PROBING ACTINOMYCETES AND THEIR METABOLITES FOR AGRICULTURAL AND MEDICINAL USE

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For the Award of the Degree of

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in
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by

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Certificate

This is to certify that the work incorporated in this Ph.D. thesis entitled **"Probing actinomycetes and their metabolites for agricultural and medicinal use"** submitted by **Ms. Jubi Jacob** to **Academy of Scientific and Innovative Research (AcSIR)** in fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy in Biological Sciences**, embodies original research work carried out by her under mysupervision. I further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

It is also certified that this work done by the student, under my supervision, is plagiarism free.

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DECLARATION

I hereby declare that the work presented in the thesis entitled "**Probing actinomycetes and their metabolites for agricultural and medicinal use**" is the result of the work carried out by my student **Ms. Jubi Jacob** (AcSIR Registration No. 10BB13A39010)under my guidance and supervision at the CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram, Kerala, India. I also declare that all suggestions made by the audience during the Pre-synopsis presentation and those recommended by the Doctoral Advisory Committee have been incorporated in the thesis. The work incorporated in the thesis or any part of this has not been submitted elsewhere for any other degree or diploma.

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DECLARATION

I hereby declare that the matter embodied in the thesis entitled "**Probing actinomycetes and their metabolites for agricultural and medicinal use**" is the result of the work carried out by me at the Agro-Processing and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram, under the supervision of **Dr. Dileep Kumar B.S.** and the same has not been submitted elsewhere for any other degree or diploma.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other researchers.

Jubi.

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Thiruvananthapuram
March 2020

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Chapter 1

Introduction and Review of literature

1.1. Introduction

Infectious diseases are really a challenge throughout many years. After the discovery of antibiotic "Penicillin" by Flemming while experimenting influenza virus in 1928, many antibiotic molecules released in the market. Still, most of the antibiotics were discovered over fifty years ago and there were no new discoveries reported. In 2017, a molecule called "Teixobactin" was discovered which was the first new antibiotic discovered after long years. After a "golden age" of discovery (figure 1.1) in the 1940s, 50s and 60s, antibiotic development was ceased even though many of new molecules has been discovered. These early era of research provided discovery of molecules such as streptomycin (Waksman et al., 1946) from *Streptomyces griseus*, cephalosporin C (Newton and Abraham, 1955) from *Cephalosporium acremonium*, chloramphenicol (Duggar, 1948) from *Streptomyces venezuelae* , erythromycin (Pettinga et al., 1954) from *Saccharopolyspora erythraea* (*Streptomyces erythreus*) and vancomycin (Geraci et al., 1956) from *Amycolatopsis orientalis*.

Currently, nearly 60% of approved medicines are derived from natural products and among them, 69% are antibacterial molecules (Matsumura et al., 2018; Pham et al., 2019). Natural compounds from microbial origin are the major sources of antibiotics used today (Mohanta et al., 2019) and many of those microbial strains have made a tremendous contribution in the field of drug discovery and development (Demain and Sanchez, 2009). The conventional sources of microbial metabolites are: *Bacillus*, *Pseudomonas*, actinomycetes, Cyanobacteria, Myxobacteria, fungi etc. (Katz and Baltz, 2016).

