ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM MARINE BACTERIA

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In

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By

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CERTIFICATE

This is to certify that the thesis entitled ***"Isolation and characterization of bioactive compounds from marine bacteria"*** submitted by **Mr. Suresh. T.,** I.D. No. **PFK - 413** in partial fulfillment of the requirements for the award of **Doctor Of Philosophy** in **Fishery Microbiology** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonfide research carried out by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship or fellowship and other similar titles.

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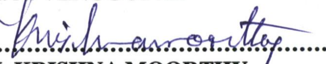

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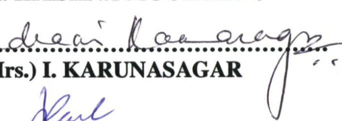

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Dedicated to

My Parents, Teachers

and

Beloved Friend

Harish

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Micro litre
µm	Micro metre
AMP	Antimicrobial property
APS	Ammoniumpersulphate
BLIS	bacteriocin-like inhibitory substance
CC	Column Chromatography
Cfu	Colony forming unit
CO2	Carbondioxide
D	Day
DAPG	2, 4- Diacetylphloroglucinol
G	Gram
H	Hour
HIV	Human Immunodeficiency Virus
HPLC	High Pressure Liquid Chromatography
IC_{50}	Inhibition concentration
IR	Infrared
kDa	Killo Dalton
Lt	Litre
MDR	Multiple Drug Resistant
Mg	Milligram
MIC	Minimum Inhibition Concentration
Min	Minute
mM	Milli moles
MRSA	Multiple resistant Staphylococcus aureus
MS	Mass Spectrometry
NCCS	National Centre for Cell Science
NMR	Nuclear Magnetic Resonance
^{o}C	Degree centigrade
PAGE	Poly Acrylamide Gel Electrophoresis
PCA	phenazine-1-carboxylic acid
Pg	Pico gram

Phl	2, 4-diacetylphloroglucinol
Plt	pyoluteorin
Prn	pyrrolnitrin
R_f	Relative factor
Rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
TEMED	N,N,N’,N’-tetramethylethylenediamine
TLC	Thin Layer Chromatography
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume

I. INTRODUCTION

Marine bacteria are normal biota of the marine ecosystems and are found in various environmental niches such as marine sediments, open oceans and other marine surfaces including marine living organisms (Wilson and Stevenson, 1980; Nair and Simidu, 1987; Austin, 1992). Over the past decade, microorganisms have been recognized as an important and untapped resource for many unique novel bioactive compounds of clinical significance (Rosenfeld and Zobell, 1947; Grein and Meyers, 1958). Majority of such compounds that have found wide applications are basically isolated from terrestrial sources. However, microorganisms isolated from various marine sources are known to synthesize unique metabolites that are totally different from the terrestrial counterparts. In recent years marine bacteria are becoming a major source for several novel biologically active compounds (Fenical, 1993 and 1997; Kelecom, 1999).

All organisms in nature compete with each other for their survival in their biological niches. This survival strategy is very well established in the case of microorganisms. Such survival mechanisms are achieved by the production of toxin, inhibitory enzymes and several antimicrobial agents that inhibit the growth of the other bacteria in their surrounding habitat. Such inhibitory compounds are generally secondary metabolites and are synthesized primarily for their survival against the other microorganisms (Rosenfeld and Zobell, 1947; Grein and Meyers, 1958). These secondary metabolites however exhibit several important properties including antibacterial, anticancer and antitumor properties. Some of these secondary metabolites are also the main source for many antibiotics. These antibiotics are chemical compounds that can inhibit the growth or kill the bacterial cell (Gillespie, 2002) exhibiting various modes of antibacterial action. Generally they interfere with biological processes of microorganisms such as replication, protein and cell wall synthesis (Gardener *et al.,* 2000). Thus marine bacteria represent certainly a great potential

reservoir for such scientific investigations and the reports on the antibiotics and other novel metabolites from marine sources are scarce (Ruiz-Ponte *et al.,* 1999).

Antibiotic production by marine bacteria has been reported in some of the early works (Rosenfeld *et al.*, 1947; Baam *et al.,* 1966). Many of the earlier reports on novel secondary metabolites were on isolation of low molecular weight antibiotic compounds from various marine bacteria (Faulkner, 2002). Terrestrial bacteria synthesize several antibiotic proteins known as bacteriocins and many of them are well characterized. Similarly marine bacteria are also excellent source of various antibiotic proteins and polypeptides such as the nisin and subtilin (Tagg *et al.,* 1976; Klaenhammer, 1988). Antibiotic proteins were also isolated from *Alteromonas* strains with a molecular mass of approximately 100 kDa (McCarthy *et al.,* 1994). Similarly an oligomeric protein with a mass of 190-kDa was isolated from an unidentified biofilm-forming marine bacterium D2.

One of the ways of discovering novel bioactive metabolites from marine microorganisms is through the isolation of new microorganisms. But the research over the years has demonstrated that only less than ten percent of the micro-organisms are cultivable and among them only about one percent have been found to have industrial and clinical importance (Hawksworth, 1991; Whitman *et al.,* 1998).

The development of resistance to drugs by pathogenic bacteria is a major concern in the field of medical science in recent days. The overuse of antibiotics has caused an increase of multiple drug resistant organisms mainly that belong to the genera *Pseudomonas*, *Acinetobacter*, *Streptococcus* and *Staphylococcus* (Breiman *et al.,* 1994; Goldman *et al.,* 1996 and Chitnis *et al.,* 2000). Microorganisms develop resistance to antibiotics because of mutations caused in their genome and by incorporating foreign genomic material like plasmids. Some of these strains are resistant to most used antibiotics, including methicillin, cephalosporins, and other beta-lactams that target peptidoglycan synthesis. Others have

gained resistance toward neomycin and streptomycin which attack the bacterial ribosome. Some of the strains of *Mycobacterium sp* that cause tuberculosis (TB) have been reported to be resistant to drug treatment. This is an event of great concern for the medical community since pathogenic organisms are becoming resistant to a large quantity of antibiotics. However the yield of novel metabolites is also decreasing and new sources of bioactive natural products must be investigated (Iwai and Takahashi, 1992). Isolation of new bacterial strains is being attempted using particular habitats and various techniques to screen for new bioactive compounds. Hence considerable research is necessary in order to find new chemotherapeutic agents from marine bacteria. Against this background the study was conducted to isolate microorganisms that produce antimicrobial compounds and to characterize the compound. The objectives of the present study are as follows.

1. Isolation and screening of marine bacteria for antimicrobial activity.
2. Extraction and purification of bioactive molecules produced by the bacteria with antimicrobial property.
3. Identification of bioactive molecules from crude extracts prepared from the marine organisms.

II. REVIEW OF LITERATURE

2.1. Antimicrobial producing marine bacteria

The Ocean is called the "mother of origin of life" and occupies more than seventy percent of the Earth's surface. Due to their depth they encompass about three hundred times the habitable volume of the terrestrial habitats on Earth. It includes life forms from the microscopic including Plankton and Phytoplankton to the huge Cetaceans like Whales exists in the Oceans. In the past, the Oceans has been considered as rich source of extremely potent compounds (Gochfeld *et al.*, 2003; Newman *et al.*, 2005) which represent a considerable number of drug candidates (Haefner, 2003). Although macro organisms of the Oceans have proved to be good sources of novel bioactive metabolites, large scale production of these bioactive metabolites has been difficult (Bernan *et al.,* 1997). Marine microorganisms such as bacteria and fungi have been reported to produce antibacterial (Rosenfeld and Zobell, 1947), antifungal, antiviral and antitumor substances (Bernan *et al.*, 1997). The biodiversity of marine microbes and the versatility of their bioactive metabolites have not been fully explored. Many studies have also suggested that such bacteria can also be used to combat epizootic in aquaculture systems (Maeda and Liao, 1992; Maeda, 1994; Douillet and Langdon 1994; Abraham *et al.,* 2001).

Due to the complex nature of the Oceans, marine bacteria have developed sophisticated physiological and biochemical systems with which they uniquely adapt to extreme habitats and various unfavorable marine environmental conditions. They live in a biologically competitive environment with unique conditions of pH, temperature, pressure, oxygen, light, nutrients and salinity, which is especially rich in Chlorine and Bromine elements. Microbes can sense, adapt and respond to their environment quickly and can compete for defense and survival by the generation of unique secondary metabolites. Even though these compounds are produced in response to stress, many have shown value in biotechnological

and pharmaceutical applications (Wenzel and Müller, 2005). The marine microbial metabolites exhibit unique biological activities compared with terrestrial bacteria (Blunt *et al.,* 2004; Berdy, 2005).

2.2. Ecological interactions of antimicrobial compounds producing marine bacteria

The knowledge of the distributions of various bacterial groups in the complex marine environment is essential for searching and developing a new chemical resource. However, the studies on the distributions of marine bacteria in the marine habitat are limited. Gram-negative bacteria, such as those of the common marine genus *Vibrio* spp., are found in abundance in seawater and they comprise approximately ninety percent of the marine bacterial flora. The Gram-positive forms a variety of taxonomic affiliations including representatives of the genus *Bacillus* spp. (Jensen *et al.,* 1996). The Actinomyctes or filamentous bacteria, which have been the single most important source of exciting metabolites from soil bacteria, are also members of the Gram-positive. Marine bacteria are found distributed in sediment, (Russell, 1892; 1893; Johnson *et al.,* 1968), animate (Lemos *et al.,* 1985; Burkholder *et al.,* 1966; Andersen *et al.,* 1974), inanimate surfaces (Davidson and Schumacher, 1993; Weyland and Helmke, 1988; Jensen *et al.,* 1996) and the internal spaces of invertebrate (Distel *et al.,* 1995). Bacteria have also been isolated from deep-sea mud and benthic organisms such as amphipods and sea cucumber in the bathypelagic zone (Kaye and Baross, 2004; DeLong and Yayanos, 1985).

2.3. Early studies of bioactive compounds from marine bacteria

In the past decades, many studies were carried out to understand the diversity of true marine bacteria in their natural marine environment (Jensen and Fenical, 1996). Marine bacteria are generally involved in the mineralization of organic matter, nutrient cycling, and energy transfer in aquatic environments (Azam and Worden, 2004). However, their potential to synthesize novel chemical compounds with antimicrobial properties was first recognized by Rosenfeld and ZoBel1 (1947) and Grein and Meyers (1958). The bactericidal property of seawater was observed during the same period of study which was subsequently realized that it was due to the production of antibiotics by Planktonic Algae and marine bacteria (Baam *et al*., 1966; Baslow, 1969) found abundantly distributed in the sea water.

Microorganisms, particularly bacteria have tremendously influenced the development of medical science. Since the discovery of Penicillin, intensive studies on marine bacteria have shown that they are a rich source of structurally unique, bioactive compounds. About fifty thousand natural products have been discovered from micro organisms in the past six decades. More than ten thousand of these are biologically active and about eight thousand are recognized as antibiotics and antitumor agents (Berdy, 1989; Stierle *et al.* 1993; Tomaz, 1995). Nearly hundred microbial products have now found clinical applications as antibiotics, antitumor agents and agrochemicals (Demain, 1983; DiMasi *et al.*, 1994; Clark, 1996; Cragg *et al.,* 1997).

Despite these early observations, relatively little attention was directed towards the study of natural products from marine bacteria. This was due to the difficulties encountered during the isolation and cultivation of marine bacteria. Only a small percentage of the viable bacterial cells in marine samples ultimately grow under standard culture conditions. However, in the recent decades more common and well known genera are being isolated

and cultivated under standard culture conditions. Not many studies about the diversity and distribution of marine bacteria have been conducted and only a small percentage has been explored. Hence only few marine bacteria have been the subject of comprehensive chemical analysis and most of the structurally unique metabolites of them have been discovered through fermentation studies (Oki and Yoshimoto, 1979; Clark, 1996; Cragg *et al.,* 1997). This was mainly to establish significant Pharmacological applications of the novel metabolites in order to replace failed antibiotics (Umezawa, 1972 and 1982).

2.4. Marine bacteria as a source of new novel metabolites

The difficulty and high cost of isolating novel structures and antimicrobial agents with new modes of action led to the phase of decline in this field of research. However, in the recent decades Chemistry of marine natural products has emerged as a mature field after years of intensive research. As a result studies on secondary metabolites from microorganisms are a rapidly growing field. Secondary metabolites are those chemical compounds in organisms that are not directly involved in the normal growth, development or reproduction of organisms. The function and importance of these compounds to the organism is usually of an ecological nature as they are used for defense against predators, parasites and diseases, for interspecies competition, and to facilitate the reproductive processes. Secondary metabolites also include several antibiotics. Typically primary metabolites are found across all species within broad phylogenetic groupings, and are produced using the same pathway or similar pathway in all these species.

However microorganisms have received very little attention in the field of drug discovery. This is mainly due to the non-cultivability of the majority of bacteria (Hugenholtz *et al.*, 1996). Several studies showed that the marine bacteria are capable of producing unusual bioactive compounds that are not isolated from terrestrial sources (Fenical *et al.,* 1993 and 1997).

Many unusual compounds like thermostable Proteases, Lipases, Esterases, Starch and Xylan degrading enzymes have been isolated mainly from bacterial and archael hyperthermophilic marine microorganisms (Bertoldo, 2002). Unusual gram-positive bacteria from deep- sea sediment, which produced a series of new natural products, Macrolectin A-F of C24 linear acetogen origin has been isolated (Gustafson *et al.,* 1989). The major metabolite, Macrolectin A inhibits B16-F10 Murine Melanoma cells in *In vitro* assays, showing significant inhibition of Mammalian Herpes Simplex Virus (type I and II) and protecting T Lymphocytes against Human Immuno-deficiency Virus (HIV) replication (Carte, 1996). In another study, a microbial metabolite from *Alteromonas* spp with anti-HIV potential as reverse transcriptase inhibitor has been reported. Production of variety of extra cellular proteases also been reported from some *Vibrio* species. *Vibrio alginolyticus* is known to produce six proteases including an unusual detergent resistant, alkaline serine exoprotease. This marine bacterium also produces collagenase, an enzyme with a variety of industrial and commercial applications, including the dispersion of cells in tissue culture studies (Fenical *et al.,* 1993).

2.4.1. Antibiotic producing bacteria derived from sea water

The bacteria in seawater are mainly Gram-negative rods belonging to various Taxonomic groups. Some of the first novel metabolites were obtained from marine bacteria that were isolated from seawater. The first marine bacterial metabolite to be reported was the highly brominated pyrrole antibiotic. This was isolated by Burkholder and co-workers through fermentation studies of a Gram-negative bacterium obtained from the surface of the Caribbean Sea grass Thalasia (Burkholder *et al.,* 1966). The highly unique metabolite was identified by X-ray crystallographic methods which were known to be composed of more than seventy percent Bromine by weight. The molecule showed impressive *in vitro* antibiotic properties against Gram-positive bacteria, with minimum

inhibitory concentrations (MICs) ranging from 0.0063 to 0.2 pg/ml. Although this bacterium was first assigned as *Pseudomonas bromoutilis,* the biochemical characteristics of this isolate confirmed it as *Alteromonas* sp (Skerman *et al.,* 1980). The compound synthesized by this bacterium was known to be pentabromopseudiline and its antitumor properties were also reported (Laatach *et al.,* 1989).

The Faulkner group in California isolated a purple-pigmented bacterium which also provided potent antibiotic. The strain originally defined as a *Chromobacterium* sp and later confirmed as *Alteromonas* sp was isolated from seawater samples collected in the North Pacific Ocean (Baumann *et al.,* 1981). Chemical analysis showed that this organism produces several antimicrobial compounds like pyrrole, tetrabromopyrrole, hexabromo-2, 2'-bipyrrole and several simple phenolics including 4-hydroxybenzaldehyde and n-propyl 4-hydroxybenzoate. Tetrabromopyrrole showed moderate antimicrobial activity *in vitro* against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans.* It was even more active against several groups of marine bacteria and showed auto toxicity against the producing *Chromobacterium* sp.

The same group later isolated an antibiotic producing bacterium from a La Jolla, CA, tide pool seawater sample (Wratten *et al.,* 1977). The yellow strain was identified as a *Pseudomonas* sp, and careful analysis of its metabolic products showed the production of 6-bromoindolecarboxaldehyde, its debromo analog, and a mixture of 2-n-pentyl and 2-n-heptylquinolinol.The 2-n-heptylquinolinol is a known antibiotic produced by strains of *Pseudomonas aeruginosa*. The most potent of these simple antibacterial agents was 2-n-pentylquinolinol, which showed its greatest activity against *Staphylococcus aureus.*

Another marine bacteria *Alteromonas rubra* was isolated and identified by researchers at the Roche Research Institute in Australia. This bacterium provided a novel new target for the isolation of unique natural products. Under fermentation study, the bacterium

produced a series of C16 aromatic acids, which are acetogenins of fatty acid synthetic origin. The acids showed interesting pharmacological properties in bronchodilator assays and in neuromuscular assays designed to detect relaxant effects. *P. magnesiorubra* isolated from the surfaces of the tropical marine green algae *Caulerpa peltata* also provided novel metabolite. Two antibiotic pigments, the magnesidins, were isolated as a 1: l mixture of methylene homologs (4-and 6-methylene groups) as minor metabolites of this marine bacterium (Gandhi *et al.,* 1976). These unique pigments were considered to be oxidation products of prodigiosin, a common tripyrrolic pigment produced by marine as well as terrestrial bacteria.

A structurally unprecedented lactone, oncorhyncolide was also produced by a seawater-derived bacterium obtained from samples taken near a Chinook salmon (*Oncorhyncus tshawytscha)* net-pen farm (Needham *et al.,* 1991). Oncorhynoclide is a unique lactone of unknown biosynthetic origin. Biosynthetic studies indicated that it could be of at least partial terpenoid origin, or derived by unique methylation reactions not yet observed in nature. The seawater-derived bacteria studied so far have been taxonomically very limited and seemingly driven mostly by screening processes for new antibiotics.

2.4.2. Bacteria derived from sediment

Sediments from sea, estuaries and bays are rich in organic compounds and vary greatly in organic content from place to place. In shallow coastal waters, including temperature and tropical oceans, geologically diverse sediments are found with different chemical characteristics. There are also diverse and dynamic regions such as Mangroves, Coral

reefs, Hydrothermal vents and deep- sea sediments which supports the growth of microbes (Knight *et al .,* 2003). Unlike terrestrial soil, marine sediments are generally not considered to be closely related microenvironments for several reasons. Like the soil, marine sediments are a more nutrient-rich microhabitat which varies greatly in organic content. Such microhabitat provides a diversity of bacterial flora not found in more nutrient limited habitats. The presence of free surfaces in these microhabitats is known to stimulate and support enhanced bacterial colonization and growth of chemically prolific marine bacteria.

The antimicrobial agent producers present in marine sediments are generally Gram-positive bacteria which include genus *Bacillus* and the *Actinomycetes* (Pandey *et al*., 2002) and Gram-negative bacteria mainly *Psuedomonas spp., Alteromonas spp.* The *Actinomycetes* group comprises the major sources of antimicrobial compounds. About ten to thirty three percent of the total bacterial community present in soil is comprised by these bacteria, being the genera *Streptomyces* sp and *Nocardia* sp the most abundant actinomycetes found in soil (Osborne *et al.,* 2000). They are a group of gram-positive bacteria that exhibit characteristics of both bacteria and fungi. These microbes produce filamentous structures forming pseudo-mycelia. These are also spore forming microbes, characteristic shared with fungi. Some of the characteristics that they share with bacteria are the formation and composition of the cell wall, the flagella and the ribosome.

The genus *Streptomyces* spp is responsible for the synthesis of the majority of antimicrobial agents with clinical importance (Roy *et al.,* 2006; Sasaki *et al.,* 2001a, 2001b, 2002; Igarashi, *et al.,* 2000, 2002; Furumai *et al.,* 2003). Some of the important antimicrobial compounds produced by *Streptomyces* spp are amphotericin, erythromycin, streptomycin, tetracycline, and rifamycin (Omura *et al.,* 2001). Inhibition of protein synthesis is the mode of action of all the previously mentioned antibiotics whereas the

amphotericin attacks the cell membrane. Also the majority of these antibiotics have a broad spectrum. These microbes exhibit a vast metabolic versatility. Hence they have many physiological cycles that produce intermediate molecules such as enzymes or secondary metabolites with antibacterial, antifungal and antiviral capabilities.

A slow-growing, Gram-positive bacteriumC-237 was isolated from deep-sea sediment sample obtained from along the California coast. Fermentation studies in a salt-based medium yielded a series of novel cytotoxic and antiviral Macrolides, the MacrolactinsA-F (Gustafson *et al.,* 1989). Under standard fermentation at atmospheric pressure, this bacterium produced the six macrolide and two open-chain hydroxy acids in varying amounts. Macrolactin A was produced as the major metabolite (ca. 4-8 mg/L) in most of the fermentations. The majority of the biological properties were due to Macrolactin A, which showed modest antibacterial activity, but was active against B16-FlO murine melanoma *in vitro* with IC_{50} values of 3.5 µg/ml. More importantly, Macrolactin A inhibited several viruses including *Herpes simplex* (IC_{50} = 5.0 µg/ml), and Human Immunodeficiency Virus, HIV, (IC_{50} = 10 µg/ml).

