# ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM MARINE MICROBES

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# ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM MARINE MICROBES

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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 MICROBES" submitted by Mr. VISHWAS M., for the degree of MASTER OF SCIENCE (AGRICULTURE) in MOLECULAR BIOLOGY AND BIOTECHNOLOGY, to the University of Agricultural Sciences, Dharwad, is a record of research work done by him during the period of his study in this University under my guidance and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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# 1. INTRODUCTION

Ocean is the mother of life. Most primitive forms of life originated from this “primordial soup”, covers 70 % of the earth surface. As life has originated from the ocean, there is a vast ecological, chemical, and biological diversification in the life of microorganisms to vertebrates. Thus, the rich biological and genetic diversity of marine organisms has been raised due to the harsh chemical and physical nature of marine environmental and adaptations to different habitats including stressful environments under cold, high pressure, and lightless conditions (Blunt *et al*., 2004).

The marine microbes are marginally explored domain, having a great potential. Presently, under exploitation of marine microorganisms have been investigated for bioactive metabolites. Among them, the marine bacteria, actinomycetes (Bull and Stach, 2007; Fenical and Jensen, 2006) and fungi (Bhadury *et al*., 2006; Newman and Hill, 2006), are attracting attention as new microbial resource and could serve as potential source to isolate bioactive compounds. Microbes can sense, adopt and respond to their environment quickly and can compete for defence and survival by the generation of unique secondary metabolites. These compounds are produced in response to stress and many have shown value in biotechnological applications. The marine environment was once thought to have high salt, poor nutrition and less microbial growth. On the contrary, soil microbes are widely regarded to live in much more crowded and competitive environment. The ecology of marine natural products actually reveals that many of these compounds are chemical weapons and have evolved into highly potent inhibitors of physiological processes in prey predators or competitors of the marine organisms that utilize them for survival (Venter *et al.,* 2004).

The marine environment is extremely complex and contains a huge diversity of life forms. Culture-independent methods have demonstrated that the marine sediments contain a wide range of unique microorganisms not present in the terrestrial environment. Surprisingly, a large number of species with high diversity survive under such conditions and produce fascinating and structurally complex natural products (Magarvey *et al*., 2004 and Kin, 2006). They produce variety of compounds with unique structural and chemical properties showing various biological activities, and hence, they feature a great potential to pharmaceutical industry and also in Agricultural crops (Blunt *et al*., 2004).

Marine microbial secondary metabolites include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immune modulating agents, receptors antagonistic and agonists, pesticides, antitumor agents and growth promoters of animals and plants. They have a major effect on the health, nutrition and economy of our society (Davatia *et al*., 2013). Nature produces an amazing variety and number of products. About 1,00,000 secondary metabolites of molecular weight less than 2500 have been characterized, mainly produced by microbes and plants (Roessner and Scott, 1996); some 50,000 are from microorganisms (Fenical and Jensen, 1993; Berdy, 1995). Out of the 12,000 known antibiotics in 1995, about 55 % have been produced by filamentous bacteria (Actinomycetes) of the genus Streptomyces, 11 % from other Actinomycetes, 12 % from non filamentous bacteria and 22 % from filamentous fungi (Berdy, 1995; Strohl, 1997).

Among microbial resources, large group of marine Actinomycetes have been able to synthesize wide varieties of biologically active secondary metabolites such as antibiotics, bio-pesticides and anti-parasitic compounds and enzymes like cellulase, xylanase, proteinase and chitinase. The Actinomycetes have produced 70 to 80 % of bioactive secondary metabolites, among which approximately 60 % of antibiotics were developed for agricultural purpose which were mainly isolated from *Streptomyces spp*. (Sowndhararajan *et al.,* 2012). Bacteria are known to produce bioactive substances in the marine environment, predominantly protection themselves from their predators. Marine bacterial resources have become important in the study of novel microbial products exhibiting antibacterial, antiviral, antitumor as well as anticoagulant and cardio active properties (Devi *et al*., 2011). Fungi are providing plentiful and diverse source of unique and often bioactive metabolites, and they have produced a number of medicinally important compounds, including penicillin, mevinolin (Lovastatin) (Gloer, 2007), fingolimod (Strader *et al*., 2011), and caspofungin (Keating and Figgitt, 2003).

The search for new and active compounds from microbial sources is a pursuit for many natural products laboratories. Typically, these efforts will employ a standard culture procedure that most or all microbial strains pass through as a preliminary step to the natural products discovery process. Since the discovery of penicillin in 1928 (Fleming, 1929), intensive studies, mainly on soil-derived bacteria and fungi which has demonstrated that the microorganisms are a rich source of structurally unique bioactive substances (Fenical, 1993). The increasing need for new antimicrobial agents able to control emerging diseases or resistant strains of microorganisms inspired a growing number of research groups to explore the oceans for new bioactive compounds. Throughout the years, extensive screening programs were developed worldwide and great efforts have been devoted aiming of the isolation of new metabolites from marine microorganisms.

Identification of diversity amongst microbes and also the hotspots of diversity in terms of niches they are present in, it is logical to hypothesize that more novel metabolites await discovery. Characterization of unknown compounds for its biological activity involves separation, purification and identification of the active compounds from the secondary metabolites produced by the microbes. It is complicated, labour intensive and time consuming task which is further complicated by failure to separate and identify the bioactive compounds. Recently, the progress in the robotic and automated methods in chromatographic isolation methods (LC-MS, GC-MS, LC-MS-ELSD, HPLC-UV-VIS, HPLC-ELSD, HPLC-PDA (Photo array detectors), HPCCC, CZE etc. and spectroscopic identification techniques (multi-dimensional NRM, X-Ray crystallography, NOESY, electro spray MS, HR-MS) are more or less driving high through-put screening and discovery (Berdy, 2005).

Most of the secondary metabolites produced by these microbes are chemically either polyketide or non-ribosomal peptide (Llerena *et al*., 2007; Ruiz *et al.,* 2010).Non-ribosomal peptide synthetases (NRPS) and type I polyketide synthases (PKS-I) are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds produced by microorganisms. A broad range of biologically active polyketide and peptide compounds with applications in medicine, agriculture, and biochemical research are synthesized by type-I polyketide synthases (PKS-I) and non-ribosomal peptide synthetases (NRPS). These are structurally diverse metabolites include among

others antibiotics (e.g., penicillins, vancomycin, and erythromycin), antifungal (e.g., nystatin), antitumor agents (e.g., ansamitocin, bleomycinsbleomycins), anthelmintics (e.g., avermectin) and immunosuppressive agents (e.g., rapamycin and FK506). PKS-I and NRPS biosynthetic systems have been extensively describe not only in Actinomycetes but also in myxobacteria (Beyer *et al*., 1999) and cyanobacteria (Christiansen *et al*., 2001) and among other bacterial taxa and in filamentous fungi (Bingle *et al*., 1999*;* Nicholson *et al*., 2001).

In the Department of Biotechnology, University of Agricultural Sciences, Dharwad, significant progress has been made with respect to the isolation of marine microbes from different aquatic environments and screening for antimicrobial activity. In continuation of the previous work and considering the above circumstances potent isolates with great potential for antimicrobial property were selected for the investigation. The present study will be focus on isolation, extraction and biochemical characterization of potent bioactive compounds from marine microbes. Therefore, the following objectives have been investigated:

1. Isolation and extraction of bioactive compounds from marine microbes.
2. Biochemical characterization of potent bioactive compounds.
3. Molecular characterization of potent isolates for the genes encoding bioactive compounds.

# 2. REVIEW OF LITERATURE

The marine microbes are a marginally explored domain, having a great potential. The marine environment is extremely complex and contains a huge diversity of life forms. Culture-independent methods have demonstrated that marine sediments contain a wide range of unique microorganisms not present in the terrestrial environment. Surprisingly, a large number of species with high diversity survive under such conditions and produce fascinating and structurally complex natural products (Magarvey *et al*., 2004 and Kin, 2006). The search for new and active compounds from microbial sources is a pursuit for many natural products laboratories. Characterization of unknown compounds for its biological activity involves Separation, purification and identification of the active compounds from the secondary metabolites produced by the microbes (Berdy, 2005).

A review of scientific literature which deals with reports on isolation of bioactive compounds from marine microbes, antiphytopathogenic activity of bioactive compounds and biochemical and molecular characterization of bioactive compounds are reviewed in this chapter under following headings: Marine microbes, Biological factores affecting agricultutral productivity, Biological control of plant pathogens, Biological control of plant pathogens by marine microbes, production extraction and screening of bioactive compounds producing isolates against pathogens, bioactive compounds from marine microbes, origin activity of some bioactive compounds from marine microbes, biochemical characterization of bioactive compounds and molecular identification of marine microbes.

## 2.1 Marine microbes

Life on Earth most likely originated as microorganisms in the sea. Over the past ~3.5 billion years, microorganisms have shaped and defined Earth's biosphere and have created conditions that have allowed the evolution of microorganisms and complex biological communities, including human societies (Karl, 2007). The world ocean with a coastline of 312,000 km (193,000 miles) and a volume of 137 x $10^6$ $km^3$ is the largest ecosystem on earth. Because of its large volume and vast area, influence of the world ocean on world climate is profound (Das *et al*., 2006).

Microorganisms occur nearly everywhere in nature and occupy an important place in human view of life (Das *et al*., 2006). Microbiology in marine ecosystem analysis is viewed from extremely different positions. Combined descriptive and experimental approaches have elucidated key functions of microorganisms that challenge the traditional, microorganisms dominated concepts in ecosystem ecology (Reichardt, 1995).

Generally, the prokaryotes occur in the open ocean, soil, oceanic and terrestrial sub surfaces, whose the numbers of cells are 1.2 $X10^{29}$, 2.6 $X10^{29}$, 3.5 X $10^{30}$ and 0.25–2.5 X $10^{30}$ respectively. The numbers of heterotrophic prokaryotes in the upper 200 m of the open ocean, the ocean below 200 m and soil are consistent with average turnover times of 6–25 days, 0.8yr and 2.5yr respectively. The large population size and rapid growth of prokaryotes provides an enormous capacity for genetic diversity (Whitman *et al.,* 1998).

Numerically, microbial species dominate the oceans, yet their population dynamics, metabolic complexity and synergistic interactions remain largely uncharted. The understanding of life in the ocean requires more than knowledge of marine microbial taxa and their genome sequence (DeLong,

2009). The microbes rely on mutation and the processes of horizontal gene transfer (HGT; conjugation, transformation, and transduction) to acquire new traits (McDaniel *et al*., 2012). Recent advances in technology have been highlighted the vast and previously unknown genetic information that contained in present marine microorganisms from new protein families to novel metabolic processes (Karl, 2007).

Marine organisms represent a valuable source of new compounds. The biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of new active substances in the field of the development of bioactive products (Aneiros, 2003).Marine microbes represent a potential source for commercially important bioactive compounds and their bioremediation capabilities are also remarkable (Das *et al.,* 2006).

### 2.1.1 Marine actinomycetes

Marine actinomycetes are aerobic, Gram positive bacteria with branched filaments. It is the transitional group between simple bacteria and fungi with majority of isolates being streptomycetes (Williams, and Cross, 1971). These organisms are classified as bacteria in strict sense, all being Schizomycetes of order Actinomycetals. They produce slender and branched filaments or hyphae producing asexual spores and can even produce inciting bodies. They are diverse and widespread in producing varieties of pigments in different media (Alexander, 1961). Sediments are the best sources for marine Actinomycetes and their distribution varies depending on the depth from which the samples were collected. They are termed saprophytic and known to turnover biopolymers such as lignocelluloses, hemicelluloses, pectin, keratin, chitin and nitrogen fixing Actinomycetes of the genus (Dhevendaran, 2008) need recent articles on molecular based characterization.

### 2.1.2 Marine bacteria

The bacteria are a ubiquitous class which colonizes any habitat of the planet having the great active biomass than any other group of organisms. They are also the most metabolically diverse group that obtain energy from oxidizing organic carbon, parasitism, chemoautotrophy and photoautotrophy (Alexopoulos *et al.,* 2009). Most of the marine bacteria are halophilic, *i.e.* they need sodium chloride for optimal growth and development. Majority of marine bacteria are Gram negative and their cell wall is better adapted for survival in marine environment. On the other hand sediment contains heavy load of Gram positive bacteria especially *Bacillus* (Dhevendaran, 1984). Marine bacteria can grow even at temperature of 0-4$^0$C and are the deep sea psychrophilic bacteria (Certes, 1884).

### 2.1.3 Marine fungi

Though the existence of fungi in aquatic habitat is known from early times, their significance has only been overlooked. Marine fungi comprise of an estimated 1500 species, excluding those that form lichens (Hyde *et al.,* 1998). This number is low compared to the number of described and estimated terrestrial fungi (over 250,000). So far, less than 500 filamentous higher marine fungi have been described and only 79 are associated with algae as parasites or symbionts, and 18 with animal hosts (Tarman *et al.,* 2011). The representatives of all four class *viz*., Myxomycetes, Ascomycetes, Phycomycetes and Basidomycetes were found in sea water and some of them require NaCl for their growth. Phycomycetes are lower fungi and aquatic in habitat. They are unicellular, able to produce

myceta through without septa. A few forms have no cell wall with either chitin or cellulose. Deep-sea fungi that inhabit the sea and the sediment at depths of over 1000 m below the surface, have become an important source of industrial, agricultural and nutraceutical compounds based on their diversities in both structure and function (Wang *et al.,* 2015).

## 2.2 Biological factors affecting agricultural productivity

Crop production is an important source of food and is expected to play a key role in meeting the needs of the world population (Hoisington *et al*., 1999). Biotic stresses, mainly represented by pests and diseases, constitute the single greatest threat to crop production. These include many thousands of species and types of fungi, insects, bacteria, viruses, nematodes and other organisms (Haggag *et al.,* 2015). Losses to pests and diseases are a serious limitation to the productivity of farming with 50 % of all crops in some cases being lost to pre and post harvest pests (Jonathan, 2002).

Pathogenic microorganisms affecting plant health are major and chronic threat to food production and ecosystem stability worldwide (Compant *et al*., 2005). Identification of the cause of disease is the most important step towards its eradication, cure and management. India being an agro-based country, plant diseases alone cause immense economic loss to the tune of rupees 500 crores every year (Sarkar and Chaudhuri, 2016). Diseases caused by viruses, bacteria, and fungi as well as physiological disorders lead to serious losses in agricultural productivity in world over (Jonathan, 2002; Cook, 2000).

Modern farming practices, with their reliance on agrochemical pest and disease control, are responsible for considerable pollution and can have harmful effects on human health. Pests control strategies in crops included the fungicides and other cultural control measures, particularly against airborne fungi pathogens, are continually eroded in their effectiveness by adaptations in the pathogen. Resistance against pests and stress is one of the key factors for plant varieties used in production systems (Haggag *et al*., 2015). Plant diseases need to be controlled so as to enhance the quality and abundance of food, feed and fiber production around the world. The current practice of the use of the agrochemicals/chemical fungicides in agriculture has led to human health and environmental problems (Vijayakumar *et al.,* 2012). Small-scale farmers sometimes lack access to the technologies they need to control plant disease problems. In some cases, reliance on heavy overuse of broad-spectrum chemical pesticides is a major cause of ill health due to toxicity, environmental damage as well as high residues on food crops (Elizabeth and Handelsman, 1998). The challenge is to seek alternatives to supplement existing control strategies, and to improve crop management systems so that chemical pesticides are used only where necessary and in an effective and safe manner (Benbrook *et al*., 1996).

### 2.2.1 Plant fungal pathogens

Plant diseases caused by plant pathogenic fungi which continuously threaten the sustainability of global crop production (Elizabeth and Handelsman, 1998). Fungal diseases are common and cause significant economic loss of crops and ornamental plants (Ligon *et al*., 2000). Soil-borne plant pathogenic fungi are of major concern in agriculture which affects yield and quality of agricultural products (Farah and Nasreen, 2016).The capacity to cause plant disease is, however,

clearly a complex phenotype, and phytopathogenic fungi exhibit considerable diversity both in their developmental biology and in the types of disease symptoms they induce (Agrios, 1988; Bowyer 1999). Pathogenic fungi have diverse growth lifestyles that support fungal colonization on plants. Successful colonization and infection for all lifestyles depends upon the ability to modify living host plants to sequester the necessary nutrients required for growth and reproduction (Selin *et al*., 2016).

2.2.1.1 Colletotrichum sp.

*Colletotrichum* is one of the most important plant pathogens worldwide causing the economically important disease anthracnose in a wide range of hosts including cereals, legumes, vegetables, perennial crops and tree fruits (Bailey and Jeger, 1992). *Colletotrichum gloeosporioides* is an anthracnose causing fungal pathogen of various fruits and vegetables and causes both pre and post harvest losses to crops leading to huge losses to farmers (Sharma and Kulshrestha, 2015). This pathogen infects about 470 different host genera and which also causes post-harvest problems (Prusky and Plumbley, 1992).

*C. gloeosporioides* is most important pathogen and belongs to order melanconiales, which follows the hemibiotrophic mode of infection where biotrophic and necrotrophic phases are sequentially occur. In biotrophic phase, infection vesicles and primary hyphae are formed and secondary hyphae developed and spread to kill the host cell in necrotrophic phase. The pathogen produced lesions on leaves, fruit and other parts of plant, finally the lesions become dark and form concentric ring pattern (Sharma and Kulshrestha, 2015).

*C. gloeosporioides* requires optimum temperature of 25-28℃, pH range of 5.8-6.5 and high humidity for better growth. This pathogen is inactive in dry season and switches to active stages when encountered favourable environmental conditions (Sharma and Kulshrestha, 2015).

2.2.1.2 *Fusarium oxysporum*

*Fusarium oxysporum* belonging to phylum Ascomycota comprises of a group of soil inhabitants, a fungus with no known sexual stage. They exist as saprophytes in the soil debris and also as pervasive plant endophytes (Garrett, 1970). *F oxysporum* is a saprophytic fungus occurring in the rhizosphere of many plant species. The pathogen has a broad range of host species but host specialization of isolates is more circumscribed. It is widespread and leads to substantial yield losses in both greenhouse as well as soil production systems (Szczechura *et al*., 2013). Fusarium wilt is fungal disease that attacks potato, tomato, eggplant and pepper which enter through roots and interfere with the water conducting vessels of the plant. As the infection spreads up into the stems and leaves it restricts water flow causing the foliage to wilt and turn yellow (Poozad and Kariminik, 2015).

The strains of *F. oxysporum* causing wilt diseases are accountable for severe destruction to economically important plant species. *Fusarium* wilts are mainly managed through fumigation of soil with the help of chemicals or by the usage of resistant varieties. Chemicals such as methyl bromide with broad spectrum activity are used to fumigate the soil before planting. Unfortunately, these are environmentally detrimental and difficulties in controlling *Fusarium* wilt have encouraged research in biocontrol of this phytopathogen (Fravel *et al*., 2003).

2.2.1.3 *Sclerotium rolfsii*

*Sclerotium rolfsii* is soil borne plant pathogen causing root rot, stem rot, collar rot, wilt and foot rot diseases on more than 500 plant species of agricultural and horticultural crops throughout the world and also great economic loss on various crops. It has been reported that the *S. rolfsii* caused about 25 % seedling mortality in the groundnut cultivar JL-24. In tomato, it was responsible for crop loss of 30 % (Muthukumar *et al*., 2013). The crops that are mostly affected by *S. rofsii* include soybean, peanut, sugar beet, pepper, tomato and potato while sorghum, wheat, rice, lentil, betel vine, alfalfa, cotton, sugarcane, tobacco, sunhemp, sunflower, chrysanthemum, gladiolus and other ornamental species are less affected (Ansari, 2005).

Control of *Sclerotium* stem rot, may be achieved by applying tremendous volume of fungicides every alternative day during the crop season. However, the problems regarding the efficacy of the chemicals and fungicide residues are of increasing concern and need to be solved, as it has direct influence on human health and the environment. Biological control of phytopathogens by microorganisms has been considered to be more natural and an environmentally acceptable alternative to the existing chemical treatment methods (Baker and Paulitz, 1996).

### 2.2.2 Plant bacterial pathogens

Plant diseases caused by bacteria are major economic liability to agricultural production and its control has been major challenge for many bacterial diseases (Civerolo, 1982). This challenge is direct result of pathogen variability, high probability for mutation or gene transfer in the pathogen when confronted with resistance genes or bactericides, high pathogen multiplication rate during optimal conditions for disease development and lack of adequate chemical-based approaches for control (Norelli *et al*., 1991).

2.2.2.1 *Ralstonia solanacearum*

*Ralstonia solanacearum* is an aerobic non-sporing, gram-negative plant pathogenic bacterium, which causes the wilts of tomato, pepper, eggplant and Irish potato. It colonizes the xylem, causing bacterial wilt in very wide range of potential host plants (Denny, 2006). *R. Solanacearum* is one of the devastating and a widespread disease that can affect more than 450 plant species in 54 families and occurred in tropical, subtropical, and warm temperate regions of the world (Melanie *et al.,* 2007). Management of wilt mainly briefs the methods adopted against *R. solanacearum*. The management of the disease by use of antagonistic bacteria and fungi has been found to be the recent trend than physical and chemical methods (Sarkar and Chaudhuri, 2016).