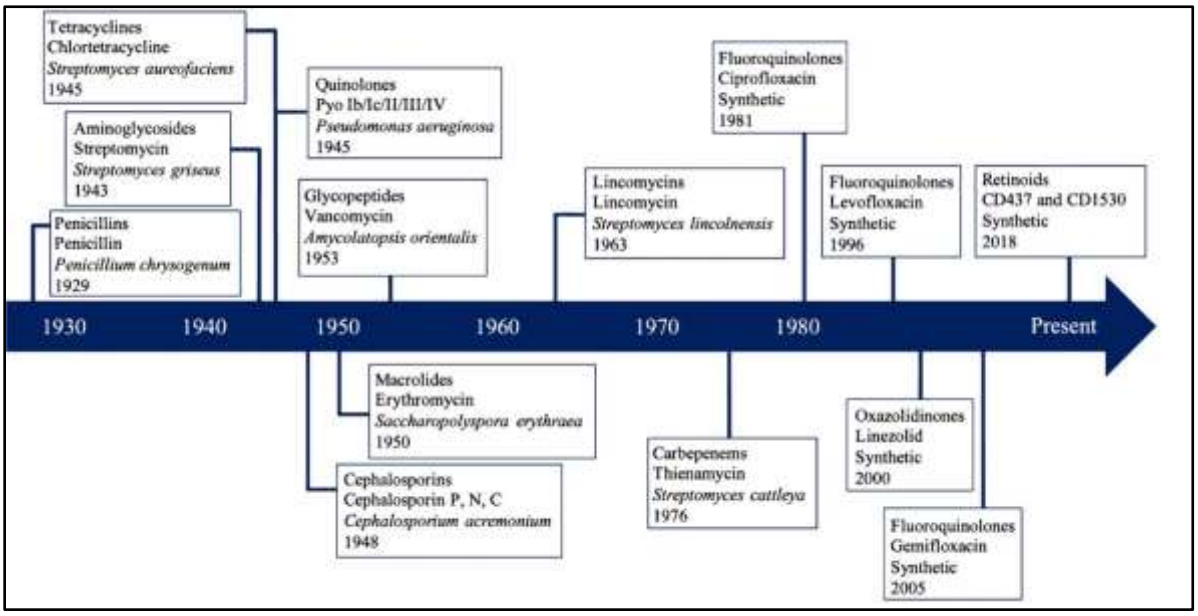

Figure 1.1: Timeline of clinically important antibiotics (Mullis et al., 2019).

Due to inappropriate pharmaceutical knowledge in the people, antibiotics have been taken incorrectly during illness conditions, which lead to the resistance in pathogenic microorganisms. Moreover, persistent usage of antibiotics has aggravated the development of multidrug resistant (MDR) pathogens. This resistance lead to the ineffectiveness of drugs which were used previously for the illness. Furthermore, there were an emergence of extended spectrum β-lactamase and carbapenemase producing pathogenic bacteria. These Gram-negative pathogens have emerged as a major therapeutic challenge. The term "ESKAPE" comprises of six such pathogens viz. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (Santajit and Indrawattana, 2016). These microorganisms acquire antibiotic resistance by horizontal gene transfer which incorporates drug resistant genes in their genomes. This phenomenon causes the development of various resistant mechanisms in pathogens contribute to inactivate the powerful antibiotics (Wright, 2012). In recent years, there were several outbreaks of infectious diseases, for example, those caused by *Candida*

auris (Chowdhary et al., 2013), Zika virus (Campos et al., 2015), Ebola virus (Carroll et al., 2015), SARS virus (Zheng et al., 2004), Nipah virus (Kumar and Kumar, 2018) and the new COVID-19 (Ahmad and Rodriguez-Morales, 2020). Hence, there is an urgent need for the discovery of bioactive metabolites against the emerging multidrug-resistant microbes (Nasfi et al., 2018).

Soil harbours diverse ecological niches and its inhabitants produce different biologically active metabolites including antimicrobial molecules with clinical importance. Actinomycetes represent a major proportion of most of the forest soil microbiota (Radhakrishnan et al., 2010). They are filamentous, Gram-positive with high G + C DNA. This group has a remarkable feature of largest bacterial phyla. The potential of actinobacteria to produce various natural bioactive metabolites like antibiotics, antioxidants, enzyme inhibitors, immunomodifiers, pigments, plant growth promoting compounds, herbicidal substances and other compounds of biotechnological interests make them a predominant microorganism in biotechnology industry (Dholakiya et al., 2017). The importance of these organisms relies on the fact that more than 6000 compounds have been discovered from actinomycetes, first in 1940s. These compounds have contributed to the development of most of the antibiotics being used for either research or clinical purposes (Baltz, 2008). Recent studies have shown that actinomycetes are the known producers of polyketides and non-ribosomal polyketide peptides through different pathways such as type I, type II and non-ribosomal peptide synthetases. These are the dominant pathways for the production of secondary metabolites in these organisms (Passari et al., 2015).

The largest genus of actinobacteria comprises of 826 species of *Streptomyces* which are reported as the major producers of natural compounds with a huge record of bioactivities. Several commercially available drugs like nystatin, ivermectin,

tetracycline and streptomycin etc. were isolated from these organisms (Puttaswamygowda et al., 2019). From *Streptomyces*, several compounds with antibacterial, antifungal, antioxidant, antitumor capacities were purified and characterized during the past years. Use of those compounds as such or their synthetic modifications were successfully employed in the treatment of various diseases.