Another group of gram-positive bacteria, the genus *Bacillus* sp present in soil also have the ability to produce antimicrobial agents with clinical and agricultural significance (Ahern *et al.,* 2003; Bechard *et al.,* 1998; Bizani and Brandelli, 2002; Cladera-Olivera *et al.,* 2004; Oscariz, 2006; Paik *et al.,* 1997; Pattnaik, *et al.,* 2001).This genus is gram-positive and spore forming rods. The type of antimicrobial agent produced by this particular genus is dependent on its growth phase as it is known to interfere in their metabolite production. These microbes usually produce antimicrobial agents in various stages of their growth curve (De Vuyst *et al.,* 1996). This is demonstrated in the case of active growth of *Bacillus subtilis* 168. In case of *B. subtilis* 168 strain, known to produce non ribosomal oligo-peptides with antifungal and antimicrobial properties which include

surfactins, intrinsic and bacilysin (Oskay *et al.,* 2004; Thomashow *et al.,* 2000). This strain is also known to synthesize ribosomal antibiotics such as subalancin and subtilosin. This strain produces ribosomal peptides in its active growth phase while non-ribosomal peptides are synthesized when bacterial growth ceases (Tamehiro *et al.,* 2002).

It is also demonstrated that some of the members of *Bacillus* have plasmids arranged in multiple gene operons which encode for toxins that are being used as biocontrol agents in the recent years (Jaben *et al.,* 2004). This phenomenon is found in the case with *Bacillus thuringiensis. Bacillus thuringiensis* secrete certain toxins with insecticidal property. This chemical has found wide application and is now applied in the biocontrol of insects. In the case of *Bacillus thuringiensis* serovar israelensis (*Bti*) bacteria synthesize toxins and toxins of this particular strain is used for the biocontrol of black flies and mosquitoes .

Among the gram negative bacteria, *Pseudomonas sp*, *Alteromonas sp* and *Vibrios sp* are known to produce antimicrobial substances. Bioactive molecules produced by *Pseudomonas* sp have found wide application mainly in the agriculture sector.

Some of the cultivable microbes most commonly isolated from soil samples belong to the genera of *Bacillus, Streptomyces* and *Pseudomonas* (Belma *et al.,* 2002; Stabb *et al.,* 1994). The Actinomycetes in particular *Streptomyces* are responsible for the production of over 70% of the antibiotics that have been isolated and reported (Dairi *et al.,* 1999; Lo *et al.,* 2002.). The genus *Pseudomonas* is comprised of a gram-negative bacteria and is vastly involved in biological control of many plant pathogens.

Another group of gram-positive bacteria present in soil and responsible for the production of antimicrobial agents with clinical and agricultural importance is the genus *Bacillus*. This genera is characterized by being gram-positive, spore forming rods. It has been demonstrated that these microbes produce antimicrobial agents in various stages of their growth curve. For example, *B. subtilis* 168 can produce non ribosomal oligopeptides

with antifungal and antimicrobial properties such as surfactins, inturinics and bacilysin (Oskay, 2004; Thomashow *et al.,* 2000). Ribosomal antibiotics are also synthesized by this strain which include subalancin and subtilosin. Previous research has demonstrated that the growth phase interferes with the type of antimicrobial agent produced. For example, in active growth *Bacillus subtilis* 168 produces ribosomal peptides while non-ribosomal peptides are synthesized when bacterial growth ceases (Tamehiro *et al.,* 2002). It is also documented that some of the members of *Bacillus sp* have plasmids arranged in multiple gene operons which encode for toxins that are currently being used as biocontrol agents (Jaben *et al.,* 2004).

Bacillus is an interesting genus to be investigated for antimicrobial activity because *Bacillus sp* produces a large number of peptides with biological activities. Biological peptides such as cerecin, produced by *Bacillus cereus* Bc7, has a molecular weight of 3.94 kDa and inhibits a wide range of gram-positive bacteria (Oscariz, 1999). Tochicin, isolated from *Bacillus thuringiensis,* appears to be exclusively active against other *Bacillus thuringiensis* strains (Paik, 1997). Thuricin and entomocidus were also isolated from members of the genus Bacillus (Ahern, 2003; Cherif *et al.,* 2000 and 2001). Thuricin and entomocidus are active against some severe pathogenic and spoilage organisms, such as *Listeria monocytogenes, Streptococcus pyogenes,* and *Pseudomonas aeruginosa.*

Pseudomonas sp encompasses gram-negative rods that have the ability of producing antimicrobial agents in soil. Most of the biomolecules that they produce are of agricultural importance. The antibiotics pyoluteorin (Plt), pyrrolnitrin (Prn), phenazine-1-carboxylic acid (PCA), and 2, 4-diacetylphloroglucinol (Phl) are currently a major focus of research in biological control. Strains of *Pseudomonas sp* have been isolated from soils that exhibited suppressive effects against plant diseases such as Take all of wheat, Black root rot of

tobacco, *Fusarium* wilt of tomato and damping off of tomatoes caused by *Rhizoctonia solani* (Gardener *et al.,* 2000).

2.5. Separation of bioactive compounds

Marine organisms produce variety of bioactive secondary metabolites. These bioactive metabolites are chemically divided into amino acids, peptides, nucleosides, alkaloids, terpenoids, sterols, saponins, polycyclic ethers etc. Since the chemical nature of bioactive compounds of the complex mixture is unknown, it is not possible to follow any specific technique for the separation of the constituents of the complex mixture. However, a broad separation of the mixture can be achieved by fractionation with organic solvents.

The ethanolic or methanolic extracts of marine organisms exhibiting biological activities could be a mixture of several classes of compounds. In accordance with the diverse properties of the components of the bioactive fractions, two different procedures are generally followed. In the first step the fractions of low or medium polarity contain lipophilic organic compounds that can usually be separated by standard normal or reverse phase column chromatography and MPLC and finally HPLC to get the individual components.

The second method is based on high-polarity fractions contain the water-soluble organic compounds and cannot be treated as mentioned previously. A good procedure is to first get rid of the Sodium Chloride and other mineral salts present in large amounts in the water extract. This is easily achieved by retention of the organic components on a nonionic resin (i.e., CC with Amberlite XAD). The reunited organic material is then chromatographed on Sephadex, and the individual components of the active fraction are further separated by countercurrent chromatography (i.e., DCCC) and HPLC on the appropriate column packing (C18, amino, cyano, etc.).

The initial extraction of natural compounds from the sources is done with an adequate solvent system (usually methanol or acetone). The first step in the isolation of a bioactive compound from the main extract or broth usually consists of a sequential gradient partition with solvents such as chloroform, hexane, ethyl acetate, dichloromethane and carbon tetrachloride. The fractions so obtained contain compounds distributed according to their polarity. In the case of a bioactive extract, this process can be guided by the appropriate assay to localize the active component. Thus the water-soluble organic material is represented mainly by alkaloid salts, amino acids, polyhydroxysteroids, and saponin is found in the *n*-Butanol fraction. The Dichloromethane fraction affords compounds of medium polarity such as peptides and depsipeptides, while in the hexanes and Carbontetrachloride, only low polarity metabolites (hydrocarbons, fatty acids, acetogenins, terpenes, etc.) are found. However the growth of bacteria and fungi in the aqueous extract obtained from marine organisms or aqueous fraction of the ethanolic or methanolic extract, often degrades the active constituents.

The active extract at early stages is fractionated by solvent partitions which eliminates much of the weight of inactive material. However the active fractions thus obtained from these partitions are chemically complex in nature. The broad fractions, thus obtained, are further fractionated by column chromatography of several types (absorption on silica gel or alumina, ion change, partition, gel permeation) using a wide variety of solvent systems adapted to the polarity of the active fraction. Thus multiple chromatographies are necessary before the active fraction can be concentrated to a state of purity.

Other techniques, such as preparative thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC), counter-current distribution, electrophoresis, and fractional crystallization are required in the final phases of isolation of pure compounds. The processes involved in isolation of active principles are complicated by lack of

knowledge of the chemical nature of the active material. This makes the design of isolation procedure aimed at a particular chemical entity impossible in many cases. Besides, they require many modifications of the isolation procedures so that the activity can be concentrated in a single fraction, and a pure material or materials can be isolated. The presence of multiple active compounds, which are closely related and are extremely difficult to separate, also complicate the isolation procedures.

The separation of water soluble bioactive compounds from water extracts is difficult due to the presence of salts carried from the seawater. The presence of large amount of inorganic salts gives rise to false results in bioassays. It also interferes in all chromatographic separations including gel-filtration.

2.6. Isolation of pure bioactive compound

The preliminary procedure in the isolation of a natural compound from the main extract or broth is with an adequate solvent system. The ethanol or methanol extract is successively extracted with hexane, chloroform, ethyl acetate and then divided into water soluble and water insoluble fractions. Each of these fractions is then subjected to biological assay. If the separation is good the biological activity may concentrate in a particular fraction. Sometime the biological activity may be in more than one fraction.

The isolation of pure compound from hexane and chloroform soluble fraction is comparatively easier than from the water soluble fraction. The non-polar compounds that are extracted in hexane, benzene and chloroform are generally esters, ethers, hydrocarbons of terpenoids, sterols, fatty acids etc. as mentioned earlier. The mixture of these compounds is resolved by standard chromatographic techniques over SiO2, Al2O3, and HPLC etc.

2.7. Identification of antimicrobial compounds

Antimicrobial compounds can be identified by Nuclear Magnetic Resonance (NMR) Spectroscopy and Mass Spectrometry (MS), the latter being often used for the determination of Molecular mass. The active fraction is first obtained from gel filtration of the culture filtrate. When the individual components are isolated in a pure form, and then the efforts are focused on the disclosure of their structure. This is carried out by spectroscopic methods, mainly MS and NMR (1D and 2D), although chemical transformations and comparison to known products are classical ways to corroborate the spectroscopic results to obtain additional information. This is particularly important in oligomeric compounds (i.e., peptides, depsipeptides and glycosides) where selective hydrolysis may facilitate identification of the monomers (monosacharides, amino acids, etc.) or the position of sulfate groups on the sugar or a glycone. The usefulness of those spectra, i.e., the size and quality of the information obtained, is critically dependent on certain experimental details.

Although no general rules can be formulated, the MS of compounds found in the more polar fractions are subjected to soft ionization techniques such as CI, FAB, thermospray, and electrospray. Tandem MS-MS is particularly well suited for identification of fragments from molecules formed by several individual units such as peptides, depsipeptides, oligosacharides, and saponins. Similarly, the NMR of water-soluble compounds such as saponins, sugars, and other polyhydroxy compounds are frequently more informative if taken in pyridine-d5 than in other solvents (methanol, etc.).

2.8. Structural elucidation of bioactive compounds

The elucidation of the structure of a natural product with high biological activity is both stimulating and challenging. The first step in structure elucidation is to ascertain the skeleton of the molecule and then narrowed down by reference to phytochemical literature on related genera and species. Knowledge of biosynthesis of secondary metabolites is very

helpful in deducing the most logical substitution patterns once the basic structural nucleus is established. Spectral data, such as 1H NMR, 13C NMR, Infrared (IR), Ultraviolet (UV) and Mass spectra, are determined and compared with those reported for compound which may be related on the basis of chemical and biosynthetic reasoning. All the compounds isolated by activity-directed fractionation may not be novel and new. Some known compounds are often detected. The classical method of structure determination requires degradation of the molecule to establish the nucleus, and various transformation reactions combined with rigorous analysis of spectral data of the derivatives. X-Ray crystallographic studies are finally undertaken either on the compound itself or a heavy atom containing derivative to establish the structure and stereochemistry.

2.9. Antimicrobial resistance

The search for novel antimicrobial agents with clinical importance is significant since many clinical pathogens such as *Mycobacterium tuberculosis*, *Enterococcus spp.*, *Pseudomonas sp.*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* are developing resistance to routine used antimicrobials. Many researchers have identified bacteria in biological samples that resist all currently available antibiotic drugs. The emergence of antibiotic resistant bacteria can be a natural process and a human mediated one (Daley, 2002; Tarantino, 1999).

The misuse of antibiotics is one of the major factors that have caused development of antibiotic resistance. About 10,200 tons of antibiotics were used in the USA in 1996, of which approximately fifty percent was applied in veterinary medicine and as growth promoters (Swartz, 2000). In a study conducted by Chitnis *et al* (2000) it was determined that MDR strains spread through hospital effluents to the municipal sewage system, and that the MDR strains in a total of ten hospitals ranged from 0.58-49% of the total bacterial

communities isolated. Over twenty four percent of nosocomial infections are complicated by the intrinsic resistance of the MDR strains to many antibiotics.

In the agricultural industry the antibiotics are used for the treatment of animals and also to promote their growth. Various strains of *Enterococcus sp* have been recovered that are resistant to multiple antibiotics such as penicillin, macrolides and tetracycline specifically in Poultry culture. Over twenty four percent of nosocomial infections are complicated by the intrinsic resistance of this group of organisms to many antibiotics. Hence the discovery of the new metabolites to control the MDR strains is very much essential.

III. MATERIALS AND METHODS

3.1. Sampling site and sample collection

Water and sediment samples were collected from five sampling areas, Someshwar, Surathkal, Ullala, Nethravathy estuary and Mangrove region along the West Coast of India. The water samples were collected in sterile glass bottles and the sediment samples were collected into sterile plastic sachets and sealed. All samples were collected under aseptic conditions and were placed on ice until processing .These samples were processed within 2 h of collection.

3.2. Sample processing

3.2.1. Water samples

Water samples in water bottles were mixed thoroughly by shaking vigorously and appropriate dilutions were made with sterile physiological saline. 0.1ml of water samples were spread on the pre dried Tryptic Soy Agar (TSA) plates and incubated for 24h at room temperature. After incubation, colonies were enumerated and subjected to further screening of antimicrobial property (AMP) showing strains.

3.2.2. Sediment samples

Ten grams of sediment was carefully weighed using sterile butter paper after proper mixing of the sample. The sediment samples were transferred to 90ml of filter-sterilized sea water and mixed thoroughly by vigorous shaking. From this, a sub sample of 10 ml was taken and mixed thoroughly by using vortex mixer for 5 min. The vortexed sample was allowed to settle for 30 min. From this 1 ml of supernatant was taken, diluted appropriately and 0.1 ml of diluted sample was plated on pre dried TSA plates and the plates were incubated for 24 h at ambient temperature. After incubation, the colonies were enumerated and screened for AMP showing bacteria.

3.3. Isolation of bacteria with AMP by replica plating method

The water and sediment samples were plated on TSA plate and the plates with well isolated colonies were considered as master plates. These colonies were transferred from master plates to the plates with a lawn of sensitive test strain by replica plate method (Lechevalier and Corke, 1952). The gram positive bacterium *Staphylococcus aureus* and the gram negative *Vibrio harveyi* were used as indicator strains in the initial antimicrobial screening method. The bacterial lawns were prepared by mixing 25ml of molten TSA (44°C) with 30μL of a suspension of overnight culture of indicator strains, vortexed and poured on Petri plates. The plates were dried and the replica of the master plates was carefully stamped on these plates. The plates were incubated at 30^{0} C for 24 to 48h and observed for inhibition zone. The bacterial colonies on the master plates which produced clear inhibitory zone against the indicator strains were considered as bacteria with AMP. After the incubation period, the colonies with antimicrobial activity were isolated from the master plates and stored at -80°C for further studies.

3.4. Isolation of bacteria

Tryptone Soya Agar with 1% NaCl (TSAS)

Tryptone	1.7%
Soya peptone	0.3%
Sodium chloride	1.0%
K_2HPO_4	0.25%
Agar	1.5%
pH	7.3 ± 0.2

The ingredients were dissolved in 100 ml of distilled water, sterilized at 121°C for 15 min and poured in sterile Petri plates.

3.5. Bacterial identification

A series of biochemical tests were performed to identify the selected bacterial isolates (Mac Faddin, 1980). The bacterial identification was done according to the scheme for Gram positive bacteria (Le Chevallier *et al*., 1980) and the Gram negative bacteria (Bain and Shewan, 1968 and Le Chevallier *et al.,* 1980). The identification schemes are schematically represented in Fig.1, 2, 3 & 4.

3.5.1. Gram staining

Hucker's modification of gram staining procedure was followed. After staining, slides were observed under oil immersion objective to record the gram reaction of each isolate.

3.5.2. Motility test

16 to 18h young cultures of bacteria, grown in TSB containing 1 % NaCl were tested for motility by hanging drop technique using cavity slide.

Tryptone Soya broth with 1% NaCl (TSBS)

Tryptone	1.7%
Soya Soya peptone	0.3%
NaCl	1.0%
K_2HPO_4	0.25%

These ingredients were dissolved in 100 ml distilled water and by autoclaving at 121°C for 15 min.

3.5.3. Oxidation fermentation test (O/F test)

Hugh and Leifson's O/F medium was used to test whether the organism was fermentative, oxidative or inert. About 3 ml medium was poured into a set of two tubes and autoclaved. Organisms were stabbed into the butt and one of the tubes was overlaid with liquid paraffin. Fermentative organisms produced acid in both tubes and oxidative organisms produced acid in the tube that is not overlaid liquid paraffin. Inert organisms failed to produce acid and hence medium colour was unchanged.

Hugh Leifson O/F medium

Soya peptone	0.2%
Yeast extract	0.5%
Sodium chloride	1.0%
Glucose	1.0%
Bromocresol purple	0.0015%
Agar	0.3% - 0.4%
pH	7.2 ± 0.1

The ingredients were added to 100 ml of distilled water and boiled to dissolve the agar. The medium was then distributed in 2.5 ml volume in test tubes and sterilized at 110°C for 15 min.

Liquid paraffin

This was sterilized at 160-180°C for $1^1/_2$ - 2 h in a hot air oven. Liquid paraffin was used to create anaerobic environment in the fermentative tubes.

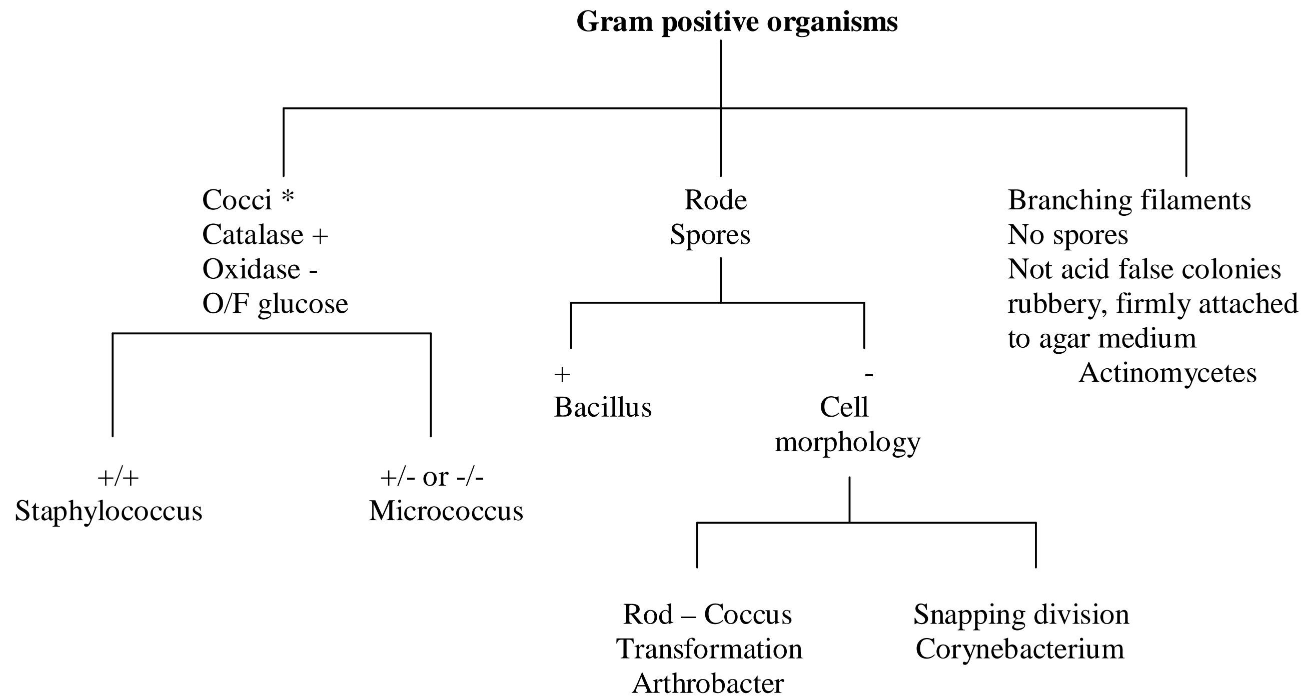


Fig. 1. Flow chart for identification of Gram positive bacteria
* Excludes catalase negative streptococci
Source : Lechevallier *et al*. (1980)

Gram negative organisms
O/F Glucose
+/+
(Fermentative)

Oxidase test

\+ : Sensitivity to 0/129

\- : Enterobacteriacease [C]

Sensitivity to 0/129: + Vibrio; - Aeromonas; - Plesiomonas

	Vibrio	Aeromonas	Plesiomonas
Arginine	-	+	+
Lisine	+	-	+
Ornithine	+	-	+

	Escherichia	Klebsiella	Enterobacter	Salmonella	Hafnia	Serratia	Ptoteus
Catalase	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Motility	+	-	+	+	+	+	+
Indole	+	-	-	-	-	D	D
M.R.	+	-	-	+	-	D	D
V.P.	-	-	+	-	D	+	-
Citrate	-	+	+	+	+	+	D
Urease	-	+	D	-	-	-	+

Fig. 2. Flow chart for identification of Gram Negative bacteria. *(Note: D. Some strains positive and some negative)*
Source: *Carpenter (1966) (C) Cruickshank *et al.* (1975)

