2 µl of PCR product was mixed with 1 µl 6X DNA loading dye (Takara) and loaded on agarose gel. The agarose gel (1.5%) was run at constant voltage of 90 V and current of 45A till the bromophenol blue has reached the extreme

opposite side of the wells. 100 bp DNA ladder (Takara) was run along with the samples to identify the molecular size of the amplicon. The gel was then visualized and photographed on BioRad gel documentation system.

3.2.7.4. Sequencing of positive amplicons

3.2.7.4.1. Purification of amplicons

PCR product was purified using a gel extraction kit (GenElute – SigmaAldrich). 1.5% agarose gel was prepared and 100 µl of DNA with 20 µl 6X DNA loading dye was loaded on the gel and run at 90 V for 40 min. The gel was then viewed on UV transilluminator and DNA band was excised from the gel using a clean sterile razor blade. Weight of the gel slice was noted and three gel volumes of gel solubilisation solution was added to it and incubated at 55°C for 10 min.The mixture was vortexed every 3 min for complete dissolution of the gel. Column was prepared prior to elution by adding a column preparation solution. It was centrifuged at 12000 rpm for 2 min. After complete dissolution of the gel 10 µl of 3 M sodium acetate was added for maintaining the pH of the solubilization buffer. Two gel volume of isopropanol was added, mixed well and 700 µl of this mixture was loaded into the binding column that is assembled in the collection tube. It was centrifuged at 12000 rpm for 2 min, discarded the flow through and repeated the steps until complete mixture is loaded into the column. Then 700 µl of wash buffer was added and centrifuged at 12000 rpm for 2 min. Additional centrifugation was carried out to remove the remaining residues present in the column. The column was then transferred to a new collection tube and 25 µl of elution buffer was added, incubated for 5 min at 37°C and centrifuged at 12000 rpm for 5 min. Eluted product was stored at -20°C.

The purified products of PKS were directly sequenced at a custom DNA sequencing facility (Agrigenome, Kochi). In case of NPRS gene, sequencing was done after cloning into plasmid as follows:

3.2.7.4.2 Cloning of Adenylation 'A' domain of NRPS gene in pMD20 –T vector

3.2.7.4.2.1 Ligation of A domain to pMD20 –T vector

The PCR purified product was ligated to pMD20 –T vector using Mighty TA-cloning kit (TaKaRa). The ligation mix include 1 µL vector, 3 µL NFW and 5 µL ligation mighty mix to which 1 µL of purified PCR product was added. The mix was then incubated at 4^0C overnight.

3.2.7.4.2.2 Preparation of competent E. coli DH5α cells

Competent cells were prepared following the standard protocol (Sambrook and Russel, 2006). Briefly, the *E. coli* DH5α cells from glycerol stock were streaked on LB agar plates and incubated at 37°C overnight. A single colony of *E. coli* DH5α was then inoculated in 5 ml LB broth and grown overnight at 37°C. On the next day 1 ml of this overnight culture was added to 100 ml LB broth and grown at 37°C for 3 h. Following incubation, the cells were placed on ice for 10 min. Then cells were collected by centrifugation at 6000 rpm for 3 min at 4° C. The pellet was then suspended in 10 ml of cold 0.1 M $CaCl_2$. Special care was taken while suspending the pellet to avoid mechanical disruption of the cell. It was again incubated on ice for 20 min. After incubation it was centrifuged at 6000 rpm for 3 min, discarded the supernatant and gently suspended the cells on 5 ml cold 0.1 M $CaCl_2$/15 % glycerol and distributed it in 1.5 ml microfuge tubes (300 µl in each tube)

3.2.7.4.2.3 Transformation of ligated plasmid into competent E. coli DH5α cells

Transformation was done by heat shock method using 100 µl of competent *E. coli* DH5α cells (Sambrook and Russel, 2006). Briefly, 100 µl of frozen competent cells were thawed on ice for half an hour. To this 5 µl of ligation mix was added and incubated on ice for 30 min. After that heat shock was given at 42° C for 2 min and immediately chilled on ice. Then 900 µl of Luria Bertani broth was added to it and kept in a shaker incubator for 30 min at 37°C. Following incubation, transformed *E. coli* culture was plated on LB agar containing ampicillin (100 mg/ml), IPTG (24 mg/ml) and X Gal (20 mg/ml). The plates were incubated at 37°C overnight.

3.2.7.4.2.4 Selection of recombinant clones and isolation of plasmid from recombinant clones

The white colonies obtained in LB plates after incubation were selected and suspended in 100 µl of nuclease free water. 10 µl of the same was used for crude DNA preparation. Colony PCR was done using this crude DNA with gene specific primer (MTF and MTR for 'A' domain of *NRPS* gene). 90 µl of positive colony suspensions were inoculated in 10 ml LB broth containing 100 mg/ml ampicillin and grown overnight at 37°C and plasmid DNA was isolated on the next day using GeneJet plasmid isolation kit (Thermo scientific) following the manufacturer's protocol. Briefly, the overnight culture was centrifuged at 10000 rpm for 10 min, supernatant was discarded and pellet was suspended in 300 µl of re-suspension solution. Then 300 µl of lysis buffer was added and mixed thoroughly by inverting the tubes for 4-5 times. It was then incubated at 37° C for 3 min. Following incubation, 300 µl of neutralisation solution was added and kept on ice for 15 min. After that it was mixed and centrifuged at 13000 rpm for 10 min. Supernatant was then transferred to a GeneJET spin column attached to a collection tube and centrifuged at 13000 rpm for 1 min. Flow through was discarded and 500 µl of wash buffer was added to it. Then it was centrifuged at 13000 rpm for 1 min. The washing step was repeated twice to completely remove all residues in the coloumn. Additional centrifugation was done at 13000 rpm for 1 min to remove the residual wash buffer. Column was then transferred to a new collection tube and 25 µl of elution buffer was added, incubated at 37° C for 2 min and centrifuged at 13000 rpm for 2 min. Column was discarded and purified plasmid DNA was stored at -20° C.

3.2.7.4.2.5. Sequencing

The purified plasmids were sequenced with vector specific primers at Agrigenome, Kochi.

3.2.7.5. Sequence analysis

The obtained sequence data were edited and compiled using Editseq (DNASTAR, Lasergene, USA). The sequences were compared with the *NRPS/PKS* gene sequences in GenBank using BLASTn, and closest relative sequences were retrieved. Separate phylograms were constructed for *NRPS* and *PKS* gene sequences using MegAlign. Subsequently, the *NRPS/PKS* gene sequences obtained through the present study were deposited in GenBank and got assigned with Accession numbers. Then the obtained gene sequences of selected isolates were translated to corresponding amino acid sequences and used for *insilico* analysis.

For NRPS domain prediction, *in-silico* determination of amino acid residues present in the binding pockets of 'A' domain was done followed by determination of substrate specificity of the obtained 'A' domain using Sequence Learner (SEQL)-NRPS web-server through discriminative classification method (Knudsen *et al.,* 2016). Putative functions were then predicted using structural bioinformatics approach by comparing the sequences of identified domain with corresponding sequences present in experimentally characterized secondary metabolite biosynthetic gene clusters having known metabolic products using PKS/NRPS analysis web-site (Bachmann and Ravel, 2009).

The 'KS' domains of PKS were predicted using Natural Product Domain Seeker (NaPDoS). The obtained KS domain aminoacid sequences were compared with a board set of identified and fully characterized reference domains and domain specific phylogenetic tree was constructed. This data was used to predict the putative products encoded by the test PKS domains and to anticipate the similarity or difference of the compounds produced by the same from previously known compounds of completely characterized biosynthetic pathways (Ziemert *et al.*,2012).

RESULTS

4. RESULTS

4.1. SAMPLE COLLECTION

Thirty six animals belonging to four species of crabs were collected from Cochin coast of Kerala (Plate 1). Morphometrics of crabs under study were given in Table 6. In short, average weight (W) and carapace width (CW) of the crabs were 51.89± 15.96 g and 7 ± 0.75cm, 52.89 ±12.67g and 9.05 ±1.424 cm, 43.78 ± 11.702g and 8.72 ± 1.063 plus 57.81 ± 6.79g and 10.07 ±1.652 cm for *C. feriatus, P. pelagicus, P. sanguinolentus* and *S. olivacea* respectively.

Similarly, the morphometrics of 48 bivalves belonging to *P. viridis* (Plate 2) sampled for the present study were given in the Table 7. In short, the animals having average weight and length as 26.741 ± 7.108g and 7.135 ± 1.056cm were used in the study.

4.2. ESTIMATION OF DENSITY OF CULTIVABLE MICROBIAL SYMBIONTS

Load of heterotrophic symbionts in the haemolymph of four healthy wild caught marine crab species and *P. viridis* were estimated by limiting dilution method and results were represented in terms of log colony forming units per ml/mg ($\log_{10}$ cfu/ml or mg) in Fig. 1. The abundance was in the order of *C. feriatus* > *P. pelagicus*> *S. olivacea* >*P. viridis*>*P. sanguinolentus; P. pelagicus* > *S. olivacea* > *P. viridis*> *C. feriatus* > *P. sanguinolentus* and *P. viridis*> *C. feriatus* >*P. pelagicus.*> *P. sanguinolentus* >*S. olivacea* in ZMA, BHIA and TCBS respectively.

Similarly, total viable count of cultivable heterotrophic bacteria associated with different tissues (mantle tissue, mantle fluid, gill, gut and muscle) and haemolymph of *P. viridis* was given in Fig. 2. In summary, the abundance of heterotrophic bacteria in different tissues were in the order of gut> mantle> muscle >gill >mantle fluid> haemolymph; gut> muscle > mantle > gill > haemolymph> mantle fluid and gut > muscle> mantle > gill> mantle fluid>

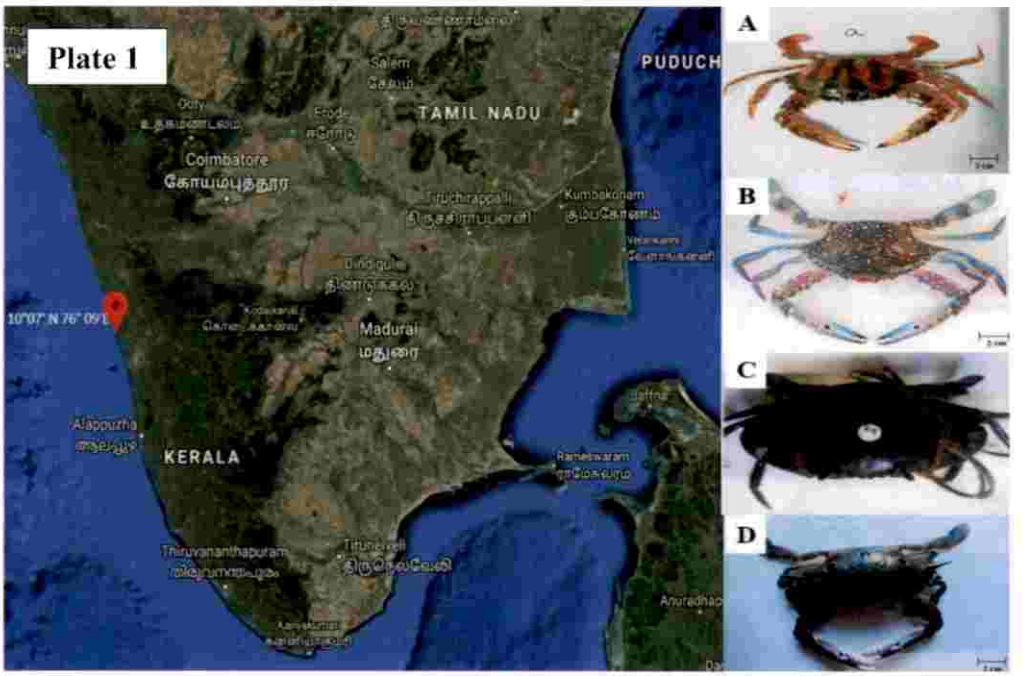

Plate 1. Sampling site and crab species under study. (A) *Charybdis feriatus* (Linnaeus, 1758) (B) *Portunus pelagicus* (Linnaeus, 1758) (C) *Scylla olivacea* (Herbst, 1796) (D) *Portunus sanguinolentus* (Herbst, 1783)

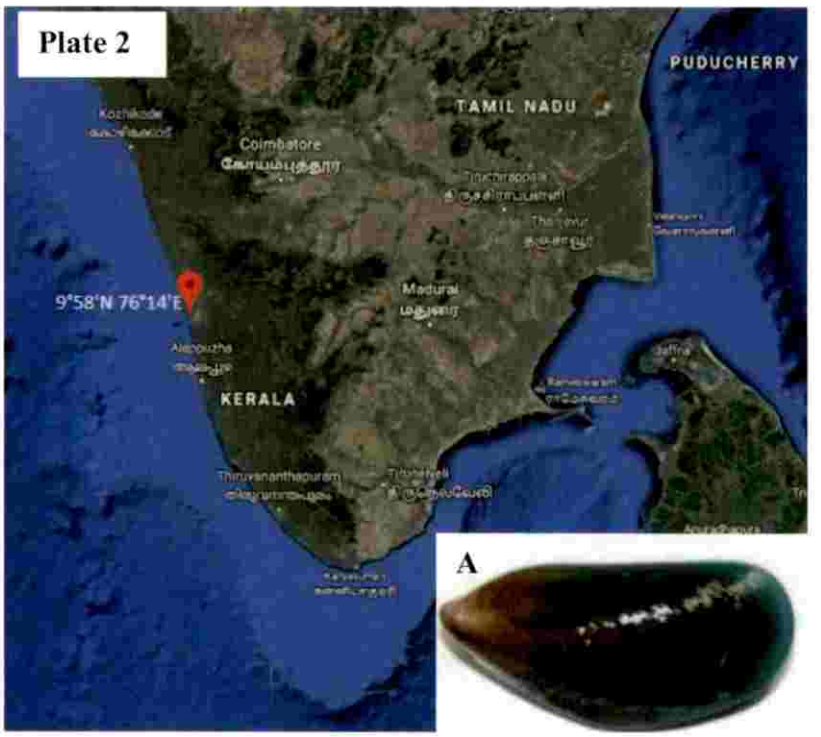

Plate 2. Sampling site and bivalve under study. (A) *Perna viridis* (Linnaeus, 1758)

Table 6. Morphometrics of crabs under study

C. feriatus (Linnaeus, 1758)	ID	1	2	3	4	5	6	7	8	9
	Sex	M	F	M	F	M	M	M	M	F
	W (g)	62	41	50	36	72	73	32	63	38
	CW (cm)	8	7	7	6.5	8	7.5	6	7	6
P. pelagicus (Linnaeus, 1758)	ID	1	2	3	4	5	6	7	8	9
	Sex	F	M	M	M	F	M	F	M	M
	W (g)	35	64	73	55	40	45	50	66	48
	CW (cm)	6	10	11	9.5	8	9	9	10	9
P. sanguinolentus (Herbst, 1783)	ID	1	2	3	4	5	6	7	8	9
	Sex	M	M	F	M	F	M	M	M	F
	W (g)	30	53	33	36	28	56	52	55	51
	CW (cm)	8.5	10	7	7.5	8	10	9.0	9.5	9
S. olivacea (Herbst,1796)	ID	1	2	3	4	5	6	7	8	9
	Sex	M	F	M	F	M	M	M	F	F
	W (g)	60	50	52	55.6	62	65	69.68	51	55
	CW (cm)	10	9	9.2	10	13	12	11	8	8.5

Table 7. Morphometrics of bivalves (*P. viridis*) under study

Groups of *P. viridis*	Average Weight (g)	Average Length (cm)
Group 1	33.33± 5.66	7.88± 0.47
Group 2	17.15 ± 2.6	5.56 ± 0.55
Group 3	21.1± 3.03	6.45 ± 0.61
Group 4	29.06± 5.04	7.75 ± 0.45
Group 5	28.6± 7.70	7.53± 0 .78
Group 6	17.56± 3.25	5.75± 0 .5
Group 7	33.83±5.44	7.91± 0.49
Group 8	33.3± 3.921	8.25± 0.25

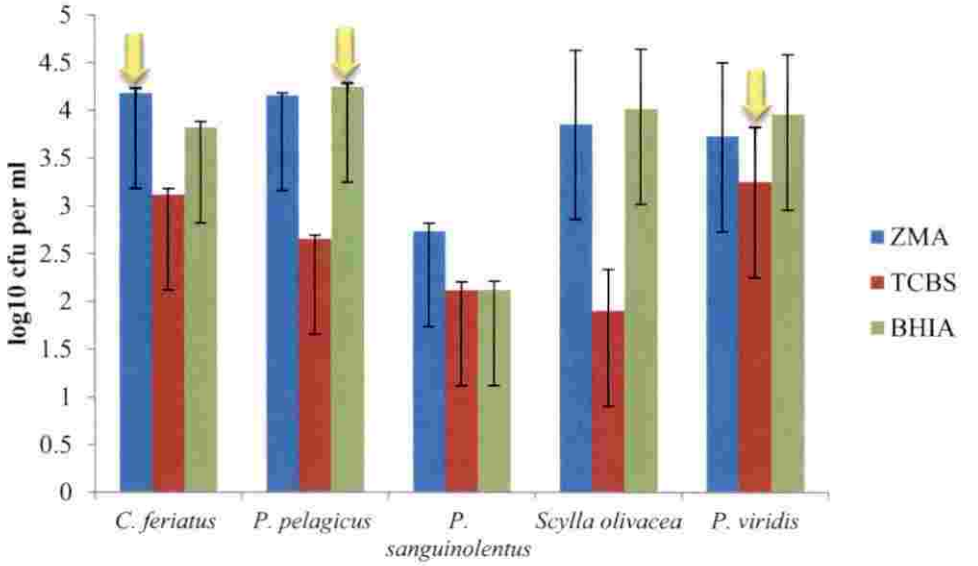

Fig. 1. Density of cultivable bacterial isolates in the haemolymph of different marine crab species. Observed means of log cfu ± SE per ml. The pictogram indicates media with higher abundance in each crab species