Many investigators have been searching for novel actinomycete strains from different habitats to explore new drug moieties to be active against many pathogens. Even though, only a small number of culturable actinomycetes have been recovered from various habitats till date. This is because of the 'great plate count anomaly' where a major portion of soil microorganisms are 'unculturable'. Therefore, the discovery of novel antimicrobial metabolites from culturable actinomycete species has declined rapidly. The problem relies on the fact that the culture crude extracts frequently provided already known metabolites. Deviating the research away from these explored habitats to underexplored ones may boost the likelihood of identifying or formulating novel compounds (Ahmad et al., 2019). Unexplored and underexplored habitats include biodiversity hotspots where these environments influence species diversity as well as the evolution of new pathways for the synthesis of new natural products (Bull and Stach, 2007). Western Ghats area of South India falls under the biodiversity hotspots and known for its diverse ecosystems and endemism. This high biodiversity area is classified as evergreen forests with tropical, temperate and wetland ecosystems (Nampoothiri et al., 2013). This protected network area in Kerala, a state in South India, occupies an area of 160,000 km^2 and covers 25 % of India's biodiversity with new flora and fauna. There are many investigations on the search of actinomycete strains from these ecosystems for antimicrobial potential (Mohandas et al., 2012). Even though, many forest ecosystems of Western Ghats are still underexplored and thus represent

effective resources for the procurement of novel microorganisms including actinomycetes.

Another challenge faced by the society in agricultural sector in India is the tremendous use of chemical fertilizers and pesticides to boost agricultural productivity. Agriculture accounts a considerable amount of India's economic development (GDP growth rate, 4.5 %), and it provides food for more than 1.37 billion people and total employment to about 43.21 %. The total food grain demand is projected to 355 million tonnes in 2030 (Gandhi and Zhou, 2014). The last report of the FAO on world fertilizer trends and its outlook to 2020 indicated that more than 200 million tonnes of chemical fertilizers and pesticides are applied to soil annually, which is 25% higher recorded in 2008 (FAO, 2011) and are found to be well correlated with the crop yield. Besides, soil biodiversity is rapidly shrinking and large farmland areas in India have become barren due to excessive use of chemical fertilizers particularly, the indiscriminate application of urea (Figure 1.2). The overwhelming use of fertilizers pose environmental pollution that leads to resource degradation and negatively impact soil ecological functions (Naher et al., 2019). However, minimized use of fertilizer or pesticide application in the fields sometimes leads to the lower productivity due to either nutrient deficiency or pathogenic attack. Therefore, it is a great challenge to search for sustainable approaches to alleviate the adverse effects of intensive farming practices which consume a large quantity of synthetic fertilizers and pesticides.