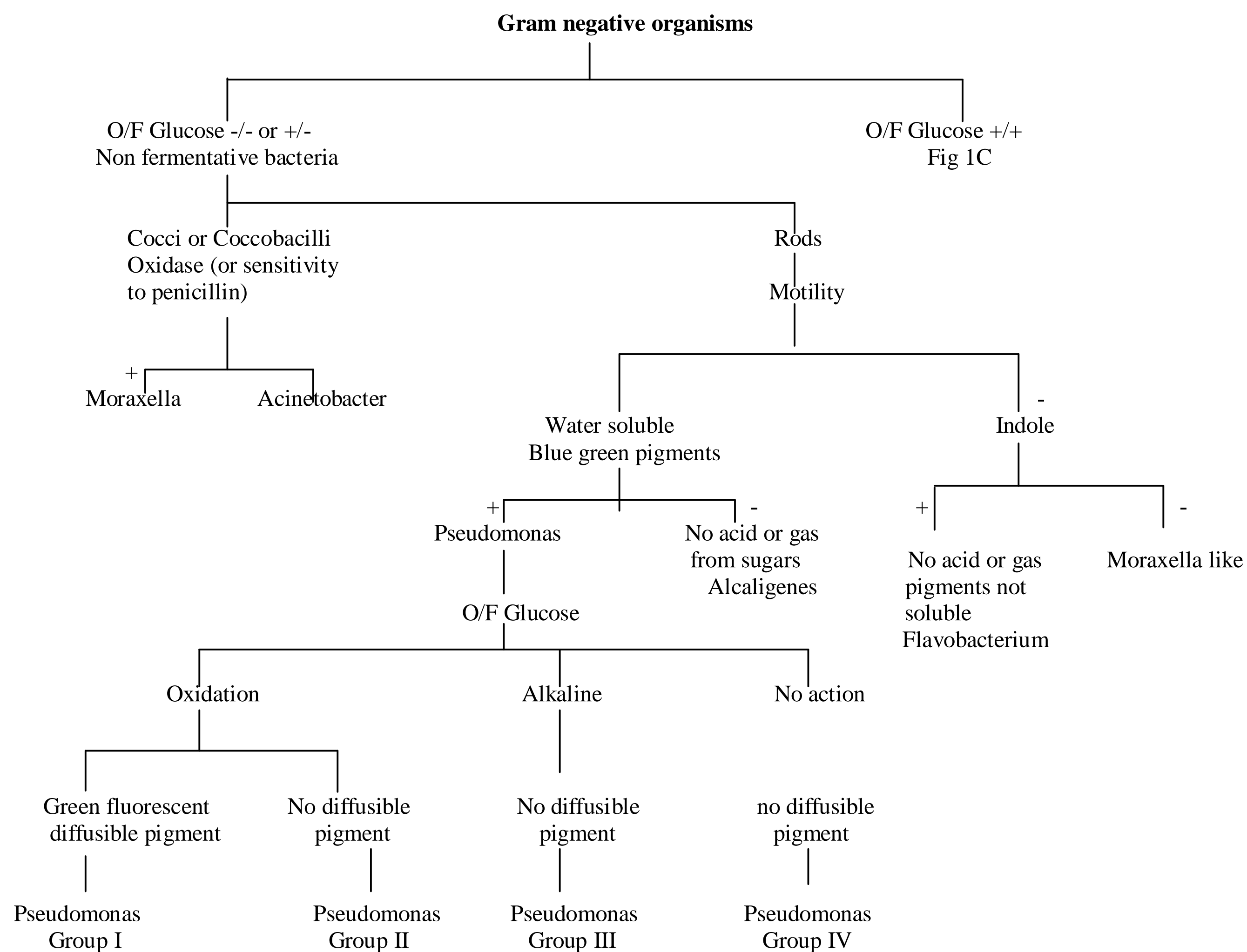


Fig 3. Flow chart for identification of *Pseudomonas* spp. Source : Modified from Bain and Shewan, 1968 and Le chavellier *et al.,* 1980.

	Catalase	V-P reaction	Growth in anaerobic agar	Growth at 50°C	Growth in 7% NaCl	Acid and gas in glucose	NO_3 reduced to NO_2	Starch hydrolyzed	Growth at 65°C	Rods 1.0µm wide or wider	pH in V-P medium <6.0	Acid from glucose	Hydrolysis of casein	Parasporal bodies
B. megaterium	+	-	-	-	+	-	V	+	-	+	V	+	+	-
B. cereus	+	+	+	-	+	-	+	+	-	+	+	+	+	V
B. thuringiensis	+	+	+	-	+	-	+	+	-	+	+	+	+	-
B. licheniformis	+	+	+	+	+	-	+	+	-	-	V	+	+	-
B. subtilis	+	+	-	+	+	-	+	+	-	-	V	+	+	-
B. pumilus	+	+	-	+	+	-	-	-	-	-	+	+	+	-
B. firmus	+	-	-	-	+	-	+	+	-	-	-	+	+	-
B. coagulans	+	+	+	+	-	-	V	+	-	V	+	+	V	-
B. polymyxa	+	+	+	-	-	+	+	+	-	-	V	+	+	-
B. macerans	+	-	+	+	-	+	+	+	-	-	-	+	-	-
B. circulans	+	-	V	+	V	-	V	+	-	-	V	+	V	-
B. stearothermophilus	V	-	-	+	-	-	V	+	+	V	+	+	-	-
B. alvei	+	+	+	-	-	-	-	+	-	V	+	+	+	-
B. laterosporus	+	-	+	+	-	-	+	-	-	-	-	+	+	-
B. brevis	+	-	-	+	-	-	V	-	-	-	-	+	+	-
B. larvae	-	-	+	-	+	-	V	-	-	-	-	+	+	-
B. popilliae	-	-	+	-	+	-	-	-	-	-	-	+	-	-
B. lentumorbus	-	-	+	-	-	-	-	-	-	-	-	+	-	-
B. sphaericus	+	-	-	-	V	-	-	-	-	V	-	-	V	-

Fig. 4. Identification of *Bacillus sp*. Source: Gordon, Haynes, and Pang (1973). Note: V, variable character

3.5.4. Sensitivity to O/129 compound

A lawn culture of test strain was prepared on TSAS and O/129 disc (150 µg) was placed at the centre of the lawn. Development of a clear zone of inhibition around the disc indicated that the strain is sensitive and growth around the disc indicated resistance to this compound.

O/129 reagent

O/129 (2-4,Diamino 6-7 Di isopropyl pteridine)	15 mg
Acetone	1 ml

O/129 compound was dissolved in acetone and 100 discs of 6.5 mm diameter each (pre-sterilized at 140°C for 1h) were soaked in the solution, dried gently to evaporate the acetone and stored in a dark bottle at 4°C. Each disc contained 150 µg of the pteridine compound.

3.5.6. Oxidase test

Cytochrome oxidase test was performed using moistened filter paper strips soaked with 1% oxidase reagent. Young colonies of bacteria were spotted on the oxidase paper using sterile toothpicks. Development of dark purple colour within 10 sec indicated positive reaction.

Oxidase reagent

Oxidase reagent (N, N, N', N' – tetramethyl)

p-phenylene diamine dihydrochloride	1.0 g
Distilled water	100 ml

Whatman filter paper No.1 was cut into strips of 2.5 x 1.0 cm, sterilized in hot air oven at 140°C for 1h. The strips were later dipped in oxidase reagent, allowed to absorb and then dried. The strips were stored in dark bottle at 4°C.

3.5.7. Amino acid decarboxylase test

Ability of the microorganism to decarboxylate aminoacids lysine, ornithine and arginine was tested by inoculating test cultures into media containing amino acids in separate tubes and to a tube with only basal medium which served as a control. After overlaying with liquid paraffin, all the tubes were incubated at 30°C. The change in the colour of the indicator from purple to yellow and back to purple indicated that the strains were positive for decarboxylation.

Basal medium for amino acid decarboxylase test

Soya peptone	0.5%
Yeast extract	0.3%
Sodium chloride	1.0%
Glucose	0.1%
Bromocresol purple	0.0016%
pH	7.2 ± 0.2

The basal medium was divided into four parts and aminoacid lysine, ornithine and arginine were added individually to each quarter at a concentration of 0.5, 0.5 and 0.4% respectively. The last quarter served as control. The media was distributed into tubes, in 4 ml volumes and sterilized at 110°C for 15 min.

3.5.8. Sugar fermentation test

Different sugars viz. glucose, sucrose, arabinose, mannose and *m*-inositol were used as substrates to test the ability of the bacterial cultures to utilize them with resultant production of acid. Cultures were inoculated into pre-sterilized individual sugar media in tubes and colour change from purple to yellow indicated fermentation of the sugar.

Sugar fermentation medium

Soya peptone	1.0%
Sodium chloride	1.0%
Sugars	1.0%
Bromocresol purple	0.0016%
pH	7.1 ± 0.2

The ingredients were dissolved in distilled water and then dispensed in 4 ml volume into test tubes containing Durham's tubes and sterilized by autoclaving at 110°C for 10 min.

3.5.9. Indole test

The cultures were grown in Tryptophan broth for 24-48 h and then a few drops of Kovac's reagent were added. Formation of a pink indole ring at the surface of culture was recorded as a positive reaction.

Tryptone broth

Tryptone	1.0 %
Sodium chloride	1.0 %
pH	7.1 ± 0.2

This was distributed in 5 ml volumes into test tubes and autoclaved at 121°C for 15 min.

Kovac's reagent

p-Dimethyl aminobenzaldehyde (DMAB)	5.0 g
Amyl alcohol	75 ml
Conc. Hydrochloric acid	25 ml

DMAB was dissolved in amyl alcohol and then the Conc. HCl was added slowly. The solution was stored at 4°C in a dark bottle and used to perform the test whenever required.

3.5.10. Salt tolerance test

Test cultures were inoculated into Tryptone broth containing 0%, 3%, 8% and 11% NaCl and incubated at room temperature for 18 – 24 h. Turbidity in the tubes was considered positive.

Tryptone salt medium

Tryptone	10 g
NaCl	0% or 3% or 8% or 11%
Distilled water	1000 ml
pH	7.2 + 0.2

This medium was distributed in 5 ml volumes into test tubes and autoclaved at 121°C for 15 min.

3.5.11. Voges Proskauer (VP) test

Test cultures were inoculated into Methyl red- Voges Proskauer (MR-VP) broth and incubated for 48 h at 37°C. To 1 ml of the culture, 0.6 ml of α-Naphthol and 0.2 ml of

KOH reagent were added and mixed by shaking. Development of port wine colour indicated a positive reaction. VP negative isolates were proceeded with.

MR-VP broth (Hi Media, Mumbai)

Glucose	5.0 g
Peptone	7.0 g
K_2HPO_4	5.0 g
NaCl	5.0 g
Distilled water	1000 ml

The medium was dispensed in 5 ml volumes into tubes and autoclaved at 121°C for 15 min.

Voges - Proskauer reagent

Solution A

α- Naphthol	50.0 g
Absolute alcohol	1000 ml

Solution B

KOH	40.0 g
Distilled water	100 ml

3.5.12. Citrate utilization test

Ability of bacteria to use citrate as the sole source of carbon for metabolism was tested by growing the cultures in Simmon's citrate agar slants. Change of colour from green to Prussian blue colour and growth of bacteria was recorded as a positive reaction.

Simmon's citrate agar

Sodium chloride	1.0%
Magnesium sulphate	0.02%
Ammonium dihydrogen phosphate	0.1%
Potassium dihydrogen phosphate	0.1%
Sodium citrate	0.2%
Bromothymol blue	0.008%
Agar	2.0%
pH	6.8

The medium was boiled to dissolve, distributed into tubes in 3 ml volumes and sterilized at 121 ºC for 15 min. Slants were prepared after it was autoclaved.

3.5.13. Urease test

Cultures were grown on urea slants to see their ability to break down urea. The reaction was recorded as positive if the slants changed colour from yellow to pink after incubation with the culture.

Basal medium

Peptone	0.1%
Dipotassium hydrogen phosphate	0.2%
Glucose 0.1%	
Phenol red	0.0012%
Agar	1.5%
pH	6.9

Autoclave the basal medium at 110 ºC for 15 min. To 90 ml of this molten medium 10ml of 20% filter sterilized urea solution was added, distributed in 4 ml volume in to sterile tubes and slants prepared.

Phosphate buffered saline (Sambrook *et al.,* 1989)

Composition	**Grams/Litre**
NaCl	8.0
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

The above ingredients were dissolved in 800 ml distilled water and pH was adjusted to 7.4 with 1N HCl and the volume was made up to one liter and sterilized by autoclaving for 20 min at 15 lb/inch^2 and stored at room temperature.

Phosphate buffer (pH 7.5)

Composition	**Grams/Litre**
Monopotassium phosphate	136.09
Disodium phosphate	141.96

These ingredients were weighed and dissolved in 1000 ml distilled water, adjust pH 7.5 and sterilize by autoclaving at 121°C for 15 min.

3.6. Optimization for the production of antibacterial metabolite

The optimal medium and conditions required for the growth and production of secondary metabolite production was investigated. Media composition was modified in order to establish the optimal conditions for production of the active secondary metabolites. Different growth parameters such as NaCl salt concentration (1, 2, 3 and 4%),

temperature (30^0C and 37^0C) and pH (2.0 to 12.0) were varied to study the influence on the production of secondary metabolites. The samples were centrifuged and filtered using Millipore filters (0.2µm) and the amount of antimicrobial compound produced at various growth conditions were measured by antimicrobial assay by agar well diffusion method.

3.7. Extraction of antimicrobial compound from AMP isolates

The bacterial species were propagated in 1000ml Tryptone Soya broth supplemented with 1% NaCl (TSBS). The flasks containing 1000ml of medium were inoculated with 5ml of microbial culture and the flasks were incubated at 30^0C at 150 rpm, for 72h. The antimicrobial compound was extracted from a cell-free solution which was centrifuged at 10,000 rpm for 20 min at 4^0C.

3.8. Concentration of the antimicrobial compound

Many protocols have been described for the concentration of antimicrobial compound. Most commonly used methods are ammonium sulfate precipitation, absorption-desorption, and organic solvent extraction (Fig 5).

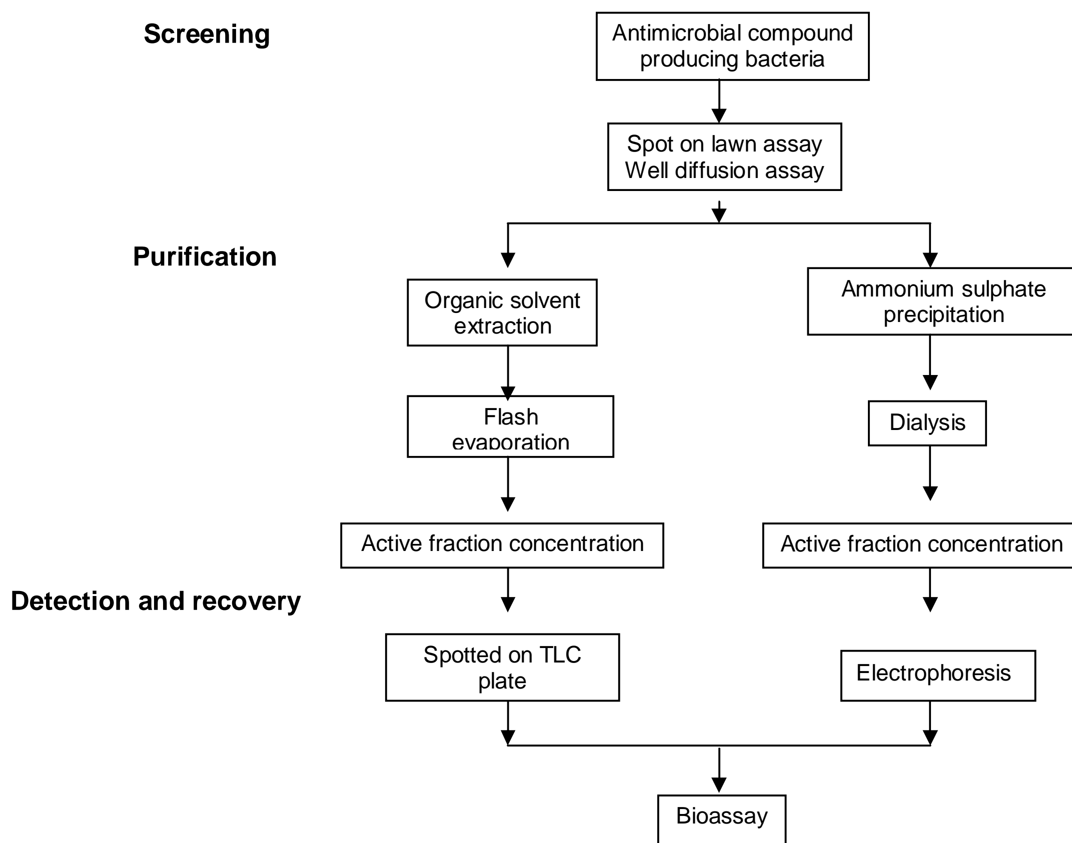


Fig 5. Protocol for the extraction and concentration of antimicrobial compounds

3.8.1. Ammonium Sulphate Precipitation

The antimicrobial compound with protein nature can be concentrated through the application of salting-out methods, the ammonium sulfate being the most frequently used. In this procedure the solid salt is added to the sample slowly until the desired saturation percentage of ammonium sulphate is reached. An 18h old culture of the SM 5 strain was centrifuged (8000×g, 10 min, 4ºC) and the cell free supernatant was used for the precipitation method. The peptide fraction from the cell-free supernatant was precipitated by gradient precipitation method using 20-70% saturated ammonium sulphate as some antimicrobial compounds can precipitate at lower ammonium sulphate concentrations. The suspension was incubated overnight at 4°C and agitated with a magnetic stirrer. Salted-out

proteins were precipitated by centrifugation (10000×g for 20 min) and dissolved in a small volume of 10 mM phosphate buffer (pH 7.0).

The suspension was desalted by dialysis with phosphate buffer at 4°C during 12h by using benzoylated membranes (molecular weight cut off 1200 Da; Sigma-Aldrich). Dialysis was carried out for 48h in a tubular cellulose membrane against and the buffer change was done at every one hour. Upon dialysis the residual sample was scraped off from the sides of the dialysis bag and stored at -20 ºC until further use. A well diffusion assay procedure was carried out to the protein precipitate obtained by this method.

3.9. Protein estimation

The protein content was also measured by the method of Lowry *et al* (1951). The procedure is based on quantitating the color obtained from the reaction of Folin Ciocalteau phenol reagent with tyrosyl residues of unknown protein and comparing this with a standard protein, usually bovine serum albumin (BSA). Five standard BSA (1mg/ml in distilled water) samples were prepared in 0.1N Sodium Hydroxide to give concentrations of 20, 40, 60, 80 and 100µg / ml, respectively, in glass tubes in duplicates. Test samples were taken in 2 different dilutions of 20µg and 40µg along with neat. Equal volume of alkaline copper reagent was added to all tubes and the mixture was incubated for 15 min at room temperature. Folin's reagent (Sigma, USA) was added at a concentration of 50µl/ml. The entire mixture was mixed well and incubated for another 30min at room temperature. Optical density was measured at 690nm. The protein concentration of the sample was obtained from the graph plotted for the standard BSA concentrations as optical density values.

Copper sulphate (1%) solution

Copper sulphate	0.1 g
Distilled water	50 ml

Sodium potassium tartarate (2%) solution

Sodium potassium tartarate	1 g
Distilled water	50 ml

Sodium carbonate (4%) solution

Sodium carbonate	2 g
Distilled water	50 ml

Sodium hydroxide (0.1 N) solution

Sodium hydroxide	0.2 g
Distilled water	50 ml

Alkaline copper reagent (ACR)

Copper sulphate (1%)	1 ml
Sodium potassium tartarate (2%)	1 ml
Sodium carbonate (4%)	48 ml

Bovine serum albumin (1%)

Bovine serum albumin	0.01 g
Distilled water	1 ml

3.10. Antimicrobial assay

3.10.1. Spot-on-lawn method

In this method agar culture plates were pre inoculated with overnight culture of the test strain. A volume of 20µl of each supernatant (or extract) of 16h cultures of AMP isolates were spotted onto this lawn and plates incubated in upright position. The inhibition zone was evaluated after overnight incubation.

3.10.2. Well diffusion assay method

The agar culture media was inoculated with the test strain (top agar) and plated over the agar base. Wells were punched with a sterile well borer and inoculated either with the cell supernatants or purified extracts. About 20µl of the samples to be assayed were pippeted into the wells and incubated in upright position and the inhibition zone was measured after overnight incubation.

3.11. Cell assay

Reagents

All the reagents used in this study were of analytical grade. Culture medium, protein reagent and neutral red reagent were purchased from Sigma Chemical Company

Cell Cultures

HeLa cells were obtained from the National Centre for Cell Science (NCCS), Pune. The cells were maintained as a monolayer and routinely passaged in Eagle's minimum essential medium supplemented with L- glutamine (0.292 g/ml) and 10% heat-inactivated Fetal bovine serum (Sigma). The cells were grown at 37^0C in an atmosphere of 5% CO_2. Three replicate cultures were used for each treatment per experiment.

Evaluation of growth inhibition

To determine the effects of the dialyzed crude protein on cell growth, the cells were seeded at 5 x 10^5 cells per well in the 25cm^2 tissue culture flasks with 5ml of the medium. One day after incubation, the medium was exchanged with fresh medium containing the crude protein with a concentration of 6.58mg/ml and the cells were incubated for 24h. For the control experiment, the cells were incubated in the fresh medium with phosphate buffer (without the test compound) in the same manner as described above. After 24h of incubation, the number of viable cells was counted by the tryphan blue exclusion test using a haemocytometer and the growth-inhibitory ratio, Y was calculated using the equation,

$$Y (\%) = (C\text{-}T)/C\text{-}Co) \times 100$$

Where T is cell count for each chemical dose after 24h incubation, C is the cell count for the control after 24h incubation, and Co is the cell count at the start of antimicrobial compound treatment.