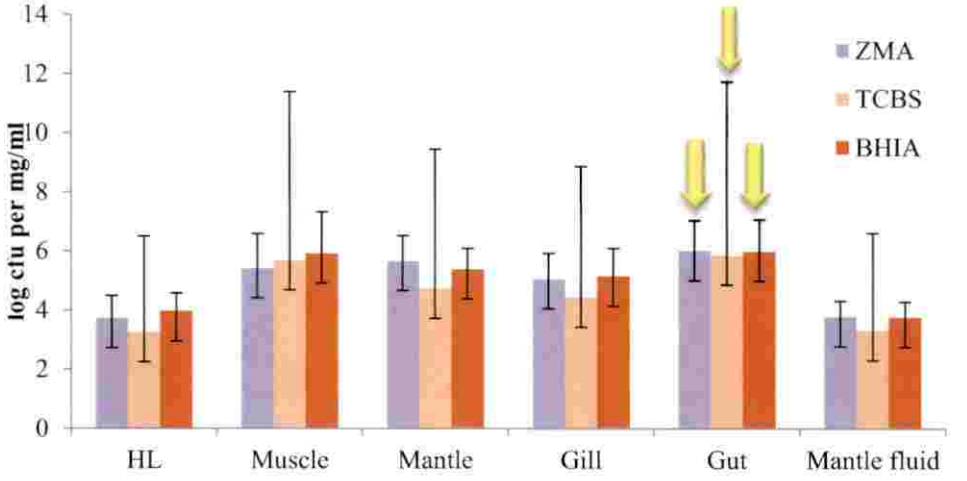

Fig. 2. Density of cultivable bacterial species in different tissues and haemolymph of *P. viridis*. Observed means of log cfu ± SE per ml.The pictogram indicates abundance tissue with higher abundance in each media

haemolymph in ZMA, BHIA and TCBS respectively.

4.3. DIVERSITY OF SYMBIONTS AND THEIR STORAGE

Morphologically unique colonies from each tissues/species were selected and stored at 4^0C and at -80^0C. Overall, 34 morphologically unique colonies were obtained from the haemolymph of different marine crab species and abundance was in the order of *C. feriatus* = *P. pelagicus* > *P. sanguinolentus* > *S. olivacea* (Fig. 3). Similarly, there were a total of 89 morphologically unique colonies in different tissues and haemolymph of *P. viridis* which were in the order of mantle > haemolymph> muscle> gut> gill (Fig. 4).

4.4. ANTIMICROBIAL ACTIVITY OF THE ISOLATED SYMBIONTS

All the 123 isolates were monitored for inhibitory activity against 12 indicator pathogens by spot on lawn method (Rojo-Bezares *et al.*, 2007) (Plate 3). Among these, 27 isolates (21.95%) showed zone of clearance around inoculation spot for at least one pathogen. In short, activity against human pathogens was more pronounced in microbial symbionts from bivalve than from crabs. Another noteworthy observation was that none of the isolates of the present study showed activity against *E. coli* 25922 and *S. flexneri*.

4.4.1. Antimicrobial activity of the symbionts associated with *P. viridis*

Of the 89 isolates screened from *P. viridis* 17 exhibited (19.101%) antagonistic activity against one or more pathogens with inhibition zone diameter ranging from 1-26 mm. The zone observed for the positive isolates were represented in Fig. 5. The maximum zone (26 mm) was observed against *V. alginolyticus* by isolate 136b followed by a zone of 18mm against *V. anguillarum* by 6b and 36b. However, the maximum versatility was shown by the isolate having the code 228b having activity against 6 number of tested pathogens. In case of human pathogens maximum zone (8mm) was observed by 36b against *S.* Typhimurium and among the MDR pathogens the maximum zone (8 mm) was observed by 185b against MRSA.

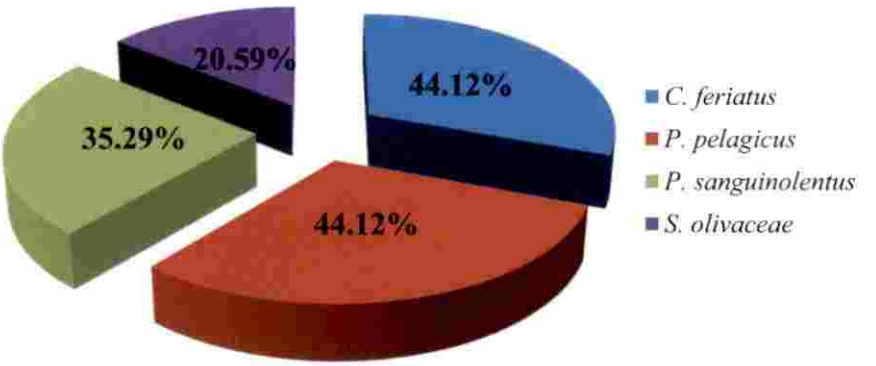

Fig. 3. Diversity of bacterial isolates (%) in the haemolymph of different marine crab species

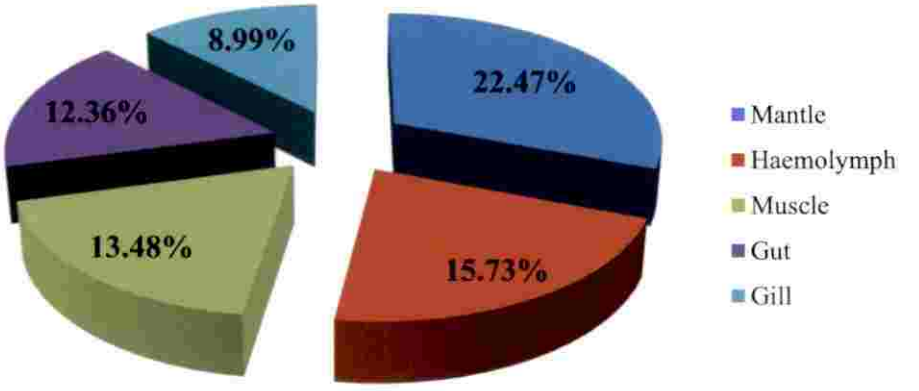

Fig. 4. Diversity of bacterial isolates (%) in different tissues and haemolymph of *P.viridis*

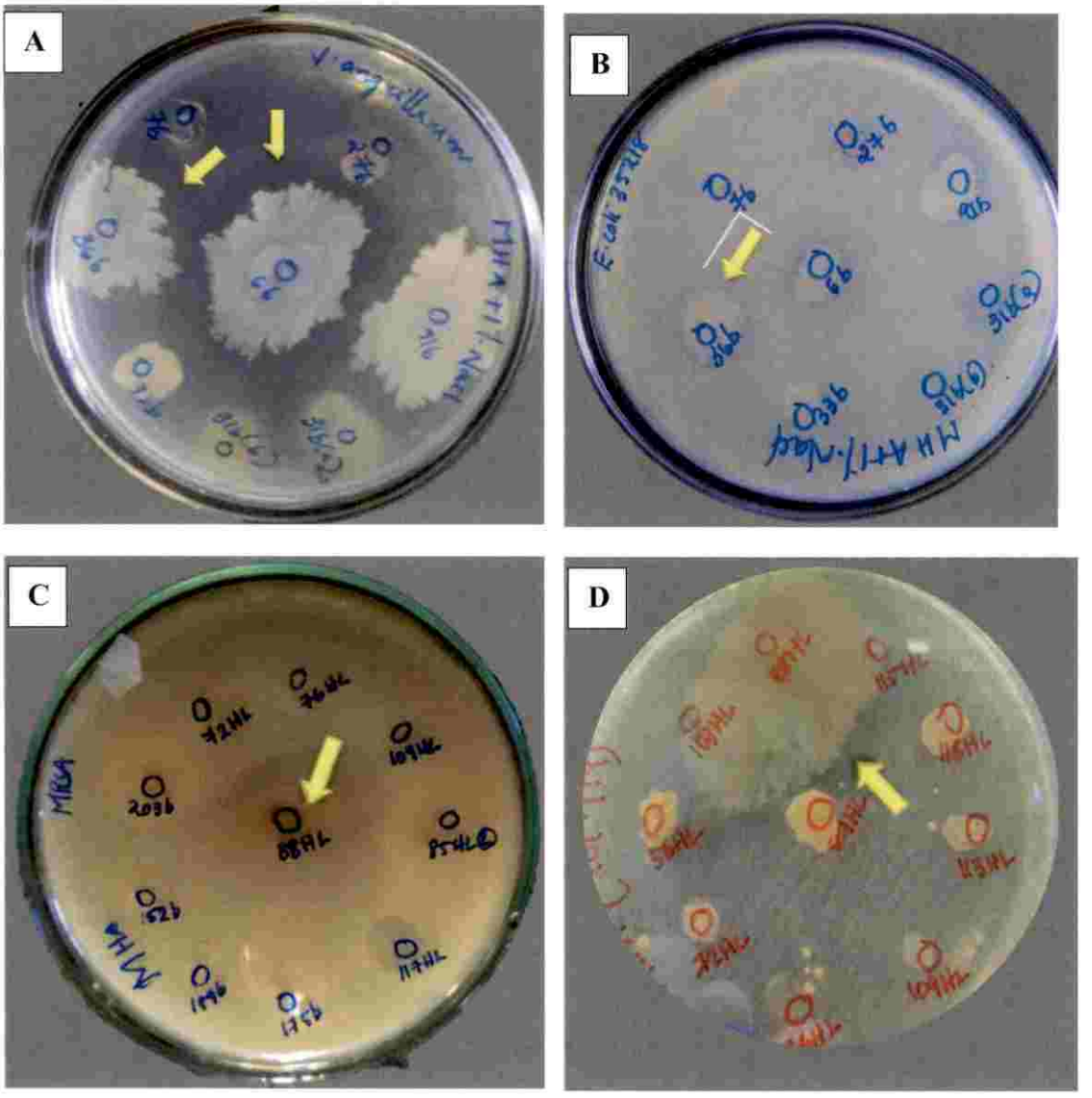

Plate 3. Screening for antibacterial activity in bacterial isolates from bivalves and different marine crabs by spot on lawn assay. Pictogram indicates (A) inhibition zone diameter shown by isolates 36b and 6b against *V. anguillarum*. (B) Activity of 36b against β lactamase producing *E. coli*. (C) 88HL exhibiting inhibition zone diameter against MRSA (D) Activity of 88HL against *V. alginolyticus*.

The activity score obtained for each isolate was represented in the Fig. 6. Based on activity score, isolate with code 136b was identified as the most promising bacteria from *P. viridis* (score 11), followed by 228b (score10). The most potential isolate was 136b showing activity against 4 pathogens namely, *P. damselae, V. alginolyticus, V. anguillarum and V. harveyi.*

Subsequently, the pathogens inhibited maximum by the isolates from *P. viridis* was calculated and represented in Fig. 7. Among the 12 indicators pathogens tested, *V. harveyi* was the most susceptible to inhibitory activity (64.71%), followed by *V. anguillarum* (43.75 %) and *P. damselae* (37.5 %). More interestingly, none of the isolates showed activity against *E. coli* and *S. flexneri.*

To identify the species-wise distribution of antagonistic isolates in bivalves, the percentage of antagonistic isolates carried in each tissue were found out and given in Fig. 8. The order of antagonistic isolates were gut = mantle> gill = muscle > haemolymph.

4.4.2. Antimicrobial activity of symbionts associated with haemolymph of marine crabs

Of the 34 isolates screened from marine crabs 10 exhibited (29.411%) antagonistic activity against one or more pathogens with inhibition zone diameter ranging from 1-40 mm. The zone observed for the positive isolates were represented in Fig. 9. The maximum zone (40 mm) was observed against *V. vulnificus* by 88HL isolate followed by a zone of 30mm against *V. alginolyticus* by the same isolate. However, the maximum versatility was shown by the isolate with the code 39HL having activity against 7 of tested pathogens. Among the MDR pathogens the maximum zone (8 mm) was observed by 88HL against MRSA. The activity score obtained for each isolate was represented in the Fig. 10. Based on activity score, 88HL isolate was identified as the most promising bacteria from marine crabs (score 13), followed by 78 HL and 39 HL (score 8). The most potential isolate 88HL was showing activity against 6 pathogens.

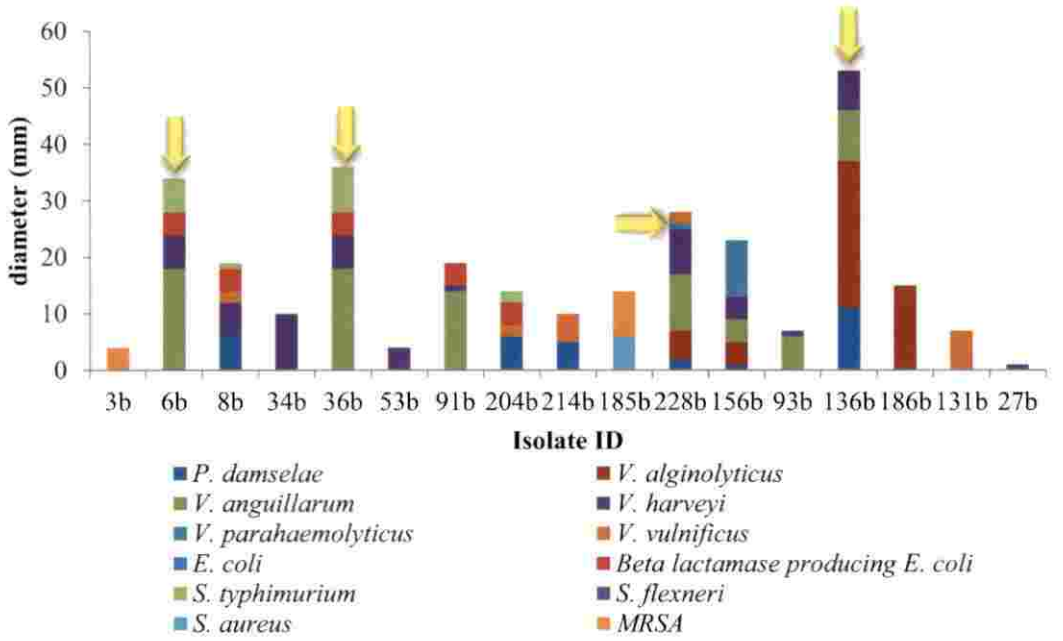

Fig. 5. Graph representing inhibition zone diameter (mm) for positive bacterial symbionts from bivalves. Downward arrows indicate the isolates with maximum inhibitory activity. Rightwards arrow indicates the isolate with most versatility

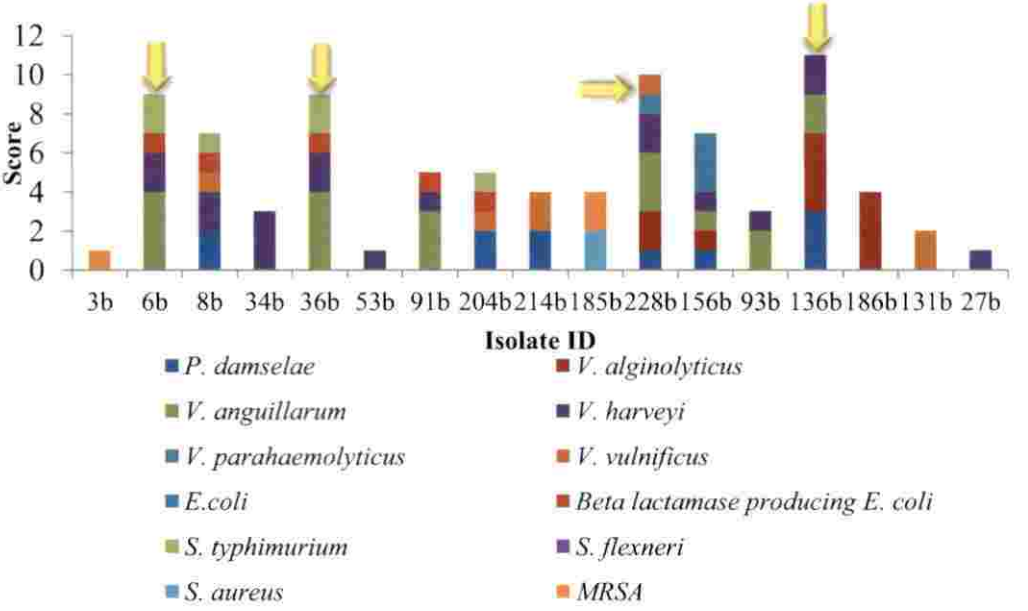

Fig. 6. Graphical representation for scoring of antagonistic isolates of bivalves. Downward arrows represents the isolates with higher scores. Rightwards arrow indicate the score of the most versatile isolate