## 2.3 Biological control of plant pathogens

Plant diseases have been controlled using various strategies. Cultural methods of control include crop rotation, intercropping and use of clean planting materials. Though many of the cultural practices are labour intensive, they have less adverse effects on the environment and are readily available without extra investment on equipment (Elizabeth and Handelsman, 1998).

Disease control is best achieved using an integrated management approach by combining proper cultural practices, chemicals such as bactericides or plant activators where applicable, introgression of plant resistance genes, and biological control strategies (Obradovic *et al*., 2005).

To overcome from the harmful and hazardous impacts of agrochemicals on global ecosystems have made scientists think seriously about alternative strategies, to make a safer and acceptable environment. In this context, the biological control methods have become a viable alternative then the chemical control methods. The biocontrol agents are persistent and minimize the deleterious side effects caused by synthetic fungicides. In modern agriculture, microbe-based biocontrol agents, as a replacement or supplement for agrochemicals among the microbes, actinobacteria could be the attractive candidate for biological control agents against plant pathogens (Vijayakumar *et al*., 2012).

Biological control, a component of integrated pest management (IPM) strategy, involves control of pests and diseases by natural enemies and antagonists respectively. Antagonists of plant diseases, also known as biological control agents, include inhibitors, competitors and pathogens (Fatope *et al*., 2000). Biological control of plant disease can occur through different mechanisms, which are generally classified as; antibiosis, competition, suppression, direct parasitism, induced resistance, hypo-virulence and predation. The antagonistic activity has often been associated with production of secondary metabolites (Silva *et al*., 2001).

Natural and agricultural ecosystems harbour a wide variety of microorganisms that play an integral role in plant health, crop productivity, and preservation of multiple ecosystem functions (Duffy *et al*., 2003). In order to achieve an increase in agricultural productivity in a sustainable manner, there will be an increased reliance on manipulation of microorganisms that are beneficial to soil and plant health (Conn and Franco, 2004). Interactions within and among microbial communities are numerous and range from synergistic and mutualistic to antagonistic and parasitic. Antagonistic and parasitic interactions have been exploited in the area of biological control of plant pathogenic microorganisms. Biocontrol is typically viewed from the perspective of how antagonists affect pathogens (Duffy *et al*., 2003).

The future of sustainable agriculture will increasingly rely on the integration of biotechnology with traditional agricultural practices. Most sustainable and environmentally acceptable control may be achieved using bio-control agents due to the effort to reduce the use of agrochemicals and their residues in the environment and in food. Identifying, understanding and utilizing microorganisms or microbial products to control of plant diseases and to enhance crop production are integral parts of sustainable agriculture. Biological control has the potential to control crop diseases while causing no or minimal detrimental environmental impact (Haggag and Mohamed, 2007).

## 2.4 Biological control of plant pathogens by marine microbes

Marine actinobacteria are the most economically as well as biotechnologically valuable prokaryotes. Many representatives of the order Actinomycetes are prolific producers of thousands of biologically active secondary metabolites. With increasing advancement in science and technology, there would be greater demands in future for new bioactive compounds synthesized by actinobacteria from various marine sources (Manivasagan *et al*., 2013).

More than 22,000 known microbial secondary metabolites, 70 % of which are produced by Actinomycetes, 20 % from fungi, 7 % from Bacillus spp. and 1–2 % by other bacteria. Among the Actinomycetes, streptomycetes group are considered economically important because out of the

approximately more than 10,000 known antibiotics, 50–55 % are produced by this genus (Subramani and Aalbersberg, 2012). The known actinomycetes produce 70 % to 80 % of bioactive secondary metabolites, where approximately 60 % of antibiotics developed for agricultural use were isolated from *Streptomyces* spp (Sowndhararajan and Kang, 2012). *Streptomyces spectabilis* showed very effective in producing bioactive metabolites against the fungi *S. rolfsii*. The culture filtrate of *S. spectabilis* was extracted using various solvents (chloroform, n-butanol, diethyl ether, ethyl acetate, and n-hexane). The n-butanol extract showed the highest activity against the test organisms, with a minimum inhibitory concentration (MIC) of 0.781 to 6.250 mg/ml (Khamna *et al*., 2009).

A study on the enzyme activities and antibacterial potential of Actinomycetes isolated from the sediments obtained from different locations of Bay of Bengal, India has been carried out. Screening for antibacterial activity revealed that 10 isolates exhibited antibacterial activity. The marine Actinomycete isolates exhibited more antimicrobial activity against gram positive bacteria than the gram negative bacteria (Sirisha *et al*., 2013). The morphological, biochemical and physiological characters of the isolate conformed to the characteristics of the genus *Actinopolyspora,* which contained three species, *viz*. *A. halophila, A. Mortivallis* and *A. iraqiensis.* It showed good antibacterial activity against *Staphylococcus aureus, S. epidermidis*, *Bacillus subtilis* and antifungal activities against *Aspergillus niger, A. fumigatus*, *A. flavus*, *Fusarium oxysporum*, *Penicillium* species and *Trichoderma* species (Kokare *et al*., 2004).

The use of microorganisms for biological purposes has become an effective alternative to control plant pathogens. There are many examples of formulations using bacterial or fungal strains with biocontrol applications (Bernal *et al*., 2002). Marine bacteria and fungi are of considerable importance as new promising sources of a huge number of biologically active products. Some of these marine species live in a stressful habitat, under cold, lightless and high pressure conditions. Surprisingly, a large number of species with high diversity survive under such conditions and produce fascinating and structurally complex natural products. Yet a huge number of active substances with some of them featuring unique structural skeletons have been isolated (Debbab *et al*., 2010).

These marine bacteria were expected to be potential resources of natural antibiotic products. It can be concluded that isolation of Marine bacterial samples can offer a numbers of microbial strains for sources of new biomolecules from Marine sources (Dhinakaran *et al*., 2012). Antibiotics are metabolic by-products of complex biosynthetic pathways in microorganisms. They are usually produced by aerobic spore-forming bacteria in the genera *Bacillus* and *Streptomyces* and in the Fungi *Penicillium* and *Cephalosporium*. Antimicrobials such as bacteriocins are inhibitory peptides or proteins, which have bactericidal effects on microorganisms (Okulate, 2009).

Marine fungi are known to produce structurally unique secondary metabolites, and more than 1000 marine fungal-derived metabolites have already been reported (Gomes *et al*., 2015). Deep-sea fungi that inhabit the sea and the sediment at depths of over 1000 m below the surface, have become an important source of industrial, agricultural, and nutraceutical compounds based on their diversities in both structure and function. These include compounds with antimicrobial, antifungal, antiprotozoal, and antiviral activities (Wang *et al*., 2015).

## 2.5 Production, extraction and screening of bioactive compounds producing isolates against pathogens

There has been growing interest in bioactive natural products and the molecular basis of their activity (Fatope *et al*., 2000). Three technologies are involved for sourcing bioactive compounds such as separation method, structural elucidation and bioassay methods (Ligon *et al*., 1999; Fatope, 1995). Bioassay technology for finding bioactive compounds has undergone major changes during the past decade. These methods can be grouped into two: the *in vivo* and *in vitro* methods. The *in vivo* method involves testing the efficacy of the metabolite or product on target organisms while the *in vitro* method is a mode of action or mechanism-based approach that involves testing in the laboratory set up of the compounds on the target pest or pathogen (Fatope *et al*., 2000). The adoption of bioassay methods, which correlate with prevention, inhibition and reversal of diseases or elimination of pests in screening natural products for bioactive compounds, is critical to the discovery of new bio-pesticides (Fatope *et al*., 2000).

Secondary metabolite production may be either intercellular or extracellular. In case of extracellular production secondary metabolites are released into the medium. But this is not the case in intercellular production, where the metabolites are released inside the cell. The conditions favourable for efficient extraction of the metabolites are strain specific. Not every strain produced metabolites efficiently under same condition. Optimisation with regards to the composition of culture medium use, number of days of incubation and the method of extraction should be carried out (Khan, 2011). The formation of secondary metabolites is regulated by nutrients, growth rate, feedback control, enzyme inactivation and enzyme induction. Regulation is influenced by unique low molecular mass compounds. Transfer RNA, sigma factors and gene products are formed during post exponential development (Davatia *et al*., 2013). Those are very much sought after in order to increase the diversity of cultivable isolates, to shorten time of cultivation needed to achieve appreciable cell mass and production of secondary metabolites, and to produce the latter in higher yields this approach will require considerable effort. Its major advantage is that, if successful, it will result in the production of a compound which structure, novelty and biological activity can be assessed straight away (Zotchev, 2012).

Extraction of the metabolites is generally carried out either by solvent- solvent extraction or extraction using salt precipitation. Organic microbial metabolites are soluble in organic solvent. But their solubility differs from solvent to solvent selection of a particular solvent which can dissolved almost all the organic compounds present in the secondary metabolites is necessary (Vijayakumar *et al*., 2012). Generally used solvents include ethyl acetate, butanol, chloroform, methanol, etc. Ethyl acetate seems to be the most common for extraction using salt precipitation (Khan, 2011).

The great physiological and biochemical diversity of marine microbes makes the ocean as rich source of biological material for biotechnology application. A variety of secondary metabolites, enzymes, polymers, organic acids and metabolic processes can be found only in the microbes that inhibit the oceans and it is likely that many more useful products will be found as research continues. The biotechnology potential of these organisms is a major driving force behind the push to characterize marine microbial diversity.

## 2.6 Bioactive compounds from marine microbes

Microorganisms, though very small and microscopic, play a major role not only in the external environment but also in plants and animals, including human beings. One of the major roles is the production of bioactive secondary metabolites. Oceans have borne most of the biological activities on our planet. The marine environment also represents a largely unexplored source for isolation of new microbes (bacteria, fungi, actinomycetes, microalgae-cyanobacteria and diatoms) that are potent producers of bioactive secondary metabolites. Extensive research has been done to unveil the bioactive potential of marine microbes (free living and symbiotic) and the results are amazingly diverse and productive. Some of these bioactive secondary metabolites of microbial origin with strong antibacterial and antifungal activities. Research is also being conducted on the general aspects of biophysical and biochemical properties, chemical structures and biotechnological applications of the bioactive substances derived from marine microorganisms (Bhatnagar and Kim, 2010).

Naine *et al*., 2015 isolated and screened the bioactivity of *Streptomyces sp.* VITJS9 strain isolated from Kanyakumari, South East coast of India. The morphological, physiological, biochemical and phylogenetic properties of the strain was confirmed by conventional methods. The ethyl acetate crude extract tested for antibacterial activity showed better zone of inhibition at 30 mg/ml concentration against bacterial pathogens. The GC–MS analysis revealed the presence of three major chemical constituents namely N-Hexadecanoic acid, Octadecanoic acid and Dimethylsulfoxoniumformylmethylide.

Cyanobacteria has gained much importance due to the presence of secondary metabolites possessing several biological activities like antibacterial, antifungal, anti algal, antiprotozoal, and antiviral activities. *N. muscurum, a* member of family Nostocaceae, known to have components with potent bioactive properties. GC-MS analysis revealed that Phthalicacid and 9-Octadecenoic acid, ethyl ester was found in higher concentration in the total polar lipid of *Nostoc muscurum* as compared to other bioactive compounds. Identified compounds with bioactive properties were mostly organic acids. The biological activities of some of the components ranged from antimicrobial, antioxidant and antitumoral activities (Ananya and Kamal, 2016).

The fungus was identified as *Fusarium solani* based on morphological and molecular characterization. The metabolite showed activity against both bacterial and fungal pathogens. The metabolite was characterized and identified by Gas-Chromatography Mass-Spectrophotometry (GC-MS) analysis due to its volatile nature. The main components were 1-tetradecene, 8-octadecanone, 8-pentadecanone, octylcyclohexane and 10-nonadecanone. Phylogenetic analysis of the fungus with other endophytic fungi producing bioactive metabolites revealed its close affinity with the *F. solani* isolate that produced taxol. The metabolite produced by the endophytic fungus could be an alternative source of antimicrobial agents against clinical pathogens (Tayung *et al*., 2010).

## 2.7 Origin, activity of some bioactive compounds from marine microbes

| Compound | Source | Activity | Literature |
|---|---|---|---|
| Actinomycetes | | | |
| Carboxamycin (benzoxazole) | *Streptomyces* sp. | Antibacterial, cytotoxic | Hohmann *et al.,* 2009 |
| Ayamycin | *Nocardia* sp | Antifungal | El-Gendy *et al*., 2008 |
| Marinopyrroles A | *Streptomyces sannurensis* | Cytotoxic | Hughes *et al*., 2008 |
| Bacteria | | | |
| Tauramamide, Tauramamide ethyl ester | *Brevibacillus laterosporus* | Antimicrobial | Desjardine *et al*., 2007 |
| Lipoxazolidinone A, B, C | *Marinispora* sp. | Antimicrobial | Macherla *et al*., 2007 |
| Lynamicins B, C | *Marinispora* sp. | Antimicrobial | McArthur *et al*., 2008 |
| Zafrin | *Pseudomonas stutzeri* | Antimicrobial | Uzair *et al*., 2008 |
| Fungi | | | |
| Dehydroxychlorofusarielin B | *Aspergillus* sp. | antibacterial | Nguyen *et al*., 2007 |
| Chlorohydroaspyrones A, B | *Exophiala* sp. | antibacterial | Zhang *et al*., 2008 |
| Macrolides (+)-brefeldin A | *Penicillium* sp. | Antimicrobial | Trisuwan *et al*., 2009 |
| Ascochytatin | *Ascochyta* sp. | Cytotoxic & antimicrobial | Kanoh *et al*., 2008 |
| bis (2-ethylhexyl) phthalate | *Cladosporium* sp. | Antifouling & antimicrobial | Qi *et al*., 2009 |
| Chaetocyclinones A | *Chaetomium* sp. | Antifungal | Lösgen *et al*., 2007 |
| Apralactone A | *Curvularia* sp. | Cytotoxic | Greve *et al*., 2008 |
| Spicellamide A, B | *Spicellumroseum* | Cytotoxic | Kralj *et al*., 2007 |
| Carbonarones A, B | *Aspergillus carbonarius* | Cytotoxic | Zhang *et al*., 2007 |
| Aspergiolide A | *Aspergillus glaucus* | Cytotoxic | Sun *et al*., 2009 |
| Chromanone A | *Penicillium* sp. | Cytotoxic | Gamal-Eldeen *et al*., 2009 |

## 2.8 Biochemical characterization of bioactive compounds

The identification of active compounds, especially a new compound, from fermentation samples (extracts, whole broths) is one of the most complicated, labour intensive, time consuming steps of the screening protocols (Berdy, 2005). In general, the crude extracts are evaluated by chemical screening approaches. The later mostly focused on the compounds with highly sensitive biological activity. Identification of a known or an unknown compound requires separation of the individual compound from the crude extract. It should be followed by screening of each separated compound for the bioactivities. Recently, the great progress in the robotic and automated methods in chromatographic isolation methods (LC-MS, LC-MS-ELSD, GCMS, HPLC-UV-VIS, HPLC-ELSD, HPLC-PDA-photodiode array detectors, HPCCC, CZE, *etc*.) and spectroscopic identification techniques are more or less satisfying the requirements of the modern HTS (high through put screening) tasks (Berdy, 2005).

Gas chromatography–mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Analytical methods development and validation play an important role in the discovery, development and Manufacture of pharmaceuticals. GC-MS has been widely heralded as a "gold standard" for forensic substance identification because it is used to perform a *specific test*. A specific test positively identifies the actual presence of a particular substance in a given sample. A *non-specific test* merely indicates that a substance falls into a category of substances. Although a non-specific test could statistically suggest that the identity of the substance, this could lead to false positive identification (HimaBindu *et al.,* 2013). Gas chromatography (GC) is recognized as the most suitable technique to find out number of components with proportion in a complex mixture of volatile compounds. When it is coupled to mass spectrometry (GC-MS), an additional information arises about each separated compound molecular mass, elemental composition when high resolution mass spectrometry is used, functional groups, and in certain cases, molecular geometry and spatial isomerism (Padmanabhan *et al.,* 2016).

## 2.9 Molecular identification of marine microbes

The use of ribosomal DNA as molecular marker to identify microbial population is now routinely used in microbial ecology. By the pioneering work of (Woese, 1987), it is now clear that all organisms on earth can be classified by comparative sequence analysis of their small subunit ribosomal RNA. Cloning of PCR- amplified 16s rDNA is the most successful approach to explore microbial diversity and to determine the species composition of bacteria (Charyulu *et al*., 2009) and Actinomycetes (Maldonado *et al*., 2009) in mixed microbial community, whereas 18S rDNA and internal transcribed spacer (ITS) regions are increasingly used to study fungal communities (Nilsson *et al.,* 2008)

## 2.9.1 Molecular characterization of gene encoding bioactive compounds

NRPS and PKS 1

Microorganisms are ubiquitous in all habitats and are recognized by their metabolic versatility and ability to produce many bioactive compounds, including toxins. These toxins are secondary metabolites, presenting a vast diversity of structures and variants. Most of microbial secondary metabolites are peptides or have peptidic substructures and are assumed to be synthesized by non-ribosomal peptide synthesis (NRPS), involving peptide synthetases, or NRPS/PKS involving peptide synthetases and polyketide synthases hybrid pathways (Valerio *et al*., 2010). Exploring new sources of bioactive natural products the marine environment warrants particular attention, in view of the remarkable diversity of microorganisms and metabolic products (Bull and Stach, 2007).

Considering that 70 % of our planet's surface is covered by oceans, it is likely that undiscovered biodiversity is still enormous. A large portion of marine biodiversity consists of microbiomes. They are very attractive targets of bioprospecting because they are able to produce a vast repertoire of secondary metabolites in order to adapt in diverse environments. In many cases secondary metabolites of pharmaceutical and biotechnological interest such as non-ribosomal peptides (NRPs) and polyketides (PKs) are synthesized by multi-modular enzymes named nonribosomal peptide synthetases (NRPSs) and type-I polyketide synthases (PKSs-I), (Amoutzias *et al*., 2016).

The genes encoding for PKSs and NRPSs have been exposed to complex evolutionary mechanisms, which have determined the great number and diversity of metabolites (Gallo *et al*., 2013). Structurally unique metabolites with biological and pharmacological activities have been isolated from the marine-derived fungi, such as polyketides, alkaloids, peptides, lactones, terpenoids and steroids. Some of these compounds have anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, antibiotic and cytotoxic properties (Jin *et al.,* 2016).

Biosynthetic pathways for secondary metabolites are often quite complex and many congeners are produced due to the substrate flexibility of certain biosynthetic enzymes, interference from other pathways, etc. The study by Kalaitzis *et al*., (2009) demonstrates the utility of the biosynthetic studies that may help both in directing the biosynthesis toward specific natural congener with desired properties, and generation of completely new "unnatural" derivatives for bioactivity screening (Zotchev, 2012).

Novel findings regarding the mechanisms underlying NRPS and PKS evolution demonstrate how microorganisms could leverage their metabolic potential. Moreover, these findings could facilitate synthetic biology approaches leading to novel bioactive compounds (Amoutzias *et al*., 2016).

More sophisticated identification techniques arose when it was realized that many of the structural genes involved in secondary metabolism are highly conserved and could be cloned by hybridization probing or amplified from the microbial genome by use of degenerate primers; the latter technique was especially fruitful in procuring polyketide synthases (Bok *et al*., 2006).

Ongoing advances in bioinformatics and next-generation sequencing (NGS) technologies are driving the discovery of NRPs and PKs derived from marine microbiomes mainly through two strategies: genome-mining and metagenomics. Microbial genomes are now sequenced at an unprecedented rate and this vast quantity of biological information can be analysed through genome mining in order to identify gene clusters encoding NRPSs and PKSs of interest (Amoutzias *et al*., 2016).

Metagenomic libraries totaling more than four gigabases of sponge associated bacterial genomes were screened for type I modular PKS gene clusters. More than 90 % of the clones in total sponge DNA libraries represented bacterial DNA inserts, and 0.7 % harboured PKS genes. Metagenomic libraries made from fractions enriched for unicellular or filamentous bacteria differed significantly, with the latter containing numerous nonribosomal peptide synthetase (NRPS) and mixed NRPS-PKS gene clusters. An unculturable sponge-specific taxon previously implicated in the biosynthesis of bioactive peptides (Schirmer *et al*., 2005).