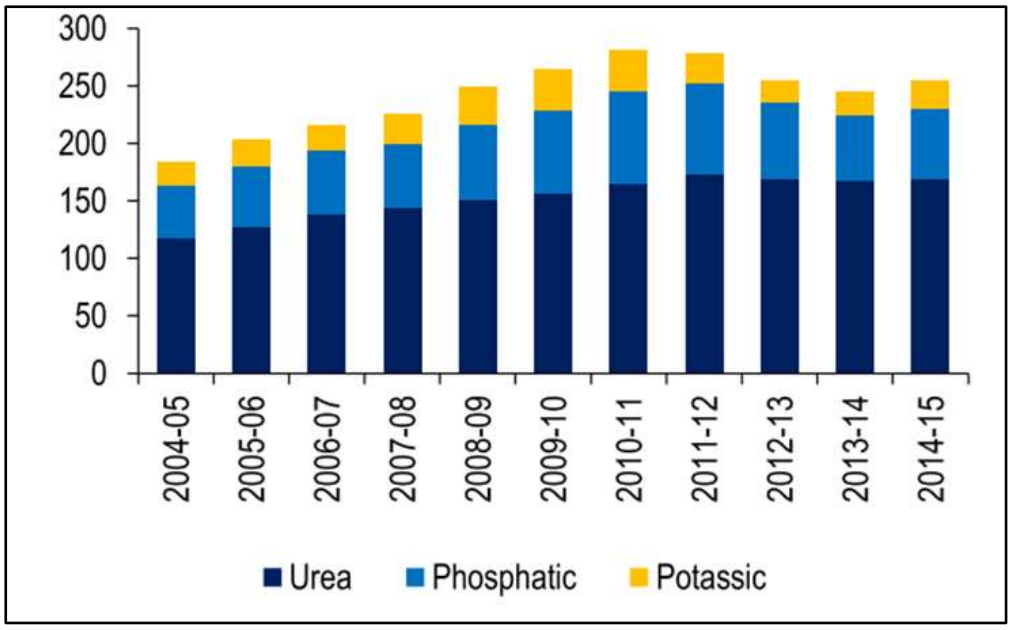

Figure 1.2: Consumption of fertilizers in agricultural fields in lakh tonnes (FAO, 2015).

Sustainable agriculture makes use of organic farming to get rid of the consequences posed by chemical compounds applied in agricultural practices to a notable level. These sustainable strategies include the application of beneficial soil microorganisms as a formulation in the fields. These beneficial soil microbes collectively known as plant growth promoting rhizobacteria (Kloepper et al., 1980) are being used for enhancement of crop yield in a sustainable manner to reduce the chemical fertilizer input in various agricultural fields. These organisms have the potential to enhance plant growth under normal as well stress conditions through a number of direct and indirect mechanisms (Bhattacharyya and Jha, 2012). These mechanisms include nitrogen fixation, production of ammonia, indole acetic acid, volatile organic compounds, production of siderophores, phosphate solubilization, various enzymatic activities namely, chitinase, protease, pectinase and antagonism through the production of antibiotics (Kannojia et al., 2019). These microbes can aggressively colonize the rhizosphere or plant roots thereby stimulating plant growth and yield. Some

actinomycete strains can be used as biocontrol agents (BCA) which can exert a positive effect on plants by alleviating both biotic and abiotic stresses (Palaniyandi et al., 2013). The antagonistic nature may be due to the production of antibiotics which make them a valuable tool in the management of various diseases.

1.2. Objectives

With this background, present investigation aimed to explore actinomycetes with antimicrobial potential from the soils of different parts of Kerala including microbiologically underexplored forest ecosystems located in the Western Ghats Biodiversity hotspot. The soil actinomycetes present in these locations were isolated in pure cultures. Furthermore, the selected strains were evaluated for the potency of antimicrobial metabolite production, plant growth promotion and disease resistance with the following objectives.

1. Isolation, screening and identification of actinomycetes from different parts of Kerala soils.
2. Optimization of fermentation conditions and media for enhanced antibacterial production from two selected strains A30 and A32.
3. Exploitation of strain A30 for medicinal applications.
4. Modulation of plant growth and induction of systemic resistance against fusarial wilt in Mung bean (*Vigna radiata* L.) employing strain A32.

1.3. Review of literature

1.3.1. Introduction to actinomycetes

Actinomycetes are a group of bacteria belong to the order *Actinomycetales* (super kingdom, Bacteria; phylum, Firmicutes; class, Actinobacteria; subclass, Actinobacteridae). They are aerobic, spore-forming, filamentous and Gram-positive.

Ecologically, they are found in terrestrial, fresh water and marine habitats and majority are free living in nature (Bérdy, 2012). They are considered as a treasure house of bioactive secondary metabolites. The most common bioactive metabolite producers fall under the genera *Streptomyces*. These microbes possess similar characteristics with bacteria as well as fungi. Their morphological similarity with fungi is based on the following characteristics: (i) Higher genera of actinomycetes possess an extensively branching mycelium like fungi. (ii) Many actinomycetes form fungal like conidia known as spores. (iii) in liquid cultures, most of it occurs as clumps of pellets except some strains which result in turbidity as in case of unicellular bacteria (Ahmad et al., 2019). On agar surface, they form extensive branching of hyphae with aerial and substrate hyphae. They are either chemoautotrophic or heterotrophic, but majority them are chemoheterotrophic. They can utilize a wide range of nutritional sources, including complex polysaccharides. Actinomycetes can be phylogenetically analyzed by 16S and 23S rRNA genes and characteristic gene arrangements (Ludwig et al., 2012). As producers of 80% of known antibiotics, actinomycetes possess the highest position in microbial world with several biotechnological applications (Alharbi et al., 2012). Other than antibiotics, they secrete various enzymes such as amylases, cellulases, lipases, chitinases, proteases, peroxidases, ureases, L-asparaginases of biological importance (Sharma et al., 2014).