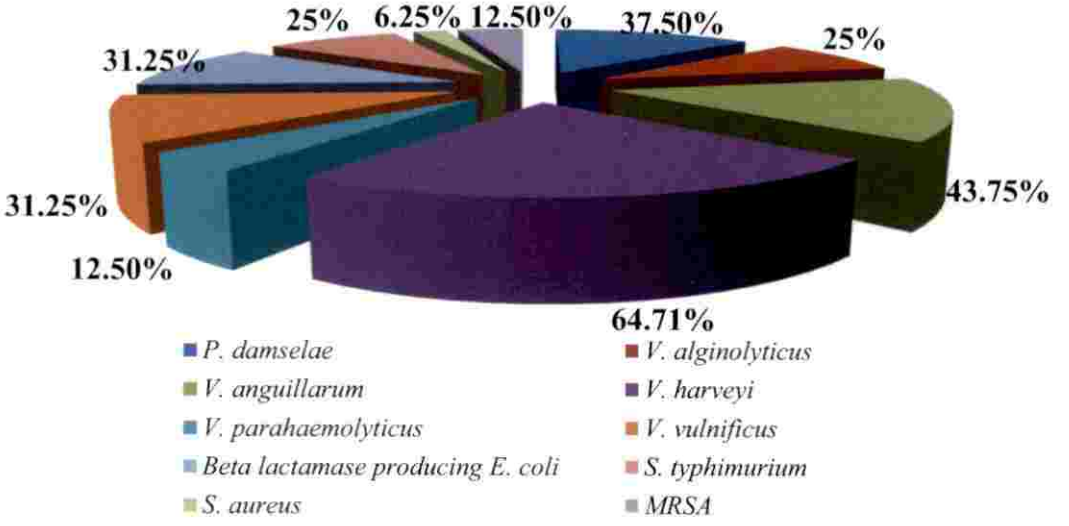

Fig. 7. Inhibitory activity of bacterial isolates (%) from *P. viridis* in terms of indicator pathogens

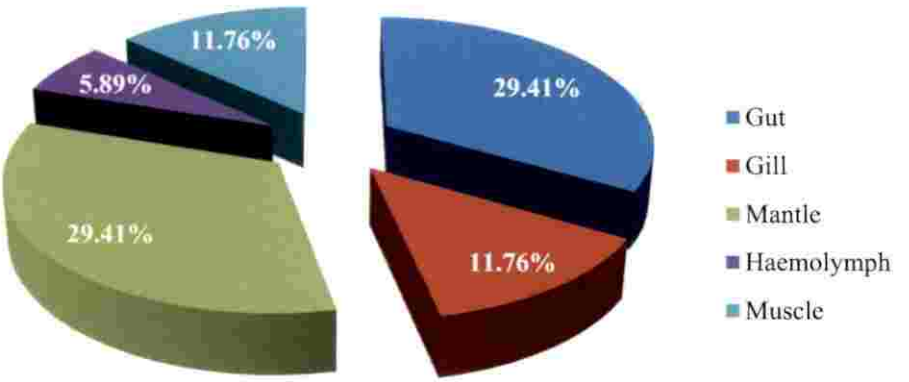

Fig. 8. Tissue wise distribution (%) of antagonistic isolates in *P. viridis*

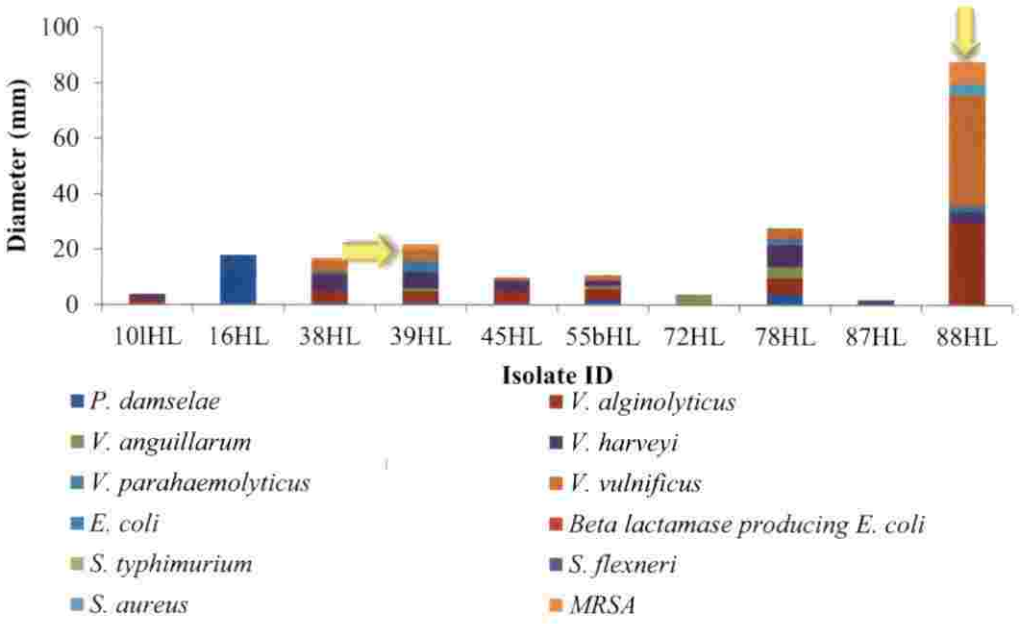

Fig. 9. Graph representing inhibition zone diameter (mm) of antagonistic bacterial symbionts from different marine crab species. Rightwards arrow and downwards arrows indicate the isolate with maximum and second maximum versatility

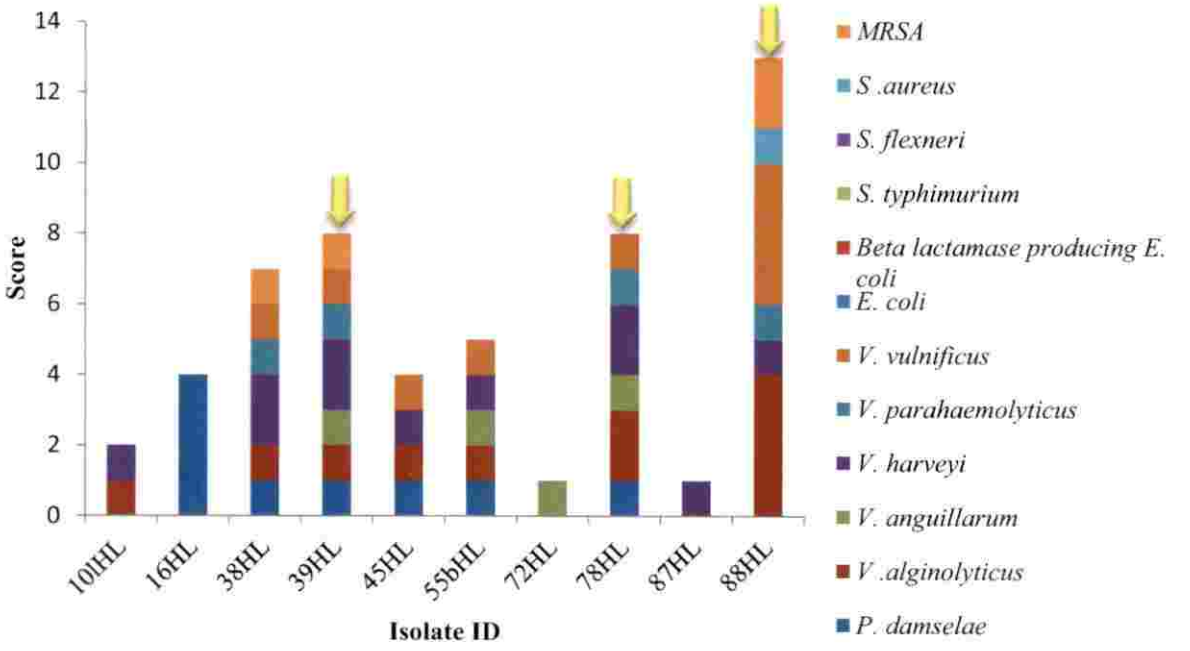

Fig. 10. Graphical representation for scoring of antagonistic bacterial isolates from different marine crab species. Arrows indicate the isolates with maximum scores

namely, *V. alginolyticus, V. harveyi, V. parahaemolyticus, V. vulnificus, S. aureus* and MRSA.

Subsequently, the pathogens inhibited maximum by the isolates from marine crabs was calculated and represented in Fig. 11. Among the 12 indicators pathogens tested, *V. harveyi* was the most susceptible to inhibitory activity (80%), followed by *V. alginolyticus* (70%) , *V. vulnificus* and *P. damselae* (both 60%). More interestingly, none of the isolates showed activity against *E. coli* 25922, *E. coli* 35218, *S.* Typhimurium, *S. flexneri.*

To identify the species-wise distribution of antagonistic isolates in marine crabs, the percentage of antagonistic isolates carried in each crab species were found out and given in Fig. 12. The distribution of antagonistic isolates in crabs were in the order: *P. sanguinolentus>P. pelagicus>C. feriatus>S. olivacea.*

4.5. IDENTIFICATION OF ANTAGONISTIC ISOLATES

All the 27 antagonistic isolates of the present study were identified using polyphasic taxonomic approach that includes morphological, cultural, biochemical and molecular characterisation.

4.5.1. Morphological characterization

During the morphological characterization by Gram's staining (Plate 4), 12 of the 27 isolates retained the pink colour of safranin and 15 were stained purple by crystal violet representing gram negative and positive character of their cell wall respectively. Further, KOH string test was done to confirm the nature of cell wall which differentiated gram negative bacteria from gram positive microbes based on the formation of viscous bacterial suspension and stringing out of the same when the loop was lifted. Thus, out of 27 antagonistic isolates 55.56% & 44.44 % were classified as gram positive and gram negative respectively.

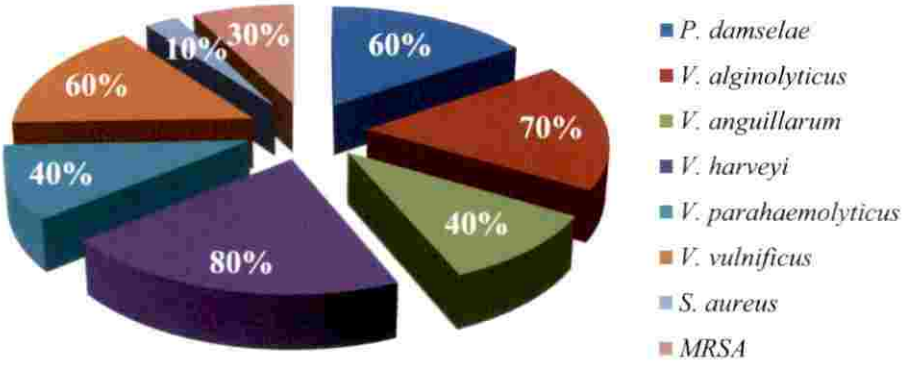

Fig. 11. Inhibitory activity of bacterial isolates (%) from different crab species in terms of indicator pathogens

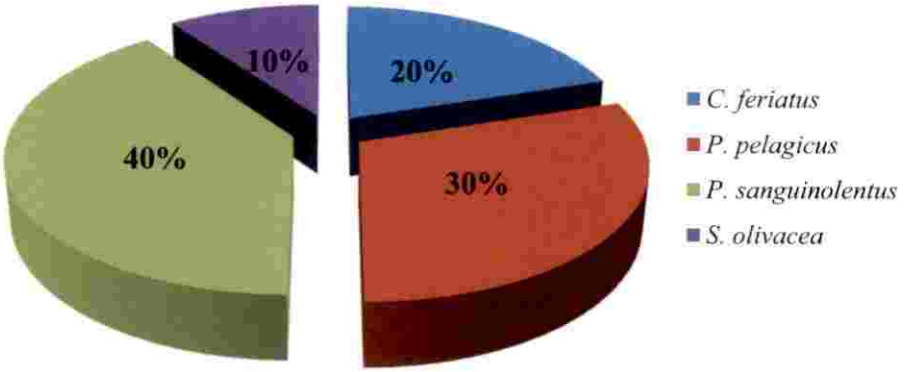

Fig. 12. Species wise distribution (%) of antagonistic isolates in different crab species.

4.5.2. Cultural characterization

During cultural characterisation, the isolates were sub-cultured onto 6 different media namely, ZMA, TCBS, BHIA, MCA (MacConkey agar), MSA (Mannitol Salt Agar) and the features were noted down (Table 8).

4.5.3. Preliminary biochemical characterization

Further, a series of 6 preliminary biochemical tests (Plate 5) were carried out, the results of the same (Table 9) were interpreted as follows:

a. Catalase test: Positive reaction was evident by the formation of immediate effervescence (bubble formation).
b. Oxidase test: Appearance of a deep blue or purple colour within 5-10 sec was considered as positive reaction while no color change was read as negative.
c. Indole test: The positive reaction was indicated by the formation of red colour in the surface layer of the broth on addition of Kovac's reagent.
d. Methyl red (MR) test: The positive reaction was observed as the colour change of MR-VP broth from yellow to red on addition of methyl red indicator.
e. Vogues Proskaeur (VP) test: Positive test was indicated by the appearance of pinkish red colour on addition of alpha naphthol and potassium hydroxide.
f. Citrate utilization test: The colour change of the media from green to blue within 48 h of incubation was regarded as positive reaction.

4.5.4 Molecular characterization by *16S rRNA* gene sequencing

During molecular characterisation by 16S *rRNA* gene sequencing, an amplicon of ~ 1499 bp was obtained in all the isolates (Fig. 13) by PCR using universal prokaryotic primers. The band size was consistent with all isolates as analysed by 0.7% agarose gel electrophoresis. The PCR products were then sequenced and the obtained sequences were analysed by NCBI, blast for the identification of genus.

4.5.5 Biochemical characterization up to species level

The species level was confirmed by different biochemical tests (Plate 6 and 7) based on the genus identified, the results of the same were given in Table 10 and 11 for bivalves and crabs respectively. The outcomes were interpreted as follows:

a. Sugar fermentation test: The positive reaction was indicated by a colour change to yellow due to the acid formation by the respective sugar fermentation.
b. Amino acid decarboxylation tests: The positive test was indicated by a colour change from yellow to purple indicating the utilization of the corresponding amino acids (arginine, ornithine and lysine).
c. Sensitivity to vibrio static agents: (O129) (10 mcg and 150 mcg): An inhibition zone diameter is observed against the vibrio static agent in the disc differentiating vibrios from non -vibrios.
d. Esculin hydrolysis test: The positive reaction was indicated by the black colour formation.
e. Urease test: The pink colour formation after 48h incubation was considered as positive.
f. ONPG test: Formation of yellow colour was taken as positive reaction.
g. Gelatinase test: The positive isolates were identified by the formation of a clear zone around the colonies.
h. Nitrate reduction test: The tubes showing red colour after adding the reagents were considered as positive.
i. Growth of bacteria at different temperatures: The turbidity/pellet/sediment formation at the end of incubation was taken as positive.
j. Caseinase test: A clear zone formation surrounding the colonies after 24h was taken as positive test.
k. Salinity tolerance test: The growth of bacteria at the end of incubation was noted down.

l. Haemolysis test: Isolates that produced haemolytic enzymes discoloured the blood agar in the proximity of their colonies at the end of incubation.

m. Triple Sugar Iron test (TSI test): The ability to produce acid slant (yellow) and alkaline butt (pink) were noted down. Black colour formation was taken as indicative of H_2S production.

n. Antibiotic sensitivity testing: The zone diameter around the culture spot was measured and interpreted based on the manufactures guidelines (Himedia)

4.5. 6. Identification of the antagonistic isolates up-to the species level

Based on the polyphasic taxonomic approach, 27 isolates showing antagonistic potential were identified and were found to be the members of 11 different species. The *16S rRNA* gene sequence of the representative species were deposited in the NCBI, GenBank and got assigned with accession numbers (Table 12).

By the combined analysis of the results of antagonism and species identification, the isolate with the highest score (88HL) from marine crabs was found to be *Pseudomonas aeruginosa* followed by *Staphylococcus sciuri* and *Staphylococcus haemolyticus* (39HL and 78 HL respectively) having a score of 8. The isolate having the maximum versatility (39HL) from marine crabs was identified as *S. sciuri.* Similarly, among the isolates from *P. viridis,* the isolate with the highest score (11) was found to be *Staphylococcus pasteuri* (136b) followed by *S. sciuri* (228b) having a score of 10. The isolate having the maximum versatility (228b) from *P. viridis* was identified as S. *sciuri.*

4.6. PHYLOGENETIC TREE CONSTRUCTION OF ANTAGONISTIC ISOLATES

The neighbour-joining phylogram demonstrating the phylogenetic relationships of the various antagonistic isolates was constructed and given as Fig. 14.