3.12. Characterization of antimicrobial compounds

The preliminary characterization of the dialyzed protein compound was carried out to investigate the effects of heat, pH and proteolytic enzymes on the compound. A sample volume of 600µl was used in the characterization study mentioned earlier. The tested compounds were assayed by well diffusion method against the test strains *Staphylococcus aureus* and *Vibrio harveyi.*

3.12.1. Effect of temperature on the antimicrobial compounds

The compound was subjected to heat treatment from 40 to 100^0C. Aliquot volumes of each fraction were then removed after 0, 30, 60 or 90min. The heat treated samples were assayed for antimicrobial activity by well diffusion assay.

3.12.2. Effect of pH on antimicrobial activity

The samples were adjusted to various pH levels 2.0 to 12.0 with either 1N HCl or 1N NaOH. The sterile media without culture adjusted with pH was used as control. The samples were then tested for antimicrobial activity.

3.12.3. Effect of enzymes on antimicrobial compound

The crude dialyzed compound was digested with 100mg/ml of trypsin (sigma) and 50mg/ml pepsin (Hi Media) for about 1h at 37^0C. Samples was then boiled for 2min to inactivate the enzyme reaction. After the enzymatic treatments, the samples were tested for antimicrobial activity.

3.13. Partial purification of Antimicrobial protein by TLC method

The PBS extract was spotted on TLC silica gel and the chromatogram was developed in different mobile phase in which n- butanol/acetic acid/water (60:15:25) solvent v/v showed better resolution. The developed TLC plate sprayed with 1% Ninhydrin in Acetone to locate the chromatogram. The bands were scraped from the TLC plate dissolved in PBS vortexed and centrifuged. The silica gel free supernatant was collected in sterile Eppendorf tubes for bioassay.

3.14. Glycine-SDS-PAGE

Glycine-SDS-PAGE (Laemmli, 1970) was used to analyze the crude dialyzed compound. The various buffers and reagents used are as follows:

A. Acrylamide-bisacrylamide mixture

Add 30 g of acrylamide and 0.8 g of bisacrylamide (N, N'-methylene bisacrylamide) in 80 ml of distilled water. After dissolving completely, volume was made up to 100ml and stored at 4°C.

B. Separating gel buffer (1.5 M Tris-Cl, pH-8.8)

181.7g of Tris base was dissolved in 800ml of distilled water and pH was adjusted using concentrated HCl. Then final volume was made up to 1 litre with distilled water and stored at 4°C.

C. Stacking gel buffer (1 M Tris-HCl, pH-6.8)

121.1g of Tris base was dissolved in 800ml of distilled water and pH was adjusted using concentrated HCl. Then final volume was made up to 1 litre with distilled water and stored at 4°C.

D. 10 % SDS solution

10g of SDS was dissolved in 100ml of distilled water and stored at room temperature.

E. 10% APS solution

1g of APS was dissolved in 10ml of the distilled water and stored at 4°C temperature. Ammonium persulfate decomposes slowly so fresh solution was prepared weekly.

F. N,N,N',N'-tetramethylethylenediamine (TEMED)

Electrophoresis grade TEMED (Bangalore Genie, Bangalore) stored at 4°C was used.

G. Electrode buffer (5× solution)

5× stock solution was prepared by dissolving 15.1g of Tris base (Himedia, Mumbai), 94g of glycine (Himedia, Mumbai) in 900ml distilled water and then 50ml of 10% SDS solution of electrophoresis grade was added and the final volume was completed to 1 litre with distilled water and stored at room temperature. 1× buffer solution was prepared from the stock solution and used for electrophoresis.

H. SDS gel loading buffer (2×)

2× gel loading buffer contained 100 mM Tris-Cl (pH 6.8); 4% (w/v) SDS; 20% (v/v) glycerol; 0.1% (w/v) bromophenol blue (Himedia, Mumbai); 200 mM β-mercaptoethanol (Himedia, Mumbai). Gel loading buffer lacking thiol reagent was stored at room temperature. 200 mM β-mercaptoethanol was added to buffer from 14 *M* stock just before use.

I. Staining solution

2.5g of Coomassie Brilliant blue R250 was dissolved in 450ml of methanol and 100ml of acetic acid, mixed and filtered. The volume was made to 1 litre with distilled water. Solution was stored at room temperature.

J. Destaining solution

300 ml of methanol and 100 ml of acetic acid mixed with distilled water and completed to 1 litre with distilled water. Solution was stored at room temperature.

K. Standard protein molecular weight marker

Lower range protein weight marker (PMW-L) (Bangalore Genei, Bangalore) was also used for determination of molecular weights of desire protein. The molecular weight protein standards included Ovalbumin (43,000 Da), Carbonic anhydrase (29,000 Da), Soyabean trypsin inhibitor (20,100 Da) and Lysozyme (14,300 Da), Aprotinin (6,500 Da) and insulin (α and β chains (3,000 Da).

3.14.1. Preparation of SDS-PAGE apparatus (Sambrook *et al*., 1989)

Depending on the proteins to be separated, 10-15% resolving gel and 5% stacking gels were used in this study. Glass plates were rinsed by absolute alcohol and cleaned using tissue paper. Spacers (1 mm) were placed on both sides between two plates in such a way that any bubbles could not move through that and seated in stand and checked by water.

10-15% resolving gel was prepared and poured up to 3/4 portion, and kept for solidification. After solidification, 5% stacking gel was prepared, poured and 1 mm comb was inserted into the staking gel. Gel was allowed to solidify. Comb was removed and the solidified gel with plates was fixed in gel running apparatus. Gel running tank was filled with 1X electrode buffer and prepared samples were loaded into wells. Samples were resolved by applying constant current of 20 mA for 2 hr. After electrophoresis, the gel was transferred to a clean container and stained overnight at room temperature with shaking. After staining, the gel was destained using destaining solution until a clear background was obtained. Photographs of the gels were taken with gel documentation system (Herolab, Germany).

Resolving gel mixture was prepared in a small beaker with a magnetic stirrer by mixing the components for a desired concentration of acrylamide as below (Harlow and Lane, 1998).

Composition for resolving gels (pH 8.8)

Solution component	**15% gel**	
	Component Volumes (ml)	
Total volume	5.0	10.0
Deionised water	1.2	2.3
30% acrylamide mix	2.5	5.0
1.5 M Tris-HCl pH 8.8	1.3	2.5
10% (w/v) SDS	0.05	0.1
10 % (w/v) APS	0.05	0.1
TEMED	0.002	0.004

Immediately upon addition of APS and TEMED, the solution was poured into the assembled plates and overlaid with iso-butanol to prevent diffusion of oxygen into the gel and for obtaining a uniform margin of the gel. It was allowed to polymerize for about 15 min.

In a similar manner, stacking gel mixture with 5% acrylamide mix was prepared by mixing the components as below (Sambrook *et al.*, 1989).

Composition for stacking gels (pH 6.8)

Component	1 ml	3 ml	5 ml	8 ml	10 ml
H_20	0.68	2.1	3.4	5.5	6.8
30% Acrylamide Mix	0.17	0.5	0.83	1.3	1.7
1.0 M Tris (pH-6.8)	0.13	0.38	0.63	1.0	1.25
10% SDS	0.01	0.03	0.05	0.08	0.1
10% APS	0.01	0.03	0.05	0.08	0.1
TEMED	0.001	0.003	0.005	0.008	0.01

Sample preparation for SDS-PAGE

One ml of crude dialyzed compound was pelleted by centrifugation at 11,000 × g for 3 min and supernatant was discarded. The pellet was resuspended in 30 µl distilled water and 30 µl of 2X sample buffer, vortexed and lysed at 95 °C for 5-10 min in a dry bath. The samples were loaded to the SDS polyacrylamide gel.

3.15. Extraction of antimicrobial compounds with organic solvents:

The cell free supernatant was extracted with organic solvents such as like Petroleum ether, Dichloromethane, Ethyl acetate and Hexane. Extraction of supernatant was carried out with 3 volume of solvent for 2h by using a rotary shaker supernatant fraction was flash evaporated at 45° C temperature to ensure complete removal of solvent and the extracts were evaporated to dryness. The resulting residue was dissolved in small amount of respective solvents and stored at –20°C until further purified.

3.15.1. Identification of the active compounds

TLC method was used for obtaining the active substances contained in the Ethyl acetate extracts with biological activities.

Thin layer Chromatography (TLC) for determination of the active compound

Thin layer chromatography (TLC) was carried out using TLC aluminium sheets silica gel. Separation of the active compounds from the ethyl acetate was achieved by spotting by 5µl of the extract to a silica gel TLC plate, and immersed in the appropriate solvent TLC of the Ethyl acetate was carried out in three different solvent mixtures (Table 1). The plate was then covered with soft agar, inoculated with the target strain and incubated over night at 30^{0} C. Inhibition zones resulted in the area in which biologically active compounds diffused. These inhibition zones were used to define the areas to scrape out of a parallel TLC carried out in the same conditions. The active compound was eluted with Ethyl acetate.

Solvents used for TLC

Mixture	Concentrations
Ethyl acetate: methanol: water	100:2.5:2
Methanol: water	80:20
$CHCL_3$:METHANOL:NH_3^+	75:20:5

Ninhydrin Reagent Solution

Composition	g/ml
Ninhydrin	200 mg
Acetone	100 ml

200mg of Ninhydrin reagent was dissolved in acetone with constant stirring to produce a clear, light yellow solution. The solution was thoroughly mixed and poured into a Nebulizer and sprayed on the TLC plates. In a fume hood, the TLC plates were sprayed with Ninhydrin solution until plate was covered with reagent. The TLC plate was heated until spots developed completely. Primary amine containing compounds will appear as pink spots on white background.

IV. RESULTS

4.1. Isolation of bacteria having anti microbial property (AMP)

4.1.1. Someshwar

4.1.1.1. Water sample

The water samples were collected from twenty four different sampling sites along the sandy beach of Someshwar. The samples showed varying heterotrophic count in different stations. The minimum bacterial count that was observed was 2.20 x 10^2 cfu/ml with a maximum of 2.67 x 10^4 cfu/ml. The mean bacterial count was found to be 5.68 x 10^3 cfu/ml. Among the total sampling sites only about six sites yielded the bacteria with antimicrobial property. Antimicrobial property (AMP) possessing bacteria were isolated from stations 2, 7, 15, 17, 19 and 21. As many as 7868 bacteria were screened and only 11 isolates with antimicrobial property were isolated from the water samples obtained from these sampling sites (Table 1).

4.1.1.2. Sediment sample

The sediment samples were also collected from the sampling sites along the Someshwar sandy beach. The maximum bacterial count was found at the level of 2.79 x 10^7 cfu/g in sediment sample collected from station no. 21 and minimum count was 2.49 x 10^3 cfu/g in station no. 14 (Table 2). The mean bacterial count of the sediment sample of this region was 5.18 x 10^6 cfu/g. In general sediment samples yielded more bacterial count than water samples. Among 13372 bacteria screened, 18 isolates showed antimicrobial property.

Table 1. Bacteriological analysis of water samples collected from Someshwar

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC (cfu/ml)
1	2.08.05	130	0	6.5×10^2
2	16.08.05	673	1	2.67×10^4
3	2.09.05	586	0	2.25×10^3
4	16.09.05	587	0	2.48×10^3
5	2.10.05	562	0	2.20×10^2
6	16.10.05	412	0	1.68×10^3
7	2.11.05	262	3	1.10×10^4
8	16.11.05	165	0	4.5×10^2
9	2.12.05	525	0	1.25×10^3
10	16.12.05	304	0	1.23×10^3
11	2.01.06	178	0	7.25×10^3
12	16.01.06	496	0	2.10×10^3
13	2.02.06	253	0	1.05×10^4
14	16.02.06	74	0	3.30×10^2
15	2.03.06	248	1	1.60×10^4
16	16.03.06	138	0	5.50×10^2
17	2.04.06	65	2	3.25×10^3
18	16.04.06	360	0	1.80×10^4
19	2.05.06	242	3	1.06×10^3
20	16.05.06	120	0	6.0×10^3
21	2.06.06	586	1	2.46×10^3
22	16.06.06	68	0	3.4×10^2
23	2.07.06	272	0	1.86×10^4
24	16.07.06	562	0	2.19×10^3
	Total	**7868**	**11**	**5.68×10^3 (Mean)**

Table 2. Bacteriological analysis of sediment samples collected from Someshwar

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC (cfu/g)
1	2.08.05	619	0	2.60×10^{5}
2	16.08.05	682	1	2.79×10^{7}
3	2.09.05	584	1	2.24×10^{5}
4	16.09.05	560	0	2.19×10^{5}
5	2.10.05	820	0	2.77×10^{6}
6	16.10.05	699	0	2.59×10^{5}
7	2.11.05	567	2	2.32×10^{6}
8	16.11.05	540	0	2.25×10^{5}
9	2.12.05	655	2	2.69×10^{6}
10	16.12.05	572	4	2.49×10^{3}
11	2.01.06	580	0	2.86×10^{5}
12	16.01.06	540	2	2.29×10^{5}
13	2.02.06	536	0	2.25×10^{6}
14	16.02.06	242	0	1.21×10^{7}
15	2.03.06	529	1	2.16×10^{6}
16	16.03.06	318	1	1.59×10^{7}
17	2.04.06	765	0	2.78×10^{5}
18	16.04.06	233	2	2.40×10^{5}
19	2.05.06	663	0	2.69×10^{6}
20	16.05.06	710	1	2.56×10^{5}
21	2.06.06	694	0	2.92×10^{6}
22	16.06.06	556	1	2.4×10^{6}
23	2.07.06	308	0	2.59×10^{7}
24	16.07.06	400	0	2.00×10^{7}
	Total	**13372**	**18**	**5.18×10^{6} (Mean)**

4.1.2. Surathkal

4.1.2.1. Water sample

Bacterial count ranged from 6.20×10^2 cfu/ml to 8.45×10^4 cfu/ml (Table 3). The mean bacterial count was found to be 1.79×10^4 cfu/ml. As many as 7885 bacteria were screened for AMP possessing isolates from 24 water samples obtained from this sampling site. Many pigmented bacteria were also encountered in the water samples analyzed. The AMP possessing isolates were isolated from the eight samples. AMP possessing bacteria could not be isolated from the other 16 samples.

4.1.2.2. Sediment samples

The mean bacterial count of the sediment sample was found to be 3.75×10^8 cfu/g. The maximum bacterial count 2.81×10^9 cfu/g was found in sediment sample obtained from the station no.18. And the station no. 3 showed the lowest bacterial count that of 6.90×10^5 cfu/g (Table 4). As many as 12885 bacteria were isolated from 24 sediment samples. All these isolates were screened for the isolation of AMP possessing bacteria. Thirty five of 12885 bacterial strains which possess AMP were isolated. The AMP possessing bacteria were not isolated from the stations no. 13, 15, 16, 20, 23 and 24. However, they were isolated from the remaining 18 sampling sites. The maximum of four isolates AMP possessing bacteria were isolated from the sediment sample obtained from station no.10. The sediment samples analyzed from these regions yielded more isolates of AMP possessing bacteria than the water samples.

Table 3. Bacteriological analysis of water samples collected from Surathkal

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC cfu/ml
1	7.08.05	423	0	$1.61 x 10^3$
2	22.08.05	476	0	$2.16 x 10^3$
3	7.09.05	93	1	$4.65 x 10^4$
4	22.09.05	219	0	$8.80 x 10^3$
5	7.10.05	647	0	$2.75 x 10^4$
6	22.10.05	463	0	$1.78 x 10^3$
7	7.11.05	471	1	$6.10 x 10^4$
8	22.11.05	198	0	$8.10 x 10^3$
9	7.12.05	222	1	$9.20 x 10^2$
10	22.12.05	648	0	$2.45 x 10^3$
11	7.01.06	161	1	$6.75 x 10^2$
12	22.01.06	174	0	$7.40 x 10^3$
13	7.02.06	189	0	$8.15 x 10^2$
14	22.02.06	247	1	$9.80 x 10^3$
15	7.03.06	146	0	$6.40 x 10^2$
16	22.03.06	181	0	$7.40 x 10^3$
17	7.04.06	726	1	$8.45 x 10^4$
18	22.04.06	219	0	$9.10 x 10^3$
19	7.05.06	178	0	$7.10 x 10^2$
20	22.05.06	192	0	$8.40 x 10^3$
21	7.06.06	573	1	$6.10 x 10^4$
22	22.06.06	194	1	$9.70 x 10^3$
23	7.07.06	148	0	$6.20 x 10^2$
24	22.07.06	697	0	$7.00 x 10^4$
	Total	**7885**	**8**	**$1.79 x 10^4$ (Mean)**

Table 4. Bacteriological analysis of sediment samples collected from Surathkal

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC cfu/g
1	7.08.05	615	2	2.35×10^{6}
2	22.08.05	204	1	8.70×10^{7}
3	7.09.05	685	1	6.90×10^{5}
4	22.09.05	215	3	8.30×10^{7}
5	7.10.05	487	1	7.4×10^{8}
6	22.10.05	200	2	8.20×10^{7}
7	7.11.05	668	2	2.81×10^{8}
8	22.11.05	472	1	2.36×10^{7}
9	7.12.05	575	2	1.14×10^{8}
10	22.12.05	202	4	8.70×10^{7}
11	7.01.06	674	1	6.20×10^{8}
12	22.01.06	690	2	8.20×10^{8}
13	7.02.06	567	0	2.14×10^{8}
14	22.02.06	651	2	8.70×10^{8}
15	7.03.06	691	0	7.20×10^{7}
16	22.03.06	482	0	1.81×10^{8}
17	7.04.06	681	4	2.67×10^{7}
18	22.04.06	712	3	8.90×10^{8}
19	7.05.06	478	1	1.90×10^{7}
20	22.05.06	488	0	1.70×10^{8}
21	7.06.06	653	1	2.60×10^{7}
22	22.06.06	618	2	8.00×10^{8}
23	7.07.06	531	0	1.97×10^{7}
24	22.07.06	646	0	2.56×10^{8}
	Total	**12885**	**35**	**3.75×10^{8} (Mean)**

4.1.3. Mangrove

4.1.3.1. Water sample

Mangrove region is a distinct ecological niche which harbors diverse forms of microbes. Among the twenty four sampling sites analyzed from the Mangrove area, only about ten sampling sites yielded AMP possessing bacteria. The remaining fourteen stations did not yield any AMP possessing isolates. The water samples analyzed from these regions yielded 8551 bacteria which were screened for the isolation of AMP possessing bacteria. 13 isolates were found to be AMP producing bacteria. The bacterial count was highest in station no.17 with a value of 3.55×10^4 cfu/ml and lowest at station no 11 with a value of 2.35×10^2 cfu/ml. The mean count of the bacteria analyzed from the water sample of Mangrove area was found to be 5.77×10^3 (Table 5).

4.1.3.2. Sediment samples

About twenty four sediment samples were collected from the Mangrove region. The bacteriological analysis of this region produced about 12233 bacteria and was screened for AMP possessing bacteria. Forty two of 12233 isolates were AMP possessing bacteria. Only 9 of 24 sampling stations did not yield any AMP possessing bacterial isolates. Sediment samples collected from station no.6 and 13 yielded maximum number of AMP possessing isolates that is 6 and 7 isolates, respectively. The mean bacterial count of the sediment sample was 8.72×10^7 cfu/g. Maximum bacterial count of 5.20×10^8 cfu/g was found at station at no.10 and the minimum count of 1.28×10^6 cfu/g was found at station no 13(Table 6).

Table 5. Bacteriological analysis of water samples collected from Mangrove

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC cfu/ml
1	13.08.05	600	0	2.49×10^3
2	28.08.05	511	0	2.15×10^3
3	13.09.05	635	0	2.60×10^3
4	28.09.05	675	0	2.81×10^3
5	13.10.05	664	2	2.80×10^3
6	28.10.05	216	0	8.00×10^2
7	13.11.05	80	1	4.00×10^2
8	28.11.05	680	1	2.80×10^3
9	13.12.05	70	0	3.50×10^2
10	28.12.05	86	1	4.30×10^2
11	13.01.06	87	0	2.35×10^2
12	28.01.06	107	0	5.35×10^2
13	13.02.06	78	0	3.90×10^3
14	28.02.06	68	2	3.40×10^2
15	13.03.06	85	0	4.25×10^2
16	28.03.06	120	1	6.00×10^2
17	13.04.06	608	0	3.55×10^4
18	28.04.06	502	2	1.81×10^3
19	13.05.06	651	0	2.48×10^4
20	28.05.06	98	0	4.90×10^2
21	13.06.06	611	1	2.54×10^3
22	28.06.06	613	0	2.30×10^4
23	13.07.06	64	1	3.20×10^2
24	28.07.06	542	1	2.16×10^4
	Total	**8551**	**13**	**5.86×10^3 (Mean)**

Table 6. Bacteriological analysis of sediment samples collected from Mangrove

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC cfu/g
1	13.08.05	347	3	1.30×10^{8}
2	28.08.05	524	4	2.40×10^{7}
3	13.09.05	585	2	4.05×10^{6}
4	28.09.05	146	0	7.3×10^{6}
5	13.10.05	650	2	2.46×10^{8}
6	28.10.05	689	6	2.75×10^{7}
7	13.11.05	288	0	1.21×10^{8}
8	28.11.05	646	3	2.33×10^{7}
9	13.12.05	527	0	2.13×10^{8}
10	28.12.05	630	3	2.67×10^{8}
11	13.01.06	535	2	2.27×10^{7}
12	28.01.06	689	0	2.51×10^{7}
13	13.02.06	264	7	1.09×10^{7}
14	28.02.06	500	2	2.08×10^{8}
15	13.03.06	496	0	1.89×10^{7}
16	28.03.06	489	0	1.70×10^{7}
17	13.04.06	423	2	1.68×10^{7}
18	28.04.06	701	3	2.43×10^{7}
19	13.05.06	556	0	2.36×10^{7}
20	28.05.06	355	0	1.31×10^{8}
21	13.06.06	600	1	2.62×10^{6}
22	28.06.06	618	1	5.20×10^{8}
23	13.07.06	334	1	1.28×10^{6}
24	28.07.06	641	0	2.48×10^{7}
	Total	**12233**	**42**	**8.72×10^{7} (Mean)**

4.1.4. Nethravathy Estuary

4.1.4.1. Water samples

Water samples collected from 24 sampling sites along the Nethravathy estuary showed a minimum bacterial count of 9.05×10^2 cfu/ml and a maximum of 2.23×10^5 cfu/ml. The bacterial count was found high in the station 15 and lowest at station 9(Table 7). The mean bacterial count was found to be 2.31×10^4 cfu/ml. Only six AMP possessing bacteria could be obtained from the 9636 bacteria screened. Only six stations yielded AMP possessing isolates among the twenty four sampling sites.