1.3.2. Ecology and diversity of actinomycetes

Actinomycetes can be found in soil, fresh water and marine habitats. They are found in free living form in these habitats (Figure 1.3). Based on their diversity, they are classified close to bacteria. In actinomycete group, *Streptomyces* contribute to 70%, followed by *Nocardia* and *Micromonospora*. Some other genera such as *Actinomyces ,*

Actinoplanes, Microbiospora, Thermomonospora and *Streptosporangium* are also found in soil ecosystem (Ahmad et al., 2019). Actinomycetes are responsible for many ecological functions in the soil. They take part in the turnover of organic materials. Their major role is to decompose complex organic materials including humic acids. Some of the actinomycetes also found in symbiotic association with fungi, insects, animals and plants. Most of these interactions are beneficial, where actinomycetes produce bioactive metabolites that render their host to seek protection against pathogens or pests. Moreover, their association provide them enzymes to degrade complex natural polymers such as lignocellulose.

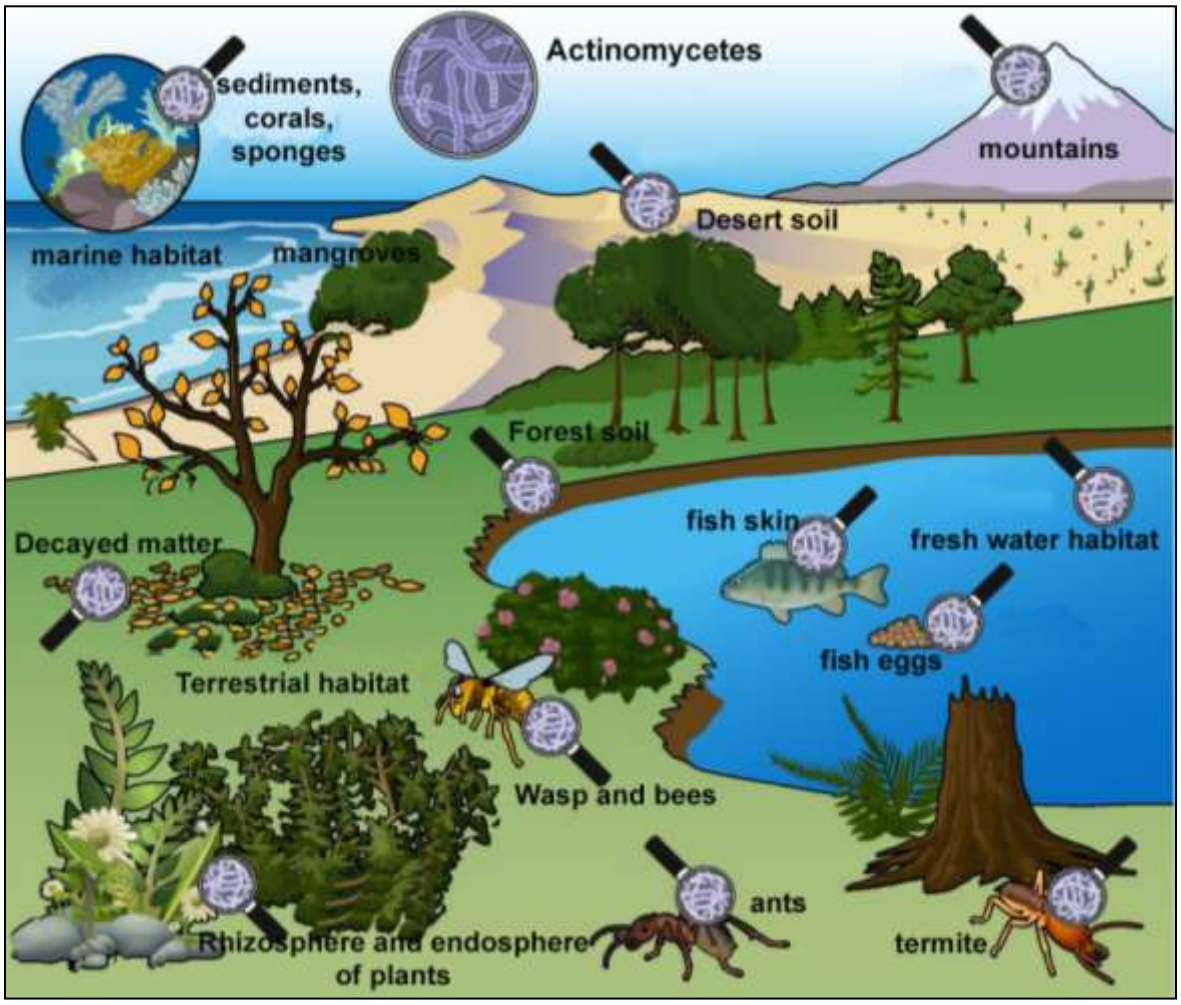

Figure 1.3: Ecology of actinomycetes (Modified from van der Meij et.al., 2017).

1.3.3. General features

The "earthy" or musty smell of fresh soil is the distinctive occurrence of actinomycetes due to a chemical substance known as geosmin (Gerber and Lechevalier, 1965). When grown on agar surface, actinomycetes produce a network of hyphae in both directions *viz*. aerial hyphae (seen on the surface) and substrate hyphae (on under surface). The aerial hyphae are capable of asexual reproduction and spread above the substratum. The hyphae are divided into cells of about 20 mm and separated by the septa and comprise many bacterial chromosomes (nucleoids). Usually, the members of actinomycetes do not have motility. Conversely, if motile, the ability is restricted to the presence of flagellated spores. The developmental life cycle of sporulating actinomycete is depicted in figure 1.4. The lifecycle begins with the germination of a spore with the development of germ tubes. The germ tubes mature into hyphae which further grow by branching and extension of tip. This will establish a network of hyphae that together form the vegetative mycelium. At the time of unfavorable conditions such as nutrient depletion, a portion of mycelium undergo autolytic degradation *via* programmed cell death. This is accompanied by the nutrient release from the cell to the environment and utilized for further generation of aerial hyphae and spores. Furthermore, the initiation of cell differentiation coincides with antibiotic production. This provides protection against competing microorganisms attracted by the nutrients released during programmed cell death. The onset of development of aerial mycelium is regulated by the *bld* (bald) genes (Merrick, 1976) while genes that are specifically associated with the formation of fully maturated spores are called *whi* (white) genes (Hopwood et al., 1970).