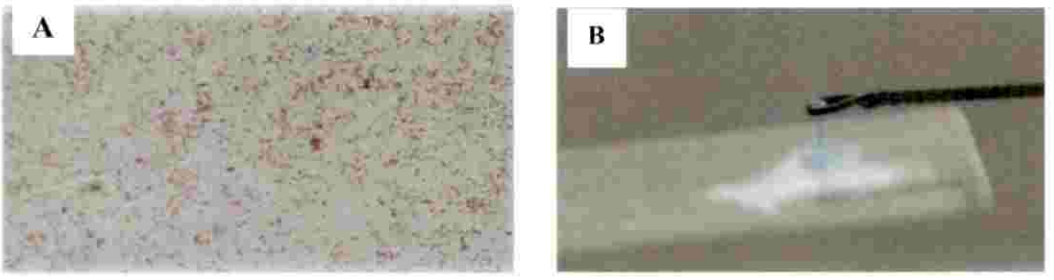

Plate 4. Morphological characterisation of antagonistic isolates by (A) Gram staining- Gram negative bacilli (B) KOH string test

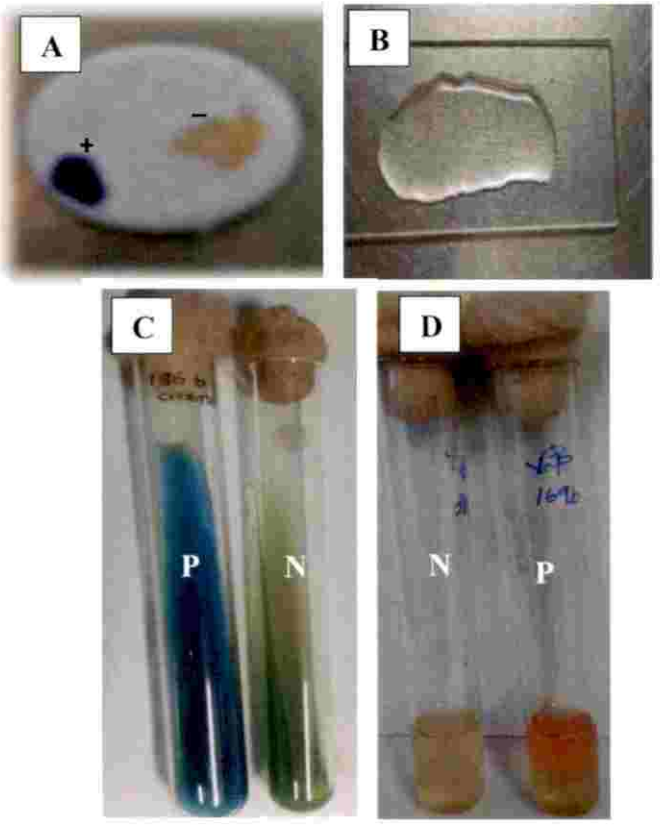

Plate 5. Preliminary biochemical characterisation for antagonistic isolates. (A) Oxidase test indicating both positive (+) and negative (-) activity (B) Catalase test (C) Citrate utilization test (D) Voges- Proskauer test

Table 8. Cultural characterization of the antagonistic isolates of crabs and bivalves.

Isolate ID	Cultural characteristics				
	BHIA	ZMA	TCBS	MSA	MCA
3b	ND*	WOM-L*	YO*	ND	ND
6b	ND	WSp.*	ND	ND	ND
8b	ND	WSp.	NG*	ND	ND
34b	ND	OWCNTl.*	ND	ND	ND
36b	ND	WTSp.*	ND	ND	ND
53b	ND	OTM-L*	NG	ND	ND
91b	ND	WOMu.*	ND	ND	ND
204b	ND	WSp.	NG	ND	ND
214b	ND	Pi.ROS-M*	ND	ND	ND
185b	ND	YO	ND	ND	ND
228b	ND	BCo.D*	ND	ND	ND
156b	ND	FYC*	ND	ND	ND
93b	ND	PT*	Y*	ND	ND
136b	ND	PT	ND	ND	ND
186b	ND	PT	ND	ND	ND
131b	ND	Mu.L*	Y	ND	ND
27b	ND	Ma.OCo.M-L*	ND	ND	ND
10lHL	ND	Cr.Sw.*	ND	ND	ND
16HL	ND	WOI*	ND	ND	ND
38HL	ND	PY	YMu.*	ND	ND
39HL	ND	PY	ND	ND	ND
45HL	ND	WOS-M*	ND	ND	ND
55bHL	ND	WOR*	ND	ND	ND
72HL	ND	WTl.S*	GFSm.*	ND	ND
78HL	ND	WODP*	ND	ND	ND
87HL	ND	WOS-M*	ND	ND	ND
88HL	YTl.*	FSp.MS*	ND	ND	NLF*

Abbreviations: *ND- Not Done; NG- No growth; Y- Yellow; YO- Yellow, opaque; GFSm.- Green, flat, smooth; YTl.- Yellow, translucent; WOM-L- White, opaque, medium to large; WSp.- White Spreading; OWCNTl.- Off white, circular, non-translucent; WTSp.- White, translucent, spreading; OTM-L- Opaque, Transparent, medium to large; WOMu.- White, opaque, mucoid; Pi.ROS-M- Pink, round, opaque, small to medium; BCo.D- Buffy coloured, convex, dry; FYC- Flat, yellow, circular; PT- Pinpoint, transparent; Mu.L- Mucoid, large; Ma.OCo.M-L - Marron, opaque,convex, medium to large; Cr.Sw.- Creamy, swarming; WOI- White,opaque,irregular; PY- Pinpoint, yellow; WOS-M- White, opaque, small to medium; WOR- White, opaque, round; WTl.S- White, translucent, small; WODP- White, opaque, dry, pinpoint; FSp.MS- Flat, spreading, metallic sheen; YMu.- Yellow, mucoid; NLF- Non lactose fermenting.

Table 9. Preliminary biochemical characterization of the antagonistic isolates of crabs and bivalves.

Isolate ID	Biochemical tests						
	CT*	OT*	Indole	MR*	VP*	Citrate	O/F*
3b	ND*	ND	ND	ND	+	ND	ND
6b	ND	+	ND	ND	+	-	ND
8b	+	ND	ND	ND	ND	ND	ND
34b	ND	+	ND	ND	ND	ND	ND
36b	ND	+	ND	ND	ND	ND	NG/NG*
53b	ND	ND	ND	ND	ND	ND	ND
91b	ND	ND	ND	ND	ND	ND	ND
204b	ND	+	-	ND	+	-	ND
214b	ND	ND	ND	ND	ND	ND	ND
185b	ND	-	+	ND	ND	+	ND
228b	ND	-	ND	ND	ND	ND	ND
156b	ND	ND	ND	ND	ND	ND	ND
93b	ND	ND	ND	ND	ND	ND	ND
136b	ND	ND	ND	ND	ND	ND	ND
186b	ND	ND	ND	ND	ND	+	ND
131b	ND	ND	ND	ND	ND	ND	ND
27b	ND	+	ND	ND	ND	ND	-
10lHL	+	+	+	ND	+	-	G/G*
16HL	+	+	-	ND	+	+	ND
38HL	-	+	-	+	+	-	NG/NG
39HL	ND	+	ND	ND	-	ND	ND
45HL	ND	ND	ND	ND	ND	ND	ND
55bHL	+	+	ND	-	-	+	ND
72HL	ND	ND	ND	ND	ND	ND	ND
78HL	ND	+	ND	ND	ND	ND	ND
87HL	ND	ND	ND	ND	ND	ND	ND
88HL	+	+	-	-	-	+	ND

Abbreviations: *CT- Catalase test; OT- Oxidase test; MR- Methyl red test; VP- Voges–Proskauer test; O/F- Oxidative/Fermentative test; ND- Not done; NG- No growth; G- Green.

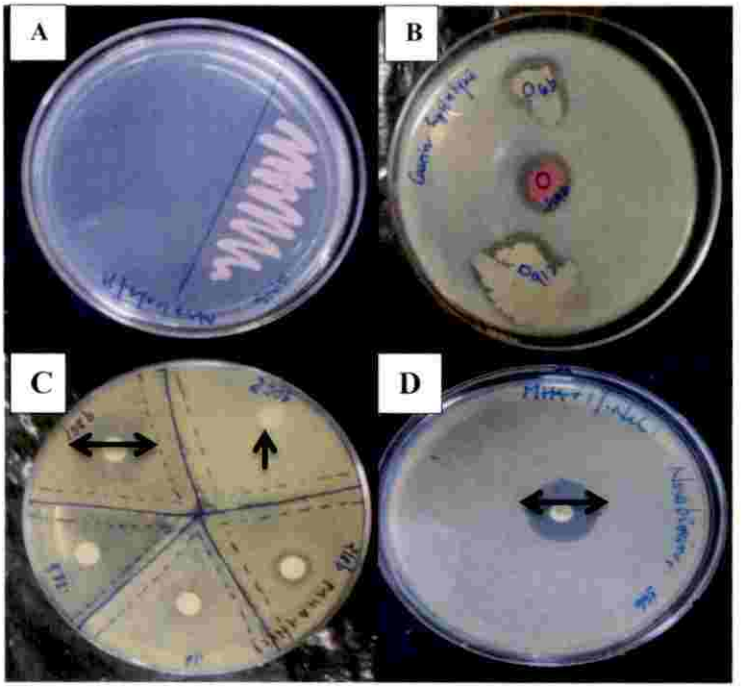

Plate 6. Confirmatory biochemical characterisation of antagonistic isolates. (A) Growth at different salt concentrations 4% NaCl (B) Casein hydrolysis test (C) Sensitivity to vibriostatic agent O/129 (150 mcg), left-right arrow indicates susceptibility and upward arrow indicates resistance to the agent (D) left-right arrow indicates susceptibility to Novobiocin

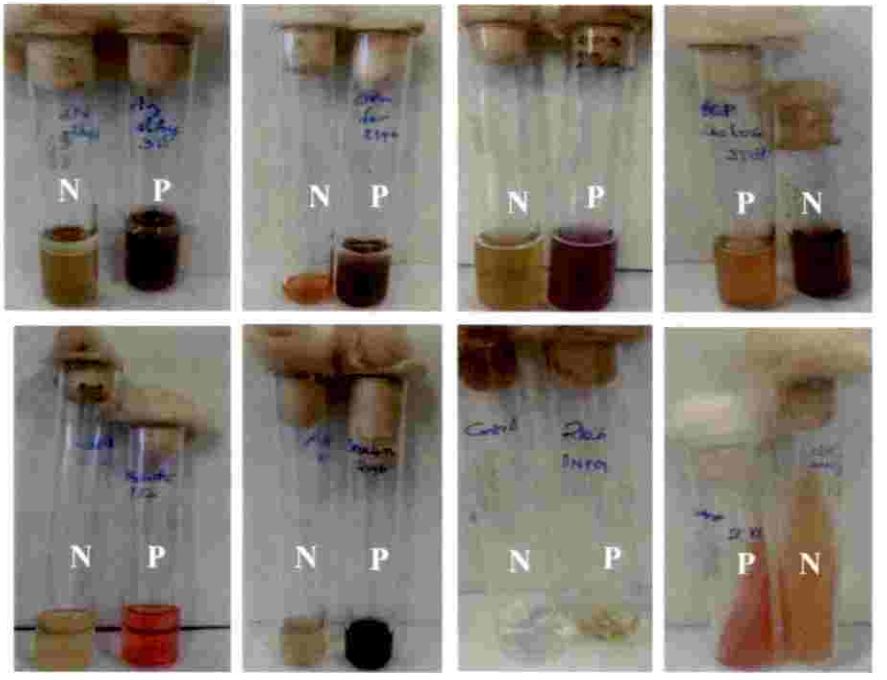

Plate 7. Confirmatory biochemical characterisation of antagonistic isolates. (A)Arginine dihydrolase test (B) Ornithine decarboxylase test(C) Arginine decarboxylase test (D) Sugar fermentation test (E) Nitrate reduction test (F) Esculin hydrolysis test (G) ONPG test (H) Urease test. 'N' indicates negative control and 'P' indicates positive test

Table 10. Biochemical characterization up to species level for isolates from *P. viridis*

Biochemical test	3b	6b	8b	34b	36b	53b	91b	204b	214b	185b	228b	156b	93b	136b	186b
Gelatinase	-	ND*	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	+	ND
Urease	+	-	ND	ND	-	ND	ND	+	-	+		+	ND	ND	ND
Esculin hydrolysis	-	ND	ND	ND	ND	ND	-	ND	+	ND	-	ND	ND	ND	-
Casein hydrolysis	-	+	ND	ND	ND	ND	+	ND	+	ND	ND	ND	ND	-	ND
Blood agar haemolysis	ND	ND	ND	ND	ND	-	ND	+	ND	ND	ND	ND	ND	ND	ND
Tween 80 hydrolysis	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND
Amylase test/Starch hydrolysis	ND	+	+	ND	ND	ND	+	+	ND	ND	ND	ND	ND	ND	ND
Nitrate reduction	ND	+	ND	ND	ND	ND	+	ND	ND	ND	ND	+	ND	+	ND
H_2S gas production	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND
ONPG*	-	-	ND	ND	ND	ND	ND	-	+	+	ND	ND	ND	ND	ND
O/129 (150 mcg)	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	S	ND	ND
Resistant to Novobyocin (5 mcg	ND	ND	ND	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Resistant to Polymixin B (50 units)	ND	ND	ND	ND	ND	R	ND	ND	ND	ND	ND	ND	ND	ND	ND
ADi.T*	+	ND	ND	-	ND	ND	-	ND	-	+	ND	ND	ND	+	+
Amino acid decarboxylation test															
ADT*	-	ND	ND	ND	ND	ND	ND	-	-	-	ND	ND	+	ND	ND
LDT*	-	ND	ND	ND	ND	ND	ND	-	-	-	ND	ND	-	ND	ND
ODT*	-	ND	ND	ND	ND	-	ND	-	-	+	ND	ND	-	ND	-

Sugar fermentation test															
Sucrose	ND	ND	-	+	-	ND	ND	ND	ND	+	ND	ND	ND	ND	+
Arabinose	+	-	ND	ND	ND	ND	-	-	+	+	ND	ND	ND	ND	ND
Salicin	ND	ND	-	-	ND	-	ND	-	+	ND	ND	ND	ND	ND	-
Inositol	ND	-	ND	ND	ND	ND	ND	-	ND	+	ND	ND	ND	ND	ND
Sorbitol	ND	ND	ND	ND	ND	ND	ND	-	ND	+	ND	ND	ND	ND	ND
Mannose	ND	ND	-	+	-	-	-	ND	+	ND	+	ND	ND	-	ND
Cellobiose	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mannitol	-	ND	ND	ND	-	ND	ND	ND	+	+	ND	ND	ND	+	ND
Mellibiose	ND	-	ND	ND	ND	ND	ND	-	+	+	ND	ND	ND	ND	+
Maltose	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	+	ND	ND	-	ND
Rhamnose	ND	-	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND
Raffinose	ND	-	ND	ND	ND	ND	+	-	+	ND	+	ND	ND	ND	+
Glycerol	ND	ND	-	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glucose	ND	-	-	+	ND	+	ND	ND	ND	+	ND	ND	ND	ND	ND
Galactose	ND	-	ND	ND	ND	ND	ND	-	+	ND	ND	ND	ND	ND	ND
Xylose	ND	-	ND	-	ND	ND	-	-	+	ND	ND	ND	ND	ND	ND
Trehalose	ND	ND	ND	-	-	ND	ND	-	+	ND	+	ND	ND	+	ND
Lactose	ND	ND	ND	ND	ND	ND	ND	-	+	ND	ND	ND	ND	-	ND
Dulcitol	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	-
Growth at different salt concentrations															
0%	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4%	ND	ND	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND

8%	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9%	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
10%	ND	ND	ND	ND	ND	-	ND	-	ND	ND	ND	ND	ND	ND	ND
Growth at different temperatures															
4^0C	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND
42^0C	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
45^0C	ND	ND	+	+	ND	-	+	ND	ND	ND	ND	ND	ND	ND	ND
55^0C	ND	ND	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND	ND	ND
4^0C	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviations: *ND- Not done; ONPG- Ortho nitrphenyl hydrazine; ADi.T- Arginine dihydrolase test; ADT- Arginine decarboxylase test; LDT- Lysine decarboxylase test; ODT- Ornithine decarboxylase test.