4.1.4.2. Sediment samples

The sediment samples were collected from the 24 sampling sites along the Nethravathy estuary. The minimum bacterial count that was observed was 2.08×10^4 cfu/g and the maximum was 2.87×10^8 cfu/g. The highest bacterial count was found in the station no 18 and lowest at the station 1. The mean bacterial count that was found in this region was 5.85×10^7cfu/g. As many as 13530 bacteria were isolated from 24 samples and 11 isolates were AMP possessing bacteria isolated from 10 sampling sites (Table 8).

4.1.5. Ullala

4.1.5.1. Water samples

Water samples were collected from 24 sampling sites. Bacterial count ranged from 3.5×10^2 cfu/ml to 8.20×10^4 cfu/ml. The mean total plate count was observed to be 1.93×10^4 cfu/ml. Of 12076 bacteria screened, only 10 isolates possessed AMP and these isolates were obtained from six sampling stations. Remaining 18 stations did not yield any AMP possessing isolates (Table 9).

Table 7. Bacteriological analysis of water samples collected from Nethravathy estuary

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC cfu/ml
1	10.08.05	630	0	5.5×10^3
2	25.08.05	127	1	6.35×10^3
3	10.09.05	69	0	3.45×10^3
4	25.09.05	156	0	6.50×10^3
5	10.10.05	390	1	1.68×10^4
6	25.10.05	410	0	1.82×10^4
7	10.11.05	166	1	6.9×10^3
8	25.11.05	589	0	2.11×10^3
9	10.12.05	219	0	9.05×10^2
10	25.12.05	693	0	2.67×10^3
11	10.01.06	795	1	2.79×10^3
12	25.01.06	657	0	2.58×10^3
13	10.02.06	74	0	3.55×10^3
14	25.02.06	446	0	2.23×10^5
15	10.03.06	108	1	5.40×10^4
16	25.03.06	696	0	2.59×10^4
17	10.04.06	582	0	2.21×10^4
18	25.04.06	93	0	4.65×10^4
19	10.05.06	65	1	3.25×10^3
20	25.05.06	673	0	2.80×10^4
21	10.06.06	239	0	1.09×10^4
22	25.06.06	598	0	2.10×10^4
23	10.07.06	620	0	2.45×10^4
24	25.07.06	541	0	1.89×10^4
	Total	**9636**	**6**	**2.31×10^4 (Mean)**

Table 8. Bacteriological analysis of sediment samples collected from Nethravathy estuary.

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC cfu/g
1	10.08.05	559	0	2.08×10^4
2	25.08.05	563	2	2.10×10^6
3	10.09.05	369	1	1.52×10^7
4	25.09.05	440	0	1.81×10^7
5	10.10.05	672	1	2.86×10^8
6	25.10.05	662	0	2.91×10^7
7	10.11.05	469	0	1.83×10^7
8	25.11.05	332	0	1.31×10^7
9	10.12.05	344	0	1.30×10^7
10	25.12.05	650	1	2.81×10^6
11	10.01.06	443	0	1.92×10^8
12	25.01.06	633	1	2.78×10^7
13	10.02.06	730	0	2.90×10^7
14	25.02.06	637	1	2.78×10^7
15	10.03.06	738	0	2.80×10^7
16	25.03.06	697	0	2.74×10^7
17	10.04.06	652	1	2.24×10^7
18	25.04.06	672	0	2.87×10^8
19	10.05.06	548	1	2.30×10^6
20	25.05.06	524	1	2.12×10^8
21	10.06.06	646	0	2.72×10^6
22	25.06.06	608	1	2.71×10^7
23	10.07.06	278	0	1.21×10^8
24	25.07.06	664	0	2.59×10^7
	Total	**13530**	**11**	**5.85×10^7 (Mean)**

Table 9. Bacteriological analysis of water samples collected from Ullala

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC cfu/ml
1	4.08.05	280	0	1.17×10^3
2	19.08.05	618	0	2.60×10^3
3	4.09.05	326	0	1.23×10^4
4	19.09.05	467	0	5.40×10^3
5	4.10.05	70	1	3.5×10^2
6	19.10.05	683	0	2.69×10^4
7	4.11.05	367	0	4.15×10^4
8	19.11.05	595	0	2.45×10^4
9	4.12.05	643	2	8.20×10^4
10	19.12.05	620	0	2.41×10^4
11	4.01.06	683	1	2.83×10^4
12	19.01.06	599	0	6.00×10^3
13	4.02.06	623	2	5.20×10^4
14	19.02.06	545	0	2.27×10^4
15	4.03.06	292	1	1.43×10^4
16	19.03.06	324	0	1.19×10^4
17	4.04.06	675	0	6.00×10^4
18	19.04.06	600	3	4.15×10^3
19	4.05.06	629	0	2.41×10^3
20	19.05.06	685	0	2.86×10^3
21	4.06.06	339	0	1.19×10^4
22	19.06.06	536	0	2.28×10^3
23	4.07.06	491	0	1.86×10^4
24	19.07.06	386	0	5.30×10^3
	Total	**12076**	**10**	**1.93×10^4 (Mean)**

Table 10. Bacteriological analysis of sediment samples collected from Ullala

Sampling station no	Date of sampling	Total no of bacteria screened	No of isolates showing AMP	TPC Cfu/g
1	4.08.05	467	0	1.90×10^6
2	19.08.05	748	2	2.77×10^6
3	4.09.05	471	0	1.80×10^8
4	19.09.05	611	3	2.63×10^7
5	4.10.05	717	0	2.90×10^7
6	19.10.05	615	0	2.24×10^8
7	4.11.05	576	4	2.44×10^7
8	19.11.05	730	0	2.80×10^7
9	4.12.05	143	0	7.15×10^6
10	19.12.05	638	3	2.30×10^6
11	4.01.06	174	0	8.7×10^6
12	19.01.06	674	2	2.50×10^6
13	4.02.06	634	0	2.74×10^7
14	19.02.06	466	0	1.86×10^7
15	4.03.06	385	3	1.43×10^8
16	19.03.06	723	0	2.70×10^7
17	4.04.06	720	2	2.82×10^6
18	19.04.06	655	0	2.43×10^7
19	4.05.06	589	0	2.46×10^7
20	19.05.06	508	1	1.80×10^7
21	4.06.06	542	0	1.98×10^8
22	19.06.06	570	2	2.25×10^7
23	4.07.06	479	0	1.69×10^4
24	19.07.06	406	0	1.58×10^7
	Total	**13241**	**22**	**3.73×10^7 (Mean)**

4.1.5.2. Sediment samples

The sediment samples were also collected from twenty four different stations along the sandy beach of Ullala region. The samples collected were subjected to bacteriological analysis. The bacterial count ranged from 1.69 x 10^4 cfu/g to of 2.24 x 10^8 cfu/g. The mean total plate count was 3.73 x 10^7cfu/g. The sediment samples analyzed produced about 13241 number of bacteria of which only 22 exhibited antagonistic property. Station 7 yielded 4 isolates producing anti microbial compounds. Similarly station 4, 10 and 15 yielded three isolates each possessing AMP (Table 10).

4.2. Isolation of anti microbial property (AMP) possessing bacteria

The samples were collected from five different sampling regions along the West Coast of India (Table 11). As many as 46016 bacterial colonies from water and 65261 colonies from sediment were screened. The initial screening of water sample resulted in 48 AMP possessing isolates and the sediment samples analyzed yielded 128 AMP possessing isolates. Total of 176 isolates were obtained after initial screening. The antimicrobial properties by AMP possessing isolates against the test strains *S. aureus* and *V. harveyi* are indicated by clear zones around the colonies (Plate 1& Plate 2). Of 176 isolates, only 28 isolates produced prominent inhibitory activity which was confirmed by spot assay method against the *S. aureus* and *V. harveyi*. These AMP possessing isolates showed inhibition zone of more than 6mm. The rest of the isolates showed weaker inhibitory activity with inhibition zone less than 4mm. The results of the inhibitory zone shown by the 28 AMP possessing isolates reconfirmed by spot assay are shown in the Table 12.

Table 11. AMP bacteria isolated from the west coast of India

Sl no.	Sampling site	Source of sample	No. of samples	No of bacteria screened	Isolates obtained with antimicrobial activity	Total
1	Someshwar	Water	24	7868	11	**29**
		Sediment	24	13372	18	
2	Surathkal	Water	24	7885	8	**43**
		Sediment	24	12885	35	
3	Mangrove	Water	24	8551	13	**55**
		Sediment	24	12233	42	
4	Nethravathy Estuary	Water	24	9636	6	**17**
		Sediment	24	13530	11	
5	Ullala	Water	24	12076	10	**32**
		Sediment	24	13241	22	
	Total		**240**	**111277**	**176**	

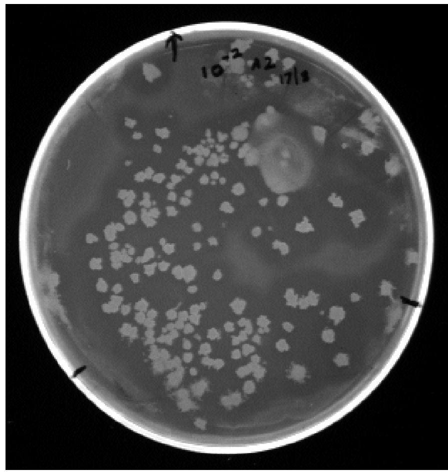


Plate 1. The inhibitory zone around the AMP possessing colonies against *Staphylococcus aureus*

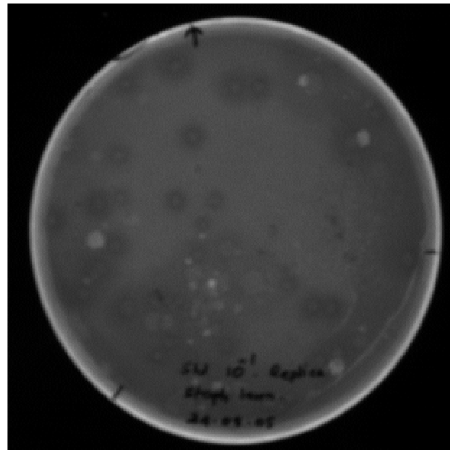


Plate 2. The inhibitory zone around the AMP possessing bacterial colonies tested against *Vibrio harveyi.*

Table 12. Confirmed AMP isolates from water and sediment samples

Sl. no.	Strain code	Source	Sample type	Inhibition zone (cm)	
				V. harveyi	*S. aureus*
1	SM5	Someshwar	sediment	1.1	1.2
2	SM9	Someshwar	Water	0.8	0.8
3	SM15	Someshwar	sediment	0.7	0.8
4	SM25	Someshwar	Water	0.7	0.8
5	SK2	Surathkal	sediment	0.8	0.8
6	SK6	Surathkal	sediment	0.9	0.9
7	SK9	Surathkal	sediment	1.0	1.2
8	SK8	Surathkal	Water	0.8	0.8
9	SK14	Surathkal	sediment	1.0	0.9
10	SK20	Surathkal	sediment	0.7	0.7
11	SK31	Surathkal	Water	0.7	0.8
12	NE10	Nethravathy estuary	sediment	0.8	1.0
13	NE12	Nethravathy estuary	sediment	0.8	0.8
14	NE15	Nethravathy estuary	sediment	0.6	0.7
15	MG1	Mangrove	sediment	0.8	1.0
16	MG2	Mangrove	Water	0.8	0.8
17	MG5	Mangrove	sediment	0.7	0.8
18	MG8	Mangrove	Water	1.0	1.0
19	MG10	Mangrove	sediment	0.8	0.8
20	MG12	Mangrove	sediment	1.0	1.1
21	MG14	Mangrove	Water	0.8	0.8
22	MG16	Mangrove	sediment	0.7	0.7
23	MG29	Mangrove	sediment	0.7	0.8
24	UL4	Ullala	sediment	0.8	1.0
25	UL7	Ullala	Water	0.8	0.8
26	UL2	Ullala	sediment	0.7	0.8
27	UL9	Ullala	sediment	0.8	0.7
28	UL28	Ullala	Water	0.7	0.8

4.3. Biochemical identification of AMP possessing bacteria

The 176 AMP possessing bacteria were identified up to genus level and some were identified up to species level. *Bacillus* and the *Pseudomonas* sp were the dominant group identified (Plate 3 & Plate 4). The results of the identification of AMP possessing bacteria are indicated in Table 13. *Pseudomonas sp* accounted for 36% and *Bacillus* spp, 64%. Among the species that could be identified as *P. aeruginosa* accounted for 20.5%, *B. subtilis* 14.8%, *B. cereus,* 13.6%, *B. pumilus,* 11.4%. Of the 176 AMP producing bacteria (Table 13), 28 were showing high potential. The identification of these 28 active AMP possessing isolates is given in Table 14. Of these 8 were *Pseudomonas* spp and others were *Bacillus* spp. These isolates came from different regions and were derived from water or sediment

4.4. Optimization of the production of AMP

The AMP possessing isolates which showed maximum antimicrobial property was selected for the optimization of the antimicrobial compound production. The inhibitory activity indicated by clear zone was measured by following well diffusion method. The culture isolates were subjected to various growth temperatures at 30, 37 and 42^0 C to study the optimum temperature required to produce antimicrobial compounds. All the isolates produced maximum antimicrobial compound at 30^0 C when compared to the compound produced at 37^0C (Table 15). However the isolates did not produce antimicrobial compound at 42^0C. The concentration of Sodium Chloride (NaCl) was also varied in the TSB broth from 0%, 1%, 2%, 3% and 4% to find the required level to produce the optimum amount of antimicrobial compounds (Table 16). The antimicrobial compound synthesis was optimum at 1 and 2% NaCl concentration. Even though growth of the AMP possessing isolates were observed no inhibitory activity was found at 3 and 4% of NaCl concentration. The AMP possessing isolates were also grown in TSB broth with different

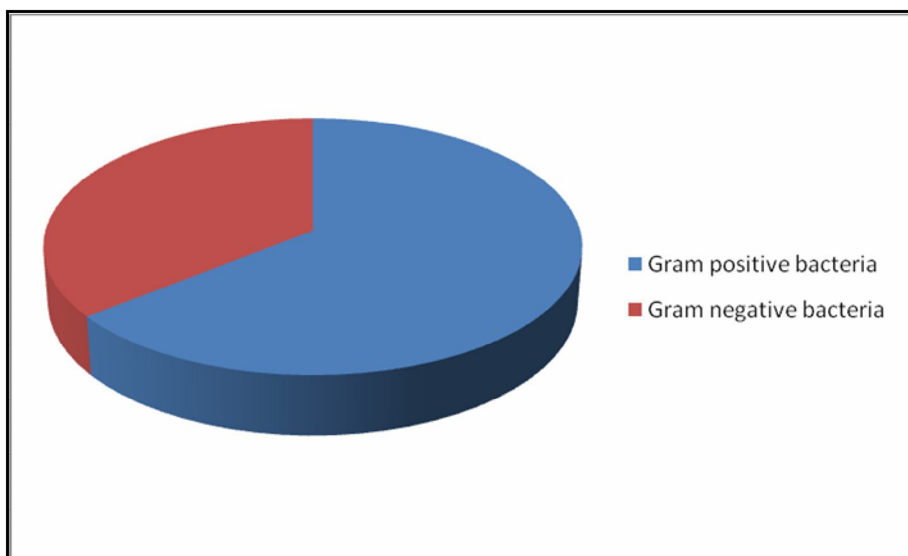


Plate 3. Distribution of Gram positive and Gram negative bacteria among AMP possessing isolates

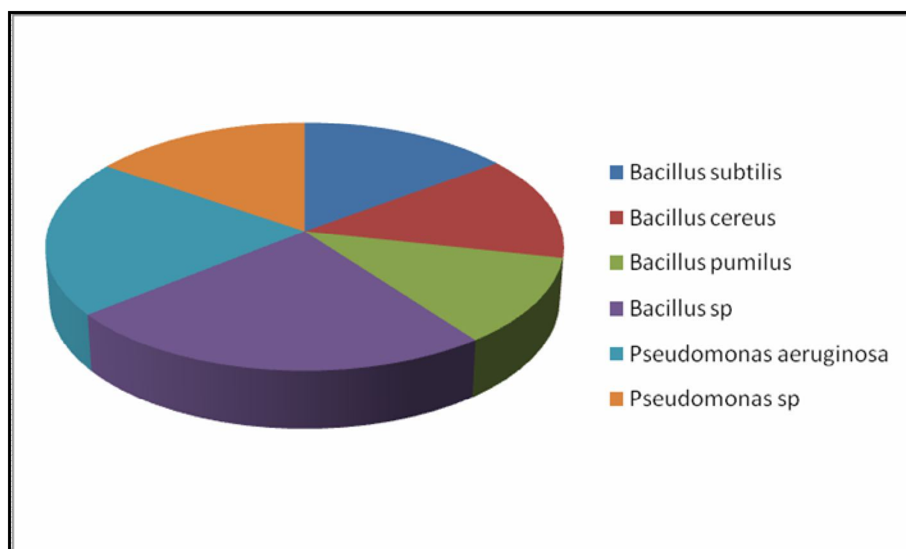


Plate 4. Species composition of AMP possessing bacteria

Table 13. Biochemical identification of AMP possessing isolates

Sl. No	Bacteria identified	Total no bacteria(n=176)	%
1	*Bacillus subtilis*	26	14.77
2	*Bacillus cereus*	24	13.63
3	*Bacillus pumilus*	20	11.36
4	*Bacillus sp*	43	24.43
5	*Pseudomonas aeruginosa*	36	20.45
6	*Pseudomonas sp*	27	15.34
	Total	**176**	

Table 14. Biochemical identification of the 28 AMP possessing isolates with potential activity.

Sl. no.	Strain code	Source	Sample type	Bacteria identified
1	SM5	Someshwar	sediment	*Bacillus sp*
2	SM9	Someshwar	water	*Bacillus sp*
3	SM15	Someshwar	sediment	*Pseudomonas sp*
4	SM25	Someshwar	water	*Bacillus sp*
5	SK2	Surathkal	sediment	*Bacillus sp*
6	SK6	Surathkal	sediment	*Pseudomonas sp*
7	SK9	Surathkal	sediment	*Pseudomonas aerogenosa*
8	SK8	Surathkal	water	*Bacillus cereus*
9	SK14	Surathkal	sediment	*Pseudomonas aerogenosa*
10	SK20	Surathkal	sediment	*Pseudomonas aerogenosa*
11	SK31	Surathkal	water	*Bacillus sp*
12	NE10	Nethravthi estuary	sediment	*Bacillus cereus*
13	NE12	Nethravthi estuary	sediment	*Bacillus sp*
14	NE15	Nethravthi estuary	sediment	*Bacillus subtilis*
15	MG1	Mangrove	sediment	*Bacillus subtilis*
16	MG2	Mangrove	water	*Bacillus pumilus*
17	MG5	Mangrove	sediment	*Bacillus pumilus*
18	MG8	Mangrove	water	*Bacillus sp*
19	MG10	Mangrove	sediment	*Bacillus cereus*
20	MG12	Mangrove	sediment	*Bacillus pumilus*
21	MG14	Mangrove	water	*Bacillus subtilis*
22	MG16	Mangrove	sediment	*Bacillus pumilus*
23	MG29	Mangrove	sediment	*Bacillus pumilus*
24	UL2	Ullala	sediment	*Bacillus subtilis*
25	UL4	Ullala	water	*Pseudomonas aerogenosa*
26	UL7	Ullala	sediment	*Pseudomonas aerogenosa*
27	UL8	Ullala	sediment	*Pseudomonas aerogenosa*
28	UL28	Ullala	water	*Bacillus cereus*

Table 15. Effect of temperature on the production of antimicrobial compounds by different AMP isolates

Sl no.	Strain code	Zone of inhibition (cm)		
		30^0C	37^0C	42^0C
1	SM5	1.2	1.1	-
2	SM9	0.8	0.8	-
3	SM15	0.7	0.7	-
4	SM25	0.7	0.6	-
5	SK2	0.8	0.7	-
6	SK6	1.0	0.9	-
7	SK9	1.0	0.9	-
8	SK8	0.7	0.7	-
9	SK14	1.0	0.9	-
10	SK20	0.8	0.7	-
11	SK31	0.7	0.7	-
12	NE10	0.8	0.8	-
13	NE12	0.8	0.8	-
14	NE15	0.7	0.6	-
15	MG1	0.9	1.0	-
16	MG2	0.8	0.8	-
17	MG5	0.7	0.7	-
18	MG8	1.0	0.8	-
19	MG10	0.8	0.8	-
20	MG12	1.0	1.0	-
21	MG14	0.8	0.7	-
22	MG16	0.7	0.7	-
23	MG29	0.7	0.6	-
24	UL2	0.8	0.8	-
25	UL4	0.8	0.7	-
26	UL7	0.7	0.7	-
27	UL8	0.8	0.7	-
28	UL28	0.7	0.8	-

Table 16. Effect of salt (NaCl) concentration on the production of antimicrobial compounds by the AMP isolates.