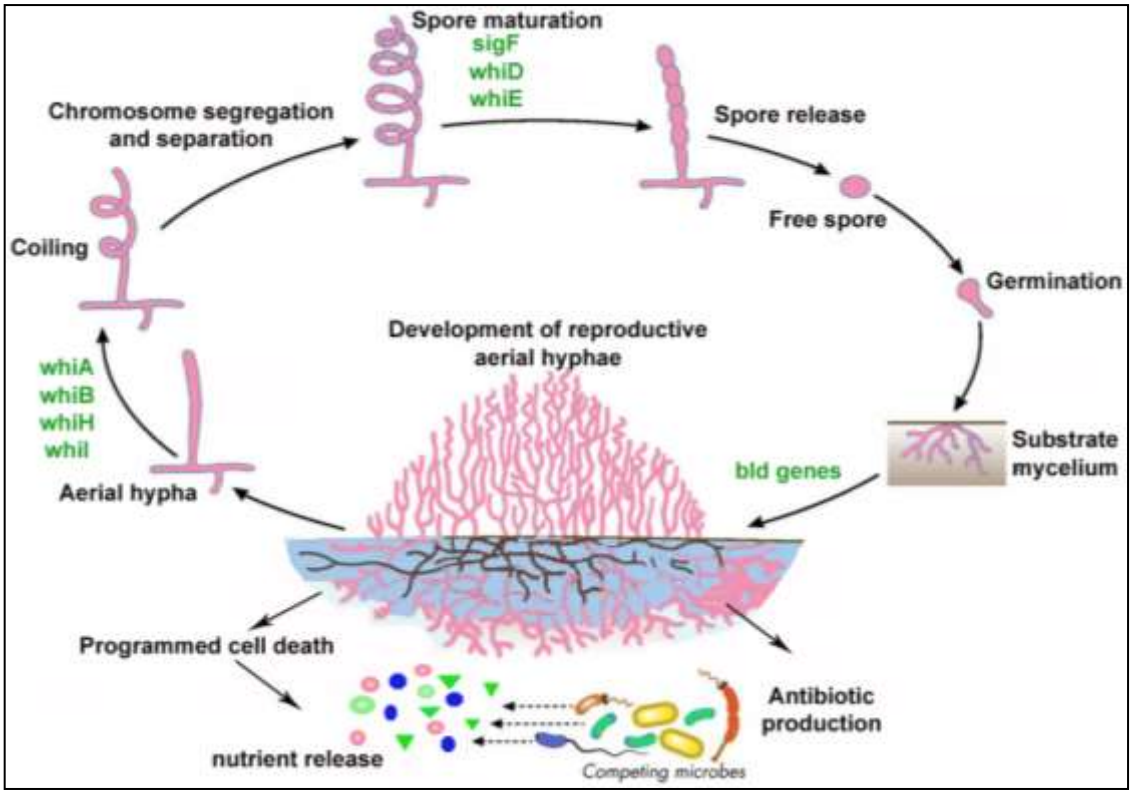

Figure 1.4: Developmental cycle of sporulating actinomycetes.

1.3.4. Bioactive metabolite production

In history, during 1940s, Waksman and his colleagues isolated more than fifteen antibiotics, the most important of which was streptomycin, the first effective molecule for the treatment of tuberculosis (Bose et al., 2012). Antibiotics are referred as wonder drugs because of their success to destroy pathogenic microorganisms. This group of compounds has diversified structures and mode of action (Table 1.1). Antibiotics acts on virtually every type of microbial activities like DNA, RNA and protein synthesis, electron transport, sporulation, germination of spores, microbial membrane functions and so on. As stated earlier, members of the order *Actinomycetales* are responsible for producing 90% of all known secondary metabolites, many of them are antibiotics (Bérdy, 2012). Out of all antibiotics, two third are produced by actinomycetes group and the majority are produced by the genus *Streptomyces* (Cragg and Newman, 2009).

Mode of action	Antibiotic group	Description	Examples
1. Inhibition of cell wall synthesis	Beta-lactam antibiotics	Penicillin binding protein (PBP) interacts with β-lactam ring	Penicillin Cephalosporins Carbapenems Monobactams
	Glycopeptides	Bind to D-alanyl D-alanine portion of peptidoglycan	vancomycin
2. Inhibition of protein synthesis	Aminoglycosides,	Bind to 30S subunit of bacterial ribosomes	gentamicin, amikacin, tobramycin,
	Tetracyclines		neomycin,
	Chloramphenicols,	Bind to 50S subunit of	streptomycin.
	Macrolides,	bacterial ribosomes	Tetracycline, oxycyclines, minocyclines Azithromycin, clarithromycin
	Oxazolidinones		Linezolid, posizolid
3. Inhibition of DNA replication	Quinolones	inhibit the enzyme bacterial DNA gyrase	ciprofloxacin