Table 11. Biochemical characterization up to species level for isolates from marine crabs

Biochemical test	10lHL	16HL	38HL	39HL	45HL	55bHL	72HL	78HL	87HL	88HL
Gelatinase	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
Urease	+	-	ND	-	-	-	+	-	+	+
Esculin Hydrolysis	-	ND	ND	+	ND	ND	ND	ND	+	ND
Casein hydrolysis	ND	+	ND	ND	ND	+	ND	ND	ND	-
Tween 80 hydrolysis	ND	ND	ND	ND	ND	ND	ND	ND	+	ND
Amylase test	ND	ND	ND	ND	ND	ND	+	ND	ND	-
Nitrate reduction	+	ND	ND	ND	+	+	+	ND	_	+
H_2S gas production	ND	ND	ND	ND	ND	-	ND	ND	ND	-
ONPG*	-	ND	ND	-	ND	ND	ND	ND	ND	ND
O/129 (150 mcg)	ND	ND	ND	ND	ND	ND	ND	ND	ND	R
Resistant to Novobiocin (5 mcg	ND	ND	R*	R	R	ND	ND	R	ND	ND
ADi.T*	ND	+	ND	-	ND	ND	ND	ND	ND	ND
Amino acid decarboxylation test										
ADT	-	ND	ND	ND	ND	ND	ND	ND	ND	+
LDT	+	-	ND	ND	ND	ND	ND	ND	ND	-
ODT	+	-	ND	-	ND	ND	ND	ND	ND	-
Sugar fermentation test										
Sucrose	-	-	-	ND	-	+	ND	-	ND	ND
Arabinose	+	-	ND	ND	ND	ND	ND	ND	+	ND

Salicin	-	ND	ND	ND	ND	ND	ND	ND	_	ND
Inositol	-	-	ND	ND	ND	ND	ND	ND	ND	-
Sorbitol	-	-	ND	ND	ND	ND	ND	ND	ND	ND
Mannose	+	ND	ND	ND	+	ND	ND	-	ND	-
Cellobiose	-	ND	ND	ND	ND	ND	+	ND	_	ND
Mannitol	+	-	ND	ND	-	+	ND	-	ND	_
Mellibiose	ND	-	ND	-	ND	ND	ND	ND	ND	ND
Maltose	ND	ND	ND	+	+	+	+	ND	ND	-
Rhamnose	ND	-	ND	ND	ND	ND	ND	ND	ND	ND
Raffinose	-	ND	-	-	-	ND	ND	ND	ND	ND
Glycerol	ND	ND	ND	ND	ND	ND	+	ND	ND	_
Glucose	ND	ND	ND	ND	ND	+	ND	ND	ND	ND
Galactose	ND	ND	ND	ND	ND	_	ND	ND	_	+
Xylose	-	ND	ND	ND	-	+	-	ND	ND	ND
Trehalose	+	ND	+	+	+	ND	ND	-	ND	-
Lactose	ND	ND	ND	ND	ND	-	ND	ND	ND	-
Fructose	ND	ND	ND	+	ND	ND	ND	ND	ND	ND
Ribose	ND	ND	ND	-	ND	ND	ND	ND	ND	ND
Growth at different salt concentrations										
0%	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
5%	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
8%	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
10%	-	ND	ND	ND	ND	ND	ND	ND	ND	ND

Growth at different temperatures										
4^0C	-	ND	ND	ND	ND	ND	ND	ND	ND	ND

Abbreviations: *ND- Not done; ONPG- Ortho nitrphenyl hydrazine; ADi.T- Arginine dihydrolase test; ADT- Arginine decarboxylase test; LDT- Lysine decarboxylase test; ODT- Ornithine decarboxylase test; R- Resistant.

Table 12. Final identification of the antagonistic isolates

Isolate ID	Identification	GenBank accession number
3b	*Vibrio japonicus*	MK696615
6b	*Bacillus endophyticus*	MK696616
34b	*Bacillus paraanthracis*	MK696974
53b	*Schewanella haliotis*	MK713653
91b	*Pseudomonas oleovarans*	MK713655
204b	*Bacillus amyloliquifaciens*	MK713818
214b	*Serratia marscences*	MK713840
185b	*Enterobacter asburiae*	MK713758
228b	*Staphylococcus sciuri*	MN240456
156b	*Micrococcus luteus*	MN094370
93b	*Vibrio fluvialis*	MK713849
136b	*Staphylococcus pasteuri*	MN094366
186b	*Citrobacter amalonaticus*	MK713850
131b	*Vibrio alginolyticus*	MK713761
27b	*Staphylococcus epidermidis*	MK696596
10lHL	*Vibrioalginolyticus*	MN240447
16HL	*Bacillus albus*	MN240455
38HL	*Staphylococcus saprophyticus*	MG792290
39HL	*Staphylococcus sciuri*	MN240456
45HL	*Aeromonas caviae*	MG792292
55bHL	*Bacillus megaterium*	MN240460
72HL	*Photobacterium damselae*	MK696615
78HL	*Staphylococcus haemolyticus*	MG792305
87HL	*Staphylococcus arlettae*	MG792307
88HL	*Pseudomonas aeruginosa*	MG792308

Fig. 13. (A)Gel profile of *16 SrRNA* gene amplification products (B) NCBI blast results of *16 SrRNA* gene sequences.

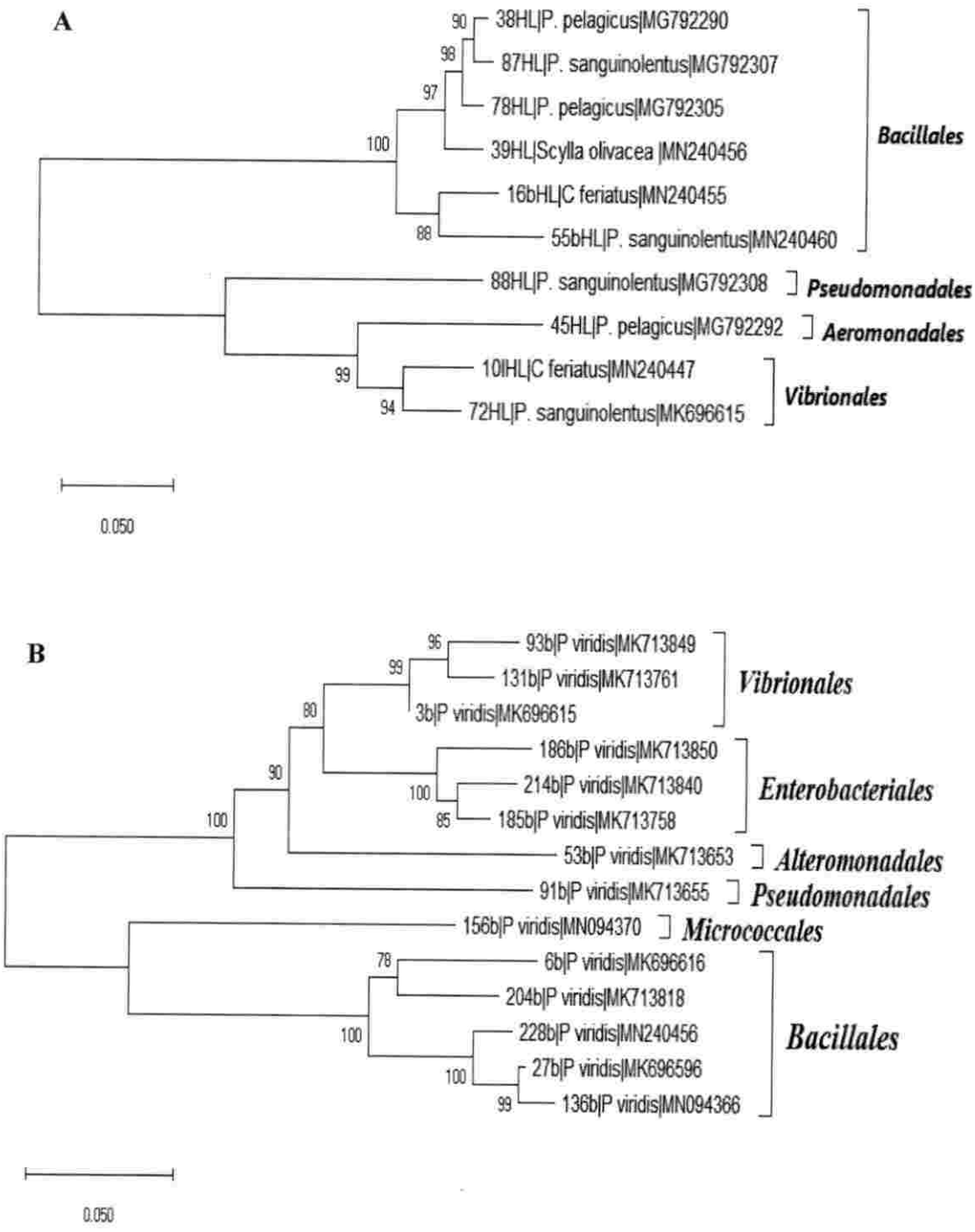

Fig. 14. Phylogenetic tree based on *16 SrRNA* gene sequences for (A) antagonistic bacterial isolates from marine crabs (B) antagonistic isolates from bivalves. The isolate ID is followed by its source and accession number. The values near the nodes indicate the bootstrap values of 1000 replicates

4.7. AMPLIFICATION PROFILE OF BIOSYNTHETIC GENES

The antagonistic isolates exhibiting inhibitory action against at least one indicator pathogens were then screened for the presence of two biosynthetic genes namely, *NRPS* and *PKS*. Amplification profiles of both *NRPS* genes and *PKS* genes were given in Table 13. Out of the 27 antagonistic isolates screened, 13 showed positive amplification for *NRPS* gene. *NRPS* genes of isolates produced amplicon of size ~1000 bp (Fig. 15(A)).In case of *PKS* gene, of the total 27 screened 6 showed the presence of the same. The gene size was obtained as ~ 700bp (Fig. 15(B)).

4.8. ANALYSIS OF RESULTS OF ANTAGONISM WITH THE RESULTS ON THE EXISTENCE OF BIOSYNTHETIC GENES

Statistical analysis showed that the presence of *NRPS/PKS* gene has statistically significant influence on the inhibitory zone diameter against 3 pathogens namely, *P. damselae*, *S.* Typhimurium ($p<0.1$) and β-lactamase producing *E. coli* ($p<0.05$) (Table 14). Additionally, the inhibitory zone diameter against these three indicator pathogens was statistically significantly higher in *NRPS/PKS* positive isolates than the negative ($U =$ 57, 66 and 60.0 respectively, $p =$ .070, 0.058 and 0.030 respectively). More interestingly, the inhibitory zone diameter against all other indicator pathogens was higher in *NRPS/PKS* positive isolates than the negative even though the difference is not statistically significant (Table 15).

4.9. SEQUENCE ANALYSIS

4.9.1. Sequence analysis of *NRPS* gene

For sequencing, 5 *NRPS* positive isolates were randomly selected and cloned. The sequences were submitted in GenBank, NCBI and got assigned with accession numbers (MN372397-MN372401). After editing the obtained sequence data from both forward and reverse primers from each clones using Editseq (DNASTAR, Lasergene, USA), the vector sequences were removed.

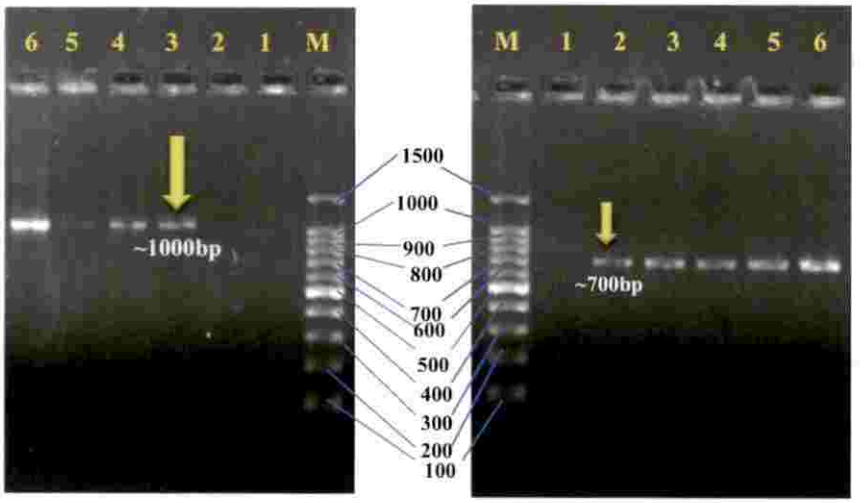

Fig. 15. AGE results of (A) *NRPS* adenylation domain amplification. (B) *PKS* ketosynthase domain amplification.

Table 13. Profile of *NRPS/PKS* biosynthetic genes of bacterial isolates in both crabs and bivalves.

Isolate ID	10lHL	16HL	38HL	39HL	45HL	55bHL	72HL	78HL	87HL	88HL
NRPS	✓	✓	✓	✓	×	×	×	✓	✓	×
PKS	×	×	×	×	×	✓	×	×	×	×
Isolate ID	3b	6b	8b	34b	36b	53b	91b	204b	214b	185b
NRPS	×	✓	✓	✓	✓	×	✓	✓	✓	×
PKS	×	✓	×	×	✓	×	✓	✓	×	×
Isolate ID	228b	156b	93b	136b	186b	131b	27b			
NRPS	×	×	×	×	×	×	×			
PKS	×	×	×	×	×	×	✓			

✓- represents the presence of corresponding genes
×- represents the absence of *NRPS/PKS* genes

Table 14. Descriptive statistics analysing the antagonistic activity with the existence of BGCs.

Pathogens	BGS	N	Mean Rank	Sum of Ranks
PD	1	15	16.20	243.00
	2	12	11.25	135.00
	Total	27		
Val	1	15	13.73	206.00
	2	12	14.33	172.00
	Total	27		
VAng	1	15	14.47	217.00
	2	12	13.42	161.00
	Total	27		
VH	1	15	16.10	241.50
	2	12	11.38	136.50
	Total	27		
VPara	1	15	13.67	205.00
	2	12	14.42	173.00
	Total	27		
Vvul	1	15	15.43	231.50
	2	12	12.21	146.50
	Total	27		
BLEcol	1	15	16.00	240.00
	2	12	11.50	138.00
	Total	27		
ST	1	15	15.60	234.00
	2	12	12.00	144.00
	Total	27		
Sau	1	15	13.00	195.00

	2	12	15.25	183.00
	Total	27		
MRSA	1	15	13.10	196.50
	2	12	15.12	181.50
	Total	27		

Table 15.Representation of the significance level of antagonism against each pathogen towards presence/absence of BGCs

Test Statistics[b]										
	PD	Val	VAng	VH	VPara	Vvul	BLEcoli	ST	Sau	MRSA
Mann-Whitney U	57.000	86.000	83.000	58.500	85.000	68.500	60.000	66.000	75.000	76.500
Wilcoxon W	135.000	206.000	161.000	136.500	205.000	146.500	138.000	144.000	195.000	196.500
Z	-1.813	-.234	-.395	-1.576	-.335	-1.214	-2.174	-1.894	-1.611	-.972
Asymp. Sig. (2-tailed)	.070	.815	.693	.115	.737	.225	.030	.058	.107	.331
Exact Sig. [2*(1-tailed Sig.)]	.114[a]	.867[a]	.755[a]	.126[a]	.829[a]	.300[a]	.152[a]	.256[a]	.486[a]	.516[a]
a. Not corrected for ties.										
b. Grouping Variable: BGS										

Abbreviations: PD- *P. damselae*, Val- *V. alginolyticus*, VAng- *V. anguillarum*, VH- *V. harveyi* , VPara- *V. parahaemolyticus*, Vvul- *V. vulnificus*, BLEcoli- β- lactamase *producing E. coli*, ST- *S.* Typhimurium Sau- *S. aureus* , MRSA- Methicillin-resistant *S. aureus*, Asymp. Sig.- Asymptotic Significance , Exact Sig.- Exact significance

The final length of *NRPS* gene present in isolates16HL, 38HL, 39HL and 78HL was 1.01 kb and for 87HL it was 1.007kb. The final gene sequences were then compared with those in GenBank database, NCBI and closest relative sequences and their coding products were retrieved (Table 17). The phylogenetic analysis of the *NRPS* sequences obtained through the present study is given in Fig 16.

The coding sequences of the obtained *NRPS* were then converted into corresponding amino acid sequences and were used for insilico analysis. The results of substrate prediction and detailed description of 'A' domains of the present study with respect to other characterized *NRPS* sequences with known metabolic products were depicted in Table 16. For 16HL strain, the binding pocket may integrate a glutamine residue into a non-ribosomal peptide. The signature obtained for all other strains may integrate a serine residue. Putative structural classes of antimicrobial products produced by the identified NRPS genes of the present study were also defined (Table 16). Results showed that the sequence of 16HL showed 99% ID with Lichenycin, surface-active lipopeptides with antibiotic properties produced by *B. licheniformis*. The low ID percentages of other four sequences (42–50%) could suggest their novelty or uncharacterized nature of antimicrobial products from these strains (Table 16).

4.9.2. Sequence analysis of *PKS* gene

Of the 6 isolates positive for *PKS*, 5 were randomly selected and sequenced. The obtained sequence data from both forward and reverse primers was edited using Editseq (DNASTAR, Lasergene, USA). The final gene sequences were then compared with those in GenBank database, NCBI and closest relative sequences and their coding products were retrieved (Table 17). The phylogenetic analysis of the *PKS* gene sequences obtained through the present study with published *PKS* gene sequences were given in Fig. 17.