Sl no.	Strain code	Zone of inhibition (mm) at various NaCl concentrations				
		0%	1%	2%	3%	4%
1	SM5	-	1.0	1.1	-	-
2	SM9	-	0.7	0.8	-	-
3	SM15	-	0.8	0.7	-	-
4	SM25	-	0.6	0.6	-	-
5	SK2	-	0.8	0.8	-	-
6	SK6	-	1.0	0.9	-	-
7	SK9	-	1.0	1.0	-	-
8	SK8	-	0.7	0.6	-	-
9	SK14	-	0.8	0.9	-	-
10	SK20	-	0.8	0.7	-	-
11	SK31	-	0.7	0.7	-	-
12	NE10	-	0.8	0.8	-	-
13	NE12	-	0.7	0.7	-	-
14	NE15	-	0.6	0.6	-	-
15	MG1	-	1.0	1.0	-	-
16	MG2	-	0.8	0.7	-	-
17	MG5	-	0.7	0.7	-	-
18	MG8	-	1.0	0.9	-	-
19	MG10	-	0.8	0.7	-	-
20	MG12	-	1.0	1.0	-	-
21	MG14	-	0.8	0.8	-	-
22	MG16	-	0.7	0.7	-	-
23	MG29	-	0.6	0.6	-	-
24	UL2	-	0.8	0.7	-	-
25	UL4	-	0.8	0.7	-	-
26	UL7	-	0.7	0.6	-	-
27	UL8	-	0.8	0.7	-	-
28	UL28	-	0.7	0.7	-	-

Table 17. Effect of pH on the antimicrobial compound produced by the AMP isolates.

Sl. no.	Strain code	Zone of inhibition (mm) at various pH			
		6	7	8	9
1	SM5	0.6	1.2	1.2	-
2	SM9	0.7	0.8	0.8	-
3	SM15	0.7	0.7	0.8	-
4	SM25	0.5	0.7	0.8	-
5	SK2	0.7	0.8	0.8	-
6	SK6	0.7	0.9	0.8	-
7	SK9	0.6	1.1	1.0	-
8	SK8	0.6	0.8	0.8	-
9	SK14	0.5	1.0	1.0	-
10	SK20	0.7	0.7	0.6	-
11	SK31	0.6	0.7	0.7	-
12	NE10	0.5	0.8	0.8	-
13	NE12	0.7	0.8	0.8	-
14	NE15	0.6	0.6	0.7	-
15	MG1	0.6	0.8	0.8	-
16	MG2	0.6	0.8	0.8	-
17	MG5	0.7	0.7	0.8	-
18	MG8	0.6	1.0	1.0	-
19	MG10	0.6	0.8	0.8	-
20	MG12	0.7	1.0	1.1	-
21	MG14	0.6	0.8	0.7	-
22	MG16	0.6	0.7	0.7	-
23	MG29	0.7	0.7	0.8	-
24	UL2	0.6	0.8	0.9	-
25	UL4	0.6	0.8	0.8	-
26	UL7	0.6	0.7	0.8	-
27	UL8	0.7	0.8	0.7	-
28	UL28	0.6	0.7	0.7	-

Table 18. Effect of time on antimicrobial activity produced by the AMP isolates.

Sl. No.	Strain code	Zone of inhibition (mm) at different time points						
		6h	12h	24h	30h	36h	48h	72h
1	SM5	-	0.9	1.1	1.2	1.5	1.7	1.7
2	SM9	-	0.8	0.8	0.8	1.0	1.0	1.0
3	SM15	-	0.7	0.7	0.8	1.1	1.2	1.2
4	SM25	-	0.6	0.7	0.8	0.8	0.8	1.0
5	SK2	-	0.8	0.8	1.0	1.3	1.5	1.5
6	SK6	-	0.9	0.9	0.9	1.0	1.1	1.1
7	SK9	-	1.0	1.0	1.2	1.2	1.3	1.3
8	SK8	-	0.8	0.8	0.8	1.2	1.1	1.2
9	SK14	-	0.6	1.0	0.9	1.0	1.0	1.0
10	SK20	-	0.7	0.8	0.8	1.0	1.1	1.1
11	SK31	-	0.6	0.7	0.8	1.0	1.0	1.0
12	NE10	-	0.7	0.8	1.0	1.2	1.2	1.2
13	NE12	-	0.7	0.8	0.8	1.3	1.2	1.2
14	NE15	-	0.6	0.7	0.7	1.2	1.2	1.2
15	MG1	-	0.7	0.8	1.0	1.3	1.3	1.2
16	MG2	-	0.8	1.0	1.2	1.4	1.5	1.6
17	MG5	-	0.6	0.8	1.0	1.3	1.3	1.3
18	MG8	-	0.8	1.0	1.0	1.2	1.2	1.2
19	MG10	-	0.7	0.8	0.8	1.2	1.2	1.2
20	MG12	-	0.8	1.0	1.1	1..0	1.1	1.1
21	MG14	-	0.7	0.8	0.8	1.4	1.6	1.6
22	MG16	-	0.6	0.7	0.7	1.0	1.3	1.2
23	MG29	-	0.7	0.7	0.8	1.0	1.2	1.2
24	UL2	-	0.7	0.8	1.0	1.0	1.1	1.2
25	UL4	-	0.6	0.8	1.0	1.2	1.3	1.5
26	UL7	-	0.6	0.7	0.8	1.2	1.2	1.2
27	UL8	-	0.7	0.8	1.0	1.3	1.3	1.3
28	UL28	-	0.6	0.7	0.8	1.2	1.2	1.2

pH value (6, 7, 8 and 9) and the optimum inhibitory activity was found between pH 7 and 8 (Table 17). At pH 9 only growth of the bacteria was observed whereas the production of the antimicrobial compound and the inhibitory activity produced was not observed for most of the isolates while some produced very mild inhibitory activity. Among the 28 AMP possessing isolates tested SM5, SK9, SK14, MG 8 and MG 12 showed optimum activity at 30^0 C supplemented with 1% NaCl at a pH of 7.

The AMP possessing isolates were also grown for different hours and the samples were collected at time interval of 6, 12, 24, 30, 36, 48 and 72 h and tested for their inhibitory activity by well diffusion method. All the AMP possessing strains identified as *Pseudomonas sp* and *Psudomonas aerugenosa* showed maximum activity at 24h at 30^0 C. The AMP possessing isolates confirmed as *Bacillus subtilis* and *Bacillus cereus* showed maximum activity during incubation at 24 to 48h and much variation was not observed when they were cultured for 72h. SM5, SK 2, MG 2, MG 14 and UL 4 showed increase in their inhibitory activity up to 72h. The results of the inhibition zone of the isolates tested at different growth time are given in the Table 18.

4.5. Extraction of the antimicrobial compound.

The AMP possessing isolates which showed maximum inhibitory activity were used for extraction and purification of the compound for further analysis. To characterize the antimicrobial compound, the culture supernatant that contains the antimicrobial substance, was concentrated by two different methods. The method used in the study was ammonium sulfate precipitation and organic solvent extraction. The AMP possessing strain SM 5 was used for protein extraction by ammonium sulfate precipitation method, as the extracts of this strain showed maximum inhibitory activity when compared to other AMP possessing isolates. Similarly the AMP possessing isolate SK 9 produced maximum inhibitory activity when the active compound was extracted by organic solvent extraction method.

4.5.1. Ammonium sulfate precipitation.

All the 28 AMP possessing isolates were subjected to extraction to isolate antimicrobial compound by Ammonium sulfate precipitation. The bioactive protein fraction was precipitated from the cell-free supernatant by gradient precipitation method from 20 - 70% saturated ammonium sulphate. The protein fraction was dialyzed and subjected to inhibitory assay to check for the fraction with maximum activity. Among the AMP possessing isolates tested the isolate SM 5, showed maximum activity at 30% ammonium sulphate saturation (Plate 5). About 820 mg precipitate was obtained from 2lt culture of SM5 in 30% fraction with a protein concentration of 6.58 mg/ml.

4.5.2. SDS-PAGE profile of dialyzed protein

The protein profile of the dialyzed protein precipitated at various fractions was studied by 15% SDS-PAGE. The crude culture supernatant showed 12 bands. The dialyzed protein obtained at 20% saturation showed 4 protein bands. The bands were located at 3.0kDa, 14.3 kDa and 20.1kDa. However the dialyzed protein obtained at 30% saturation showed two bands at 3.0 kDa and 14.3kDa (Plate 6).

4.6. Antimicrobial assay of 30% fraction

The 30% fraction obtained from the AMP possessing isolate SM5 was subjected to antimicrobial assay by well diffusion method. About 40µl of the 30% fraction was loaded into the wells of plates previously inoculated with test strains. The dialyzed protein was tested against both gram positive and gram negative bacteria (Plate 7 to Plate 16). It showed an inhibition zone of 10- 15mm against *S. aureus, Bacillus sp, Micrococcus sp, E. coli, Salmonella sp, E. tarda* and *V. cholerae.* The activity was also observed on *Klebsiella sp* with inhibition zone between 15-20mm. Among the *Vibrios* tested, the activity was stronger against *V. harveyi, V. parahaemolyticus* and *V. fisheri* with inhibition zone of more than 20mm. The compound also showed strong inhibitory activity against *Listeria*

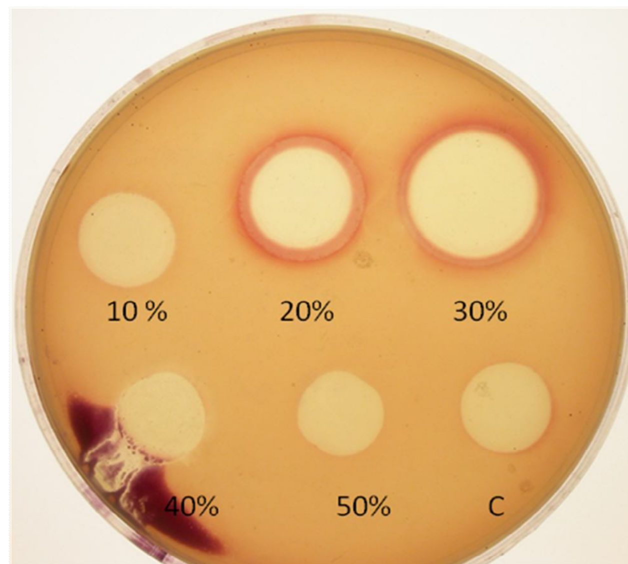


Plate 5. Spot-on-lawn assay of 30% fraction SM5 showing inhibition against the test strain *L. monocytogens*

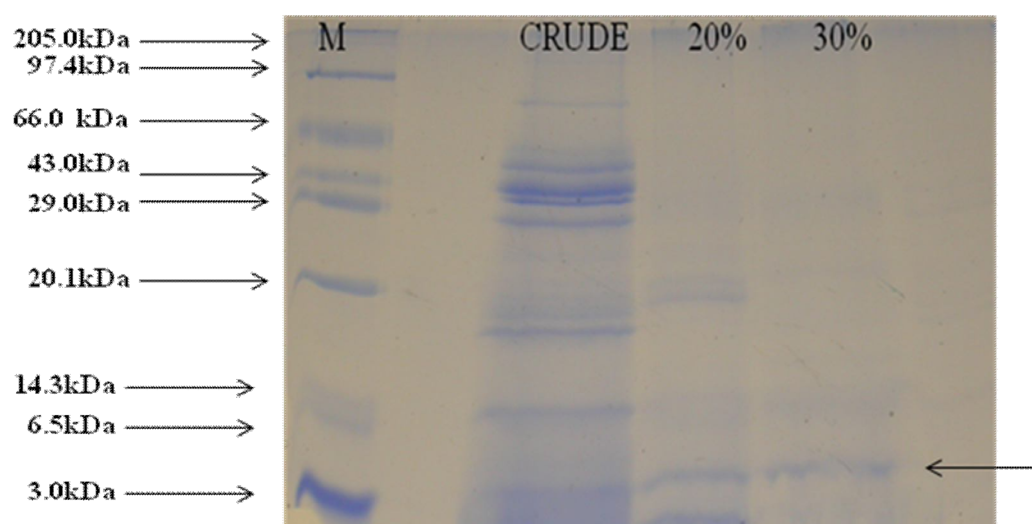


Plate 6. SDS-PAGE profile of 30% fraction obtained from SM5 at 30 % ammonium sulphate precipitation (indicated by arrow on left side of the gel).

Plate 7 to 16. Well diffusion assay of 30% fraction SM5 showing inhibition against different test strain (CFS: Cell free supernatant; CP: Crude protein; N: phosphate buffer negative control)

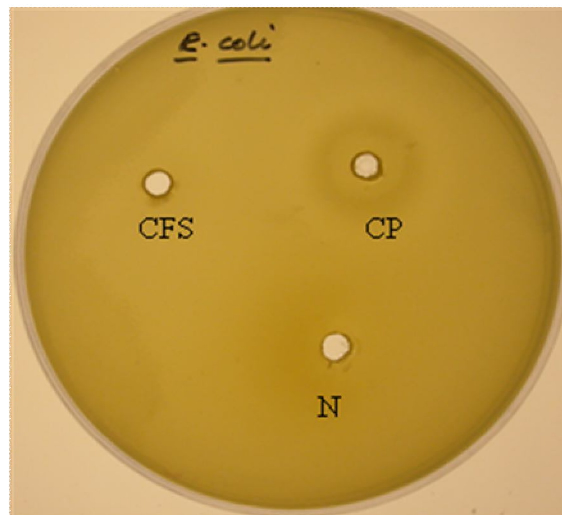


Plate 7. 30% fraction of SM5 showing inhibition against the test strain *E.coli*

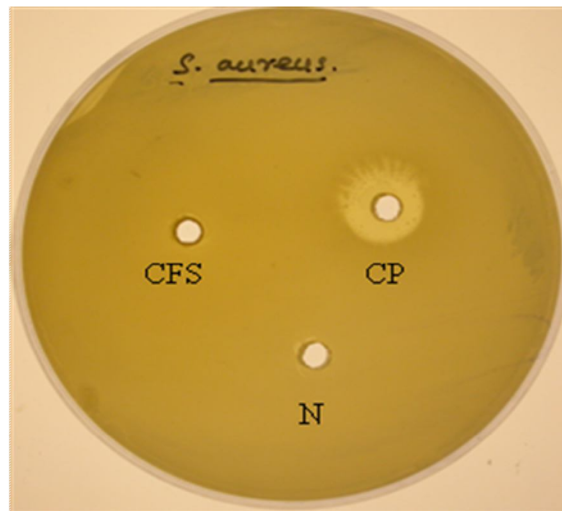


Plate 8. 30% fraction of SM5 showing inhibition against the test strain *S. aureus.*

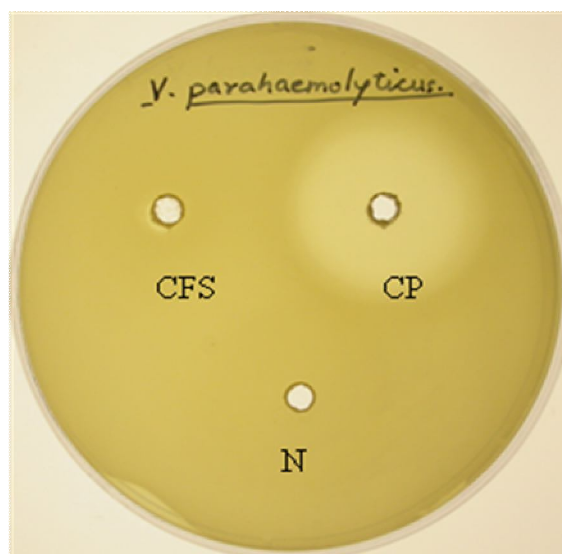


Plate 9. 30% fraction of SM5 showing inhibition against the test strain *V. parahaemolyticus.*

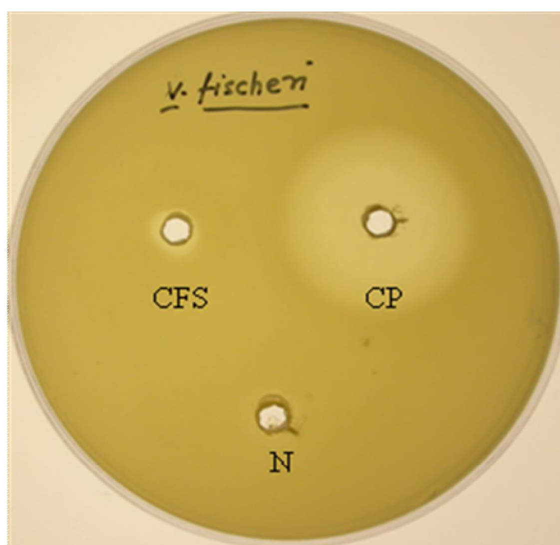


Plate 10. 30% fraction of SM5 showing inhibition against the test strain *V. fischeri.*

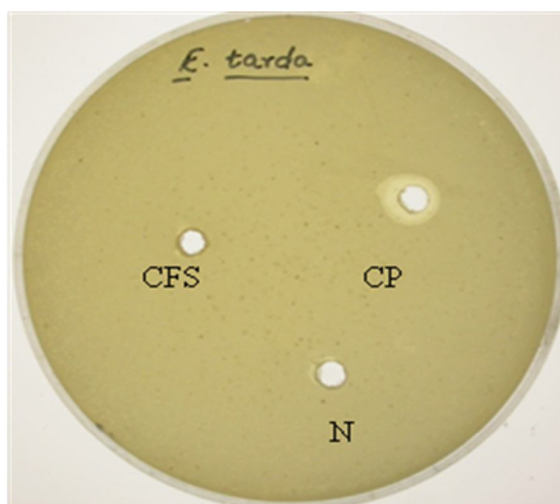


Plate 11. 30% fraction of SM5 showing inhibition against the test strain *E. tarda*.

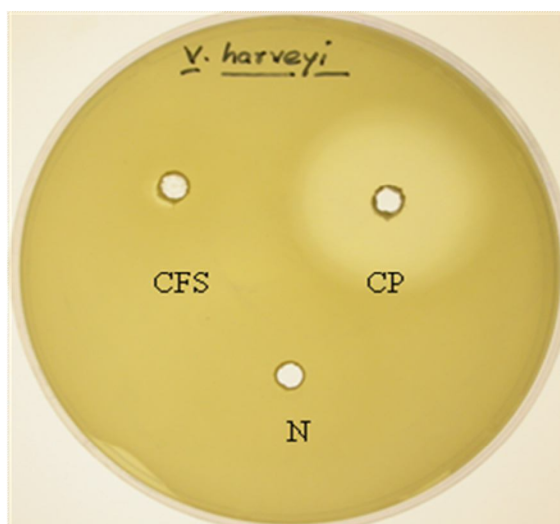


Plate 12. 30% fraction of SM5 showing inhibition against the test strain *V. harveyi.*

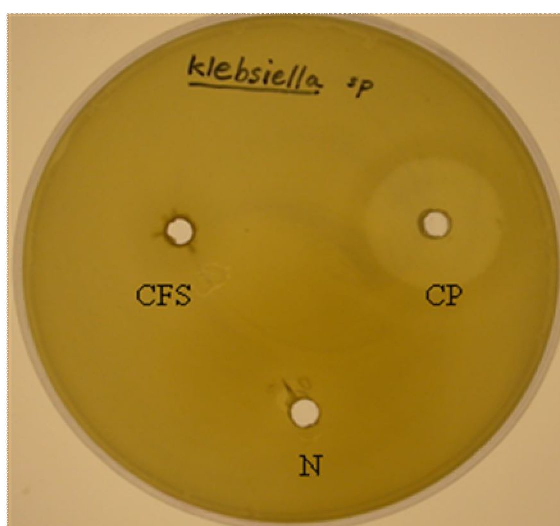


Plate 13. 30% fraction of SM5 showing inhibition against the test strain *Klebsiella* sp.

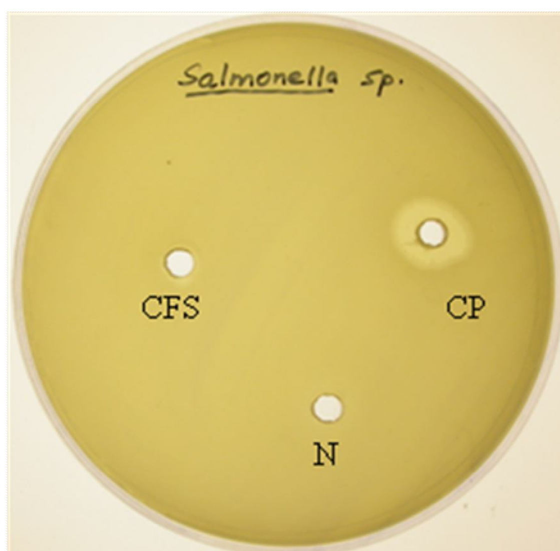


Plate 14. 30% fraction of SM5 showing inhibition against the test strain *Salmonella* sp.