4. Inhibition of Folic acid metabolism	Sulfonamides	inhibit dihydropteroate synthase	Sulfadiazine, sulfamethizole
	trimethoprim	inhibit dihydrofolate reductase	

Table 1.1: Classification of antibiotics based on their mode of action.

Among the antibiotics, tetracycline and erythromycin targeting bacterial ribosome functions, are used for treating respiratory infections, as in the case of Legionnaires' disease. On the other hand, some antibiotics like vancomycin acts on methicillin-resistant *Staphylococcus aureus* (MRSA), a devastating pathogen. Vancomycin acts on the pathogen by inhibiting bacterial cell wall synthesis. The antibiotic rifamycin, target bacterial DNA dependent RNA polymerase and used for the treatment of leprosy and tuberculosis. Amphotericin acts on ergosterols of fungal membranes and leads to death. Currently, a variety of antifungal compounds such as

kocumarin, abequines and hydrolytic enzymes are reported from actinomycetes (Jakubiec-Krzesniak et al., 2018; Girão et al., 2019).

Other than antibiotics, some of the actinomycetes are also capable of producing clinically useful drugs for tumor such as anthracyclines (aclarubicin, daunomycin, and doxorubicin), aureolic acids (mithramycin), carzinophilin, peptides (bleomycin and actinomycin D), enediynes (neocarzinostatin), mitomycins, antimetabolites (pentostatin) etc. It is also noted that metabolites from actinomycetes not only have the potential of therapeutic activities, but also have the desirable pharmacokinetic properties which are required for proper usage in clinical and healthcare scenario (Claverías et al., 2015). Even though, the metabolites produced by actinomycetes do not influence human cells, there are some reports that Adriamycin (an agent used in cancer), inhibits DNA replication and rapamycin (repress the immune response), to facilitate organ transplantation therapy.