Table 16: Genetic analysis of *NRPS* 'A' domains obtained through the study

Isolate ID	Species	Aminoacids in binding pockets	Substrate prediction	Cluster having maximum relation to the domains	Enzyme producing maximum alignment	Similarity (%)	Identity (%)	Alignment of the binding domains	
16b HL	*B. albus*	DAQDLGVVD	Gln (Glutamine)	Lichenycin	Lichenysin synthetase A of *B. licheniformis*	99	99	16HL	DAQDLGVVD
								Lichenysin	DAQDLGVVD
38HL	*S. saprophyticus*	DVWHLSLIE	Ser (D-serine)	Iturin	Iturin A synthetase C of *B. subtilis*	66	50	38HL	DVWHLSLIE
								Iturin	DVWHLSLID
39HL	*S. sciuri*	DVWHLTLIE	Ser (D-serine)	Iturin	Tyrocidine synthase 3 of *Brevibacillus brevis*	61	44	39HL	DVWHLTLIE
								Iturin	DVWHLSLID
78HL	*S. haemolyticus*	DVWHLTLIE	Ser (D-serine)	Iturin	Tyrocidine synthase 3 of *Brevibacillus brevis*	66	46	78HL	DVWHLTLIE
								Iturin	DVWHLSLID
87HL	*S. arlettae*	DVWHLTLIE	Ser (D-serine)	Iturin	Tyrocidine synthase 3 of *Brevibacillus brevis*	59	42	87HL	DVWHLTLIE
								Iturin	DVWHLSLID

Table 17. Identity of *NRPS* and *PKS* gene sequences of isolates to their closest relatives in NCBI database

Isolate	Species identified	Closest relative	NCBI Accession number	Identity %
Strains with *NRPS* sequence				
16b HL	*B. albus*	lichenysin synthetase A gene (*lchAA1*) of *Bacillus licheniformis*	LR134165.1	99.21%
38HL	*S. saprophyticus*	Non-ribosomal peptide synthetase of *Staphylococcus xylosus*	LN554884.1	95.54%
39HL	*S. sciuri*	Non-ribosomal peptide synthetase of *Bacillus subtilis*	EU399172.1	97.51%
78HL	*S. haemolyticus*	Non-ribosomal peptide synthetase of *Bacillus subtilis*	EU399172.1	97.72%
87HL	*S. arlettae*	*Staphylococcus arlettae* surfactin synthase subunit 1	AP019698.1	99.4%
Strains with *PKS* sequence				
6b	*Bacillus endophyticus*	Zinc-binding dehydrogenase gene *of Bacillus velezensis strain WRN014*	CP041361.1	97.30%
36b	*Staphylococcus epidermidis*	Zinc-binding dehydrogenase gene of *Bacillus velezensis* strain WRN014	CP041361.1	98.36%
91b	*Pseudomonas oleovarans*	Zinc-binding dehydrogenase gene of *Bacillus velezensis* strain WRN014	CP041361.1	97.55%
55bHL	*Bacillus megaterium*	Polyketide synthase gene of *Bacillus velezensis* strain L-S60	CP011278.1	98.68%

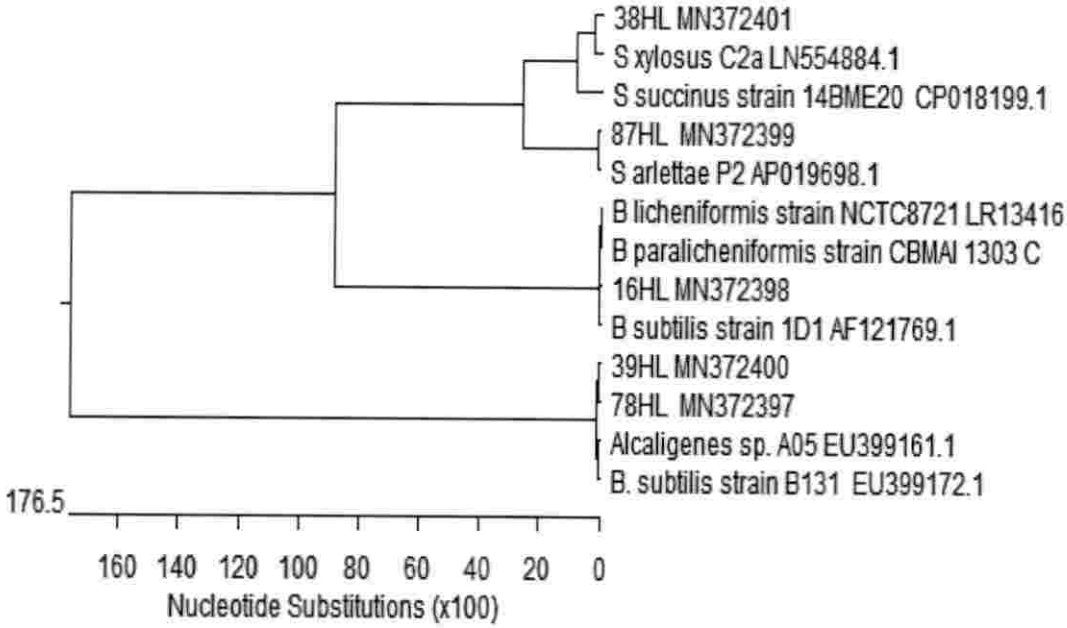

Fig. 16. Neighbour Joining tree representing *NRPS* positive isolates and their closest relatives. Isolate ID and strains are followed by their accession number.

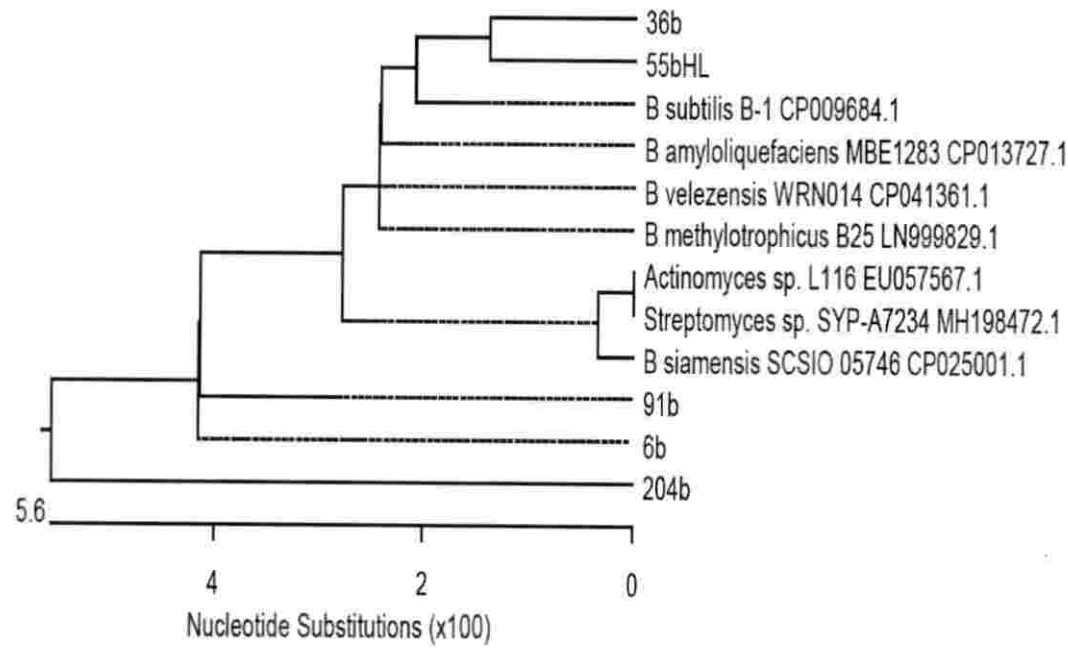

Fig. 17. Neighbour Joining tree representing *PKS* positive isolates and their closest relatives. Isolate ID and strains of closest relatives are followed by accession number.

Among the *PKS* positive isolates the gene of 55bHL was cloned and sequenced. The obtained sequence was translated to corresponding aminoacid sequence and was subjected to *insilico* analysis using NaPDoS. The results suggest that the domain shows 40% identity to the product jamicamide (JamM_AAS98784_H) coded by a hybridKS domain. The KS domain sequences of 55bHL was compared with a board set of identified and fully characterized reference domains and domain specific phylogenetic tree was constructed (Fig. 18).

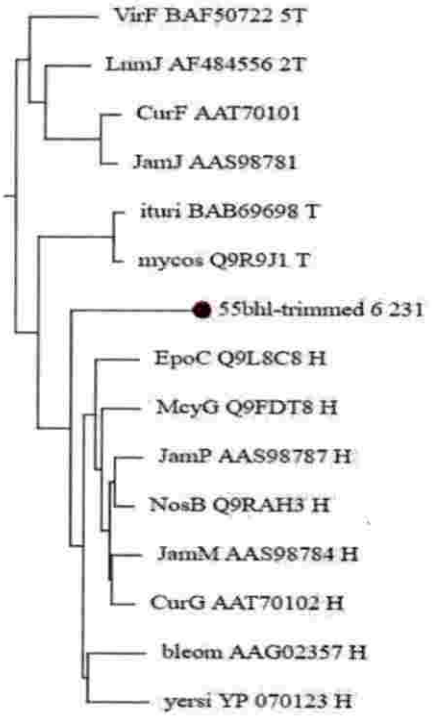

Fig 18. Phylogram representing KS domain of 55bHL and closely related domain-specific sequences. Red spot indicate the position of 55bhL with respect to reference domains sequences.

DISCUSSION

5. DISCUSSION

The present study emphasis on the exploration of marine microbial symbionts to meet the increasing demand for antimicrobial agents. The study also analyses the presence of two most significant biosynthetic genes namely, *NRPS* and *PKS* genes in the cultivable antagonistic bacterial symbionts of two classes of marine invertebrates namely, crab and bivalves by PCR targeting the conserved domains. The gene sequences found were analyzed and the antagonistic strains were identified by polyphasic taxonomic approach. The study also examined the density of the cultivable microbial symbionts in the targeted niche.

Microbial symbionts of various animals have become a growing area of research during recent years (Kohl and Carey, 2016). Insight into marine environment and symbionts associated with many marine organisms are especially important as five of the seven compounds recently approved by FDA were produced from the symbionts of marine invertebrates (Giddings and Newman, 2019). Moreover, many bio-actives which were earlier attributed to be produced from marine macro-organisms showed structural similarity to compounds of microbial origin suggesting the importance of these microbes especially, symbionts of the sessile and slow moving organisms in antimicrobial discovery (Putz and Proksch, 2018). Many marine invertebrate animals were reported to be a vital source for novel drugs especially drugs promoting activity against MDR strains (MRSA and VRE- vancomycin-resistant enterococci); thus forming the recent research hotspots in the search for novel drugs (Uzair *et al.,* 2008; Palomo *et al.,* 2013; Javed *et al.,* 2011). Even though symbiotic microbes of various marine organisms like sponges, algae and tunicates has been screened in the search for novel antibacterial compounds (Abad *et al.,* 2011), microbes associated with marine crabs and bivalves are the least unexplored. A few studies have shown the potential of mollusk symbionts as antibacterial agents such as isolation of 5DD-diketopiperazines exhibiting activity against *Vibrio anguillarum* from a marine bacterium associated with *Pecten maximus* (Fdhila *et al.,* 2003) and B-1015 isolated from *Alcaligenes faecalis* (Isono *et al.,* 1993). Similarly,

Kohayashi *et al.* (1995) isolated Flavocristamides from symbiotic *Flavobacterium* sp. associated with the marine bivalve *Crisrcuia piicutu.*

Apart from the search for antagonistic strains, information on the actual load of symbionts in apparently healthy commercially significant farmed species of marine crabs and bivalves is necessary to study the host immunity and to reveal pathogenesis as well as diagnosis of diseases during farming practices. Even though microbes of few commercially important marine crabs and bivalves have already been inspected for the presence of human pathogens (Faghri *et al.,* 1984; Peralta and Andalecio, 2011; Mahalaxmi *et al.,* 2013; Soundarapandian and Sowmiya, 2013; Teerawut *et al.,* 2017; Sorio and Inolino, 2018), its actual microbial load and role of these microbes remain mostly unknown, especially on tropical marine invertebrates. Such data will also be useful in the field of microbial ecology. Therefore, even though the study primarily aimed to explore marine microbial symbionts for antimicrobial agents, the same niche was examined for the density of the cultivable microbial symbionts as the initial step. It was found that the haemolymph of all the four species of marine crabs examined in the present study and that of *P. viridis* carried bacteria even in apparently healthy conditions. Even though existence of bacteria in the hemolymph of invertebrates had been previously inferred as pathological conditions (Lightner and Lewis, 1975), it is currently considered as a normal process (Gomez-Gil *et al.,* 1998). Corresponding to the observations on hemolymph of other crustaceans (Wang and Wang, 2015), our study also supported the non-sterile condition of hemolymph of all the four species of crabs and *P. viridis* even in apparently healthy conditions. The abundance of cultivable microbial symbionts in the haemolymph was in the order of *C. feriatus* > *P. pelagicus*> *S. olivacea* >*P. viridis*>*P. sanguinolentus; P. pelagicus* > *S. olivacea* > *P. viridis*> *C. feriatus* > *P. sanguinolentus* and *P. viridis*> *C. feriatus* >*P. pelagicus.*> *P. sanguinolentus* >*S. olivacea* in ZMA, BHIA and TCBS respectively. Also it was found that within *P. viridis*, the abundance of heterotrophic bacteria was in the order of gut> mantle> muscle >gill >mantle

fluid> haemolymph; gut> muscle > mantle > gill> haemolymph> mantle fluid and gut > muscle> mantle > gill> mantle fluid> haemolymph in ZMA, BHIA and TCBS respectively. Thus, the maximum microbial load was found in the gut of that organism in all the three media used. The average load in the gut was 6.01 ± 1.03 $\log_{10}$CFU/g of gut tissue; which was in the similar range reported for other molluscs by Galli and Giese (1959), Lesel *et al.* (1990) and Watkins and Simkiss (1990), but lower than that reported by Charrier *et al.* (1998) and Charrier *et al.* (2006). The variation in density may be attributed by the difference of species under study/area from where the animals were collected. However, such tissue specific load in different tissues of *P. viridis* was not available for further comparison.

The morphologically unique colonies isolated from all the 5 species were then purified and examined for the antagonistic activity against 12 indicator pathogens including 2 AMR strains. There were a total of 89 and 34 morphologically unique colonies from *P. viridis* and haemolymph of targeted crabs respectively. Among these, 27 isolates (21.95%) showed zone of clearance around inoculation spot for at least one pathogen. Of these 10 were belonging to different crab species (29.41%) and 17 (19.1%) were belonging to P. *viridis*. A study on hemolymph microbes of marine bivalves, Desriac *et al.,* (2014) reported that ~ 3% of hemolymph isolates showed clear inhibition against aquatic pathogens through agar diffusion assay using cell-free supernatants of culturable haemolymph-associated bacteria. Besides, 20% and 25–70% of cultivable bacteria from coral mucus (Ritchie, 2006; Shnit-Orland and Kushmaro, 2010) demonstrated antibiotic activity against pathogen strains through overlay with soft agar protocol and spot diffusion assay. Velho -Pereira and Furtado (2012) demonstrated that four symbiotic isolates from *P. viridis* were antagonistic to clinically relevant pathogens especially *E. coli* and *S.* Typhimurium eventhough they have not mentioned about the % of antagonistic isolates and tissue source of the isolates.

When the inhibitory activity of individual bacteria was examined, it was found that the maximum zone (26 mm) was observed against *V. alginolyticus* by isolate 136b (*Staphylococcus pasteuri*) followed by a zone of 18mm against *V. anguillarum* by 6b (*Bacillus endophyticus*) and 36b (*Staphylococcus epidermidis*) among the isolates from *P. viridis*. Staphylococcus and Bacillus, representatives of Firmicutes, are already known to be the producers of numerous antibacterial metabolites (Mani *et al.,* 2016). *S. pasteuri* was previously studied for the presence of asparaginase gene involved in antitumor activity (Nayak *et al.,* 2013). Similarly it was identified as a plant growth promoter and is recognized for the production of siderophores in plants (Alibrandi *et al.,* 2018). The maximum versatility was shown by 228b (*S. sciuri*) having activity against 6 number of tested pathogens. *S. sciuri* was identified as an efficient inhibitor of food borne pathogenic microbes like *S. aureus* and *Clostridium botulinum* in fermented meat (Mainar *et al.,* 2016). Among the MDR pathogens the maximum zone (4 mm and 8 mm) was observed by 36b (*Staphylococcus epidermidis*) and 185b (*Enterobacter asburiae*) against AR *E. coli strain* and MRSA respectively. *S. epidermidis* isolated from marine grass *Halodule uninervis* was recognized for its potential for antifungal activity against plant pathogens including *Phythium, Rhizoctonia, Phytophthora* and *Pyricularia* (Bibi *et al.,* 2018). The potency of *S. epidermidis* in combating human pathogen was revealed by Iwase and colleagues (2010). They identified a serine protease producing *S. epidermidis* having inhibitory effect on *S. aureus* colonizing human nasal cavity. The responses of plant epiphyte, *Enterobacter asburiae* was identified as a potent indigenous biocontrol agent which prevent pathogens like *S. aureus*, *E. coli* and *Micrococcus agilis* from colonizing *Arabidopsis thaliana* and cotton plant (Quadt-Hallmann *et al.,* 1997; Cooley *et al.,* 2003). They were also recognized for their role in producing plant growth regulators (Ahemad and Khan, 2010). Based on activity score, isolate with code 136b was identified as the most promising bacteria from *P. viridis* (score 11), followed by 228b (score10). The most potential isolate was 136b (*S. pasteuri*) showing activity against 4 pathogens namely, *P damselae, V alginolyticus, V anguillarum and V harveyi.* Even though studies have analysed

the potential of *S. pasteuri* as a plant symbiont, none have analyzed the potential of *S. pasteuri* as a source for antimicrobial compound.