Bacteria and fungi use large multifunctional enzymes, the so-called non ribosomal peptide synthetases (NRPSs), to produce peptides of broad structural and biological activity (Finking and Marahiel, 2004). Sponge-associated bacteria are thought to produce many novel bioactive compounds, including polyketides. PCR amplification of ketosynthase domains of type I modular polyketide synthases (PKS) from the microbial community of the marine sponge *Discodermia dissoluta* revealed great diversity and a novel group of sponge specific PKS ketosynthase domains (Schirmer *et al*., 2005).

Surveys for the presence of potential antibiotic encoding polyketide synthase and nonribosomal peptide synthetase genes also revealed that genes for the biosynthesis of these secondary metabolites were present in most bacterial phyla but were particularly prevalent among the Actinobacteria and Proteobacteria (Kennedy *et al*., 2009).

# 3. MATERIALS AND METHODS

In the present study, experiments were conducted to isolate and characterize bioactive compounds from marine microbes which have shown antiphytopathogenic activity against phytopathogens (*Sclerotium rolfsii, Fusarium oxysporum, Colletotrichum* sp. and *Ralstonia solanacearum*). The details of materials used and the technique adopted in the present investigation are described in this chapter under the following headings: microbial samples used in this study, collection of the phytopathogens, primary screening for antagonistic activity, extraction of antimicrobial secondary metabolites from the potent isolates, *in vitro* secondary screening for anti-phytopathogenic against test pathogens using crude extract, chemical characterizaration of the compounds produced by the isolates and molecular characterization of potent isolates.

## 3.1 Microbial samples used in this study

During this investigation, previously isolated microbial cultures from the marine water and sediment samples from coastal parts of Karnataka, Maharashtra and Goa were used (Table 3.1.2) (Alone, 2012).

Revival of the cultures: Actinomycetes, bacterial and fungal strains were revived on the following culture media.

### 3.1.1. Media for actinomycetes, bacteria and fungi

| Sl. No. | Microorganisms | Media | References |
|---|---|---|---|
| 1 | Actinomycetes | Kuster's agar | Kumar, 2005 |
| 2 | | Martin's Rose Bengal agar (prepared in distilled water) | Kostadinova *et al.,* 2009 |
| 3 | | Martin's Rose Bengal agar (prepared in sea water) | |
| 4 | | Glucose Yeast Extract agar | Maldonado *et al.,* 2009 |
| 5 | Bacteria | Nutrient agar (prepared in distilled water) | Dhevendaran, 2008 |
| 6 | | Nutrient agar (prepared in sea water) | |
| 7 | Fungi | Martin's Rose Bengal agar (prepared in distilled water) | Kostadinova *et al.,* 2009 |
| 8 | | Martin's Rose Bengal agar (prepared in sea water) | |

**Table 3.1.2. Marine microbial isolates of bacteria, fungi and actinomycetes**

| Bacteria | | | Fungi | | | Actinomycetes | |
|---|---|---|---|---|---|---|---|
| AUDI-3 | AUDI-144 | AUDI-243 | AUDI-462 | AUDI-600 | AUDI-699 | AUDI-733 | AUDI-825 |
| AUDI-8 | AUDI-165 | AUDI-244 | AUDI-478 | AUDI-607 | AUDI-700 | AUDI-735 | AUDI-830 |
| AUDI-13 | AUDI-170 | AUDI-254 | AUDI-481 | AUDI-609 | AUDI-701 | AUDI-741 | AUDI-836 |
| AUDI-18 | AUDI-172 | AUDI-257 | AUDI-485 | AUDI-615 | AUDI-703 | AUDI-743 | AUDI-841 |
| AUDI-34 | AUDI-176 | AUDI-267 | AUDI-496 | AUDI-623 | AUDI-704 | AUDI-745 | AUDI-843 |
| AUDI-38 | AUDI-177 | AUDI-271 | AUDI-501 | AUDI-626 | AUDI-705 | AUDI-751 | AUDI-847 |
| AUDI-42 | AUDI-183 | AUDI-307 | AUDI-505 | AUDI-630 | AUDI-712 | AUDI-754 | AUDI-853 |
| AUDI-49 | AUDI-188 | AUDI-317 | AUDI-506 | AUDI-632 | AUDI-722 | AUDI-755 | AUDI-862 |
| AUDI-57 | AUDI-191 | AUDI-332 | AUDI-507 | AUDI-635 | | AUDI-761 | AUDI-865 |
| AUDI-59 | AUDI-194 | AUDI-339 | AUDI-526 | AUDI-651 | | AUDI-764 | AUDI-871 |
| AUDI-65 | AUDI-201 | AUDI-347 | AUDI-544 | AUDI-666 | | AUDI-768 | AUDI-872 |
| AUDI-69 | AUDI-203 | AUDI-353 | AUDI-545 | AUDI-668 | | AUDI-769 | AUDI-876 |
| AUDI-70 | AUDI-205 | AUDI-360 | AUDI-547 | AUDI-670 | | AUDI-779 | AUDI-883 |
| AUDI-80 | AUDI-208 | AUDI-364 | AUDI-553 | AUDI-675 | | AUDI-794 | AUDI-884 |
| AUDI-100 | AUDI-220 | AUDI-367 | AUDI-563 | AUDI-676 | | AUDI-795 | AUDI-885 |
| AUDI-103 | AUDI-224 | AUDI-368 | AUDI-570 | AUDI-682 | | AUDI-799 | AUDI-887 |
| AUDI-114 | AUDI-227 | AUDI-372 | AUDI-572 | AUDI-684 | | AUDI-801 | AUDI-888 |
| AUDI-121 | AUDI-228 | AUDI-375 | AUDI-575 | AUDI-685 | | AUDI-803 | AUDI-891 |
| AUDI-130 | AUDI-229 | AUDI-376 | AUDI-583 | AUDI-692 | | AUDI-810 | AUDI-897 |
| AUDI-139 | AUDI-239 | AUDI-378 | AUDI-585 | AUDI-694 | | AUDI-820 | AUDI-902 |
| AUDI-385 | | | AUDI-590 | AUDI-695 | | AUDI-821 | |

## 3.2 Collection of the phytopathogens

The antipathogenic activities of the marine microbes were studied against the phytopathogens *Sclerotium rolfsii, Fusarium oxysporum, Colletotrichum* sp. and *Ralstonia solanacearum.* The fungal pathogen *Sclerotium rolfsii and Fusarium oxysporum* and the bacterial pathogen *Ralstonia solanacearum* were obtained from the culture collection at the Department of Biotechnology, UAS, Dharwad. The phytopathogen *Colletotrichum* sp. was collected from the Department of Plant Pathology, UAS, Dharwad.

### 3.2.1. Media for phytopathogens

| Sl. No. | Phytopathogens | Media (Prepared in distilled water) |
|---|---|---|
| 1 | Fungal phytopathogens | Potato Dextrose agar (PDA) |
| 2 | Bacterial phytopathogen | Nutrient agar (NA) |

## 3.3 Primary screening for antagonistic activity

3.3.1 *In vitro* screening of test isolates of bacteria, fungi and actinomycetes against fungal pathogens

To screen the test bacterial isolates against fungal pathogens, bacterial culture were streaked in SCAB media (Appendix I) plate on either side from centre at equidistance (25 mm). After 3 days of growth of isolates of test bacteria, ager plug of freshly revived fungal pathogen was inoculated at the centre by using sterile cork borer. Plate inoculated with agar plug of fungal pathogen was maintained as control. All plates were incubated till complete growth of fungal pathogen was observed in control plate (Appanna, 1997).

In order to check the antifungal activity, agar plug of freshly grown fungal test isolates was inoculated at the periphery of SCAB media (Appendix I) containing 90 mm plate and allowed to grow for 4-5 days on the basis of growth habit. After 4 days of incubation of test fungi, agar plug of fungal pathogen was similarly inoculated at periphery but on the opposing end of same petri dish. Media plate inoculated with agar plug of pathogen was maintained as control. All the plates were incubated at room temperature and observed for growth of inhibition of pathogen (Imtiaj and Lee, 2008).

The bioassay method was followed according to the protocol given by Soares *et al.,* (2006). All selected Actinomycetes were streaked at the middle of media plate and allowed to grow for four days to check the antifungal activity. Agar plugs of freshly grown fungal pathogens were placed on both sides of the streaked culture using cork borer. Media plate with agar plugs of pathogen was maintained as control.

All *in vitro* bioassay methods were performed in three replications and the growth of pathogen was noted in control as well as treated plates. The per cent of inhibition of pathogen was calculated by using formula.

Inhibition (%) = [(Growth radius of pathogen in untreated control plate - growth radius of pathogen in treated plates) x 100]/growth radius of pathogen in untreated control plate (Taechowisan *et al*., 2005).

Antagonistic activity of isolates showing > 70 % inhibition of growth of pathogen were subjected to analysis of variance (CRD).

### 3.3.2 *In vitro* screening of test isolates of bacteria fungi and Actinomycetes against bacterial pathogen

To check the antagonistic activity of selected bacterial isolates against *R. solanacearum,* pathogen was inoculated and mixed in soft nutrient agar containing 0.4 gm of agar powder (0.4 %) and poured in Petri plate containing hard agar. The soft agar is allowed to solidify for some time. Overnight incubated test bacterial broth culture was spot inoculated on lawn of pathogen and plate with only lawn of pathogen was kept as control. All these plates were incubated at room temperature for overnight and observed for zone of inhibition for next day after inoculation.

To screen the antagonistic activity of test fungi against bacterial phytopathogens, agar plugs of freshly revived test fungi were inoculated on soyabean-casein digest agar media (SCAB) (Appendix I) by using sterile cork borer and allowed to grow for four days after inoculation. Bacterial pathogen was streaked on either side of agar plug of test fungi. Media plate streaked with pathogens was maintained as control. All these plates were incubated at room temperature and observed for inhibition of growth pathogen.

In order to check the antibacterial activity, selected Actinomycetes isolates were streaked at the centre of the petriplate on SCAB media. These plates were allowed to incubate for four days at room temperature. Freshly revived culture of bacterial was inoculated by streaking at right angle to straight line of Actinomycetes on both sides. Media plate streaked with pathogen was kept as control. All plates were incubated for growth to occur and observed for growth inhibition of pathogen.

All *in vitro* bioassay methods were performed in three replications and the growth of pathogen was noted in control as well as treated plates.

The per cent of inhibition of pathogens was calculated by using formula (Mohapatra, 2011).

Inhibition (%) = [(mean of the growth radius in untreated control – mean of the growth radius in treatment) x100]/ mean of the growth radius in untreated control.

Antagonistic activity of isolates showing > 70 % inhibition of growth of pathogen were subjected to production media for production of bioactive compounds.

## 3.4 Extraction of antimicrobial secondary metabolites from the potent isolates

### 3.4.1. Fermentation for production of bioactive compounds

In order to produce of antimicrobial metabolites, soybean casein digest broth for Actinomycetes, yeast extract glucose broth media for bacteria, and potato dextrose broth for marine fungi (Basavaraj *et al.,* 2010; Sarker *et al*., 2015; Swathi *et al*., 2013) were used. All three production media were prepared using sea water and autoclaved. All the potent isolates were first inoculated in nutrient broth in separate flasks for seed culture and incubated at 28 $^{0}$C Actinomycetes and bacteria

were at shaking condition and for fungi in static condition for 7 days. Then the prepared inoculums were transferred aseptically into respective production media at the rate of 10 ml per 100 ml of broth and incubated at 28 $^0$C initially at shaking condition for 15 days and later at static condition for another 15 days, for Actinomycetes and bacteria, in case of fungi inoculated flasks were incubated at (28 $^0$C) under static conditions for 30 days.

### 3.4.2. Extraction of secondary metabolites from the potent isolates

Extraction of antimicrobial substance was carried out using solvent- solvent extraction method (Sharma and Parihar, 2010: Sunaryanto *et al.,* 2010). The Actinomycetes and bacterial culture broths were centrifuged at 14,000 X g for 15 minutes to remove the cell debris, in case of fungi the mycelia was separated using Whatman filter paper. The supernatant was filtered and collected into sterilized bottle. The filtrate was mixed with ethyl acetate in 1:1 (v/v) ratio and shaken vigorously for overnight in a rotary shaker. The solvent phase that contains anti-microbial compound was separated from the aqueous phase using a 500 ml capacity separating funnel. Then, solvent phase was evaporated to dryness using rotary vacuum evaporator (Heidolph) and the residue was used to check antimicrobial activity (Sharma and Parihar, 2010).

## 3.5 *In vitro* secondary screening for anti-phytopathogenicity against test pathogens using crude extract

The biological activities of the crude extract of secondary metabolites harvested from potent marine microbial isolates were monitored by agar well diffusion method (Sharma and Parihar, 2010). Wells were made on the soybean-casein digest agar plate using sterilized cork borer of 6 mm in diameter. The extracts were added into the wells at the rate of 50 µl per well. At the same time one well per plate was also added with only the pure organic solvent as control. In the middle of the plate agar plug from the freshly grown fungal plate-were inoculated. The plates were incubated at 28 ℃ for 5 days. Observation was taken on the fifth day when the pathogen showed full growth

In case *R. solanacearum,* it was first grown in tryptone glucose yeast extract broth with TTC (Tetrazolium salt) for two days and the inoculums spread on the SCAB media. Wells were then made on the spread plate extracts were added on the wells at the rate of 50 µl per well. Similarly, one well per plate was also added with pure organic solvents as control. The plates were incubated at 28 ℃ for two days (Ara *et al.,* 2011).

## 3.6 Chemical characterization of the compound produced by the isolates

### 3.6.1 Purification of the antimicrobial compounds

The crude solvent extracts were subjected to Thin Layer Chromatography (TLC) for purification of the active compounds. The TLC Silica gel 60 $F_{254}$ (MERCK) plates were used different solvent such as ethyl acetate, chloroform and methanol were used in (9:5:3) proportion to separate the crude compounds. The samples were spotted at the bottom of the silica gel plate. It was then placed inside the developing chamber containing the mobile phase. The samples were allowed to run till it reaches 3/4$^{th}$ of the plate. After running the plate was kept in room temperature for complete drying. The plate was placed in the iodine chamber to visualize the separated compounds as clear spots. Retention factor *($R_f$)* was calculated for each sample using the given formula.

Retention factor *($R_f$)* = distance travelled by the compound/ distance travelled by solvent front.

### 3.6.2 GC-MS analysis of secondary metabolites from the selected isolates

Chemical screening of the active compounds present in the crude extracts was done through GC-MS (Agilent 7890A). The GC-MS analysis was performed at Indian Institute of Sciences (IISc.), Bangalore. The analysis was conducted according to the following temperature program: 70 °C maintained for 2 min; increase at 20 °C $min^{-1}$ to 270 °C and eventually maintained for 5 min. The ions were detected in the range 30-600 m/z. The software used for data analysis was AMDIS, while NIST 11 library was used for comparison.

## 3.7 Molecular characterization of potent isolates

After secondary screening of the potent isolates against phytopathogens, only few isolates which were found to be more efficient than the reference strains in antagonistic activity were selected for further molecular characterizations. Consequently, out of total number of efficient isolates only one bacteria, one Actinomycete and one fungi were used for further characterization.

### 3.7.1 DNA isolation from potent bacterial isolates

Twenty five ml of overnight grown Nutrient broth (Appendix III) culture was centrifuged at 10,000 rpm at 4 °C for 10 minutes. The pellet was re-suspended in 10 mM Tris, 100 mM sodium chloride solution and centrifuged at 10,000 rpm for 10 min. at 4 °C. After discarding the supernatant, the pellet was re-suspended in 2.5 ml of $T_{50}E_{20}$ buffer containing 500 µl of lysozyme (50 mg/ml) and incubated at 37 °C for 40 min. to solution 25 µl of RNAase (10 mg/ml) was added and incubated at room temperature for 20 minutes. Further to this mixture 2.5 ml of SDS (2 % in $T_{50}E_{20}$) was added and incubated at 50 °C for 45 min. with proper mixing. A 50 µl of proteinase K (20 mg/ml) was added and incubated at 55 °C for 30 min. The sample was extracted in same volume of phenol, chloroform and iso amyl alcohol (25:24:1) and DNA was precipitated with one volume of isopropenol and 0.1 volume of 3 M sodium acetate. The pellet was washed with 70 % ethanol, dried and dissolved in 30 µl of $T_{10}E_1$ buffer and stored at – 20 °C for further use.

### 3.7.2 DNA isolation from potent fungal isolates

For isolation of DNA from potent fungal isolates, the mini prep method was used. Fresh fungal mat was grown on PDB (Appendix I) for 3-5 days at room temperature. About 100 mg of fungal mat was taken in 2 ml microfuge tube and to this 500 µl of lysis buffer (Appendix III) was added. The mat was macerated by using sterile micro-pestles and vortexed for 5 minutes. This mixture was extracted with equal volume of phenol: chloroform: iso amyl alcohol (25:24:1) by spinning at 10,000 rpm for 10 min. Supernatant was transferred to fresh tube and equal volume of chloroform:iso amyl alcohol (24:1) was added and centrifuged at 10,000 rpm for 10 min. Supernatant was collected in fresh tube and DNA was precipitated with one volume of isopropenol with gentle inversion and incubated for 30 min. in ice-cold condition. All tubes were centrifuged at 10,000 rpm for 10 min. at 4 °C and the pellet was washed with 70 % of ethanol, air dried and re-suspended in 30 µlof $T_{10}E_1$ buffer and stored at -20 °C.

### 3.7.3 DNA isolation from potent Actinomycete isolates

A versatile quick-prep method of DNA isolation described by Pospiech and Naumann, 1995 was followed. Mature culture of actinomycete was inoculated into 30 ml of Nutrient broth medium and incubated in rotary shaker at room temperature for 7 days at 180 rpm. The culture was centrifuged at 10,000 rpm for 10 min. and pellet was resuspended in 400 µl extraction buffer (Appendix III). 500 µl lysozyme (20 mg/ml) was added and tubes were incubated at 37 ℃ for 1 hr. To this 100 µl of 10 % of SDS and 20 µl of proteinase K (20 mg/ml) were added into tube and incubated at 55 ℃ for 45 min. in water bath. One third volume of 5M NaCl and one volume of phenol-chloroform were added and incubated at room temperature for 30 min. with frequent inversion. This mixture was centrifuged at 10,000 rpm for 10 min. and aqueous phase was transferred into fresh microfuge tube. The DNA was precipitated by using one volume of isopropenol with gentle inversion and pellet was collected by centrifuging it at 10,000 rpm for 10 min. at 4 ℃. The pellet was washed with 70 % ethanol and dissolved in 30 µl of $T_{10}E_1$ buffer and stored at -20 ℃.

After performing all these DNA isolation protocols, the purity and concentration of DNA was checked with 0.8 % agarose gel (Appendix III) by using gel electrophoresis. The gel image was documented using gel documentation system (G-BOX, SYNGENE).

### 3.7.4 PCR amplification

#### 3.7.4.1 PCR amplification of 16s rDNA gene of potent bacteria and Actinomycete

Polymerase chain reaction was done with diluted (1:20) samples of purified DNA by using Eppendorf Master Cycler (5331). 16s rDNA universal primers were used for PCR are mentioned in table below. The reaction mixture was prepared for final volume of 20 µl which contained 5 pmol of each forward and reverse primers, 2.5 mM of dNTP's, l0x Slandered *Taq* recation buffer (NEB) and 5 units of Taq DNA polymerase (NEB). PCR was performed in automated thermal cycler (Eppendorf Master Cycler, Germany) with initial denaturation of 5 min. at 95 ℃ followed by denaturation at 94 ℃ for 1 min, annealing at 57.3 ℃ for 1 min. and extension of 1 min. 30 sec. for 35 cycles followed by 10 min. final extension at 72 ℃. At the end PCR products were maintained at 4 ℃

#### 3.7.4.2 Primer details of 16s rDNA sequence

| Primers | Sequence | Reference |
|---|---|---|
| 16s rDNA | 27f- 5' AGAGTTTGATCCTGGCTCAG 3' | Lane *et al.,* 1991 |
| | 1492r- 5' ACGGCTACCTTGTTACGACTT 3' | |

#### 3.7.4.3 PCR amplification of ITS region of potent fungi

Polymerase chain reaction was done with diluted (1:20) samples of purified DNA. ITS universal primers were used for PCR are mentioned in table below. The reaction mixture was prepared for final volume of 20 µl which contained 5 pmol of each forward and reverse primers, 2.5 mM of dNTP's, 10x Standard *Taq* reaction buffer (NEB) and 5 units of Taq DNA polymerase (NEB). PCR was performed in automated thermal cycler (Eppendorf Master Cycler, Germany) with initial denaturation of 3 min. at 95 ℃ followed by denaturation at 94 ℃ for 40 sec. annealing at 64 ℃ for 40 sec. and extension of 40 sec. for 30 cycles followed by 5 min. final extension at 72 ℃. At the end PCR products were maintained at 4 ℃.