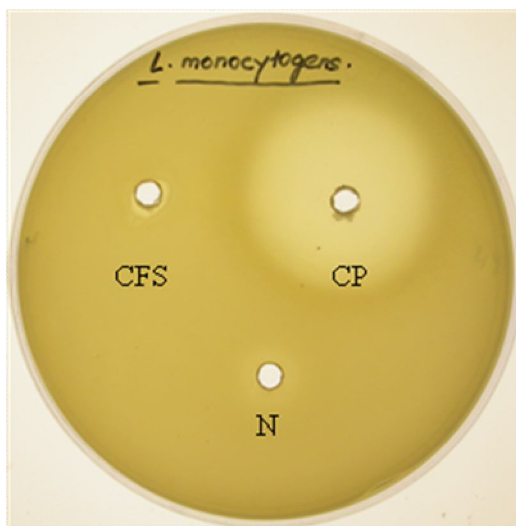


Plate 15. 30% fraction of SM5 showing inhibition against the test strain *L. monocytogens.*

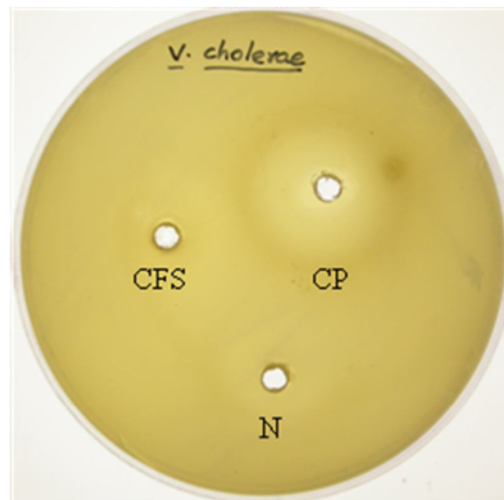


Plate 16. 30% fraction of SM5 showing inhibition against the test strain *V.cholerae.*

monocytogens with more than 25mm. The inhibitory zone of the test strain is given in Table 19.

4.7. Cell line assay

The effects of the 30% fraction were tested on HeLa cell line. The cells were exposed to the compound for 24h to look for inhibition of the HeLa cell line growth. There was nearly 100% inhibition of growth after the cells were exposed to the compound with a concentration of 6.58 mg/ml for 24h (Plate 17 & Plate 18).

4.8. Preliminary characterization of 30% fraction by TLC

The 30% fraction compound from AMP possessing strain SM5 was subjected to partial characterization. This sample was characterized with respect to thermal and pH stability and susceptibility to denaturation by enzymes. Crude dialyzed protein (600µl) was exposed to various heat treatments ranging from 40 to 100^0 C. Aliquot volumes of each fraction were then removed after 0, 30, 60 or 90 min and assayed for antimicrobial activity. The activity was observed up to 100^0 C. The protein subjected to pH 2, 4, 6, 8, 10, and 12 with Hydrochloric acid (HCl) and Sodium Hydroxide (NaOH) were incubated for 4 h at room temperature and similarly assayed. The compound showed resistance to pH variation and the activity was not lost completely. This indicates that the compound is resistant to wider pH. To study susceptibility of the antibacterial metabolite to proteolytic enzymes, the 30% fraction was treated with different enzymes Pepsin 50mg/ml and Trypsine 100mg/ml. Treated samples were tested by agar well diffusion method. The compound also showed resistance to enzymatic treatment.

4.9. Partial purification of 30% fraction by TLC

The 30% fraction was spotted on TLC and the chromatogram was developed by three different solvent mixtures. The n- butanol/acetic acid/water (60:15:25 by volume) solvent mixture showed good separation of the compound.

Table 19. Antimicrobial activity of the 30% fraction on test strain

Sl. no.	Test strain	Inhibitory zone (mm)
1	*Bacillus sp*	12
2	*S. aureus*	14
3	*Micrococcus luteus*	14
4	*L. monocytogens*	28
5	*Klebsiella sp*	18
6	*E. coli*	10
7	*Salmonella sp*	12
8	*E. tarda*	12
9	*V. harveyi*	22
10	*V. fisheri*	24
11	*V. parahaemolyticus*	23
12	*V. cholerae*	11

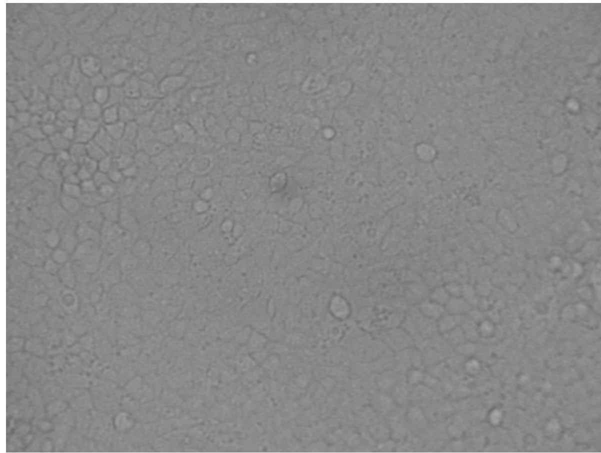


Plate 17. Normal cell line of HeLa cell line used as control.

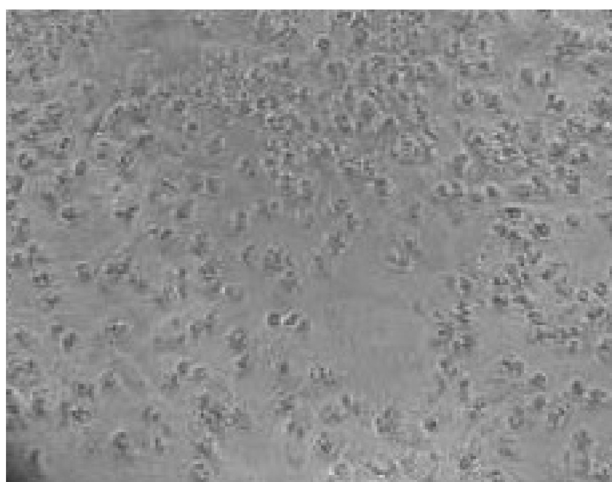


Plate 18. 30% fraction of SM5 showing inhibition against the HeLa cell line

About 6 well separated bands were observed in the chromatogram which was developed by 2% ninhydrin in acetone (Plate 19A). The separated protein/peptide fraction was scraped and tested for its activity (Plate 19B). The activity of the each fraction was tested by spot assay on the *Listeria monocytogens.* As the concentration of the active compound reduced during separation and quantification stages, only 5th fraction shows mild antibacterial activity (data not shown).

4.10. Extraction of the active compound by organic solvents

All the 28 AMP possessing isolates were studied for the extraction of the active compound by organic solvent. The isolates were grown in TSB broth and incubated for 72h at 30^{0}C. Among the 28 AMP possessing strains, strain SK9 showed maximum inhibition against indicator strains and was selected for further purification and partial characterization. The antimicrobial compounds that were produced in the culture media were extracted with different organic solvents. The solvents that were used in the study include Hexane, Dichloromethane, Chloroform and Ethyl acetate to determine the ideal solvent for extraction of the active fraction from the cultures supernatant. Extraction with solvents indicated that either Chloroform or Dichloromethane did not result in the complete extraction of inhibitory compound from the aqueous phase. Hexane also showed moderate extraction of the compound (Plate 20, 21 & 22). Ethyl acetate showed good extraction of inhibitory compound from aqueous phase and recovered from organic phase (Plate 23 & 24). The Ethyl acetate fraction was used for partial purification and characterization.

4.11. Partial purification of the crude ethyl acetate fraction by Thin Layer Chromatography (TLC)

Preliminary purification of the crude ethyl acetate fraction was conducted through TLC. A 0.2ml of crude ethyl acetate extract was spotted on TLC plate (20 by 20cm; 0.22 mm

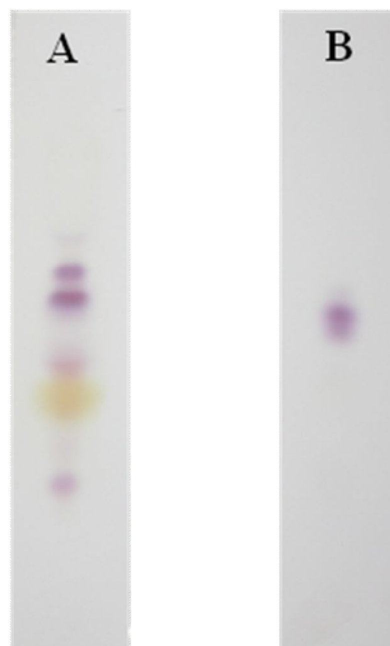


Plate 19. TLC showing the well separated bands (A) and the purified band (B) of the dialyzed protein produced by AMP possessing strain SM5.

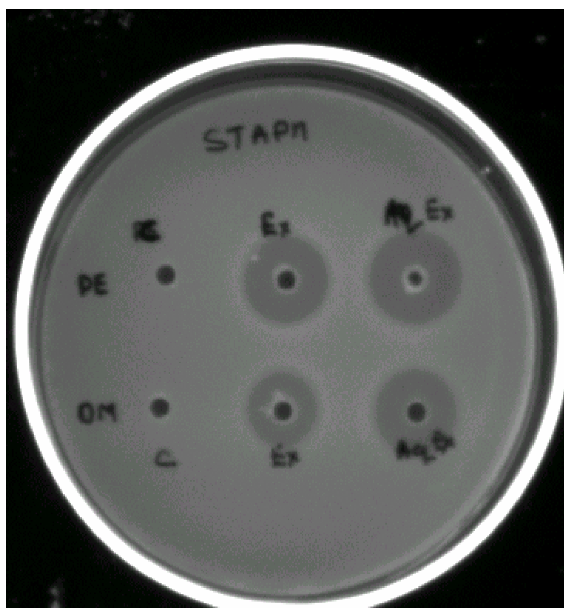


Plate 20. Antimicrobial activity of organic extracts on *S. aureus* .

Note:

PE: Petroleum ether

PE C : Control

Ex : Petroleum ether extracts

Aq Ex : Aqueous extract

DM: Dichloro methane

DM C : Control

Ex : Dichloro methane extracts

Aq Ex : Aqueous extract

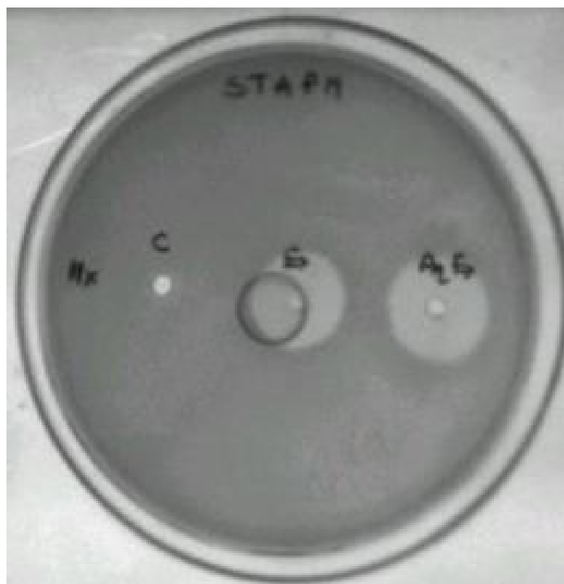


Plate 21. Antimicrobial activity of organic extracts on *S. aureus.*

Note:

HX: Hexane

HX C : Control

Ex : Hexane extracts

Aq Ex : Aqueous extract

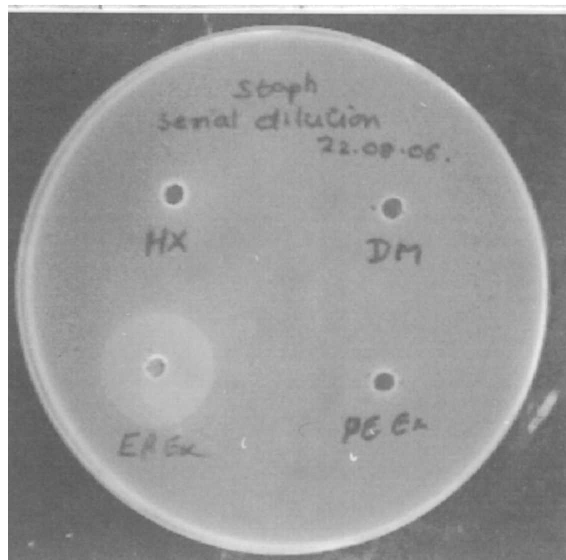


Plate 22. Antimicrobial activity of organic extracts on *S. aureus.*

Note:

HX: Hexane

HX C : Hexane extract

DM: Dichloromethane

DM Ex : Dichloromethane extract

EA: Ethyl acetate

EA Ex : Ethyl acetate extract

PE: Petroleum ether

PE Ex : Petroleum ether extract

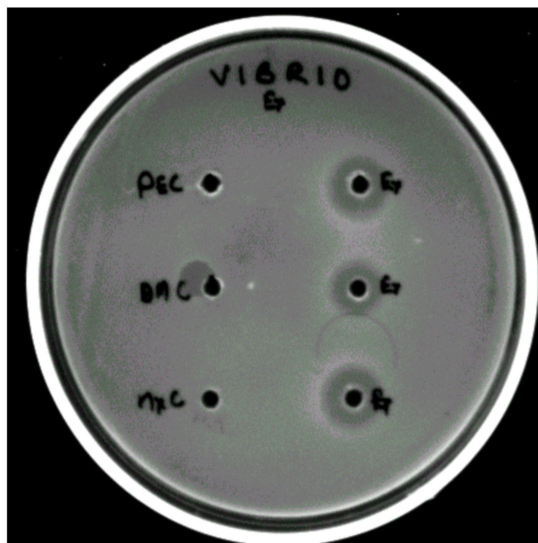


Plate 23. Antimicrobial activity of organic extracts on *Vibrio harveyi*.

Note:

HX: Hexane

PE C : Control

Ex : Petroleum ether extracts Aq Ex : Aqueous extract

HX: Hexane

DM C : Control

Ex : Dichloro methane extracts Aq Ex : Aqueous extract

Note: HX: Hexane

HX C : Control

Ex : Hexane extracts Aq Ex : Aqueous extract

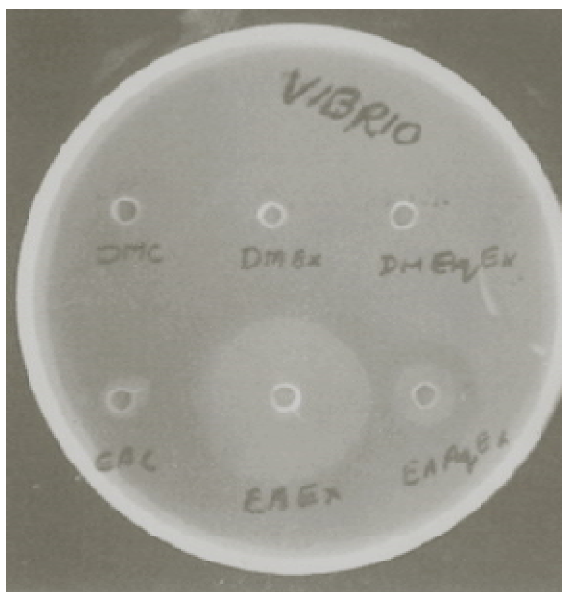


Plate 24. Antimicrobial activity of organic extracts on *Vibrio harveyi*.

Note:

DM: Dichloromethane

DM C : Control

DM Ex : Dichloromethane extracts

DM Aq Ex : Aqueous extract

EA: Ethyl acetate

EA C : Control

EAEx : Ethyl acetate extracts

EAAq Ex : Aqueous extract

thick) without any indicator. The chromatogram with good separation was obtained in solvent mixture ethyl acetate/methanol/water (100:2.5:2, by volume). When the TLC plates were observed under UV, eight bands were found. All the eight bands were marked individually and extracted separately from TLC plates by scraping silica gel of the Plate. The individual bands were then eluted with ethyl acetate. The elution was repeated twice for better extraction. The Silica residues were removed by centrifugation and supernatant was transferred into new eppendorf tubes. Each fraction was concentrated by evaporating off Ethyl acetate by vacuum evaporator. Each of these fractions was analyzed for antimicrobial activity by well diffusion method.

4.11.1. Bioassay to locate biologically active band

All the bands on TLC were scraped from the TLC plate and dissolved in 100µl ethyl acetate to confirm the active fractions. The antimicrobial activity of these eluted bands was tested by well diffusion assay against *Listeria monocytogens* and the activity could be detected from fraction 2 and 7. The activity was very less due to the poor concentrations of the eluted product (Plate 25). The bands 2 and 7 showed inhibition zones by the TLC overlay method when *Listeria monocytogens* was used as indicator strain was overlaid (Plate 26) and bands tested by 1% Ninhydrin in Acetone. There was no blue colour development indicating the absence of protein.

4.12. Antimicrobial assay

The crude ethyl acetate obtained from the AMP possessing isolate SK9 was subjected to antimicrobial assay. The compound was tested against both gram positive and gram negative bacteria. It showed strong activity against *Vibrio harveyi, Vibrio parahaemolyticus* and *Listeria monocytogens* (Plate 27A, C and D) with a MIC concentration of 20µg/ml. The mild activity was observed on *Salmonella sp* with a MIC of 80µg/ml (Plate 27B).

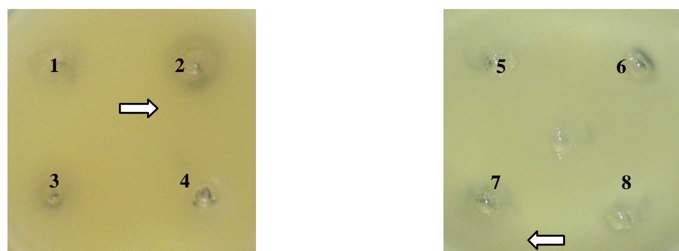


Plate 25. Bioassay of the compounds by TLC against *Staphyloccocus aureus.* (1-8: Fractions obtained from TLC plate; Arrow indicates inhibition mild zone at Fraction no. 2 and 7).

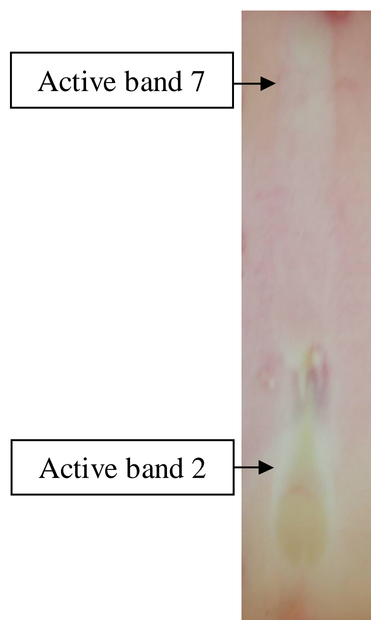
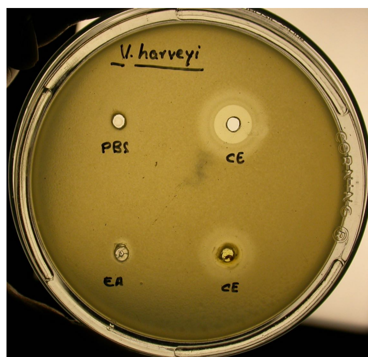


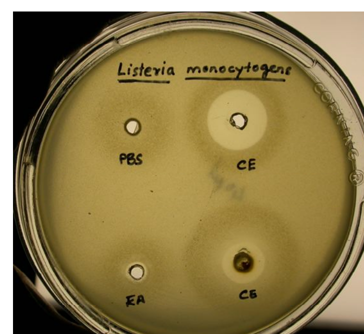
Plate 26. Bioassay of the partially purified active band (2 and 7) by TLC against *Staphyloccocus aureus.* (inhibition zone indicated by arrows).

A. *Vibrio parahaemolyticus*

B. *Salmonella sp*



C. *Vibrio harveyi*



D. *Listeria monocytogenes*

Plate 27 (A to D). Maximum antimicrobial activity of the crude ethyl acetate on different test strains

V. DISCUSSION

5.1. Isolation of positive isolates and Distribution of bacteria

The Oceans are not adequately explored for bioactive molecules from both micro and macro organisms. Most of the compounds that have been documented are derived from marine organisms and majority is from marine bacteria. Marine bacteria are widely distributed in the marine environment. These bacteria are found in the diverse environment like sediment (Russell, 1892 and 1893; Johnson, *et al.,* 1968), in association with various fauna and flora (Lemos, *et al.,* 1985; Burkholder, *et al.,* 1966; Andersen, *et al.,* 1974) and in inanimate objects (Davidson and Schumacher, 1993; Weyland and Helmke, 1988; Jensen *et al.,* 1996). They also have developed commensal and symbiotic relations with many marine vertebrates and invertebrates (Kaye, and Baross, 2004; DeLong, and Yayanos, 1985). Such diverse distribution of bacteria has emerged as a potential source of bioactive metabolites. Many such bioactive compounds have been isolated from bacteria over the last decades (Fenical, *et al.,* 1993).