Of the 34 isolates screened from marine crabs 10 exhibited (29.411%) antagonistic activity against one or more pathogens with inhibition zone diameter ranging from 1-40 mm. The maximum zone (40 mm) was observed against *V. vulnificus* by 88HL (*Pseudomonas aeruginosa*) isolate followed by a zone of 30 mm against *V. alginolyticus* by the same isolate. The same isolate was also showing activity against MRSA. *P. aeruginosa*, widely known as an opportunistic human pathogen is already recognized for its potential to synthesize antimicrobial agent (Stover *et al.,* 2000). Haba *et al.* (2001) studied the antimicrobial properties of rhamnolipids produced by *P. aeruginosa* against various bacterial and fungal pathogens. However, the maximum versatility was shown by the isolate having the code 39HL (*S. sciuri*) exhibiting activity against 7 of the tested pathogens, similar to the observation among the isolates from *P. viridis*. Based on activity score, 88HL isolate was identified as the most promising bacteria from marine crabs (score 13), followed by 78 HL and 39 HL (score 8). The most potential isolate 88HL was showing activity against 6 pathogens namely, *V alginolyticus, V harveyi, V parahaemolyticus, V vulnificus, S. aureus* and MRSA. Though *P. aeruginosa* was identified as a resistant pathogen in aquaculture (Nabi *et al.,* 2000; Mastan, 2013), their ability to combat common aquatic pathogens were not spotted. Therefore the present study provides significant details on the promising potential of this microbe in aquaculture epidemiology. Other than *P. aeruginosa*, symbionts belonging to phylum Firmicutes namely, *S. haemolyticus* (39HL) and *S. sciuri* (78HL) were recognized with promising activity against aquatic pathogens. Apart from being identified as an animal and plant pathogen, the potency of *S. haemolyticus* as an antibacterial source were not reported in any of the previous studies.

Subsequently, the pathogens inhibited maximum by the isolates from *P. viridis* was calculated. Among the 12 indicators pathogens tested, *V. harveyi* was the most susceptible to inhibitory activity (64.71%), and followed by *V.*

anguillarum (43.75 %) and *P. damselae* (37.5 %). *V. harveyi* recognized as a common perilous aquatic pathogen affecting marine invertebrates (Robertson *et al.,* 1998), is found to be the most susceptible to antagonistic activities exhibited by both bivalve and crab symbionts suggesting that the antagonistic acitivities exhibited by these symbionts might contribute to the survival of the host against pathogens as described earlier in many vertebrate animals by McFall-Ngai *et al.* (2013). Several approaches in the form of antibiotics, probiotics, phage therapy (Karunasagar *et al.,* 2007; Phumkhachorn *et al.,* 2010; Stalin and Srinivasan, 2017) were targeted to reduce the pathogenicity *V. harveyi* and the present study could able to add to this research by pointing out the potential of symbionts from marine crabs and bivalves against this pathogen. More interestingly, very small number of the isolates showed activity against human pathogens, to which they are rarely encountered. It was found that activity against human pathogens was more pronounced in microbial symbionts from bivalve than from crabs. This may be due to the filter feeding nature, development conditions, microbes present in the native habitat and susceptibility of bivalves to different pathogens (Mearns-Spragg *et al.,* 1998). The coexistence of symbiotic microbes and pathogens in the same niche may be a reason for the pronounced antagonistic activity of the studied symbiotic isolates against aquatic pathogens rather than human pathogens (Murphy *et al.,* 2009). In contrast to our findings, Mearns- Spragg *et al.* (1998) suggested the ability of marine microbes to produce novel secondary metabolites in response to previously unexposed terrestrial pathogens.

When the identification and phylogenetic analysis of the antagonistic isolates were done, 25 distinct bacterial species from 11 different genera belonging to 8 families and 3 major phyla could be identified. Gram negative isolates represented 44.44% of antagonistic microbes and that of gram positive represented 55.56% of the total antagonists. Even though diverse isolates with Gram negative cell wall were identified as antagonists, more than 50% of antagonists were Gram positive. The higher % of gram positive antagonistic

isolates have already been reported by Grossart *et al.* (2004) similar to our observation.

Further, the antagonistic isolates having inhibitory action against at least one indicator pathogens were screened for the presence of two biosynthetic genes namely, *NRPS* and *PKS*, since more than half of the drugs used in medicine today are derived from *NRPS* or *NRPS/PKS* hybrids associated with various marine microbes (Agarwal *et al.,* 2017). The potential of marine microbes for the presence of antimicrobial *NRPS* gene was recognized earlier (Barsby *et al.,* 2001; Leet *et al.,* 2003; Suzumura *et al.,* 2003; Mitova *et al.,* 2004; Desjardine *et al.,* 2007; Rungprom *et al.,* 2008; Engelhardt *et al.,* 2010; Kjaerulff *et al.,* 2013). Even though information on biosynthetic genes of marine invertebrate symbionts like corals, sponges, tunicates are available (Zhang *et al.,* 2009; Tambodu *et al.,* 2014; Samak *et al.,* 2018; Zote *et al.,* 2018), studies on such genes in bivalves and crab symbionts are limited. Thus analyzing these genes in symbionts may provide crucial information on novel *NRPS/PKS* products. Use of target - based approaches for obtaining antibacterial compounds rather than bioactivity based isolation of compounds which is rather time consuming may result in the production of already existing compounds. Zote *et al.* (2018) and Yuan *et al.* (2014) had already identified the rapidness of PCR based techniques for the screening of *NRPS/PKS* genes for the discovery of novel antimicrobial compounds. In the screening for *NPRS/PKS* genes, 13 were found to be positive for *NRPS* genes which belonged to 5 genera namely *Staphylococcus, Bacillus, Vibrio, Serratia* and *Pseudomonas*. Of which 5 of them were randomly selected for sequencing and analysis. Samak *et al.* (2018) have analyzed the symbionts associated with various invertebrates for the presence of *NRPS/PKS* genes and obtained the sequence of *PKS*, KS domains. Similarly in a study that screened the *NRPS/PKS* genes in microbes associated with mud crab, 3 isolates revealed the existence of these genes (Zote *et al.,* 2018). Some studies were able to identify novel sequences of these genes (Tambadou *et al.,* 2014). Similarly, among the 27 strains 6 were found to be positive for *PKS* genes. They were belonging to 4

genera namely *Staphylococcus, Bacillus, Pseudomonas, Vibrio* and *Citrobacter.* Among 9, 5 of them were randomly selected for *PKS* sequence analysis. Thus, out of total 27 antagonistic isolates, 15 were found positive for either *NRPS or PKS* genes. The absence of *NRPS/PKS* gene clusters in 8 isolates suggests that they may contain genes other than *NRPS/PKS* responsible for their antimicrobial activity, suggesting the need for inclusion of more biosynthetic genes in the study. Four isolates namely, 6b (*Bacillus endophyticus*), 36b (*Staphylococcus epidermidis*), 91b (*Pseudomonas oleovorans*) and 204b (*Bacillus amyloliquifaciens*) showed the presence of both genes indicating their hybrid nature. Although many studies revealed the hybrid nature of these genes in marine bacteria especially actinomycetes (Olano *et al.,* 2009 and Devi, 2011), Gram negative bacteria were scarcely identified for the same. Except in a *Vibrio* sp. involved in the production of antibiotic andrimide (Mansson *et al.,* 2011). The present study revealed the identification of hybrid gene cluster in another Gram negative species namely, *Pseudomonas oleovorans*. In the study, the presence of *NRPS* genes were more pronounced than that of *PKS* genes. In crab symbionts, the presence of *NRPS* gene in 6 isolates compared to that of *PKS* gene in one isolate (55bHL) suggested the non-ribosomal nature of the antimicrobials produced by them. In bivalve symbionts the proportions of both genes were equal. Genus *Staphylococcus* sp. showed the presence of maximum number of *NRPS/PKS* genes followed by *Bacillus* sp. According to antiSMASH database, various *Staphylococcus* sp, were analysed for the presence of biosynthetic gene clusters but insight into their *PKS* clusters are limited. *Bacillus* sp. was previously analyzed for the presence of biosynthetic gene clusters (Marahiel *et al.,* 1999; Marahiel, 2001; Czajgucki *et al.,* 2006; Bechet *et al.,* 2012). Othoum *et al.* (2018) analyzed *B. paralicheniformis* from red sea for the presence of *NRPS* genes. Similarly, Bacillus symbiotic to sponges *C. flabellata* and *R. odorabile* were identified as having *NRPS/PKS* clusters (Brinkmann *et al.,* 2017).

Then, a non-parametric statistical analysis using Mann Whitney U test was done to know whether the presence of *NRPS/PKS* gene has any influence on

inhibitory zone diameter. The analysis showed that the presence of *NRPS/PKS* gene has statistically significant influence on the inhibitory zone diameter against 3 pathogens namely, *P. damselae*, *S.* Typhimurium ($p<0.1$) and β-lactamase producing *E. coli* ($p<0.05$). This shows the influence of *NRPS/PKS* biosynthetic gene clusters in the antimicrobial activity against these three pathogens. Additionally, the inhibitory zone diameter against these three indicator pathogens was statistically significantly higher in *NRPS/PKS* positive isolates than the negative isolates ($U = 57$, 66 and 60.0 respectively, $p = .070$, 0.058 and 0.030 respectively). However, no statistical significance between the inhibitory zone diameter against all other indicator pathogens against which antagonism were demonstrated and presence of *NRPS/ PKS* genes. Similar to our observation, Samak *et al.* (2018) reported that even though only 5 of the 50 isolates associated with the invertebrates screened by them showed antimicrobial activity in well diffusion method, around 60% of the total were positive for the presence of *NRPS/PKS* genes. Thus they gave two possible theories: one is that the presence of *NRPS/PKS* genes in symbionts which showed no antimicrobial activity may be suggestive of the involvement of these clusters in other activities like anti-tumour activity (El-Moneam *et al.,* 2017) and the second states the inability of these clusters to produce antibiotics due to inappropriate conditions like repression or lack of specific signal (Ayuso *et al.,* 2005; Dashti *et al.,* 2014). The absence of both genes in some antagonistic isolates may be explained by the fact that the involvement of a different gene cluster type or novel gene cluster other than *NRPS/PKS* in the synthesis of their antimicrobials. Thus, further studies on gene clusters other than *NRPS/PKS* are required to unravel the production of antimicrobials by these organisms.

Crab symbionts namely 16HL, 38HL, 39HL, 78HL and 87HL were selected randomly and their *NRPS* genes were subjected to cloning. The plasmid DNA containing the required insert was isolated from the cloned host and sequenced at Agrigenome, Kochi. The obtained sequences of both forward and reverse primers were then subjected to editing to remove the vector sequences

using Editseq (DNASTAR, Lasergene, USA). The 'A' domain size of various *NRPS* genes was identified. For 16HL, 38HL, 39HL and 78HL the size is 1.01 kb and for 87HL it is 1.007kb. These were then subjected to NCBI Blast and their closest relatives were recognized. *NRPS* of isolate 16HL (*B. albus*) showed 99.21% similarity with that of lichenysin synthetase A gene (*lchAA1*) of *Bacillus licheniformis* (LR134165.1). Lichenysins are lipopeptide surfactins (Grangemard *et al.,* 2001) recognized for their antimicrobial activities and other industrial applications (Rey *et al.,* 2004). Studies have recognized the role of several *Bacillus* sp. for the production of such compounds (Tapi *et al.,* 2009) but none have reported the same in *B. albus*. A similarity of 95.54% was shown by 38HL (*S. saprophyticus*) to the non-ribosomal peptide synthetase gene of *S. xylosus* (LN554884.1). *S. xylosus* identified as a common inhabitant of human skin and meat was studied for their ability to thrive on salted meat (Vermassen *et al.,* 2016). Besides, their antioxidant properties for flavor production was revealed by Talo *et al.,* 2002. Isolates 39HL (*S. sciuri*) and 78HL (*S. haemolyticus*) exhibited 97.51% and 97.72% similarity respectively to non-ribosomal peptide synthetase of *Bacillus subtilis* (EU399172.1). According to antiSMASH database, (Medema *et al.,* 2011) no *NRPS/PKS* clusters have been reported from *S. sciuri*, *S. saprophyticus* and *S. haemolyticus*. This suggests the involvement of novel *NRPS* similar to that of *B. subtilis* in 78HL and 39HL respectively. Isolate 87HL identified as *S. arlettae* was blasted to have 99.40% similarity to S. arlettae P2 DNA, surfactin synthase subunit 1 (AP019698.1). So this *NRPS* gene may be attributed to the synthesis of surfactin. All the closest relatives analyzed belonged to the Phylum *Firmicutes* and 2 families namely *Staphylococcaceae* and *Bacillaceae.*

As per the three dimensional organization of gramicidin synthetase GrsA (an antibiotic having action against multiple bacteria and fungi produced by Bacillus brevis) all known *NRPS* A domains can be aligned with binding pocket residues (Conti *et al.,* 1997) which represent the specificity-conferring code of 'A' domains. Therefore, the data can be used to predict the amino acid substrate

integrated into final non-ribosomal peptide (Bachmann and Ravel, 2009). For 16HL strain, the binding pocket may integrate a glutamine residue into a non-ribosomal peptide. The signature obtained for all other strains may integrate a serine residue. It is reported that there are only 171 known peptides that integrate a serine amino acid (Caboche *et al.,* 2008). Curiously, among the five *NRPS* sequences, two new 'A' domain signatures have been determined for Firmicutes, the bacterial phylum from which *NRPS* sequences are less reported (Bragina *et al.,* 2014; Muller *et al.,* 2015). Putative structural classes of antimicrobial products produced by the identified *NRPS* genes of the present study were also defined. Results showed that the sequence of 16HL showed 99% ID with Lichenycin, surface-active lipopeptides with antibiotic properties produced by *B. licheniformis*. Numerous *B. licheniformis* strains producing different types of lichenysins are commonly exploited as enzyme and drug producers in industry (Rey *et al.,* 2004), even though lichenycin class of antimicrobial product from *B. albus* have not been reported. Hence, the similar function of the detected *NRPS* genes of *B. albus* might be proposed. The low ID percentages of other four sequences (42–50%) could suggest their novelty or uncharacterized nature of antimicrobial products from these strains, which have to be targeted in future studies, which may find out new types of lipopetide antibiotics belonging to iturin family (having antimicrobial compounds with small peptide and long fatty moiety) (Tsuge *et al.,* 2005).

Similarly *PKS* genes of 4 bivalve symbionts 6b, 36b, 91b, 204b and 1 crab isolate namely, 55bHL were sequenced and the obtained sequences followed by editing were blasted to reveal their closest relatives. The greatest similarity (97.30%) to the *PKS* gene of 6b (*B. endophyticus*) was showed by Zinc-binding dehydrogenase gene of *Bacillus velezensis* strain WRN014 (CP041361.1). Similarly 36b (*S. epidermidis*) and 91b (*P. oleovarans*) revealed 98.36% and 97.55% identity respectively to the same. Gao *et al.,* 2017 identified the antifungal properties of *B. velezensis* and its ability to produce antibiotics. Even though *P. oleovarans* have been identified with *NRPS* clusters, the *PKS* clusters were not

identified within them (antiSMASH database). The highest similarity (98.68%) to 55bHL (*B. megaterium*) was revealed by polyketide synthase of *Bacillus velezensis* strain L-S60 (CP011278.1). The insilico prediction of KS domain of 55bHL revealed 40% identity to the biosynthetic gene pathway product jamicamide. The low identity suggests novelty or uncharacterized nature of antimicrobial products from the isolate.

In conclusion, we were able to reveal the potential of crab and bivalve symbionts as potent producers of many antibacterial compounds. The study pointed out that apart from their importance in food industry, they may be beneficial in drug discovery to combat pathogens especially aquatic pathogens. The *PKS/NRPS* biosynthetic gene analysis in these antagonistic symbionts recognized the biosynthetic potential of certain microbes that were previously attributed as pathogens. Further studies on these isolates and their genes along with heterologous expression of identified *NRPS/PKS* genes in suitable host may prove advantageous and allow the rapid isolation and mass production of novel antibiotics from them.