3.7.4.4. Primer details of ITS region of 18s rDNA sequences

| Primers | Sequences | Reference |
|---|---|---|
| *ITS-1* | 5' TCC GTA GGT GAA CCT GCG G 3' | White *et al.,* 1990 |
| *ITS-4* | 5' TCC TCC GCT TAT TGA TAT GC 3' | |

### 3.7.5. Separation of amplified product by gel electrophoresis

All amplified PCR product from each tube was mixed thoroughly with 1µl of loading dye. 0.8 % of agarose gel (Appendix III) prepared in lx TAE buffer used for proper separation of bands. λ DNA *EcoR 1/ Hind* III double digest (Genei) DNA marker was run along with the samples 16s rDNA and ITS regions of potent bacteria, actinomycete and fungi respectively. The gel image was documented using gel documentation system (G-BOX, SYNGENE).

## 3.8 Cloning of the probable gene (s) involved in the inhibition of the pathogen

### 3.8.1. PCR amplification of the PKS and NRPS gene fragments

To amplify segment encoding β-ketoacyl synthase (KS) domain of type I PKS genes, the degenerated primers Ksma-F and ksmb-R were used as described by Izumikawa *et al.* (2003) with the target to amplify the PKS-1 genes of PKS gene cluster. The 20 µl of PCR mixture consisted of 10x Standard *Taq* Reaction Buffer (NEB), 5 pmol of each primer, 2.5mM dNTPs and 5U *Taq* DNA Polymerase (NEB) in a final volume of 20 µl. Polymerase chain reaction conditions were as follows: 5 min at 95 ℃ followed by 35 cycles of 1 min at 95 ℃, 1 min at 60 ℃ and 2 min at 72 ℃ followed by a 15-min extension step at 72 ℃. At the end PCR products were maintained at 4 ℃.

To amplify a segment encoding the adenylation domain of non-ribosomal peptide synthetases (NRPSs), the degenerate primers ADEdom5 and ADEdom3 were used described by Busti *et al.* (2006) were used. Polymerase chain reaction mixtures contained 10x Standard *Taq* Reaction Buffer (NEB), 5pmol of each primer, 2.5 mM dNTPs, and 5 U Tag DNA Polymerase (NEB) in a final volume of 20µl. The following PCR conditions were used: 5 min at 95 ℃ followed by 35 cycles of 1 min at 95 ℃, 1 min at 60 ℃ and 1.5 min at 72 ℃, followed by a final extension of 15 min at 72 ℃. At the end PCR products were maintained at 4 ℃.

### 3.8.2 Primers details of PKS-1 and NRPS

| Gene fragments | Primer Sequence | Expected amplicon (bp) | Reference |
|---|---|---|---|
| PKS-1 | Ksma-F (5'-TSGCSATGGACCCSCAGCAG-3') | ~700 bp | Izumikawa*e t al*., 2003 |
| | Ksmb-R (5'-CCSGTSCCGTGSGCCTCSAC-3') | | |
| NRPS | ADEdom5 (5'-ACSGGCNNNCCSAAGGGCGT-3') | 450 bp | Busti *et al*., 2006 |
| | ADdom3 (5'-CTCSGTSGGSCCGTA-3') | | |

All amplified PCR product from each tube was mixed thoroughly with 1µl of loading dye. 0.8 % of agarose gel (Appendix III) prepared in lx TAE buffer used for proper separation of bands. λ DNA *EcoRI / Hind* III double digest (Genei) DNA marker was run along with the samples of PKS-1 and NRPS regions of potent bacteria, actinomycete and fungi respectively. The gel image was documented using gel documentation system (G-BOX, SYNGENE).

### 3.8.3 Gel Elution of PCR products using Qiagen mini-elut gel extraction kit

The DNA fragment was excised in the agarose gel with clean and sharp scalpel. The size of the gel slice was minimized by removing extra agarose gel. The gel was purified according to manufacture's instructions.

## 3.9 DNA sequencing and *in silico* analysis of the sequences

The Gel eluted PCR products of 16s rDNA, ITS, PKS-1 and NRPS of potent isolates were sent for sequencing at Chromous Biotech, Bangalore. Homology search was done with BLAST algorithm available at http://www.ncbi.nlm.nih.gov.

# 4. EXPERIMENTAL RESULTS

The primary aim of this invistagation was to isolation and characterization of bioactive compounds from marine microbes against phytopathogens. The results of various experiments conducted during the course of study are presented below.

## 4.1 Antagonistic activity of selected microbial isolates against Phytopathogens

All 152 selected isolates were screened for antagonist activity against three fungal and one bacterial phytopathogens namely, *Sclerotium rolfsii, Colletotrichum sp., Fusarium oxysporum* and *Ralstonia solanacearum* respectively. During screening, when no growth of pathogen was seen, it was considered to be bactericidal and fungicidal action whereas when few colonies of bacteria and mycelial growth of fungi (compared with the control) were seen it was considered to be bacteriostatic and fungistatic activity.

## 4.2 Anti-phytopathogenic activity of test isolates of bacteria, fungi and actinomycetes against fungal pathogen

In the present study, different bioassay methods the provided evidence that aquatic test isolates effectively reduced the growth of a11 fungal pathogens. In case of pathogens, single culture of *S. rolfsii* grew actively and covered the entire plate within 5 days, whereas *Colletotrichum sp.* took 7 days and *F. oxysporum* filled the plate in 10 days in control plate.

Majority of test bacteria displayed detectable antagonistic activity. The per cent, inhibition was noticed to be better at 3 days incubation of test bacteria on SCAB media. Against*, S. rolfsii* almost 81.9 % of the total isolates showed more than 80 % inhibition of growth of pathogen. Most notable form of inhibition was the stoppage of growth and darkening of pathogen mycelium. Certain isolates such as AUDI 65, 172 and 194 resulted into complete lysis as well as gradual browning of mycelia with increasing incubation period (Table 4.2.1. and Plate 1).

Similarly, 6.5 % of test bacteria showed more than 70 % inhibition against *Colletotrichum* sp., only two isolates AUDI 172 and 194 showed strong activity with 83 % and 82 % inhibition The lowest value of inhibition was 15 % showed by AUDI 130. Remaining 93.5 % isolates displayed inhibition less than 70 %, AUDI 172 and AUDI 194 significantly differed in their against *Colletotrichum* sp.(Table 4.2.1. and Plate 1).

Among test bacteria 4.9 % isolates showed more than 70 % inhibition against *F. oxysporum* out of these isolate AUDI 172 showed strong activity with 80 % inhibition and reduction in mycelium growth was observed when grown on SCBA media (Table 4.2.1. and Plate 1).

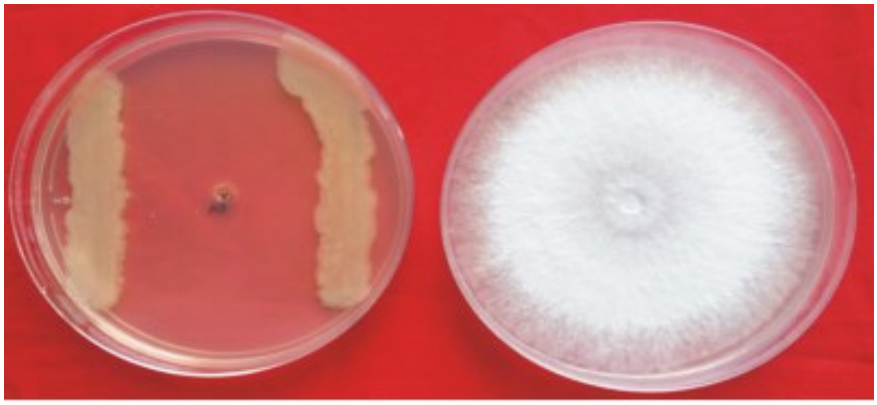
The *in vitro* antifungal activity of test fungi against three fungal phytopathogens were studied by using dual culture method. Fifty heterotrophic fungal isolates were screened against three fungal pathogens. The percentage of isolated fungi with antifungal activity against *S. rolfsii, Colletotricum* sp. and *F. oxysporum* were 74 %, 4 % and 6 % respectively showed more than 70 % inhibition.

Among the test fungi active against *S. rolfsii,* three isolates like AUDI 547, 553and 585 showed 100 % inhibition of growth of pathogen. The mode of inhibition of pathogen growth was either complete stoppage of growth or further gradual darkening of mycelia of *S. rolfsii* with increasing incubation period. 12 % of total isolates showed more than 90 % inhibition. Among them, the activity of AUDI 485, 547,553, 585and 651 resulted into darkening of mycelia of *S. rolfsii* was observed after $4^{th}$ day of incubation (Table 4.2.2 and Plate 2).

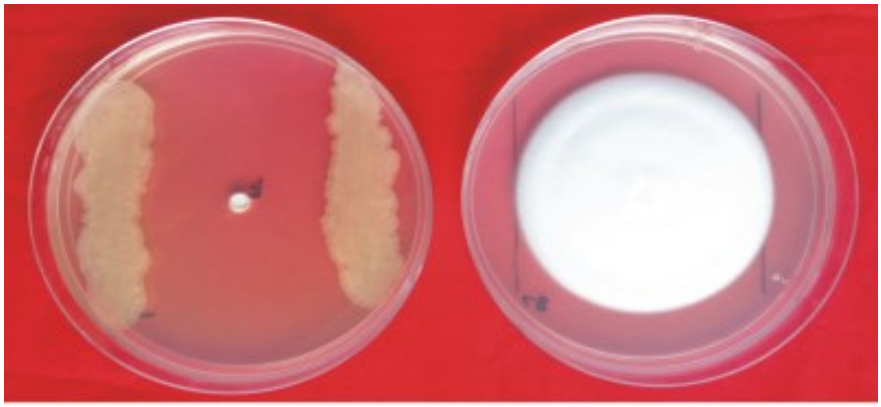
**Table 4.2.1. Inhibition of three fungal phytopathogens by marine bacterial isolates**

| Marine bacterial isolates | Per cent inhibition | | | Marine bacterial isolates | Percent inhibition | | |
|---|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | | P1 | P2 | P3 |
| AUDI-3 | 72 | 45 | 25 | AUDI-203 | 80 | 37 | 40 |
| AUDI-8 | 90 | 45 | 30 | AUDI-205 | 100 | 35 | 15 |
| AUDI-13 | 85 | 48 | 38 | AUDI-208 | 84 | 45 | 25 |
| AUDI-18 | 80 | 70 | 75 | AUDI-220 | 100 | 40 | 37 |
| AUDI-34 | 100 | 40 | 37 | AUDI-224 | 100 | 50 | 20 |
| AUDI-38 | 100 | 60 | 39 | AUDI-227 | 100 | 50 | 17 |
| AUDI-42 | 70 | 20 | 24 | AUDI-228 | 100 | 40 | 32 |
| AUDI-49 | 60 | 30 | 10 | AUDI-229 | 100 | 42 | 36 |
| AUDI-57 | 90 | 44 | 48 | AUDI-239 | 87 | 37 | 32 |
| AUDI-59 | 100 | 60 | 45 | AUDI-243 | 100 | 35 | 0 |
| AUDI-65 | 100 | 55 | 20 | AUDI-244 | 88 | 35 | 20 |
| AUDI-69 | 88 | 60 | 30 | AUDI-254 | 100 | 55 | 40 |
| AUDI-70 | 60 | 35 | 24 | AUDI-257 | 100 | 50 | 37 |
| AUDI-80 | 68 | 39 | 42 | AUDI-267 | 100 | 35 | 10 |
| AUDI-100 | 100 | 70 | 78 | AUDI-271 | 92 | 45 | 40 |
| AUDI-103 | 100 | 30 | 10 | AUDI-307 | 100 | 35 | 0 |
| AUDI-114 | 100 | 32 | 14 | AUDI-317 | 100 | 25 | 17 |
| AUDI-121 | 80 | 35 | 12 | AUDI-332 | 88 | 39 | 32 |
| AUDI-130 | 65 | 15 | 0 | AUDI-339 | 100 | 34 | 15 |
| AUDI-139 | 74 | 49 | 37 | AUDI-347 | 100 | 35 | 14 |
| AUDI-144 | 82 | 40 | 25 | AUDI-353 | 60 | 32 | 8 |
| AUDI-165 | 70 | 53 | 40 | AUDI-360 | 100 | 40 | 21 |
| AUDI-170 | 100 | 40 | 40 | AUDI-364 | 100 | 37 | 10 |
| AUDI-172 | 100 | 83 | 80 | AUDI-367 | 100 | 35 | 37 |
| AUDI-176 | 82 | 35 | 37 | AUDI-368 | 100 | 44 | 32 |
| AUDI-177 | 100 | 45 | 42 | AUDI-372 | 100 | 64 | 37 |
| AUDI-183 | 95 | 37 | 10 | AUDI-375 | 100 | 45 | 15 |
| AUDI-188 | 74 | 40 | 23 | AUDI-376 | 100 | 45 | 40 |
| AUDI-191 | 100 | 41 | 35 | AUDI-378 | 100 | 44 | 37 |
| AUDI-194 | 100 | 82 | 67 | AUDI-385 | 100 | 40 | 18 |
| AUDI-201 | 62 | 35 | 15 | | | | |

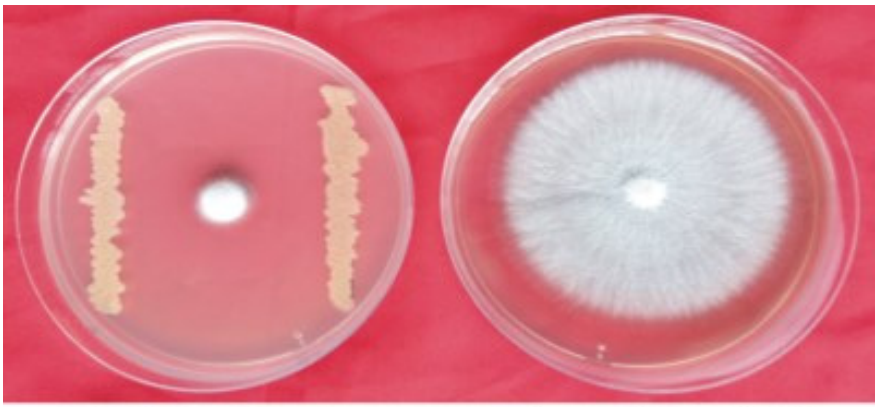
$P_1$: *Sclerotium rolfsii,*
$P_2$: *Colletotrichum* sp.
$P_3$: *Fusarium oxysporum*



I. Inhibition of *Sclerotium rolfsii* by bacterial isolate AUDI 172



II. Inhibition of *Colletotrichum* sp. by bacterial isolate AUDI 172



III. Inhibition of *Fusarium oxysporum* by bacterial isolate AUDI 172

**Plate 1: Antifungal activities by marine bacterial isolate AUDI 172**

**Table 4.2.1.1. Marine bacterial isolates showing more than 70 % inhibition against *Sclerotium rolfsii***

| AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition |
|---|---|---|---|---|---|---|---|
| 3 | 72 (58.03) | 121 | 80 (63.41) | 208 | 84 (66.40) | 317 | 100 (89.96) |
| 8 | 90 (71.54) | 139 | 74 (59.32) | 220 | 100 (89.96) | 332 | 88 (69.70) |
| 13 | 85 (67.19) | 144 | 82 (64.87) | 224 | 100 (89.96) | 339 | 100 (89.96) |
| 18 | 80 (63.41) | 165 | 70 (56.77) | 227 | 100 (89.96) | 347 | 100 (89.96) |
| 34 | 100 (89.96) | 170 | 100 (89.96) | 228 | 100 (89.96) | 360 | 100 (89.96) |
| 38 | 100 (89.96) | 172 | 100 (89.96) | 229 | 100 (89.96) | 364 | 100 (89.96) |
| 42 | 70 (56.77) | 176 | 82 (64.87) | 239 | 87 (68.84) | 367 | 100 (89.96) |
| 57 | 90 (71.54) | 177 | 100 (89.96) | 243 | 100 (89.96) | 368 | 100 (89.96) |
| 59 | 100 (89.960 | 183 | 95 (77.06) | 244 | 88 (69.70) | 372 | 100 (89.96) |
| 65 | 100 (89.96) | 188 | 74 (59.21) | 254 | 100 (89.96) | 375 | 100 (89.96) |
| 69 | 88 (69.71) | 191 | 100 (89.96) | 257 | 100 (89.96) | 376 | 100 (89.96) |
| 100 | 100 (89.96) | 194 | 100 (89.96) | 267 | 100 (89.96) | 378 | 100 (89.96) |
| 103 | 100 (89.96) | 203 | 80 (63.41) | 271 | 92 (73.62) | 385 | 100 (89.96) |
| 114 | 100 (89.96) | 205 | 100 (89.96) | 307 | 100 (89.96) | | |
| **S.Em ±**<br>**C.D. %** | | | | **0.35**<br>**1.31** | | | |
| **C.V. %** | | | | **0.75** | | | |

**Table 4.2.1.2. Marine bacterial isolates showing more than 70 % inhibition against *Colletotrichum* sp.**

| AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition |
|---|---|---|---|
| 18 | 70 (56.77) | 172 | 83 (65.63) |
| 100 | 70 (56.77) | 194 | 82 (64.87) |
| **S.Em ±** | | **0.26** | |
| **C.D. %** | | **1.35** | |
| **C.V. %** | | **0.73** | |

**Table 4.2.1.3. Marine bacterial isolates showing more than 70 % inhibition against *Fusarium oxysporum***

| AUDI No. | Per cent inhibition |
|---|---|
| 18 | 75 (59.98) |
| 100 | 78 (62.00) |
| 172 | 80 (63.41) |
| **S.Em ±** | **0.20** |
| **C.D. %** | **1.30** |
| **C.V. %** | **0.56** |

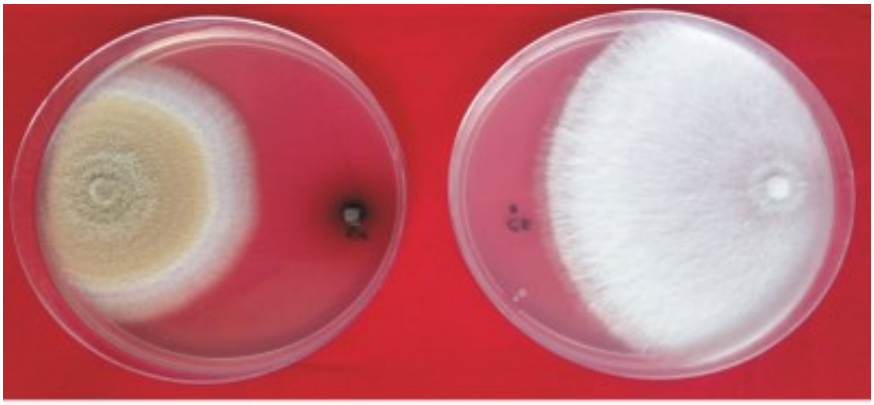
**Table 4.2.2. Inhibition of three fungal pathogens by marine fungal isolates**

| Marine fungal isolates | Percent inhibition | | | Marine fungi isolates | Percent inhibition | | |
|---|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | | P1 | P2 | P3 |
| AUDI-462 | 20 | 65 | 35 | AUDI-623 | 15 | 24 | 28 |
| AUDI-478 | 72 | 66 | 34 | AUDI-626 | 80 | 50 | 31 |
| AUDI-481 | 84 | 65 | 52 | AUDI-630 | 77 | 50 | 40 |
| AUDI-485 | 90 | 67 | 50 | AUDI-632 | 30 | 40 | 35 |
| AUDI-496 | 70 | 45 | 20 | AUDI-635 | 72 | 53 | 45 |
| AUDI-501 | 82 | 65 | 44 | AUDI-651 | 93 | 56 | 46 |
| AUDI-505 | 35 | 37 | 30 | AUDI-666 | 83 | 54 | 52 |
| AUDI-506 | 25 | 50 | 45 | AUDI-668 | 80 | 60 | 35 |
| AUDI-507 | 82 | 42 | 30 | AUDI-670 | 44 | 40 | 44 |
| AUDI-526 | 74 | 60 | 35 | AUDI-675 | 24 | 30 | 25 |
| AUDI-544 | 75 | 47 | 44 | AUDI-676 | 86 | 38 | 28 |
| AUDI-545 | 72 | 52 | 27 | AUDI-682 | 46 | 44 | 18 |
| AUDI-547 | 100 | 47 | 50 | AUDI-684 | 82 | 55 | 25 |
| AUDI-553 | 100 | 87 | 72 | AUDI-685 | 83 | 40 | 30 |
| AUDI-563 | 85 | 82 | 30 | AUDI-692 | 84 | 57 | 28 |
| AUDI-570 | 80 | 45 | 34 | AUDI-694 | 80 | 50 | 30 |
| AUDI-572 | 70 | 52 | 38 | AUDI-695 | 84 | 47 | 24 |
| AUDI-575 | 40 | 47 | 21 | AUDI-699 | 90 | 60 | 35 |
| AUDI-583 | 87 | 62 | 30 | AUDI-700 | 32 | 40 | 37 |
| AUDI-585 | 100 | 86 | 77 | AUDI-701 | 35 | 44 | 43 |
| AUDI-590 | 82 | 60 | 40 | AUDI-703 | 86 | 60 | 35 |
| AUDI-600 | 25 | 37 | 28 | AUDI-704 | 72 | 55 | 34 |
| AUDI-607 | 74 | 45 | 40 | AUDI-705 | 28 | 35 | 18 |
| AUDI-609 | 80 | 64 | 50 | AUDI-712 | 78 | 67 | 72 |
| AUDI-615 | 72 | 54 | 25 | AUDI-722 | 80 | 48 | 44 |

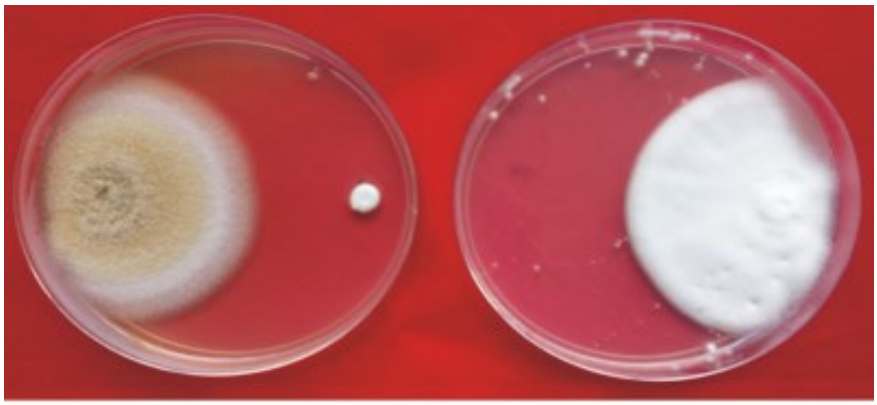
$P_1$: *Sclerotium rolfsii*

$P_2$: *Colletotrichum* sp.