Several pathogenic bacteria cause serious public health problem throughout the world. This includes multidrug resistant strains such as methicillin resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *Enterococcus*. Marine bacteria have been recognized as an important and untapped resource for novel bioactive compounds for long time which can be used to control such drug resistant bacteria. Hence greater emphasis on marine biotechnology is necessary to produce novel compounds that may contribute significantly towards new and novel drug development over the next decade (Kasanah & Hamann, 2004).

5.2. Screening for antimicrobial property possessing (AMP) marine bacteria

As can been seen from the results obtained from all the five sampling sites (Table 1 – Table 10), the total cultivable heterotrophic bacterial counts ranged between 10^2 – 10^5 cfu/ml and 10^5-10^8 cfu/g in the water and sediment samples analyzed respectively. Higher bacteria load in sediment could be due to colonization of bacteria of particulate matter and earlier workers have also observed a higher percentage of culturable bacteria in sediment compared to overlaying water (Jones, 1977). Nutrient availability around sediment particles might lead to higher proportion of bacteria being culturable. Jones *et al* (1977) reported that 0.255% of bacteria in sediment samples are culturable. However in case of water samples it is reported that only about 0.001 to 0.01% bacteria are culturable (Kogure *et al.,* 1979 and 1980; Furguson *et al.,* 1976). Total of about 240 samples were collected from all these sampling areas. In general total of 111277 isolates of bacteria were screened from all the five sampling regions and of these, 176 isolates which showed antimicrobial property was studied further. 128 (73 %) isolates with antimicrobial property were obtained from the sediment samples analyzed from all the sampling sites. However the water samples only yielded 48 (27%) isolates which showed inhibitory activity. There was marginal difference in the prevalence depending on sampling locations. On an average about 16.5% was obtained from Someshwar region, 24.3% from Surathkal region, 31.2% from Mangrove area, 9.65% Nethravathy estuary region and 18.8 % from Ullala region respectively. Higher prevalence in Mangrove area may be due to higher bacterial load in these areas. Among the screened bacteria only 28 (15.9%) strains exhibited maximum antimicrobial properties against the test strains *S. aureus* and *V. harveyi.*

5.3. Biochemical characterization of the bacterial isolates

All the 176 bacterial isolates were subjected to a battery of various biochemical tests and characterized to the Generic levels. Among the 176 bacteria characterized 117 (64.19%) were identified as gram positive and 59 (35.79%) as gram negative bacteria. There are reports that most of the cultivable microbes that are isolated from sediment and water samples belong to the genera of *Bacillus*, *Streptomyces* and *Pseudomonas* (Belma, 2002; Stabb, 1994).

Among the 117 gram positive bacteria characterized, 79 of them belong to the Genus *Bacillus*. Several reports indicate that the Gram-positive bacteria form a variety of taxonomic groups with the representatives of the genus *Bacillus* sp being the dominant group in the marine environment (Jensen, *et al.,* 1996). Similarly in this study most of the bacteria isolated belong to *Bacillus* sp. Further biochemical identification indicated that among the 117 gram positive 43 (24.43%) was unidentified *Bacillus* sp, 26(14.77%) were *Bacillis subtilis*, 24 (13.63%) were *Bacillus cereus* and 20 (11.36%) was *Bacillus pumilis* . Number of studies indicates *Bacillus* spp as source of antimicrobial compounds and these bacteria are used as probiotics in several systems including aquaculture (Verschuere *et al.,* 2000; Karunasagar *et al.,* 2005). Among the 59 gram negative bacteria 27 (15.34%) were identified as *Psuedomonas sp.* And the remaining 36 (20.45%) was identified as *Psuedomonas aeruginosa*. Several studies indicate that the genus *Pseudomonas sp* forms the major group among the other dominant among the gram negative bacteria which includes *Vibrio sp, Alteromonas sp, and Flavobacterium sp. Pseudomonas* sp have been found to be having antimicrobial activity and have been used as probiotic bacteria. Chythanya *et al.,* (2002) noted that a strain of Pseudomonas I-2 inhibited several shrimp pathogenic bacteria and suggested application of this strain in biocontrol of pathogens in aquaculture.

5.4. AMP possessing isolate with maximum antibacterial activity

Among the total 176 AMP possessing bacteria isolated only 28 (15.10 %) isolates produced significant inhibitory activity. Of these only 16 (57.14%) isolates showed inhibitory zone of more than 8mm. Other 12 (42.85%) isolates showed inhibitory activity with an inhibition zone between 5-8mm. Previous studies have shown that the antibiotic production is strain specific and the activity tends to decrease on successive transfer of the producing organism (Omura and Tanaka, 1986).

5.5. Optimization of the production of AMP.

Novel secondary metabolites like antibiotics are produced by bacteria under certain favorable growth conditions (Omura and Tanaka, 1986). The detailed mechanisms of initiation and regulation of antibiotic synthesis are not well understood. However many biochemical and physiochemical factors are known to influence the antibiotic production. Physiochemical factors such as pH, temperature, Oxygen tension and salt concentration play a significant role in the production of antibiotics from bacteria and are known to affect the antibiotic production (Higashide, 1984). Chythanya et al (2002) noted that production of anti-Vibrio factor by Pseudomonas I-2 was influenced by temperature, salt concentration and pH.

In this study, maximum antimicrobial activity was observed when the strains were cultivated as shaking cultures at pH 7.0, 30^0C and showed weak activity at pH7.0, 37^0C in shaking conditions, while no or very low activity was observed at 42 ^{0} C. In the present study among the 28 AMP isolates studied only the strains designated SM5, SK9, SK14, MG 8 and MG 12 showed inhibitory activity with an inhibition zone of more than 10mm (Table 11).

The antibiotic synthesis also follows growth associated kinetics of the bacterial strains in the medium in which it is propagated. Active antibiotic production often occurs in

association with sporulation of the producing organisms which begins with nutritional limitation in cultivation media. The antibiotic compounds are produced by the members of one or more families with many homolog and distribution of the components can also be easily varied by a minor modification of the cultivation conditions. To study the growth associated kinetics with AMP possessing isolates, they were subjected to various growth times starting from 0h to 72h. The AMP possessing isolates identified as *B. subtilis* and *B. cereus* sp showed maximum activity during incubation at 24 to 48h and much variation was not observed during further incubation time (Table 18). Only the AMP possessing strains SM5, SK 2, MG 2, MG 14 and UL 4 showed increase in their inhibitory activity when incubated up to 72h. The increase in antimicrobial activity was first detected in the exponential growth phase and increased rapidly until the end of the exponential growth phase, suggesting primary metabolite kinetics (De Vuyst *et al.,* 1996).

5.6. Extraction and concentration of the antimicrobial compound from culture supernatant

The genus *Bacillus* includes a variety of important species and some strains have a history of safe use in both industrial and food related application (Paik *et al.,* 1997). *Bacillus* is an interesting genus to investigate for activity as there are reports indicating that the *Bacillus* species produce a large number of peptide antibiotics which represent several different basic chemical structures (Mendo, 2004). There are several studies which have also reported that *Bacillus* produces polypeptide antibiotics, such as bacitracin, gramicidin S, polymyxin and tyrotricidin which are active against a wide range of Gram positive and Gram negative bacteria (Perez, *et al.,* 1992). A number of bacteriocins or bacteriocin-like substances (BLS) have been described for *Bacillus* spp., and most of them have been well characterized. Klein *et al* (1993) reported that *Bacillus subtilis* known to produce Subtilin which is a lantibiotic whose structure and mode of action are known to be similar to those

of nisin. Coagulin, an antilisterial bacteriocin belonging to the pediocin family (cystibiotic), is secreted by *Bacillus coagulans* I4 and its amino acid sequence shows homology to pediocin AcH/Pa1 produced by *Pediococcus acidilactici*. Other bacteriocinogenic strains of the genus *Bacillus* have been recently reported, such as tochin and thuricin 7 and 439, produced by *B. thuringiensis* strains (Ahern *et al.,* 2003; Paik *et al.,* 1997) and cereins from *B. cereus* strains (Bizani and Brandelli, 2002; Oscariz *et al.,* 2006).

In our study the antimicrobial compound from cell free supernatant of the AMP possessing strain SM 5 was extracted and about 820 mg was obtained from 30% ammonium sulphate saturation and this fraction showed promising antimicrobial activity. There are reports that some antimicrobial compounds like bacteriocins are known to precipitate at lower ammonium sulphate concentrations, or even in a small range of saturation (Klaenhammer, 1993). The 30% fraction was subjected to various pH, temperature and proteolytic enzyme studies. This was done to partially characterize some of the biochemical properties of the antimicrobial 30% fraction produced by the strain SM 5 strain used in this study. The 30% fraction showed resistance to pH, temperature, enzyme inactivation studies and the activity of the compound was found to be stable. The antimicrobial substance was stable from 40 to 80^0C and the activity remained even at 100^0C after 30 min of heat treatment. The residual activity could be detected in the range of pH 2-12 showing resistance to pH variation. However, the antimicrobial activity was also not inactivated by treatments with proteolytic enzymes trypsin and chemotrypsin. The failure of proteolytic enzymes to inactivate antimicrobial metabolites produced by *Bacillus* species, or other related bacteria have also been observed by previous studies (Rosado and Seldin 1993; Leddabi *et al.,* 1994; Potera 1994; von der Weid *et al.,* 2003). There are several reasons that account for the resistance to proteolytic enzymes observed. The presence of unusual amino acids in their structures or cyclic, N and or C- terminally

blocked peptides confers the resistance of the protein to proteolytic enzymes. Cyclic peptides can be resistant to hydrolysis by proteases because of their cyclic structure which renders them relatively inflexible and which may make the cleavage sites inaccessible due to their steric hindrance (Eckart, 1994). This could be a possible reason for the resistance of the crude dialyzed protein used in this study when exposed to proteolytic enzymes. The result obtained in this study supports that theory that the 30% fraction obtained from the strain SM 5 could be of protein in nature that could be a bacteriocin like compound. The stability of the 30% fraction was observed when subjected to heat treatments. Such observations are reported in case of heat stable class II non-lantibiotic small peptides described by Klaenhammer (1993). According to the SDS-PAGE analysis the active compound of the strain SM 5 showed a molecular weight of 3.5 kDa obtained from 30% ammonium sulphate saturation method. There are reports which indicate a bacteriocin-like inhibitory substance (BLIS) isolated from *Bacillus cereus* has a molecular mass of 3.4 kDa (Risoen, 2004). The BLIS was also very heat stable, and sensitive only to pronase E and proteinase K. Antimicrobial activity of this compound was stable in the pH range of 2.0–9.0, and relatively unaffected by organic chemicals. *Bacillus* spp producing bacteriocins like compound has a distinct diversity in their inhibitory activities and have been studied in different species including *B. subtilis* (Babasaki *et al.,* 1985; Zuber *et al*., 1993; Zheng and Slavic, 1999) and *B. licheniformis* (Cordovilla *et al.,* 1993; Leddabi *et al.,* 1994; Pattnaik *et al.,* 2001).

Partial purification of the 30% fraction was attempted by both gel filtration and TLC for separation and isolation of the active fraction in a suitable solvent mixture. However the activity of the compound was reduced to several folds and the concentration of the purified compound could not be measured. The compound obtained in this study thus needs further purification in order to better characterize the compound.

5.7. Antimicrobial assay of 30% fraction

In the present study 30% ammonium sulphate fraction showed large spectrum of inhibition activity. The fraction showed activity on gram negative bacteria such as *S. aureus, Bacillus sp, Micrococcus sp, E .coli, Salmonella sp, E. tarda* and *V. cholerae* with an inhibition zone of 10-15mm. The compound produced inhibition zone of more than 15mm on *Klebsiella* sp. However maximum inhibitory activity was observed on *V. harveyi, V. parahaemolyticus* and *V. fisheri* with inhibition zone of more than 20mm. The compound also showed strong inhibitory activity against *L. monocytogens* with more than 25mm. The variation among the inhibitory activity exhibited by the compound may be due to the absence of the receptors for the adsorption of the compound or due to some mechanism of antimicrobial resistance as reported earlier in case of pediocin AcH from *Pediococcus acidilacti* H (Bhunia, *et al.,* 1991). Most of the bacteriocin like compounds inhibits strains of closely related bacteria and some show a narrow spectrum of antimicrobial action. Only a few bacteriocins from gram-positive bacteria inhibit diverse groups of gram-positive bacteria, and very few inhibit gram-negative bacteria (Klaenhammer, 1988; Zheng and Slavic, 1999).

A very low activity was observed from the purified protein fraction obtained from TLC. Because of the inadequate concentration of the compound obtained from the TLC purification, the effective inhibitory concentration for the partially purified compound could not be obtained. The compound is subjected to further studies as it requires further purification with HPLC to obtain the active fraction in order to study the other associated properties of the compound in detail.

5.8. Cell assay

The 30% ammonium sulphate fraction was also observed to produce significant effect on the HeLa cell lines. This assay showed that the compound with a concentration of 6.58 mg/ml produced significant inhibition of cell line growth. There are studies concerned with anticancer compounds isolated from the genus *Bacillus* (Malkov, 2005). However, inhibition of cell cultures could be due to cytotoxins, which are known to be produced by several bacteria. Hemolysins produced by *Vibrio vulnificus*, *V. parahaemolyticus* and *Aeromonas hydrophila* are known to have cytotoxicity. Therefore, it is possible that the 30% ammonium sulphate fraction is a cytotoxin rather than being anti-cancer compound.

5.9. Extraction of crude inhibitory compound by organic solvents

The efficient recovery of antimicrobial compounds from bacterial sources is influenced by their stability, chemical and physical interactions with the sample matrix and the extraction solvent, and the handling of the sample before and during extraction. Thus the choice of solvent for extraction will depend on the solubility and charge properties of the antibiotics. A suitable method was adapted in this study by selecting appropriate solvents. Organic solvents with biocompatibility were tested for the optimum extraction of antimicrobial compound produced by the AMP possessing isolates. Among the AMP isolates tested, AMP possessing strain SK 9 identified as *Pseudomonas aeruginosa* showed interesting result with inhibitory activity of more than 20mm. The extraction of the antibiotics by organic solvents produced by fluorescent *Pseudomonas* spp. has been reported (Bonsall, 1997). In this study ethyl acetate was found to be the ideal solvent which extracted optimum antimicrobial compound from the culture extract. The antimicrobial compound was removed from the aqueous phase and recovered from the organic phase of the ethyl acetate. The use of ethyl acetate as extraction solvents has been reported by (Jayaswal, 1990) for the extraction of antifungal compounds from the strain of

Pseudomonas. Also the use of ethyl acetate as solvent for the extraction of metabolites such as phenazine, pyoluteorin, 2-4 diacetylphloroglucinol and polyketides produced by numerous strains of *Pseudomonas sp* have also been well reported (Bonsall, 1997; Rangaswamy, *et al.,* 1998).

The crude product appeared as dark brown oil and yielded about 150mg per 1000mL broth. The crude compound displayed a wide range of antimicrobial activity against Gram-positive and Gram-negative bacteria. Gram-positive pathogens like *S. aureus, Micrococcus luteus* and *Bacillus cereus* were inhibited by the crude extract with an inhibition zone of more than 20mm when assayed by well diffusion method. And the inhibitory concentration detected against these bacterial test strains was 40µg/ml. Gram negative bacteria including *Klebsiella pneumonia* and *V. fisheri* with an inhibition zone of more than 15mm with a concentration of 50µg/ml. Inhibitory activity was also observed on *Salmonella sp* and *E. coli* with an inhibition zone less than 10mm and the inhibitory concentration required to inhibit them was more than 80µg/ml. However the crude compound inhibited *L. monocytogen, V. parahemolyticus* and *V. harveyi* at 20µg/ml. Further purification of this fraction is necessary to identify the antimicrobial fraction.

5.10. Partial purification by thin layer chromatography

Thin layer chromatography (TLC) is a widely used method to fractionate antimicrobial compounds recovered from natural materials. Compounds can be separated by TLC with good resolution, and methods are readily adaptable for applications ranging from high throughput to preparative-scale work. In this study TLC was used as a method for both the evaluation of antimicrobial activity and subsequent isolation of the active fraction. Only two of the 10 fractions with an Rf value 0.16 and 0.82 exhibited activity. When an agar overlay assay was performed on the TLC it produced inhibitory zone at the band 1 and band 7 regions. This clearly indicates that there could be two compounds one which is

highly polar (band 1) and the other medium polar (band 7) in nature. These two fractions also exhibited inhibitory activity on the test strain.

Chromatogram sections were then observed for the presence of zones of inhibition. Since the product gets diluted when it is run on the TLC, clear zones could not be observed on any Chromatogram sections. However inhibition zones could be observed around the band 2 and 7. Due to the poor concentrations of the eluted product a weak inhibitory activity was observed. The compound has to be eluted in large quantities in order to obtain better activity. The purified fractions were treated with 1% Ninhydrin in Acetone. There was no blue colour development indicating the absence of protein. Inhibitory antibiotic compound by *Pseudomonas* has been reported in many studies (Fravel, 1988; Isnansetyo *et al.,* 2001). 2, 4- Diacetylphloroglucinol(DAPG) produced by *Pseudomonas* sp has drawn attention in the medical area because of its bacteriolytic activity against multidrug-resistant *S. aureus* (Bonsall, 1997). Bioactive compounds such as Micacocidin A, B, C (antimycoplasmal) and C-14, a cyclic dipeptide (chitinase inhibitor) have also been reported from marine *Pseudomonas* species (Kobayashi *et al.,* 1998; Izumida *et al.,* 1996). Anti-Vibrio compound produced by Pseudomonas I-2 was found to be chloroform soluble non-proteinaceous compound (Chythanya *et al.,* 2002). In addition to these other bioactive metabolites have been reported from marine *Pseudomonas* sp (Ponce *et al.,* 1999; Dobler *et al.,* 2002; Zeng *et al.,* 2005). In our study the compound could not be completely characterized due to the difficulty encountered in the separation and isolation of the active compound. Hence the compound extracted in our study needs further investigation to study the unique properties associated with the compound. The versatility, resolving capability and quantitative accuracy of the HPLC are the method of choice used in the most analytical studies of such antibiotics. Also the techniques such as mass spectrometry or

NMR are needed to further resolve mixtures of related compound and to provide insight into chemical structure.

VI. SUMMARY

Marine bacteria are emerging as a significant source of bioactive molecules in the recent years. But only a few studies have been conducted on marine bacteria, particularly from tropical environments. In this study, 24 samples each of water and sediment were collected from coastal areas in Surathkal, Someshwar, Ullala, from Nethravathy estuary, from Mangrove areas around Nethravathy river and subjected to bacteriological analysis using spread plate technique. Plates showing isolated colonies were replica plated to screen for production of antimicrobial compounds. Two indicator bacteria were used for screening: *Staphylococcus aureus* as a representative of gram positive bacteria and *Vibrio harveyi* as representative of gram negative bacteria. A total of 111, 2777 isolates were screened and 176 bacteria with antimicrobial property were isolated. Of these, 29 were from Someshwar, 43 from Surathkal, 32 from Ullala, 17 from Nethravathy estuary and 55 from mangrove area. These were subjected to a battery of biochemical tests for identification. 113/176 (64%) were identified as *Bacillus* spp and 63/176 (36%) as *Pseudomonas* spp.

Further screening of the 176 isolates based on zone of inhibition led to selection of 28 that showed significant activity. Of these only 16 (57.14%) isolates showed inhibitory zone of more than 8mm. Production of antimicrobial factor was optimum at 30°C and at NaCl concentration of 1-2%. All isolates of *Pseudomonas* showed inhibitory factor production at 24h, but in the case of *Bacillus*, maximum production was during 24-48h.

The strains SM 5 (*Bacillus* spp) and SK 9 (*Pseudomonas* spp) were studied in detail. The antimicrobial compounds produced by these inhibited many pathogenic bacteria and the activity was maximum against *L. monocytogens, V. fisheri* and *V.parahemolyticus* .Ammonium sulphate fractionation of culture supernatant revealed maximum activity in the 30% fraction. The dialyzed protein fraction extracted from the strain SM 5 also showed

promising inhibitory activity against HeLa cell line. In sodium dodicyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the 30% ammonium sulphate fraction showed two bands at 3.0 kDa and 14.3kDa. In the case of SK 9, the antimicrobial activity was extractable with ethyl acetate indicating different nature of compounds produced by these two strains.

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VIII ABSTRACT

Microorganisms have been recognized as an important resource for bioactive compounds. Majority of such compounds that are widely used are isolated from terrestrial sources. However, microorganisms isolated from marine sources are known to synthesize metabolites that are unique from the terrestrial counterparts. In recent years marine bacteria are becoming a major source for several novel bioactive compounds. In the present study a total of 1,11, 277 isolates were screened and about 176 bacteria with antimicrobial property were isolated. Among them 64% were identified as *Bacillus* spp and 36% as *Pseudomonas* spp. Further screening of these 176 isolates based on zone of inhibition against two indicator bacteria (*Staphylococcus aureus* as a representative of gram positive bacteria and *Vibrio harveyi* as representative of gram negative bacteria) led to selection of 28 that showed significant activity.

The antimicrobial compounds produced by the strains SM 5 (*Bacillus* spp) and SK 9 (*Pseudomonas* spp) inhibited many pathogenic bacteria and the activity was maximum against *L. monocytogens, V. fisheri* and *V.parahemolyticus*. Ammonium sulphate fractionation of culture supernatant revealed maximum activity in the 30% fraction. The dialyzed protein fraction extracted from the strain SM 5 also showed promising inhibitory activity against HeLa cell line. As the development of resistance to drugs by pathogenic bacteria is a major concern in the medical science in recent days, isolation of such antimicrobial compounds are very much essential. However the compound isolated in this study needs further study to characterize its unique chemical and antimicrobial properties.