SUMMARY

6. SUMMARY

The present study entitled "Identification and analysis of antimicrobial biosynthetic genes in marine microbial symbionts" aimed to check whether the microbes associated with unexplored marine invertebrates have antimicrobial activity against human and aquatic pathogens including some MDR (Multi Drug Resistant) strains. Additionally, antimicrobial biosynthetic genes namely, *NRPS* (Non ribosomal peptide synthetase) and *PKS* (Polyketide synthase) associated with the positive isolates was isolated and analysed. Samples of unexplored marine bivalve namely, *P. viridis* and marine crabs like *C. feriatus*, *P. pelagicus*, *P. sanguinolentus* and *S. olivacea* were used for this study. Additionally, the study examined the density of the cultivable microbial symbionts in the targeted niche, which can be used in disease management studies during cultural practices of these selected marine animal species.

When the haemolymph of targeted marine animal species were analysed for the density of cultivable microbes, haemolymph of all species were found to carry bacteria even in apparently healthy conditions. Abundance was in the order of *C. feriatus* > *P. pelagicus*> *S. olivacea* >*P. viridis*>*P. sanguinolentus*; *P. pelagicus* > *S. olivacea* > *P. viridis*> *C. feriatus* > *P. sanguinolentus* and *P. viridis*> *C. feriatus* >*P. pelagicus*.> *P. sanguinolentus* >*S. olivacea* in ZMA, BHIA and TCBS respectively. Within *P. viridis*, the abundance was in the order of gut> mantle> muscle >gill>mantle fluid> haemolymph; gut> muscle > mantle > gill> haemolymph> mantle fluid and gut > muscle> mantle > gill> mantle fluid> haemolymph in ZMA, BHIA and TCBS respectively.

Morphologically unique colonies isolated were then purified and examined for antagonistic activity against 12 indicator pathogens including 2 MDR strains. There were a total of 89 and 34 morphologically unique colonies from *P. viridis* and various crabs respectively. Among these, 27 isolates (21.95%) showed zone of clearance around inoculation spot for at least one pathogen. Of these 10 were belonging to different crab species (29.41%) and 17 (19.1%) were belonging to *P.*

viridis. Based on activity score, isolate with code 136b was identified as the most promising bacteria from *P. viridis* (score 11), followed by 228b (score10). Among the crab isolates, 88HL was identified as the most promising bacteria (score 13), followed by 78 HL and 39 HL (score 8). Among the 12 indicators pathogens, *V. harveyi* was the most susceptible to inhibitory activity (64.71%), and followed by *V. anguillarum* (43.75 %) and *P. damselae* (37.5 %). The selected isolates were then identified using polyphasic taxonomy approach; 25 distinct bacterial species from 11 different genera belonging to 8 families and 3 major phyla could be identified.

Further, antagonistic isolates were screened for the presence of two biosynthetic genes namely, *NRPS* and *PKS*. Thirteen and six bacteria were found to be positive for *NRPS* and *PKS* gene respectively. Four isolates showed the presence of both genes indicating their hybrid nature. Genus *Staphylococcus* sp. showed the presence of maximum number of *NRPS/PKS* genes followed by *Bacillus* sp. A non-parametric statistical analysis using Mann Whitney U test was done which showed that the presence of *NRPS/PKS* gene has statistically significant influence on the total activity scores ($p<0.05$) and the scores was statistically significantly higher in *NRPS/PKS* positive isolates than the negative isolates.

Five and one amplicon of *NRPS* and *PKS* genes respectively were randomly selected, cloned and sequenced. Detailed *in-silico* analyses of these amplicons were conducted to predict their activity. It was found that for one isolate namely, 16HL the binding pocket may integrate a glutamine residue into a non-ribosomal peptide. The signature obtained for all other strains may integrate a serine residue. Curiously, among the five *NRPS* sequences, two new 'A' domain signatures have been determined for Firmicutes, the bacterial phylum from which *NRPS* sequences are less reported. Putative structural classes of antimicrobial products produced by the identified *NRPS* genes of the present study were also defined. Results showed that the sequence of 16HL showed 99% ID with Lichenycin, surface-active lipopeptides with antibiotic properties produced by *B. licheniformis*. Numerous *B. licheniformis* strains producing different types of

lichenysins are commonly exploited as enzyme and drug producers in industry, even though lichenycin class of antimicrobial product from *B. albus* have not been reported. The low ID percentages of other four sequences (42–50%) could suggest their novelty or uncharacterized nature of antimicrobial products from these strains, which have to be targeted in future studies, which may find out new types of lipopetide antibiotics belonging to iturin family.

In conclusion, we were able to reveal the potential of microbial associations of marine crab and bivalves as potent producers of many antibacterial compounds. They study pointed out that apart from their importance in food industry, they may be beneficial in drug discovery to combat pathogens especially aquatic pathogens. The *PKS/NRPS* biosynthetic gene analysis in these antagonistic symbionts recognized the biosynthetic potential of certain microbes that were previously attributed as pathogens. Also, the present study successfully generated *NRPS* gene sequences from four antagonistic bacterial species for which information of *NRPS* 'A' domain was not available earlier. Detailed sequence analysis revealed two new *NRPS* 'A' domain binding signature and detected 4 antagonistic microbes with previously uncharacterized NRPS products from the marine crab haemolymph associated bacterial collections. Further studies on these isolates and their genes and heterologous expression of identified *NRPS/PKS* genes may prove advantageous and allow the rapid isolation and mass production of novel antibiotics from them.

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APPENDICES

APPENDIX-I

MEDIA FOR BACTERIOLOGICAL STUDIES

ENRICHMENT MEDIA

1. Zobell Marine Agar

Ingredients	**g L^{-1}**
Peptic digest of animal tissue	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Sodium sulphate	3.240
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.000

2. Brain Heart Infusion Agar

Ingredients	**g L^{-1}**
Calf brain, infusion from	200.000
Beef heart, infusion from	250.000
Proteose peptone	10.000
Dextrose	2.000
Sodium chloride	5.000
Disodium phosphate	2.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

3. Muller Hinton Agar

Ingredients	**g L^{-1}**
Meat, infusion solids from 300g	2.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9±0.2
Final pH (at 25°C)	6.9±0.2

SELECTIVE MEDIA

4. TCBS Agar

Ingredients	**g L^{-1}**
Proteose peptone	10.00
Yeast extract	5.00
Sodium thiosulphate	10.00
Sodium citrate	10.00
Oxgall	10.00
Sucrose	20.00
Sodium chloride	10.00
Ferric citrate	1.00
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.00
Final pH (at 25°C)	8.6±0.2

5. MacConkey Agar

Ingredients	**g L^{-1}**
Peptones (meat and casein)	3.000
Pancreatic digest of gelatin	17.000
Lactose monohydrate	10.000
Bile salts	1.500
Sodium chloride	5.000
Crystal violet	0.001

Neutral red	0.030
Agar	13.500
pH after sterilization(at 25°C)	7.1±0.2

6. Mannitol Salt Agar

Ingredients	**g L^{-1}**
Proteose peptone	10
Beef Extract	1
Sodium chloride (NaCl)	75
D-Mannitol	10
Phenol red	0.025

7. Nutrient Agar

Ingredients	**g L^{-1}**
Beef extract	10.0
Peptone	10.0
Sodium chloride	5.0
Agar	20.0
Distilled water	1000
Final pH	7.2 ± 0.2

8. LB (Luria-Bertani) broth

Ingredients	**g L^{-1}**
Bacto-tryptone	10 g
Bacto Yeast extract	5 g
NaCl	10 g
Deionized Water	

APPENDIX-II

REAGENTS FOR BIOCHEMICAL TESTS

1. Trypton broth for Indole

Ingredients	**g L^{-1}**
Casein enzyme hydrolysate	10.000
Sodium chloride	5.000
Final pH (at 25°C)	7.5 ± 0.2

2. MR-VP Medium (Glucose Phosphate Broth)

Ingredients	**g L^{-1}**
Buffered peptone	7.000
Dextrose	5000
Dipotassium phosphate	5.000
Final pH (at 25°C)	6.9 ± 0.2

3. Simmon's Citrate Agar

Ingredients	**g L^{-1}**
Yeast extract	0.500
L-Cysteine hydrochloride	0.100
Sodium citrate	3.000
Dextrose	0.200
Monopotassium phosphate	1.000
Sodium chloride	5.000
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.9 ± 0.2

4. Oxidative fermentative (OF) test

Ingredients	**g L^{-1}**
Sodium chloride	5.0
Di-potassium phosphate	0.3
Peptone	2.0
Bromthymol blue	0.03
Agar	3.0
Glucose	10
Water	1000

3. Triple Sugar-Iron Agar Medium

Ingredients	**g L^{-1}**
Beef extract	3.000
Peptone	20.000
Yeast extract	3.000
Lactose	10.000
Sucrose	10.000
Dextrose monohydrate	1.000
Ferrous sulphate	0.200
Sodium chloride	5.000
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.00

4. Sugar Fermentation test

Ingredients	**g L^{-1}**
Proteose peptone	10.000
Sodium chloride	5.000
Meat extract	1.000
Bromocresol purple	0.100
Final pH (at 25°C)	6.8 ± 0.2

5. Arginine Dihydrolase Broth

Ingredients	g L^{-1}
Peptic digest of animal tissue	1.000
Sodium chloride	5.000
Dipotassium hydrogen phosphate	0.300
L-Arginine	10.000
Bromo cresol purple	0.016
Agar	3.000
Final pH (at 25°C)	6.0±0.2

6. Lysine Decarboxylase Broth

Ingredients	g L^{-1}
Peptic digest of animal tissue	5.000
Yeast extract	3.000
Dextrose	1.000
L-Lysine hydrochloride	5.000
Bromocresol purple	0.020
Final pH (at 25°C)	6.8 ± 0.2

7. Ornithine Decarboxylase Broth

Ingredients	g L^{-1}
L-Ornithine monohydrochloride	5.000
Yeast extract	3.000
Glucose	1.000
Bromo cresol purple	0.015
Final pH (at 25°C)	6.8 ± 0.2

8. Gelatin Agar

Ingredients	g L^{-1}
Gelatin	30.000
Casein enzymic hydrolysate	10.000
Sodium chloride	10.000
Agar	15.000
Final pH (at 25°C)	7.2 ± 0.2

9. Media for Esculin Hydrolysis test

Ingredients	g L^{-1}
Esculin	0.5

Ferric citrate	0.05
Sodium chloride	0.8
Dipotassium phosphate	0.04
Monopotassium phosphate	0.1
pH	5.6

10. Urea Agar Base (Christensen)

Ingredients	**g L^{-1}**
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000
Disodium phosphate	1.200
Monopotassium phosphate	0.800
Phenol red	0.012
Agar	15.000
Final pH (at 25°C)	6.8 ± 0.2

11. Normal Saline Solution (NSS)

Ingredients	**g L^{-1}**
Sodium chloride (NaCl)	8.5
Distilled water	1000

It was autoclaved at 15 lb pressure for 20 min.

12. Buffered Peptone Water

Ingredients	**g L^{-1}**
Protease peptone	10.0
Sodium chloride	5.0
Di-sodium phosphate	3.50
Mono-potassium phosphate	1.50
Distilled water	1000
Final pH	7.2 ± 0.2

APPENDIX III

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. TBE electrophoresis buffer(10x)

Tris base	121.1g
Boric acid	61.1g
EDTA	7.4g
Triple distilled water	1000 ml

2. Ethidium bromide stock solution (10 mg/ml)

Ethidium bromide	100 mg
Distilled water	10 ml

The solution was mixed and stored at 4°C. A concentration of 0.5-1 µg/ml was used in preparing agarose gel.

3. TE buffer

Tris-HCl (pH 8)	10 mM
EDTA (pH 8)	1 mM

Sterilize solution by autoclave.

4. 6X gel loading dye

Bromophenol blue	2.5 mg
Sucrose	4 g

Dissolved in 6 ml of TE buffer. 5X TBE was added to make the final volume up to 10 ml.

APPENDIX IV

REAGENTS FOR GENOMIC DNA ISOLATION

1. TE buffer

Tris-Cl (pH 8)	10 mM
EDTA (pH 8)	1 mM

Sterilize solution by autoclave.

2. 1 M Tris-Cl

Tris base	121.1 g
Triple distilled water add upto	1000 ml

Adjust the pH with conc. HCl.

3. 0.5 M EDTA (pH 8)

Disodium EDTA-2H2O	186.1 g
Triple distilled water add upto	1000 ml

Adjust the pH with NaOH.

4. 10% SDS stock (pH 7.2)

Sodium dodecyl sulphate	200 g
Triple distilled water add upto	1000 ml

Heat to 68°C and stir. Adjust the pH by adding few drops of conc. HCl

4. 5 M Sodium chloride

Sodium chloride	292.2 g
Triple distilled water add	1000 ml

5. 7.5 M ammonium acetate

Ammonium acetate	578.1 g

Triple distilled water was added up to 1000 ml. Sterilized by filtration

7. CTAB/NaCl solution (10% CTAB in 0.7 M NaCl)

Sodium chloride	4.1 g
Triple distilled water	80 ml

Sterilize by autoclaving.

Add CTAB	10 g

Heat to 65°C to dissolve. Adjust the volume to 100 ml.

8. Proteinase K (20 mg/ ml)

Proteinase K	20 mg
Sterile triple distilled water	1 ml

Store at $^{-}20^{0}$C.

APPENDIX V

REAGENTS FOR COMPETENT CELL PREPARATION

1. 0.1 M $CaCl_2$
2. 0.1M$CaCl_2$ – 15 % glycerol

APPENDIX VI

REAGENTS FOR CLONING

1. LB (Luria-Bertani) broth

Bacto tryptone	10 g
Bacto Yeast extract	5 g
NaCl	10 g
Deionized water	950 ml

Adjust pH to 7.4 with 1N NaOH. Volume made up to 1L and sterilized by autoclaving.

Store at 4°C.

2. LB agar

Add 2.5% agar in LB broth and sterilize by autoclaving

3. Ampicillin(100mg/ml)

Ampicillin powder	100 mg
Sterile distilled water	1 ml

4. X- Gal (20mg/ml)

20 mg X gal is dissolved in 1 ml dimethyl sulfoxide (DMSO)

5. IPTG(24mg/ml)

24 mg IPTG is dissolved in 1 ml sterile distilled water.

APPENDIX VII

REAGENTS FOR PLASMID ISOLATION (Alkaline lysis method)

1. Solution I (P1) Resuspension buffer

Tris-Cl (pH 8.0)	50 mM
EDTA (pH 8.0)	10 mM

The P1 solution was autoclaved for 15 min and store at 4°C.

2. Solution II (P2) Lysis buffer

NaOH	0.2 N
SDS	1.0%

The solution was prepared fresh and stored at room temperature.

3. Solution III (P3) Neutralization buffer

5 M Potassium acetate (pH 5.5)	60.0 ml
Glacial acetic acid	11.5 ml
Triple Distilled Water	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate and stored at 4°C.

ABSTRACT

IDENTIFICATION AND ANALYSIS OF ANTIMICROBIAL BIOSYNTHETIC GENES IN MARINE MICROBIAL SYMBIONTS

By

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Abstract of the Thesis

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ABSTRACT

Contagious spread of drug resistant strains as well as newer diseases necessitates the development of novel antibacterial agents which led scientists to explore new niches like marine habitat and associated marine microbes. Therefore, the present study aimed to check whether the microbes associated with unexplored marine invertebrates namely, *P. viridis*, *C. feriatus*, *P. pelagicus*, *P. sanguinolentus* and *S. olivacea* have antimicrobial activity against human and aquatic pathogens including some MDR (Multi Drug Resistant) strains. Additionally, antimicrobial biosynthetic genes namely, *NRPS* (Non ribosomal peptide synthetase) and *PKS* (Polyketide synthase) associated with the positive isolates was isolated and analyzed. Out of 123 isolates screened against 12 indicator pathogens, 27 isolates showed antibacterial activity and the isolates with maximum activities were identified as *Staphylococcus pasteuri* and *Pseudomonas aeruginosa*. When these isolates were screened for the presence of *NRPS* and *PKS* genes, 9, 2 and 4 of them showed the presence of *NRPS*, *PKS* and both *NRPS* and *PKS* genes respectively. A non-parametric statistical analysis using Mann Whitney U test showed that the presence of *NRPS/PKS* gene has statistically significant influence on the total activity scores ($p<0.05$) and the scores was statistically significantly higher in *NRPS/PKS* positive isolates than the negative isolates. Detailed *in-silico* analysis of these amplicons to predict their activity, revealed two new *NRPS* 'A' domain binding signature and detected 4 antagonistic microbes with previously uncharacterized *NRPS* products from the marine crab haemolymph associated bacterial collections. The present study successfully generated *NRPS* gene sequences from four antagonistic bacterial species for which information of *NRPS* 'A' domain was not available earlier. Altogether, the study supports the hypothesis stating that marine invertebrate bacterial associations can provide strains having future biotechnological and pharmaceutical applications in aquaculture industry.