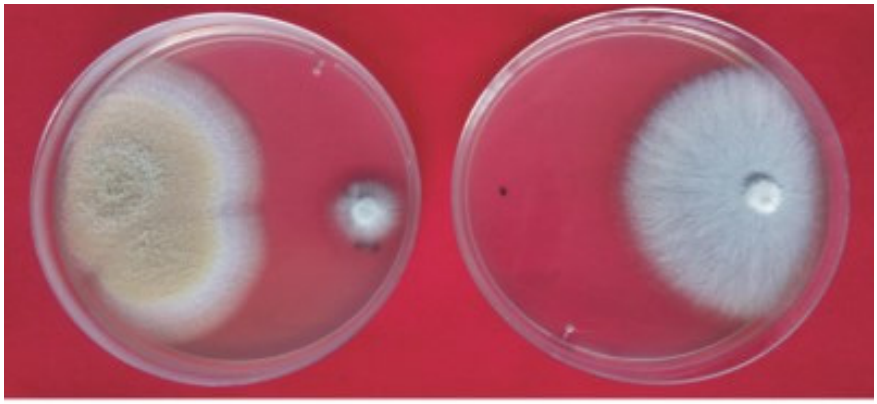
$P_3$: *Fusarium oxysporum*



I. Inhibition of *Sclerotium rolfsii* by fungal isolate AUDI 585



II. Inhibition of *Colletotrichum* sp. by fungal isolate AUDI 585



III. Inhibition of *Fusarium oxysporum* by fungal isolate AUDI 585

**Plate 2: Antifungal activities of marine fungal isolate AUDI 585**

**Table 4.2.2.1. Marine fungal isolates showing more than 70 % inhibition against *Sclerotium rolfsii***

| AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition |
|---|---|---|---|---|---|
| 478 | 72 (58.03) | 572 | 70 (56.77) | 676 | 86 (68.00) |
| 481 | 84 (66.40) | 583 | 87 (68.84) | 684 | 82 (64.87) |
| 485 | 90 (71.54) | 585 | 100 (89.96) | 685 | 83 (65.63) |
| 496 | 70 (56.77) | 590 | 82 (64.87) | 692 | 84 (66.40) |
| 501 | 82 (64.87) | 607 | 74 (59.32) | 694 | 80 (63.41) |
| 507 | 82 (64.87) | 609 | 80 (63.41) | 695 | 84 (66.40) |
| 526 | 74 (59.32) | 615 | 72 (58.03) | 699 | 90 (71.54) |
| 544 | 75 (59.98) | 626 | 80 (63.41) | 703 | 86 (68.00) |
| 545 | 72 (58.03) | 630 | 77 (61.32) | 704 | 72 (58.03) |
| 547 | 100 (89.96) | 635 | 72 (58.03) | 712 | 78 (62.00) |
| 553 | 100 (89.96) | 651 | 93 (74.63) | 722 | 80 (63.41) |
| 563 | 85 (67.19) | 666 | 83 (65.63) | | |
| 570 | 80 (63.41) | 668 | 80 (63.41) | | |
| **S.Em ±** | | | **0.22** | | |
| **C.D. %** | | | **0.83** | | |
| **C.V. %** | | | **0.59** | | |

**Table 4.2.2.2. Marine fungal isolates showing more than 70 % inhibition against *Colletotrichum* sp.**

| AUDI No. | Per cent inhibition |
|---|---|
| 553 | 87 (68.85) |
| 563 | 82 (64.87) |
| 585 | 86 (68.00) |
| **S.Em ±**<br>**C.D. %** | **0.25**<br>**1.63** |
| **C.V. %** | **0.64** |

**Table 4.2.2.3. Marine fungal isolates showing more than 70 % inhibition against *Fusarium oxysporum***

| AUDI No. | Per cent inhibition |
|---|---|
| 553 | 72 (58.03) |
| 585 | 77 (61.32) |
| 712 | 72 (58.03) |
| **S.Em ±**<br>**C.D. %** | **0.25**<br>**1.61** |
| **C.V. %** | **0.72** |

**Table 4.2.3. Inhibition of three fungal pathogen from marine actinomycetes**

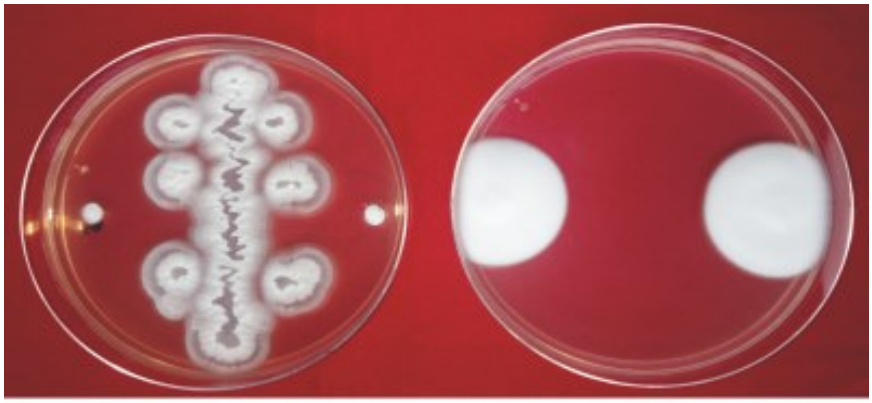
| Marine actinomycetes | Percent inhibition | | | Marine actinomycetes | Percent inhibition | | |
|---|---|---|---|---|---|---|---|
| | P1 | P2 | P3 | | P1 | P2 | P3 |
| AUDI-733 | 60 | 28 | 52 | AUDI-825 | 60 | 79 | 60 |
| AUDI-735 | 88 | 50 | 44 | AUDI-830 | 68 | 30 | 35 |
| AUDI-741 | 70 | 52 | 50 | AUDI-836 | 61 | 84 | 62 |
| AUDI-743 | 55 | 30 | 40 | AUDI-841 | 100 | 90 | 75 |
| AUDI-745 | 50 | 47 | 38 | AUDI-843 | 75 | 80 | 67 |
| AUDI-751 | 78 | 57 | 45 | AUDI-847 | 47 | 20 | 15 |
| AUDI-754 | 67 | 35 | 54 | AUDI-853 | 67 | 50 | 18 |
| AUDI-755 | 85 | 30 | 58 | AUDI-862 | 100 | 62 | 67 |
| AUDI-761 | 100 | 50 | 10 | AUDI-865 | 85 | 50 | 50 |
| AUDI-764 | 100 | 44 | 35 | AUDI-871 | 71 | 52 | 68 |
| AUDI-768 | 0 | 30 | 28 | AUDI-872 | 100 | 68 | 74 |
| AUDI-769 | 54 | 41 | 32 | AUDI-876 | 40 | 61 | 51 |
| AUDI-779 | 70 | 35 | 50 | AUDI-883 | 88 | 38 | 44 |
| AUDI-794 | 60 | 27 | 25 | AUDI-884 | 44 | 50 | 37 |
| AUDI-795 | 100 | 33 | 65 | AUDI-885 | 85 | 35 | 35 |
| AUDI-799 | 65 | 73 | 18 | AUDI-887 | 75 | 40 | 40 |
| AUDI-801 | 78 | 45 | 21 | AUDI-888 | 80 | 18 | 18 |
| AUDI-803 | 64 | 22 | 30 | AUDI-891 | 72 | 59 | 49 |
| AUDI-810 | 50 | 20 | 7 | AUDI-897 | 65 | 21 | 24 |
| AUDI-820 | 60 | 37 | 24 | AUDI-902 | 100 | 65 | 57 |
| AUDI-821 | 77 | 74 | 64 | | | | |

$P_1$: *Sclerotium rolfsii,*

$P_2$: *Colletotrichum sp.*

$P_3$: *Fusarium oxysporum*

I. Inhibition of *Sclerotium rolfsii* by actinomycete isolate AUDI 841



II. Inhibition of *Colletotrichum sp.* by actinomycete isolate AUDI 841

III. Inhibition of *Fusariumoxysporum* by actinomycete isolate AUDI 841

**Plate 3: Antifungal activities of marine actinomycete isolate AUDI 841**

**Table 4.2.3.1. Marine actinomycetes isolate showing more than 70 % inhibition against *Sclerotium rolfsii***

| AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition |
|---|---|---|---|
| 735 | 88 (69.71) | 843 | 75 (59.98) |
| 741 | 70 (56.77) | 862 | 100 (89.96) |
| 751 | 78 (62.01) | 865 | 85 (67.19) |
| 755 | 85 (67.19) | 871 | 71 (57.39) |
| 761 | 100 (89.96) | 872 | 100 (89.96) |
| 764 | 100 (89.96) | 883 | 88 (69.70) |
| 779 | 70 (56.77) | 885 | 85 (67.19) |
| 795 | 100 (89.96) | 887 | 75 (59.98) |
| 801 | 78 (62.00) | 888 | 80 (63.41) |
| 821 | 77 (61.32) | 891 | 72 (58.03) |
| 841 | 100 (89.96) | 902 | 100 (89.96) |
| **S.Em ±** | | **0.17** | |
| **C.D. %** | | **0.63** | |
| **C.V. %** | | **0.41** | |

**Table 4.2.3.2. Marine actinomycetes isolate showing more than 70 % inhibition against *Colletotrichum* sp.**

| AUDI No. | Per cent inhibition | AUDI No. | Per cent inhibition |
|---|---|---|---|
| 799 | 73 (58.67) | 836 | 84 (66.40) |
| 821 | 74 (59.32) | 841 | 90 (71.54) |
| 825 | 79 (62.70) | 843 | 80 (63.41) |
| **S.Em ±** | | **0.22** | |
| **C.D. %** | | **1.00** | |
| **C.V. %** | | **0.60** | |

Similarly, three fungal isolates against *Colletotricum* sp. and showed inhibition more than 70 %. A percentage of radial growth inhibition of *Colletotricum* sp. by AUDI 553, 563 and 585 were 87, 82 and 86 % respectively (Table 4.2.2 and Plate 2).

Out of fifty isolates of test fungi tested against *F. oxysporum,* only two isolates designated AUDI 553 and 585 had strong antagonistic activity with 72 % and 77 % inhibition of growth of pathogen (Table 4.2.2 and Plate 2).

The inhibitory effect significantly differed among the fungi and the Actinomycete isolates. Out of 41 isolates of Actinomycetes screened against three fungal pathogens, 53.6, 14.6 and 4.8 % of test Actinomycetes were found active against *S. rolfsii, Colletotrichum* sp. and *F. oxysporum* respectively showed inhibition more than 70 %.

The highest degree of inhibition was noticed in 25 % Actinomycete isolates against *S. rolfsii*. The reduction in mycelium growth and gradual darkening of mycelium of *S. rolfsii* was observed with increasing incubation period. Isolates showing more than 90 % inhibition did not differ significantly in their activity and isolates AUDI 761, 764, 795, 841, 862, 872 and 902 showed 100 % inhibition (Table 4.2.3. and Plate 3).

Against *Colletotrichum sp.* four Actinomycetes like AUDI 733, 821, 825, 836, 841 and 843, showed more than 75 % inhibition. Among these, strong activity was displayed by AUDI 841 with 90 % inhibition of growth of pathogen (Table 4.2.3. and Plate 3).

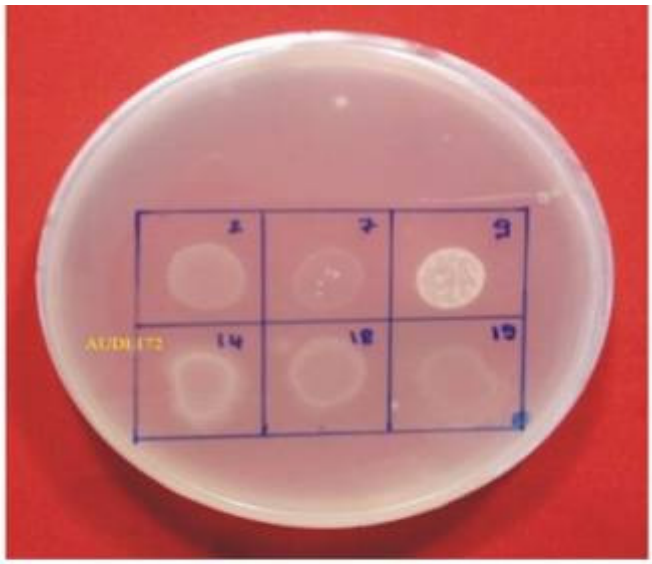
Only 4.8 % of the test actinomycetes were active against *F.oxysporum.*The highest activity was shown by AUDI 841 and 872 with 75 % and 74 % respectively showed effective against the fungal pathogen. But almost 95.2 % of total isolates showed inhibition less than 75 % (Table 4.2.3. and Plate 3).

## 4.3 Anti-phytopathogenic activity of test isolates of bacteria, fungi and Actinomycetes against bacterial pathogen

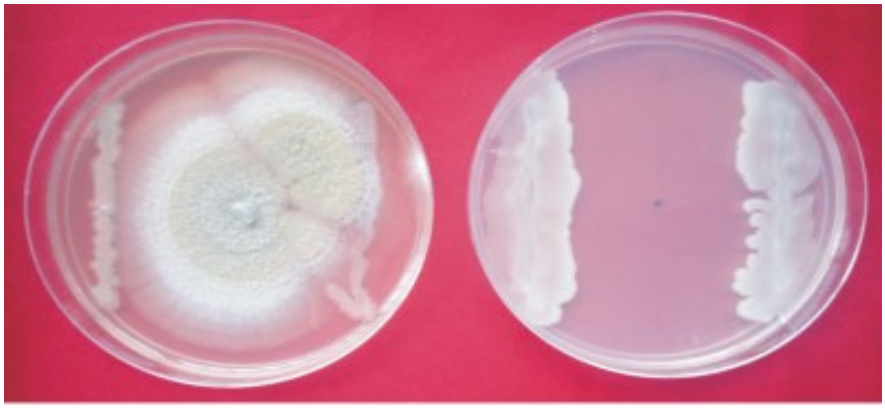
Of the 61 marine bacterial isolates tested for antibacterial activity against *R. solanacearum.* Only three isolates were found to be potentially active. Out of these, isolate AUDI 172 showed bactericidal and bacteriostatic action showed clear zone of inhibition ranging from 1-3 mm zone (Plate 4).

Out of 41 actinomycetes tested against bacterial phytopathogen, none of them showed bactericidal effect but insted promoted the growth of bacteria but isolates AUDI 755, 799, 841, 843, 862 and 872 showed bacteriostatic effect and isolate AUDI 841 showed good bacteriostatic effect and reduce the growth of the *R. solanacearum* compared with the control plate (Plate 4).

From total 50 selected isolates of marine fungi more than 90 % of isolate were not active against *R. solanacearum,* Isolate AUDI 485, 553, 570, 585 and 712 promoted the growth of bacteria but showed bacteriostatic effect when compared to control plate, isolate AUDI 585 mycelia grow over the test pathogenic bacteria and prevents the growth of the *R. solanacearum* (Plate 4).



I. AUDI 172 against *Ralstonia solanacearum*



II. AUDI 585 against *Ralstonia solanacearum*

III. AUDI 841 against *Ralstonia solanacearum*

**Plate 4: Antibacterial activity of AUDI 172, AUDI 585 and AUDI 841**

## 4.4 *In vitro* screening of the crude extract against the pathogens

The effect of production medium, duration of incubation and type of solvent used for efficient production and extraction of antimicrobial metabolites were studied. The study suggests that secondary metabolites can be efficiently produced in yeast extract glucose broth media for bacteria, and potato dextrose broth for marine fungi and soya casein digest broth for actinomycetes. Ethyl acetate was used as solvent for extraction of secondary metabolites. Agar well diffusion bioassay results revealed that the extracted metabolites show significant antimicrobial activity against the test pathogens.

Against *S. rolfsii* the metabolite extracted using ethyl acetate from the marine microbe isolates AUDI 172, AUDI 585 and AUDI 841 after 30 days of incubation in production media shows average zone of inhibition of 5 mm, 5 mm and 7mm respectively

The extracted metabolites were found to be less active against *Colletotrichum* sp. From the isolates AUDI 172, AUDI 585 and AUDI 841 the average maximum zone of inhibition of 6 mm, 5 mm and 4 mm respectively were observed in the metabolites extracted from production media after 30 days of incubation

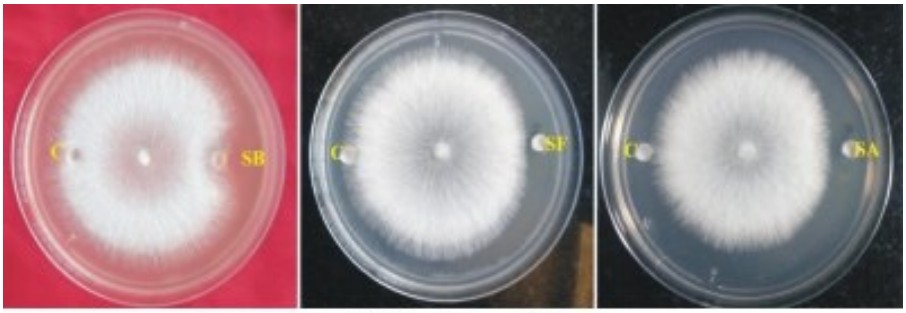
The metabolites extracted from the isolates AUDI 172, AUDI 585 and AUDI 841 were found to be less effective against *Fusarium oxysporum* and showed zone of inhibition of 2 mm, 6 mm and 5 mm respectively from the 30 days incubated isolates in production media.

Against *R. solanacearum* the metabolites extracted from the marine isolates AUDI 172, AUDI 585 and AUDI 841after 30 days of incubation in production media shows average zone of inhibition of radius 1.1 cm, 1.2 cm and 1.3 cm respectively.

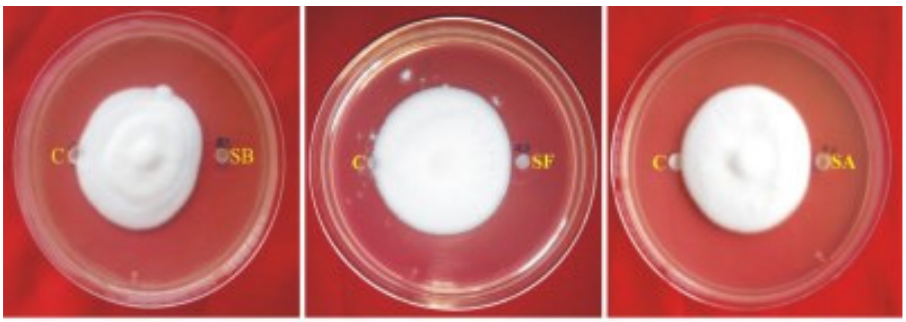
## 4.5 GC-MS analysis of secondary metabolites from the selected isolates

Chemical screening of the active compounds present in the crude extracts was done through GC-MS. The chemical compounds present in the metabolites were determined by mass spetra and retention time analysis. Compounds identified through the GCMS analysis revealed that metabolites mainly comprised of amines, aldehydes, fatty acids, alkanes and esters. Among the compounds produced by bacterial isolate AUDI 172, twelve compounds are found to possess the properties of antibacterial, antifungal and antimicrobial. (3,4-dimethoxy- phenol, diphenyl ether and propylthiouracil) compounds with purity 85, 86 and 87 % with antimicrobial activity (Furtado *et al*., 2002 ; Sun *et al*., 2015 ; Urquiza *et al*., 2016). 1-tetradecanol with purity 75 % is an antibacterial compound reported by Kumar *et al*., (2011). Tetradecanoic acid is an antifungal compound reported by Pohl *et al.,* (2011). Some of the compounds with higher purity and abundance and total ion chromatogram are given (Table no 4.5.1 and Fig. 4.5.1.1).

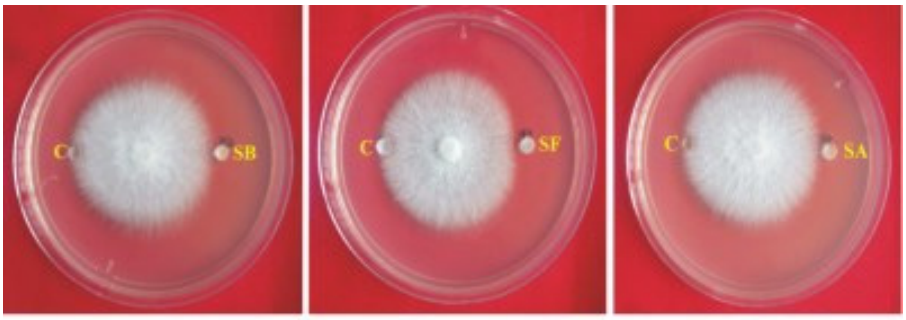
Twelve compounds were identified by GC-MS from the crud extract of metabolite produced by fungal isolate AUDI 585 among them Isoniazid, Tetradecanoic acid with 78 % and 28 % respectively with antifungal activity (Cordeiro *et al*., 2016; Pohl *et al.,* 2011). Both antibacterial and antifungal compounds includes 3-nitro- phenol, 2-nitro-phenol (Oikawa *et al*., 1985), dibutylphthalate (Roy *et al*., 2006) (Table 4.5.2) shows some of the compounds detected with higher abundance and purity and (Fig. 4.5.2.1) give the total ion chromatogram of the GCMS analysis.




I. *Sclerotium rolfsii*




II. *Colletotrichum* sp




III. *Fusarium oxysporum*

C: Control Ethyl acetate

SB : Crude extract from Bacteria AUDI 172

SF : Crude extract from Fungi AUDI 585

SA : Crude extract from Actinomycete AUDI 841

**Plate 5: Inhibition of the pathogen growth by extracted metabolites**

**Table 4.4.1. Marine microbe isolates selected for production of secondary metabolites**

| Marine microbial isolates | Test pathogens | Inhibition percentage / activity |
|---|---|---|
| **AUDI 172** | *Sclerotium rolfsii* | 100 |
| | *Fusarium oxysporum* | 80 |
| | *Colletotrichum* sp. | 83 |
| | *Ralstonia solanacearum* | Bactericidal and bacteriostatic |
| **AUDI 585** | *Sclerotium rolfsii* | 100 |
| | *Fusarium oxysporum* | 77 |
| | *Colletotrichum* sp. | 86 |
| | *Ralstonia solanacearum* | Bacteriostatic |
| **AUDI 841** | *Sclerotium rolfsii* | 100 |
| | *Fusarium oxysporum* | 75 |
| | *Colletotrichum* sp. | 90 |
| | *Ralstonia solanacearum* | Bacteriostatic |

**Table 4.5.1. Characteristics of the compounds identified from the marine bacterial isolate AUDI 172 by GC-MS**

| Retention time | Name of the compounds | Molecular formula | Molecular weight g/mol | m/z ratio | % quality | Activity | Reference |
|---|---|---|---|---|---|---|---|
| 6.1108 | 2-methylisothiazol-3(2*H*)-one | $C_4H_5NOS$ | 115.1 | 58 | 24 % | Antibacterial | Chapman and Diehi, 1994 |
| 7.5121 | Piperidine | $C_5H_{11}N$ | 85.15 | 85 | 21 % | Both Antibacterial and antifungal | Jahan*et al*., 2012 |
| 15.5596 | 2-butenal | $C_4H_6O$ | 70.09 | 97 | 47 % | Antimicrobial | Mitchell *et al.,* 2010 |
| 15.5596 | 1-tetradecanol | $C_{14}H_{30}O$ | 214.39 | 97 | 75 % | Antibacterial | Kumar *et al*., 2011 |
| 15.7895 | Naphthalene | $C_{10}H_8$ | 128.17 | 128 | 58 % | Not reported | |
| 15.9681 | Tetradecanoic acid | $C_{14}H_{28}O_2$ | 228.38 | 60 | 26 % | Antifungal | Pohl *et al.,*2011 |
| 16.0899 | 1-undecanol | $C_{11}H_{24}O$ | 172.31 | 83 | 48 % | Antibacterial | Togashi*et al*., 2007 |
| 16.0916 | 1-tridecene | $C_{13}H_{26}$ | 182.35 | TIC | 50 % | Antibacterial | Kumar *et al*., 2011 |
| 17.8474 | 3,4-dimethoxy- phenol | $C_8H_{10}O_3$ | 154.16 | 86 | 85 % | Antimicrobial | Furtado *et al*., 2002 |
| 19.3556 | 1-chloro-4-phenoxy- benzene | $C_{12}H_9ClO$ | 204.65 | 21 | 21 % | Antimicrobial | Priya*et al*., 2006 |
| 19.5621 | Phenol, 2,4-bis(1,1-dimethylethyl) | $C_{14}H_{22}O$ | 206.32 | 20 | 20 % | Antibacterial | Govindappa*et al*., 2014 |
| 20.0811 | Diphenyl ether | $C_{12}H_{10}O$ | 170.21 | 86 | 86 % | Antimicrobial | Sun *et al*., 2015 |
| 20.0811 | Propylthiouracil | $C_7H_{10}N_2OS$ | 170.23 | 87 | 87 % | Antimicrobial | Urquiza *et al*., 2016 |

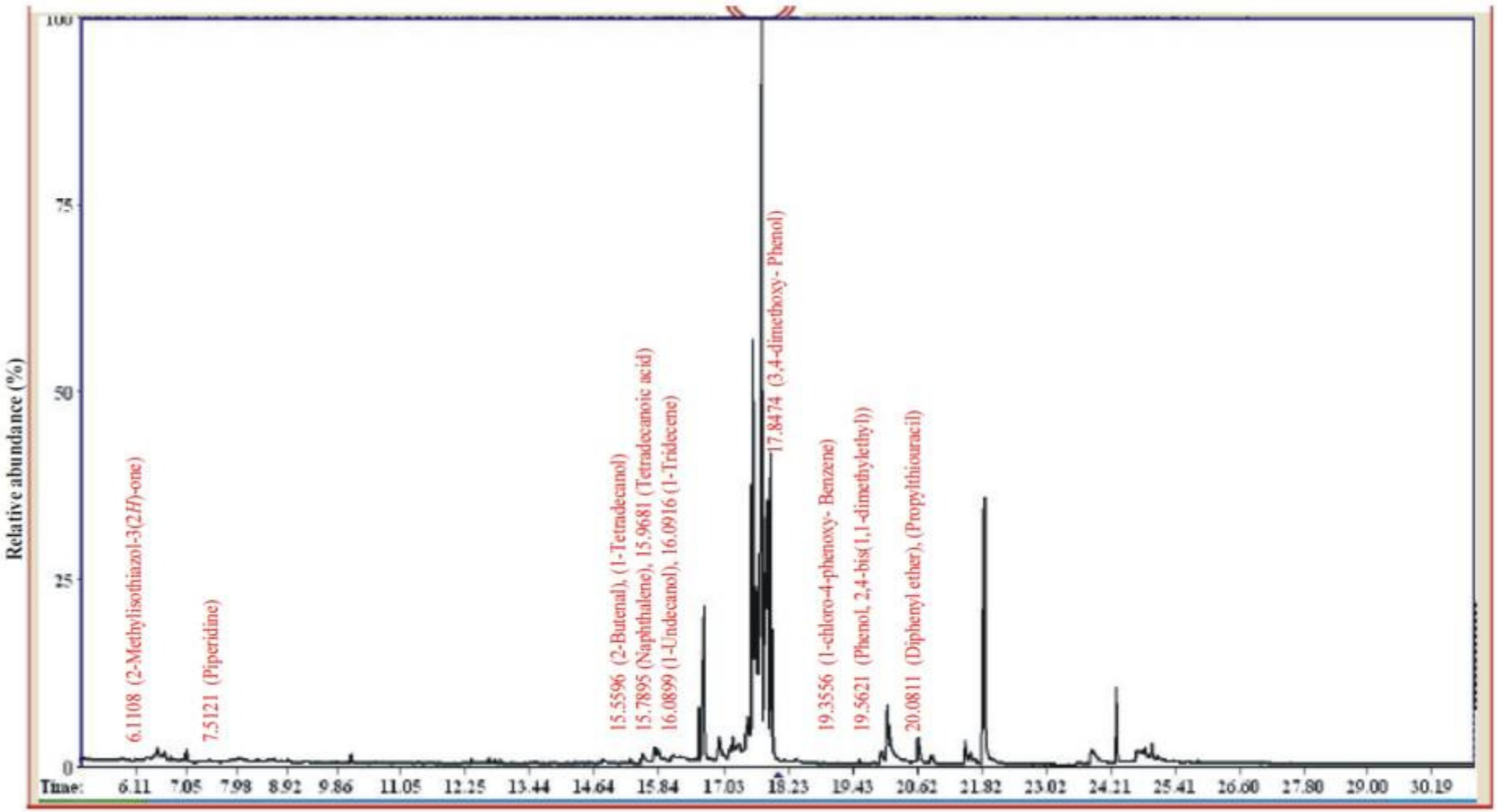



**Fig. 4.5.1.1 Total ion chromatogram of sample AUDI 172**

**Table 4.5.2. Characteristics of the compounds identified from the marine fungal isolate AUDI 585 by GC-MS**

| Retention time | Name of the compounds | Molecular formula | Molecular weight | m/z ratio | % quality | Activity | Reference |
|---|---|---|---|---|---|---|---|
| 5.9468 | Decane | $C_{10}H_{22}$ | 142.28 | 57 | 10 % | Antibacterial | Nahar*et al.*, 2016 |
| 6.8077 | 1-(4-hydroxy-3-methylphenyl) ethanone | $C_9H_{10}O_2$ | 150.17 | 151 | 8.50 % | Antibacterial | Kumar *et al.,* 2015 |
| 7.9002 | Isoniazid | $C_6H_7N_3O$ | 137.14 | 137 | 78 % | Antifungal | Cordeiro*et al.*, 2016 |
| 8.6958 | Indole | $C_8H_7N$ | 117.15 | 117 | 4.20 % | Not reported | |
| 8.8012 | Benzoic acid | $C_7H_6O_2$ | 122.12 | 105 | 5.90 % | Antifungal | Berne *et al.*, 2015 |
| 9.9941 | 3-nitro- phenol | $C_6H_5NO_3$ | 139.11 | 139 | 71 % | Antibacterial, antifungal | Oikawa *et al.*, 1985 |
| 9.9958 | 2-nitro-phenol | $C_6H_4NO_2$ | 139.109 | TIC | 74 % | Antibacterial, antifungal | Oikawa *et al.*, 1985 |
| 13.1234 | Phenol, 2,4-bis(1,1-dimethylethyl) | $C_{14}H_{22}O$ | 206.32 | 191 | 2.80 % | Antibacterial | Govindappa*et al.*, 2014 |
| 14.1483 | Diethyl phthalate | $C_{12}H_{14}O_4$ | 222.24 | 149 | 3.60 % | Antibacterial, antimicrobial | Selvin*et al.*, 2009 |
| 14.6595 | Propyl benzene | $C_9H_{12}$ | 120.19 | 91 | 13 % | Antifungal | Yuan *et al.*, 2012 |
| 15.9385 | Tetradecanoic acid | $C_{14}H_{28}O_2$ | 228.38 | 73 | 28 % | Antifungal | Pohl *et al.,*2011 |
| 18.0565 | Dibutyl phthalate | $C_{16}H_{22}O_4$ | 278.34 | 149 | 20 % | Antifungal, antibacterial | Roy *et al.*, 2006 |

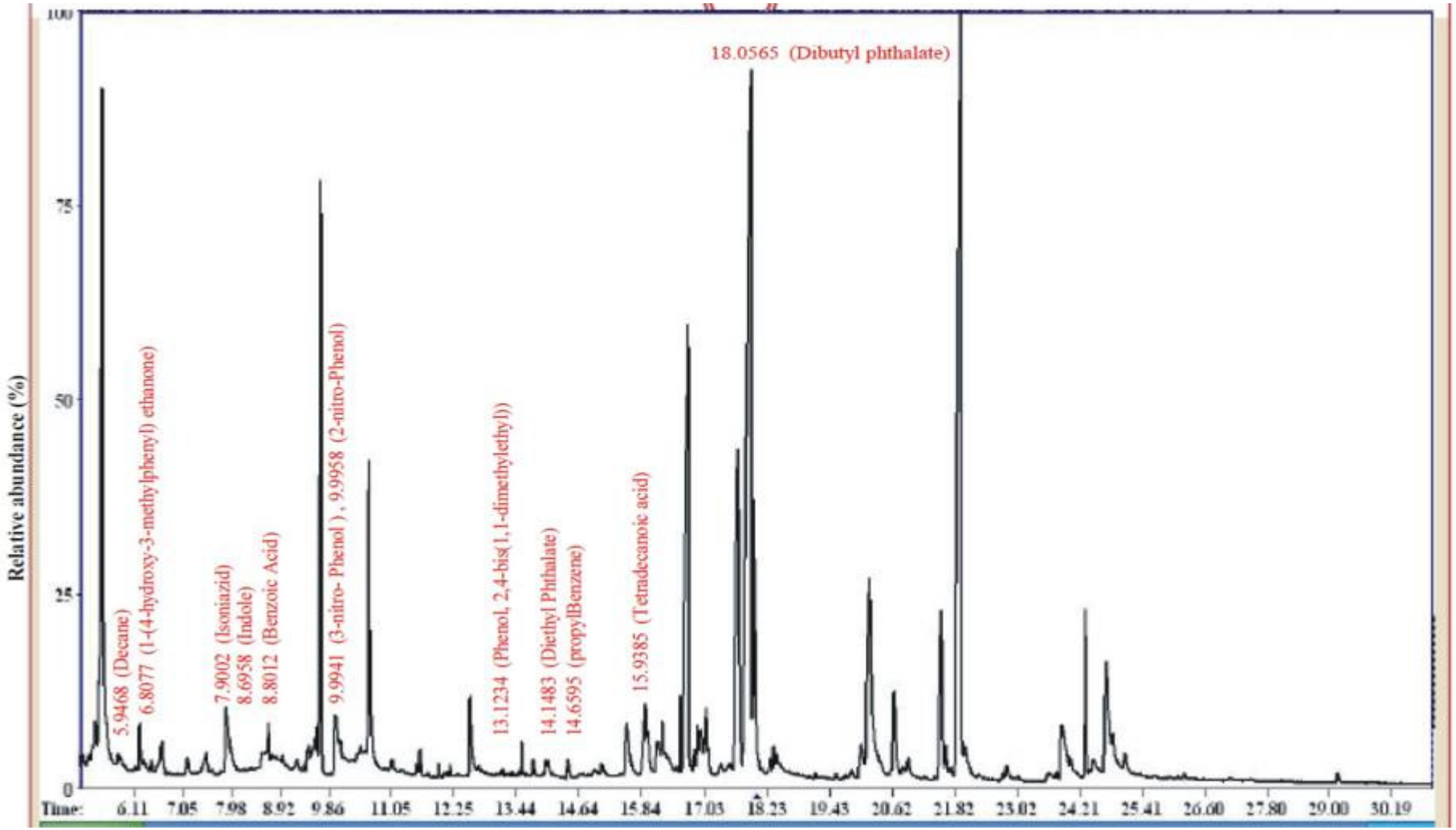



**Fig. 4.5.2.1 Total ion chromatogram of sample AUDI 585**

**Table 4.5.3. Characteristics of the compounds identified from the marine actinomycete isolate AUDI 841 by GC-MS**

| Retention time | Name of the compounds | Molecular formula | Molecular weight | m/z ratio | % quality | Activity | Reference |
|---|---|---|---|---|---|---|---|
| 6.1798 | 2-methylisothiazol-3(2*H*)-one | $C_4H_5NOS$ | 115.1 | 58 | 54 % | Antibacterial | Chapman and Diehi, 1994 |
| 6.3417 | 1-undecanol | $C_{11}H_{24}O$ | 172.31 | 71 | 32 % | Antibacterial | Togashi*et al*., 2007 |
| 7.5156 | 2-(hexyloxy)- ethanol | $C_8H_{18}O_2$ | 146.23 | 85 | 40 % | Not reported | |
| 9 | 1-methyl-2-pyrrolidinone | $C_5H_9NO$ | 99.13 | 99 | 22 % | Antimicrobial | Phaechanud*et al*., 2012 |
| 12.6279 | 1-hexadecanol | $C_{16}H_{34}O$ | 242.45 | 83 | 29 % | Antibacterial | Togashi*et al*., 2007 |
| 12.6351 | 1-dodecanol | $C_{12}H_{26}O$ | 186.34 | 70 | 23 % | Antibacterial | Togashi*et al*., 2007 |
| 15.5746 | Dibenzofuran | $C_{12}H_8O$ | 168.19 | 70 | 40 % | Antifungal | Shiu and gibbons, 2009 |
| 15.5746 | 1-tetradecanol | $C_{14}H_{30}O$ | 214.39 | 70 | 61 % | Antibacterial | Kumar *et al*., 2011 |
| 16.3331 | 1-nitroso- piperidine | $C_5H_{10}N_2O$ | 114.15 | 85 | 34 % | Antifungal | Rafiq *et al*., 2013 |
| 17.94 | 3,4-dimethoxy- phenol | $C_8H_{10}O_3$ | 154.16 | 154 | 36 % | Antimicrobial | Furtado *et al*., 2002 |
| 18.3587 | Vanillin | $C_8H_8O_3$ | 152.15 | 152 | 35 % | Both Antifungal and antibacterial | Neelakantan*et al*., 2010 |
| 20.2254 | Propylthiouracil | $C_7H_{10}N_2OS$ | 170.23 | 170 | 48 % | Antimicrobial | Urquiza *et al*., 2016 |
| 20.2427 | Diphenyl ether | $C_{12}H_{10}O$ | 170.21 | 84 | 55 % | Antimicrobial | Sun *et al*., 2015 |

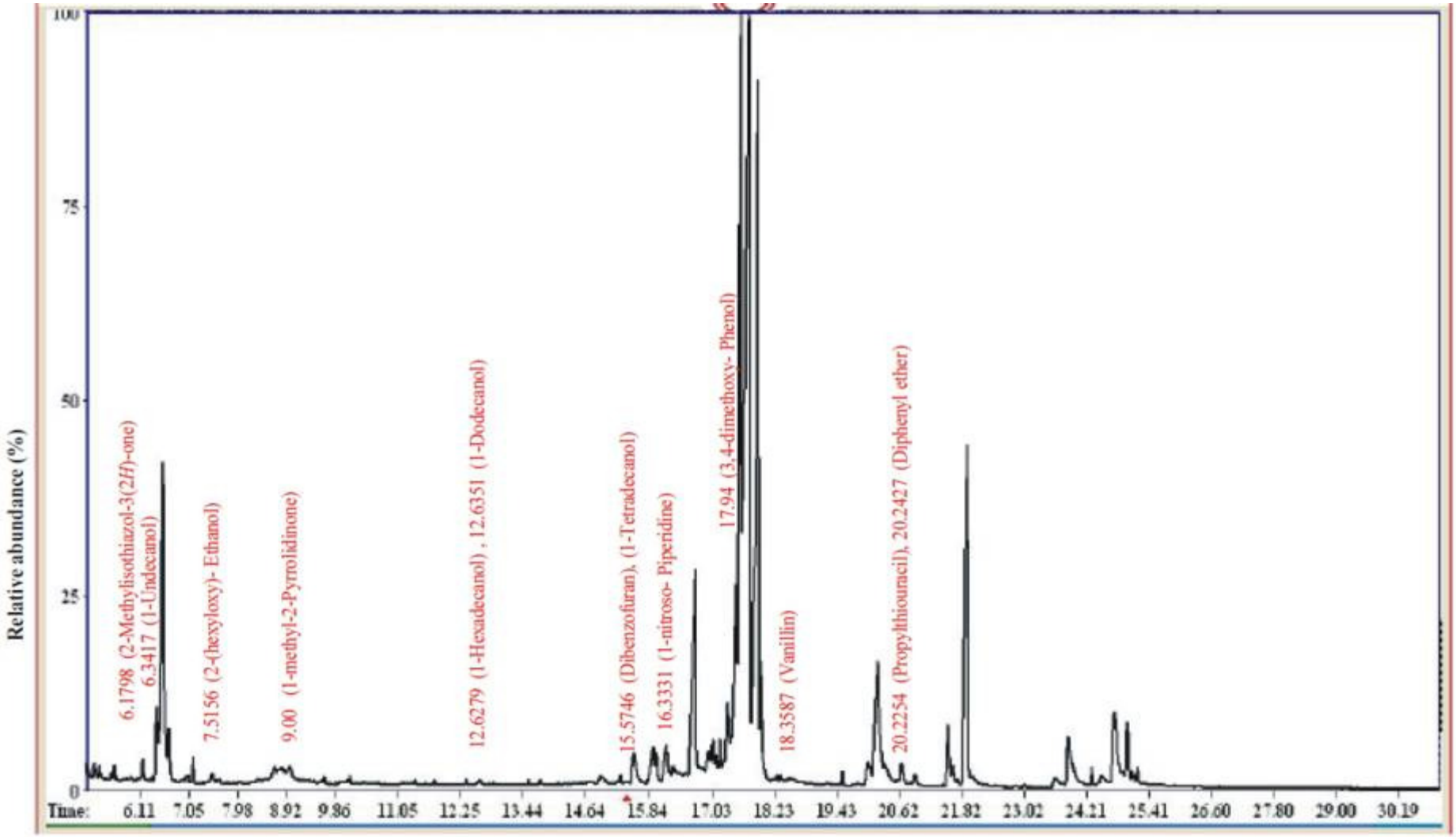



**Fig. 4.5.3.1 Total ion chromatogram of sample AUDI 841**

From the Actinomycete isolate AUDI 841 thirteen compounds were identified from the crude extract, out of which five compounds have been already reported as a antibacterial 2-methylisothiazol-3(2*H*)-one (Chapman and Diehl, 1994), 1-undecanol (Togashi *et al*., 2007), 1-hexadecanol and 1-dodecanol (Togashi *et al*., 2007), 1-tetradecanol (Kumar *et al*., 2011). Two compounds have been reported as antifungal dibenzofuran (Shiu and gibbons, 2009), and 1-nitroso-piperidine (Rafiq *et al*., 2013). And vanillin reported both antibacterial and antifungal activity. Some of the compounds with higher purity and abundance and total ion chromatogram are given Table 4.5.3 and Fig. 4.5.3.1.

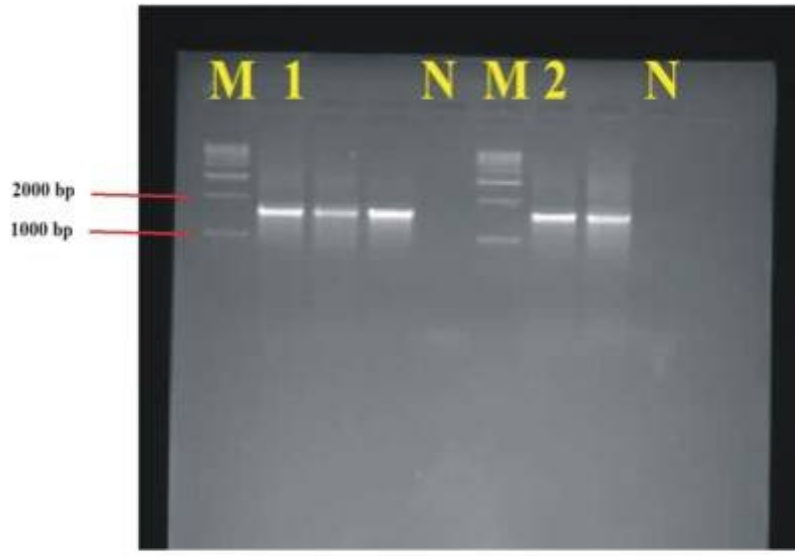
## 4.6 Molecular identification of potent isolates

### 4.6.1 PCR based amplification of 16s rDNA gene of potent bacteria and Actinomycete isolate and ITS for potent fungi

Amplification of 16s rDNA gene with universal primers was noticed (Table no 4.6.2) for potent bacteria and actinomycetes isolates. Approximately, 1.4 kb single amplicon was obtained at 57.3 $^{0}$C annealing temperature in both the isolates. A potent fungus was checked for amplification of ITS region. About 600 bp single amplicon was obtained at 64 $^{0}$C annealing temperature (Plate 7a and 7b).

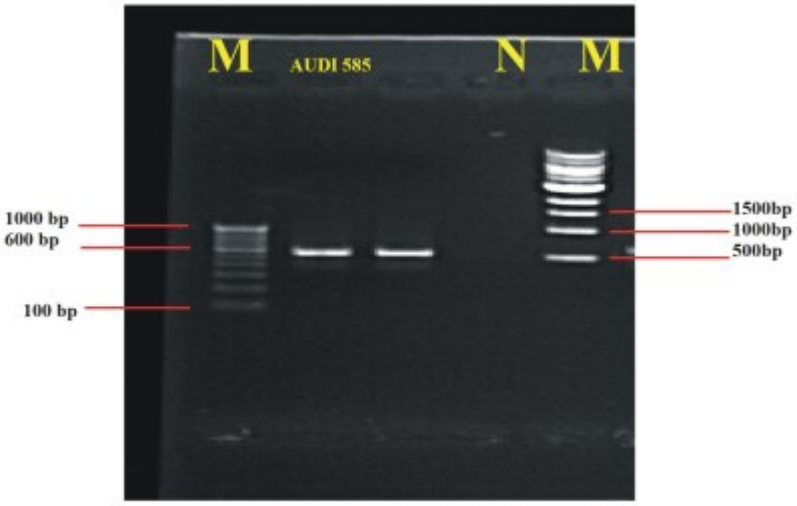
### 4.6.2 PCR amplification of PKS-1 and NRPS gene fragments

For amplification of PKS-1 and NRPS gene fragments were checked among potent marine microbial isolates using degenertative primers (Table 4.6.4) The PKS-1 primer (Ksma-F and ksmb-R), could amplify a sequence of ~700 bp from all the potent isolates and the NRPS primer (ADEdom5 and ADdom3) could amplify a sequence of ~450 bp from all the isolates (Plate 8a and 8b).




M=λ DNA *EcoR*1/*HindIII* double digest
N= Negative control
1= AUDI 172
2= AUDI 841

**Plate 7a: PCR based amplification of 16s rDNA fragment of potent bacteria**

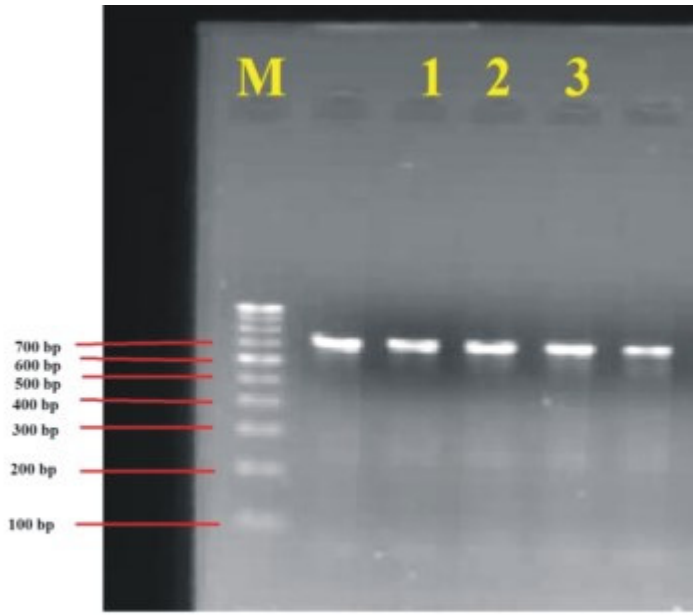



M=λ DNA *EcoR*1/*HindIII* double digest
N= Negative control

**Plate 7b: PCR based amplification of ITS fragment of potent fungi**

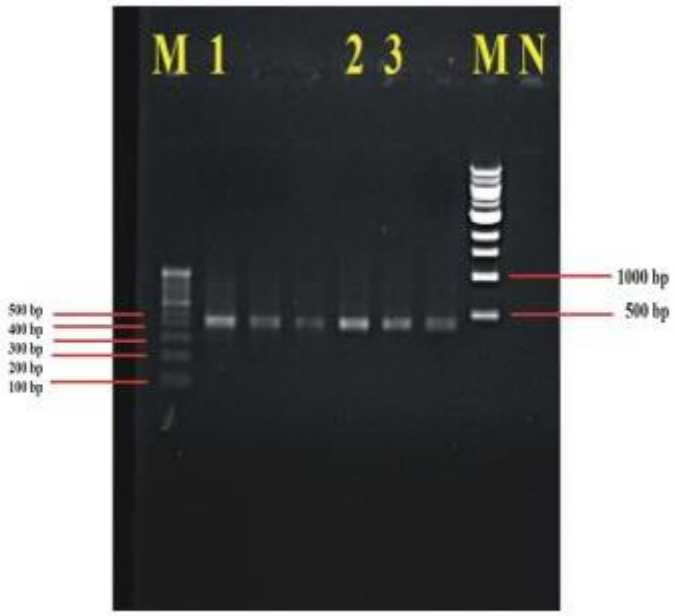
**Table 4.6.2. Identification of potent bacteria, actinomycete and fungi isolates by sequencing**

| Primers | Isolates | Type of microbe | Blast results | Accession no. | % similarity |
|---|---|---|---|---|---|
| 16s rDNA | AUDI 172 | Bacteria | *Enterobacter cloacae* | KR265465.1 | 82 |
| | AUDI 841 | Actinomycetes | *Streptomyces macrosporus* | NR_112441.1 | 99 |
| ITS | AUDI 585 | Fungi | *Debaryomyces hansenii* | KR264906.1 | 100 |




M=λ DNA *EcoR*1/*HindIII* double digest
1= AUDI 172 2= AUDI 585 3=AUDI 841

**Plate 8a: PCR based amplification of PKS-1 fragment of potent marine**




M=λ DNA *EcoR*1/*HindIII* double digest
N = Negative 1= AUDI 172 2= AUDI 585 3=AUDI 841

**Plate 8b: PCR based amplification of NRPS fragment of potent marine**

**Table 4.6.4. BLAST analysis of amplified sequences of PKS-1 and NRPS gene fragments of potent isolates**

| Sl . No. | Primer pairs | Isolates | Sequence source | Domain identified | Accession no. | % identity |
|---|---|---|---|---|---|---|
| 1 | Ksma-F & Ksmb-R | AUDI 172 | *Bacillus subtilis* isolate 2B, clone 2B8 | ketosynthase | LT555282.1 | 83 % |
| 2 | | AUDI 585 | *Dichomitus squalens* LYAD421 SS1 | ketoacyl-synt-domain | XM_007368240.1 | 92 % |
| 3 | | AUDI 841 | *Streptomyces albus* strain CCM4719 | salinomycinpolyketide synthase type I | DQ630728.1 | 92 % |
| 4 | ADEdom5 & ADdom3 | AUDI 172 | Uncultured bacterium | non-ribosomal peptide synthetase | AM492265.1 | 94 % |
| 5 | | AUDI 585 | *Aspergillus fumigatus* Af293 | non-ribosomal peptide synthase Pes1 | XM_747311.1 | 93 % |
| 6 | | AUDI 841 | *Streptomyces* sp. BSH5019 | NRPS A domain | JX008827.1 | 92 % |

# 5. DISCUSSION

Since life most likely began in the oceans, marine microorganisms are the closest living descendents of the original form of life as major pillars of the biosphere. It is now known that microorganisms live in every corner of the oceans. Their habitats are diverse and include open water, sediment, estuaries and hyper saline environments. By studying these habitats scientist has been developed ability to predict the composition of marine microbial communities. The composition as well as diversity of microbial communities is largely affected by different ecophysiological parameters in the ocean. Marine microbes are also able to adapt too many extreme environments in the ocean.

Microbial biodiversity in protected areas are often not easily accessible for sample collection for research study, limiting our understanding of the ecosystem structure. Our knowledge of microbial diversity, antibacterial, antifungal and biological control ability of the microbes isolated from these areas is therefore inadequate, yet this is critical in understanding the biotechnological potential of this microbial diversity. Their unique metabolism allows marine microorganism to carry out many steps of the biogeochemical cycles that other organisms cannot complete. The metabolic capabilities of marine microbes can be put to work in any number of biotechnology applications including the manufacture of industrial products and energy production. Marine microbes are source of novel bioactive compounds that may have medical, agricultural and industrial application. A great deal of research on the biogeography of marine microorganisms has been carried out, but many unknowns persist and more work is needed to elucidate and understand their complexity.

In the present study, an attempt was made to explore the microbial diversity of different marine habitats and analyses their potential for utility in the field of agriculture.

In this study, use of culture dependent approaches resulted in the recovery of large population of bacteria on Nutrient agar and ZoBell's 2216 marine agar than Sea water agar. This is may be due to the fact that higher plate counts from marine materials are obtained using complex rather than simple media (ZoBell, 1946). For isolation of Actinomycetes Kuster's agar, Humic acid, vitamin B media have been widely used. Due to high molecular weight and presence of polymers like starch or casein, less bacterial contamination on the media (Grey *et al.,* 1971) was observed. The second large population of fungi was isolated by using Potato dextrose agar and Rose bengal agar. These media were supplemented with dextrose and glucose which acts as carbon source and yeast extract served as source of nitrogen. The slow growth of weed associated endophytic microorganisms in earlier studies may be attributed to less competition and availability of less nutrients in the extracellular spaces of plant cells. Hence, in this study ten times diluted culture media were used for isolation of weed associated microorganisms.

Collectively 152 microbial isolates were obtained from the previously isolated potent cultures from the marine water sample from coastal parts of Karnataka, Maharashtra and Goa by Alone, 2012. All cultures were purified by using quadrant streaking method (Lakshmipathy and Kannabiran, 2010) overnight grown cultures into which 0.8 % DMSO and 20 % glycerol were added (Magarvey *et al,* 2004) and preserved as frozen stock of pure cultures. Well developed, morphologically distinct, pigmented and purified 152 isolates including 61 bacteria, 50 fungi and 41 Actinomycetes were selected for screening of antagonistic activity against four plant pathogens: *Ralstonia solanacearum, Sclerotium rolfsii, Colletotricum* sp. and *Fusarium oxysporum.*

Marine microorganisms have gained importance in recent years because of production of novel metabolites, which exhibit antimicrobial, antiviral, antitumor properties. These secondary metabolite serves as model system in discovery of new drugs (Bernan *et al*., 1997). Marine microorganisms have been known for showing antibacterial and antifungal activity against human as well as plant pathogens (Byun *et al.,* 2003).

## 5.1 *In vitro* primary screening for antimicrobial activity

In present study, 152 randomly selected microbial isolates were tested for antibacterial as well as antifungal activity against four plant pathogens: *Ralstonia solanacearum, Sclerotium rolfsii, Colletotricum.* sp and *Fusarium oxysporum.* On the basis of mode of action of test isolates against bacterial pathogens activity was considered as bactericidal or bacteriostatic. Some isolates of bacteria like isolate AUDI 172 showed bactericidal and bacteriostatic action showed clear zone of inhibition ranging from 1-3 mm zone AUDI 185, 188 and 203 showed bactericidal activity and formed clear zones ranging from 1-3 mm but AUDI 376 showed bacteriostatic action against *R. solanacearum.*

In present study, all 152 marine test isolates provided evidence that they can effectively inhibit the growth of plant fungal pathogens as well. All *in vitro* assays were conducted on Soyabean casein digest agar which contains mainly soya peptone and pancreatic digest of casein are known to induce more metabolite production. The high amount of nitrogen source allowed the organism to enter into idiophase quite early, leading to production of more metabolites with extracellular chitinase and P-1,3 glucanase activity (Basavaraj *et al.,* 2010). In this study also majority of test isolates of bacteria, fungi and actinomycetes displayed detectable antagonistic activity.

Against *Sclerotium rolfsii,* the highest degree of 100 % inhibition was noticed by 57.3 % of bacteria, 6 % of fungi and 17 % of the total actinomycetes. The mode of inhibition of *S. rolfsii* was either completely stoppage of growth or gradual darkening of mycelium. Certain isolates such as, AUDI 65, 172 and 194 of bacteria, AUDI 547, 553 and 585of fungi and AUDI 761, 764, 795, 841, 843, 862, 872 and 902 of Actinomycetes resulted into complete lysis as well as gradual browning of mycelia with increasing incubation period. The growth of *S. rolfsii* through its ability to grow much faster among the fungi thus competing efficiently for space and nutrients (Siameto *et al.,* 2010).

Antagonistic activity against *Colletotricum* sp. significantly differed among bacteria, fungi and actinomycetes isolates. Out of isolates tested, 6.5 % of bacteria, 6 % of fungi and 15 % of Actinomycete showed more than 70 % activity. Among bacteria AUDI 172 and 194 showed strong inhibition of 83 and 82 % respectively. Similarly, AUDI 585 of fungi strong inhibition of 86 % and AUDI 841 of actinomyceteshowed 90 % of inhibition of pathogen.

Antifungal activity against *F. oxysporum* by test bacteria isolate AUDI 18, 100, and 172 showed more than 70 % inhibition but the reduction in mycelium growth was observed by most of the bacterial isolates when grown on Soyabean casein digest agar media. Among 50 fungal isolates, AUDI 553 and 585 had antagonistic activity with 72 % and 77 % inhibition of *F. oxysporum.* Most of the isolates of fungi showed no activity or growth promoting activity. Similarly, out of 41 actinomycetes isolates AUDI 841 and 872 displayed 75 % AND 74 % inhibition respectively.

These results indicate that, antifungal activity exhibited by these isolates might be due to secretion of hydrolytic enzymes such as chitinase, P-1,3 glucanase, chitosanase and proteases (Yuan *et al*., 1995) which degrade the fungal cell wall or secretion of antifungal compounds (Khamna *et al.,* 2009). Cook (1985) reported that the use of antagonistic bacteria as biocontrol agents is extremely promising in reducing the spore germination of the pathogens. Among three fungal pathogens, control of mycelium growth or complete stoppage was greater for *S. rolfsii,* when compared to *Colletotricum sp* and *F. oxysporum* which suggest that *S. rolfsii* is more susceptible than *Colletotricum sp* and *F. oxysporum.* Antifungal activity of microorganisms is mostly due to the effect of lytic enzymes. This enzyme was found degrading the fungal cell wall (Sindhu & Dadarwal, 2001). However, further studies are necessary to evaluate the effect of these potential biocontrol agents in greenhouse and field conditions and also to purify and characterize the secondary metabolites produced by these microorganisms.

Among 50 fungi screened isolates AUDI 553, 585 and 712 promoted the growth of bacteria but showed bacteriostatic effect when compared to control plate, isolate AUDI 585 mycelia grow over the over test bacteria and prevents the growth of the *R. solanacearum.* This antibacterial activity may be attributed to the role of multiple enzymes like lipase, cellulose, protease (Jeffery, 2007) or some toxic secondary metabolite. In some isolates, overgrowth of fungal mycelia on bacterial pathogen was noticed which indicate the growth promoting activity of test fungi by bacterial pathogen.

Out of 41 Actinomycetes tested against bacterial phytopathogen, and isolate AUDI 841 showed good bacteriostatic effect and reduce the growth of the *Ralstonia solanacearum* compared with the control plate. And none of showed bactericidal effect and promoted the growth of bacteria but isolates AUDI 755, 799, 841, 843, 862 and 872 showed bacteriostatic effect.

## 5.2 Extraction of antimicrobial metabolites and *in vitro* secondary screening using the extract

Several factors seem to influence the production of secondary metabolite at laboratory condition. One such factor is the production medium where potent marine microbial isolates are allowed to grow and produce the metabolites. In this study, soybean casein digest broth for Actinomycetes, yeast extract glucose broth media for bacteria and potato dextrose broth for marine fungi were used for production. Wang *et al*., 2009, revealed that higher molecular weight carbon source such as starch enhances the production of secondary metabolites. They also revealed that the synthesis of antibiotic was related to phosphoenolpyruvate sugar phosphotransferase system. And controlling the concentration of carbon sources could regulate the synthesis of antibiotics. Soya peptone and pancreatic digest of casein which are known to induce more metabolite production (Burkholder *et al*., 1955). Swathi, 2013 inoculated marine fungal cultures in a conical flask containing Potato Dextrose Broth and incubated for production of antimicrobial compounds. Sarker *et* al., 2015 reported that fermentation of marine bacteria in liquid media is very important for the production of secondary metabolites and small scale liquid fermentation is the most frequent fermentation process to obtain sufficient quantity of the active secondary metabolites, fermentation was carried out in yeast-extract glucose broth medium. The higher the amount of nitrogen source helps the organism to enter

the idiophase quite early as the organism enters the idiophase, they start producing the secondary metabolites. Another factor that influences the production of secondary metabolite is the incubation. It is common knowledge that organisms when expose to stress condition producer secondary metabolites. These stresses may be achieved either by growing them in limited nutrient source, insufficient light and oxygen, or growing for more period of time till nutrient exhaustion. Overall outcome suggested that extending period of incubation is required for the microorganisms to produce significant amount of antimicrobial metabolites. This indicates that choice of production media is main responsible for the production of antimicrobial compounds from the marine isolates.

In this study, for extraction of extracellular antimicrobial metabolites solvent ethyl acetate used. The use of ethyl also are reported by earlier workers (Sunaryanto *et al*., 2010; Wu *et al., 2007*). The extraction of the active agent was processed using ethyl acetate (1:1). It showed a maximum inhibition zone (Naggar and Barakat, 2009). Sarker *et al*., 2015 releaved that using ethyl acetate solvent for extraction of diverse natural compounds from marine bacteria that play a significant role. The extracellular compounds from the cultural supernatant of marine actinomycetes were extracted by liquid- liquid extraction method using ethyl acetate and concentrated by evaporation (Bavya, 2011). Swathi, 2013 used ethyl acetate for the extraction of secondary metabolites from the marine fungi *Microascus spp.* The marine fungal strains from *Aspergillus* produced more new antibacterial and antifungal compounds extracted from EtOAc, it is the most common solvent for the extraction of marine fungal cultures, which can also extract abundant compounds from mycelia or liquid culture, especially compounds with low or medium polarity (Xu *et al*., 2015).This additionally indicates that the choice of solvent may be same for all potent isolates.

## 5.3 Chemical characterization of the compounds produced by the potent isolates

The crude extract containing the metabolites were analyzed by gas chromatography mass spectrometry (GC-MS). The used of GC-MS for compounds analysis has been widely reported. This technique has been found to be effective as it can detect every compound present even in low concentration. The availability of compound library for GC-MS makes it even easier for analysis. Compounds identified through the GC-MS analysis revealed that the crude metabolites mainly comprise of amines, aldehydes, fatty acids, alkanes and esters. Several compounds produced by AUDI 172, 585 and 841 were analysed for their biological activity. From the compounds produced form the marine bacteria isolate AUDI 172, thirteen compounds were identified, 2-methylisothiazol-3(2*H*)-one commonly called methylchloro-isothiazolone is a bacteriostatic and bactericidal compound detected with the quality of 24 per cent (Chapman and Diehl, 1995). 1-tetradecanol and 1-tridecene with quality of 75 and 50 per cent respectively was having antibacterial activity (Kumar *et al*., 2011). 3,4-dimethoxy- phenol, 1-chloro-4-phenoxy- benzene, diphenyl ether and propylthiouracil are having antimicrobial activity with quality of 85, 21, 86 and 87 respectively (Furtado *et al*., 2002; Priya *et al*., 2006; Sun *et al*., 2015; Urquizaa *et al*., 2016). Tetradecanoic acid was reported to show antifungal activity (Pohl *et al.,* 2011).

Twelve compounds identified by GC-MS of marine fungal isolate AUDI 585 were analyzed for biological activity.3-nitro- phenol, 2-nitro-phenol and dibutyl phthalate were detected with both antibacterial and antifungal activity (Oikawa *et al*., 1985; Roy *et al*., 2006) with 71, 74 and 28 per cent quality. Isoniazid, benzoic acid, propyl benzene, tetradecanoic acid detected with antifungal activity (Cordeiro *et al*., 2016; Berne *et al*., 2015; Pohl *et al.,* 2011).

From the marine Actinomycete isolate AUDI 841 thirteen compounds were identified and analyzed for biological activity. Antibacterial activity compounds were detected 2-methylisothiazol-3(2*H*)-one (Chapman and Diehl, 1995), 1-undecanol, 1-hexadecanol, 1-dodecanol (Togashi *et al*., 2007) and 1-tetradecanol (Kumar *et al*., 2011) with quality of 54, 32, 29, 23 and 61 per cent respectively. Shiu and gibbons, 2009 reported dibenzofuran with antifungal activity. 1-nitroso-piperidine with 34 per cent quality reported with antifungal activity (Rafiq *et al*., 2013). Over all study of the extract by GC-MS revealed that the isolates produced many compounds which showed different biological activity. The antimicrobial property of the isolates could be due to the individual compound detected or complex of compounds interacting among each other. Thus the crude extract has potential for many useful functions that can aid plant growth and sustainable economic yield.

## 5.4 Molecular identification of potent isolates

The molecular biology based approach like 16s rRNA and ITS region characterization, is widely used for identification and determination of evolutionary relationships of bacteria, actinomycetes and fungi at intra and intergeneric levels (Woese 1987; Stackebrandt *et al.,* 1992). In present study, the 16srRNA and ITS-PCR based tool was used for identification of potent marine isolates of bacteria, Actinomycetes and fungi.

Among 16s rRNA screened with the potent marine isolates bacteria AUDI 172 showed 82 % similarity of *Enterobacter cloacae* with accession no KR265465.1 and marine Actinomycetes showed 99 % similarity with *Streptomyces macrospores* and ITS-PCR based identification of marine fungi AUDI 585 shown 100 % similarity with *Debaryomyces hansenii*.

## 5.5 Molecular characterization of gene fragments of biosynthetic PKS-1 and NRPS gene cluster

With recent advances in understanding of the molecular genetics of natural product biosynthesis coupled with increasing access to DNA sequencing, the sequenced based approaches for natural product discovery has now become a very useful technique (Gontang *et al.,* 2010). It is known that most of the secondary metabolites are products of either polyketide synthases (PKSs) gene cluster of non-ribosomal peptide synthases (NRPSs) gene clusters. With the objective to study the genetic basis of the antimicrobial activity shown by the potent marine microbes, the gene fragments of gene clusters which are predicted to be responsible showing antimicrobial activity were targeted. PKS-1 is a multifunctional polypeptides encoded by a variable number of modules with multiple enzymatic activities. Each PKS-1 module encodes at least three domines corresponding to a ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) involved in the selection and condensation of the extender unit (Ayuso and Genilloud, 2005). Enzyme modules contain three catalytic domains: in NRPS, the adenylation (A) domain is responsible for recognition and activation

of its related amino acid or hydroxyl acid followed by transfer of the activated substrate to the same module (Nikolouli and Mossialos, 2001). The degenerative primers pair Ksma-F &Ksmb-R was designed (Izumikawa *et al*., 2003) for PKS-1 and for NRPS ADEdom5 & ADdom3 was designed (Busti *et al*., 2006) to targeting the PKS-1 and NRPS gene fragments. The primer pairs were mainly used for screening of the PKS-1 and NRPS gene clusters. The BLASTn analysis of sequences of the PKS-1 and NRPS form potent marine isolates AUDI 172, AUDI 585 and AUDI 841 revealed that the primer pairs could amplified the conserved domain of ketosynthase, ketoacyl-synt-domain and salinomycin polyketide synthase type I of the polyketide synthase-I and non-ribosomal peptide synthetase, non-ribosomal peptide synthase Pes1 and NRPS A domain of the NRPS. Stack *et* al., 2007 reported nonribosomal peptide synthetases (NRP synthetases) are large multi-functional enzymes containing adenylation, thiolation (or peptidyl carrier protein, PCP) and condensation domains.Izumikawa *et al*., 2003 cloned of polyether polyketide synthase (PKS) genes for salinomycin biosynthesis was attempted from *Streptomyces albus*. The antimicrobial bioassay of bacteria with NRPS genes were carried out to confirm the screening of NRPS genes. Nonribosomal peptide synthetase (NRPS) adenylation (A) domain genes were investigated by polymerase chain reaction (Zhang *et al.,* 2009). Isolates AUDI 172, AUDI 585 and AUDI 841 (sequences amplified with Ksma-F &Ksmb-R) were found to be 83, 92 and 92 % identity of the partial PKS-1 genes of *Bacillus subtilis* partial KS gene for ketosynthase (LT555282.1)*, Dichomitussqualens* LYAD421 SS1 (XM_007368240.1) and *Streptomyces albus* strain CCM4719 (DQ630728.1) respectively. Sequences amplified with ADEdom5 & ADdom3 for NRPS for the potent marine isolates AUDI 172, AUDI 585 and AUDI 841 were found 94 %, 93 % and 92 % identity of the partial NRPS gene of uncultured bacterium with partial nrps gene (AM492265.1), *Aspergillus fumigatus* Af293 (XM_747311.1) and *Streptomyces sp*. BSH50108827.1) respectively. The lesser % identity indicates the possibility of a novel compound being produced by the potent marine microbes.

## Future line of work

1. Fine characterization of the chemical compounds produced by the strains with a view to nail down the particular compound or metabolite showing the antimicrobial compound.
2. Evaluation of the effect of potential biocontrol agents in greenhouse and field conditions.
3. Whole genome sequencing to validate observation of this study to deduce the ability of produces other important secondary metabolites and functionally important traits.

# 6. SUMMARY AND CONCLUSION

The present investigation was carried out with the aim to exploit the antimicrobial property of marine microbes. Previously isolated microbial cultures from the marine water and sediment samples from coastal parts of Karnataka, Maharashtra and Goa of total 152 marine microbial isolates contains 61 bacterial, 50 fungal and 41 actinomycetes were selected for the studies. *In vitro* screenings of the selected isolates for anti-phytopathogenic property were studied against the phytopathogens via *Sclerotium rolfsii, Fusarium oxysporum, Colletotrichum sp. and Ralstonia solanacearum.* Among the marine microbial isolates bacteria AUDI 172, fungi AUDI 585 and Actinomycetes AUDI 841 showed good antimicrobial activity. The secondary metabolites were extracted from the above isolates using solvent-solvent extraction method and also checked for antimicrobial activity against the same pathogens. Further, characterizations of the bioactive compounds present in the partially purified crude extract of the isolates were also performed by GCMS. Finally, the investigation ended with the cloning of the genes probably involved in the secondary metabolites production pathway. Following are the outcomes of the present investigation.

1. *In vitro* screening results revealed that AUDI 172 bacteria showed 100, 83 and 80 per cent inhibition of *S. rolfsii*, *Colletotrichum sp* and *F. oxysporum* respectively.
2. Hundred per cent antagonistic effect was also shown by AUDI 585 fungal isolate against *S. rolfsii* and, 86 and 77 per cent inhibition against *Colletotrichum sp and F. oxysporum.*
3. Isolate of actinomycetes AUDI 841 shown 100, 90 and 75 per cent inhibition of *S. rolfsii*, *Colletotrichum sp* and *F. oxysporum* respectively.
4. Against *R. solanacearum* bacterial isolate AUDI 172 shown zone of inhibition ranging from 1-3 mm zone which was showed bacteriostatic and bactericidal property. Similarly isolates of Fungal AUDI 585 and Actinomycetes AUDI 841 were shown bacteriostatic properties.
5. *In-vitro* screening of the antimicrobial metabolites extracted from the isolates were showed highest activity when they are allowed to fermentation for 30 days. Ethyl acetate was used for extraction of the metabolites.
6. The metabolites extracted using ethyl acetate from the bacterial isolate AUDI 172 was shown zone of inhibition of 5 mm, 6 mm, 2 mm against *S. rolfsii, Colletotrichum sp* and *F. oxysporum* respectively.
7. Ethyl acetate extracted metabolites from the fungal isolate AUDI 585 was shown zone of inhibition of 5 mm, 5 mm and 6 mm against *S. rolfsii Colletotrichum* sp. and *F. oxysporum* respectively.
8. The isolate AUDI 841 Actinomycetes metabolites extracted from ethyl acetate was shown 7mm, 4mm and 5mm zone of inhibition against *S. rolfsii, Colletotrichum sp* and *F. oxysporum* respectively.
9. Against *R. solanacearum* the metabolites extracted from the marine isolates AUDI 172, AUDI 585 and AUDI 841 after 30 days of incubation in production media shows average zone of inhibition of radius 1.1 cm, 1.2 cm and 1.3 cm respectively.

10. GC-MS analysis of the crude extract of metabolites potent marine microbial isolates, compounds identified through the GCMS analysis revealed that metabolites mainly comprised of amines, aldehydes, fatty acids, alkanes and esters.
11. From the compounds produced form the marine bacteria isolate AUDI 172, twelve compounds were found to have the properties of antibacterial, antifungal and antimicrobial. Twelve compounds were identified by GC-MS from the crude extract of metabolite produced by fungal isolate AUDI 585, among 3 compounds shown both antifungal and antibacterial activity. Four antifungal and 3 antibacterial activity compounds were detected. From the Actinomycetes isolate AUDI 841, thirteen compounds were identified from the crude extract, including 5 antibacterial, 2 antifungal and 1 compound with both antibacterial and antifungal were detected.
12. PCR amplified and sequencing of the 16S rDNA region of the selected AUDI 172 and AUDI 841 isolates BLAST analysis were showed AUDI 172 as *Enterobacter cloacae* with accession no KR265465.1 and AUDI 841 as *Streptomyces macrospores* (NR_112441.1). A potent fungus was checked for amplification of ITS region and BLAST analysis was shown AUDI 585 as *Debaryomyces hansenii* (KR264906.1).
13. The secondary metabolites are products of either polyketide synthesis (PKSs) gene cluster of non-ribosomal peptide synthesis (NRPSs) gene clusters. The degenerative primer pair used for the amplification targeting the gene fragments from PKS-1 and NRPS gene clusters and then sequenced.
14. The sequence amplified of PKS-1 gene fragment BLAST analysis were found AUDI 172 83 % homology with ketosynthase of *Bacillus subtilis* (LT555282.1), AUDI 585 with 92 % homology with ketoacyl-synt-domain of *Dichomitus squalens* LYAD421 SS1 (XM_007368240.1) and AUDI 841 found 92 % homology with salinomycin polyketide synthase type I of *Streptomyces albus* strain CCM4719 (DQ630728.1).
15. The BLAST analysis of NRPS gene fragment amplified sequences isolate AUDI 172 found 94 % homology with non-ribosomal peptide synthetase of Uncultured bacterium (AM492265.1), AUDI 585 found 93 % homology with non-ribosomal peptide synthase Pes1of *Aspergillus fumigatus* Af293 (XM_747311.1) and AUDI 841 found 92 % homology with NRPS A domain of *Streptomyces sp*. BSH5019 (JX008827.1).

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**Appendix I: Media preparation**

Media preparation for cultivation of microorganisms

| **1. Nutrient agar (Dhevendaran, 2008)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Peptone | 5 |
| 2 | Beef extract | 3 |
| 3 | Sodium chloride | 15 |
| 4 | Agar | 15 |
| pH 7.2 | | |

| **2. Martin's rose bengal agar (Kostadinova *et al.,* 2009)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | D- Glucose | 10 |
| 2 | Peptone | 5 |
| 3 | Potassium dihydrogen orthophosphate | 1 |
| 4 | Magnesium sulphate | 0.5 |
| 5 | Rose Bengal | 0.035 |
| 6 | Agar | 15 |
| pH 6.0 | | |

| **3. Kuster's agar (Kumar, 2005)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Soluble Starch | 10 |
| 2 | Casein vitamin free | 0.3 |
| 3 | Potassium nitrate | 2 |
| 4 | Sodium chloride | 2 |
| 5 | di-potassium hydrogen phosphate | 2 |
| 6 | Magnesium sulphate | 4.3 |
| 7 | Calcium carbonate | 0.2 |
| 8 | Ferrous Sulphate | 0.01 |
| 9 | Agar | 15 |
| pH 7.2 | | |

| **4. Soyabean- casein digest agar (Basavaraj *et al.,* 2010)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Pancreatic digest of casein | 15 |
| 2 | Soya peptone | 5 |
| 3 | Sodium chloride | 5 |
| 4 | Agar | 15 |
| pH 7.3 | | |

| **5. Potato dextrose agar (Xu *et al.,* 2008)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Peeled potato | 200 |
| 2 | Dextrose | 20 |
| 3 | Yeast extract | 0.1 |
| 4 | Agar | 15 |
| pH 6.0 | | |

| **6. Nutrient broth (Vijaykumar *et al.,* 2010)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Beef extract | 2 |
| 2 | Yeast extract | 2 |
| 3 | Peptone | 5 |
| 4 | Sodium Chloride | 8 |
| pH 7.2 | | |

**Appendix II: Production media**

| **7. Soybean- Casein digest broth (Basavaraj *et al.,* 2010)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Pancreatic Digest of Casein | 15 |
| 2 | Soya peptone | 5 |
| 3 | Sodium chloride | 15 |
| pH 7.3 | | |

| **8. Yeast extract glucose broth (Sarker *et al*., 2015)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Glucose | 10 |
| 2 | Yeast extract | 10 |
| pH 7.2 | | |

| **9. Potato dextrose broth** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity (g/lit) |
| 1 | Dextrose | 20 |
| 2 | Yeast extract | 0.1 |
| 3 | Peeled potato | 200 |
| pH 6.0 | | |

**Appendix III: Lysis and extraction buffers**

a) Lysis and extraction buffers for DNA isolation

| **10. Lysis buffer for fungal DNA isolation (Sambrook , 2001)** | | |
|---|---|---|
| Sl. No. | Name of component | Concentration |
| 1 | Tris-Cl (pH 8.0) | 25 mM |
| 2 | Sodium chloride | 250 mM |
| 3 | EDTA (pH 8.0) | 25 mM |
| 4 | SDS | 0.5 % |
| 5 | Water | Make up the volume |

| **11. Extraction buffer for actinomycetes DNA isolation (Sambrook, 2001)** | | |
|---|---|---|
| Sl. No. | Name of component | Concentration |
| 1 | Tris-Cl (pH 8.0) | 100 mM |
| 2 | Sodium chloride | 250 mM |
| 3 | EDTA (pH 8.0) | 200 mM |
| 4 | Water | Make up the volume |

b) Buffers and solution for Agarose Gel Electrophoresis (Sambrook, 2001)

| **12. Recipe for 0.8 per cent agarose gel (100 ml)** | | |
|---|---|---|
| Sl. No. | Name of component | Quantity |
| 1 | Agarose | 800 mg |
| 2 | 1X TAE | 100ml |
| 3 | EtBr (10mg/ml) | 4µl |

| 13.50x TAE Buffer | | |
|---|---|---|
| Sl. No. | Name of component | Quantity |
| 1 | Tris Base | 242 g |
| 2 | Glacial acetic acid | 57.1 ml |
| 3 | 0.5 M EDTA (pH 8.0) | 100 ml |
| 4 | Water | Make up the volume to 1000 ml |

| 14.6x Loading Dye | | |
|---|---|---|
| Sl. No. | Name of component | Quantity |
| 1 | Bromophenol blue | 0.25 % |
| 2 | Sucrose | 40.0 % |
| 3 | Water | Make up the volume |

# ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM MARINE MICROBES

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## ABSTRACT

The present investigation was carried out to exploit the anti-phytopathogenic property of marine microbes. Several microbial cultures (four hundred thirty) isolated from the marine water and sediment samples from coastal parts are maintained at Department of Biotechnology, UAS, Dharwad. Among these resources sixty one bacterial, fifty fungal and forty one actinomycetes viable isolates were selected for investigation. These isolates were tested for anti-phytopathogenic activity against *Sclerotium rolfsii, Fusarium oxysporum, Colletotrichum* sp. and *Ralstonia solanacearum in vitro.* Three isolates (AUDI-172 from bacteria, AUDI-585 from fungi and AUDI-841 from actinomycete) showed good anti-phytopathogenic activity against all phytopathogens. The molecular characterization of potent microbial isolates were done based on 16s rDNA region for bacteria and actinomycete whereas internal transcribed spacer (ITS) region for fungi. BLAST analysis showed bacterial isolate as *Enterobacter cloacae*, actinomycete as *Streptomyces macrospores* and potent fungus as *Debaryomyces hansenii*.

To find out bioactive compounds, secondary metabolites from these potent isolates were extracted using ethyl acetate solvent-solvent extraction method and again tested for antimicrobial activity against the phytopathogens. Functional groups of bioactive compounds were characterized by using gas chromatography-mass spectrometry (GCMS) from the crude extract of potent isolates. Twelve different bioactive compounds were identified from the potent isolates of bacteria and fungi. Thirteen bioactive compounds were identified from potent actinomycete. Mostly, secondary metabolites are the products of polyketide synthesis (PKSs) and non-ribosomal peptide synthesis (NRPSs) gene clusters. Degenerative primer pairs were used for the amplification of gene fragments from PKS-1 and NRPS gene clusters. Presence of PKS-1 and NRPS gene fragments were confirmed in the potent isolates. The present study depicts a promising scenario to focus on marine microbial derived bioactive compounds against phytopathogens. *In vivo* analysis of these bioactive compounds can help to identify novel biocontrol agents against phytopathogens.