1.3.5. New approaches to discover novel antimicrobials

Microbial diversity is poorly exploited or explored for its industrial and therapeutic applications because majority of bacteria are non- culturable. In case of culturable microorganisms including actinomycetes, different habitats have not yet been fully understood and explored. It is supposed that screening of actinomycete diversity from unexplored habitats with improved methods (Das et al., 2018; Ouchari et al., 2019) is the only approach by which new class of metabolites could be expected. There is a demand for the development of new class of drugs since rapid emergence of drug resistance among pathogenic bacteria to all available antibacterial drugs. Therefore, it is a global concern to develop strategies to discover new drugs to defeat

the multidrug resistance problem before it takes an epidemic scenario (Soltani et al., 2019).

Apart from the conventional methods, there are several improved methods have been applied for drug discovery programs, like bioactivity-guided screening, target-based and screening chemical screening. In bioactivity-guided screening or forward pharmacology, the drug targets are not known and the extracts of purified compounds from the microorganisms have been screened for various biological activities in cell assays. On the other hand, the chemical screening denotes a chemical substance from either chemical libraries or natural sources have been screened for biological activities through the usage of sophisticated analytic techniques like Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC) and Mass Spectroscopy (MS) to avoid replication of already known compounds. In the target based screening, all the compounds will be screened for their activity against various pathogens of interest (Wohlleben et al., 2016).

1.3.6. Focusing on underexplored habitats

In addition to the great efforts to isolate novel strains from underexplored environments, there are also mining of genomes from old strains for novel metabolic pathways. Even though the biosynthetic gene clusters appear to be shared widely between actinomycetes, exploring these habitats are suitable to identify new genus of actinomycetes (Devine et al., 2017). But, most of the microbial phyla are unculturable even from terrestrial environments. New approaches, for example, metagenetic comparison of polyketide synthase and non-ribosomal peptide synthase gene clusters from microbes inhabiting in a range of environments can reveal the variation among different soil samples. Most soil microbial communities will encode specific genomes for

secondary metabolite synthesis and such studies can also be used to model discovery efforts for new natural products (Xie et al., 2018).

1.3.7. Designing new fermentation media

Nutrients, energy source and optimal physiological conditions are the main requirements for the survival of microorganisms in any environments. All microorganisms require specific nutrients for their good growth. These molecules are nontraditional electron donors and electron acceptors and signal molecules (Abdelmohsen et al., 2014). Furthermore, ionic strength in the culture medium also influence their growth. Researchers have found that the members of the genus *Salinispora*, a group of marine actinomycete require a high ionic strength for their optimal growth and metabolite production (Tsueng and Lam, 2010). Refinement of classical approaches is one of the common strategies to cultivate "not-yet-cultured" species. However, the enrichment culture techniques are employed to support the growth of slow-growing species while retarding the fast-growing species which thrive in nutrient limiting environments. Another strategy is diluting nutrient media which is successfully applied for the cultivation of previously unculturable microbes from different environments (Hahnke et al., 2015). The unculturable microorganisms can also be recovered by using a culture medium that mimics its natural environmental conditions. For instance, to culture previously uncultivated microbe SAR11, seawater has been used (Rappé et al., 2002). Moreover, helper strains can be applied to release some growth stimulating factors for the growth of unculturable strains. The cell-free extracts or extracellular material released from helper strains can acts as growth stimulants (Vartoukian et al., 2016). Another culture designing strategy is to expand the incubation time of slow-growing microorganisms at low substrate concentration in a

nutrient defined medium that have the added benefits of retarding the growth of faster growing species within mixed microbial populations.

Hence, improving the antibiotic production is indispensable to achieve adequate antibiotic yield. The ability of antibiotic production by actinomycetes is highly influenced by the culture conditions as well as media components (Rajeswari et al., 2015). Many researchers have selected various carbon and nitrogen sources using one-factor-at-a-time (OFAT) approach, a classical method, by changing one independent variable while fixing all the others at a fixed level (Xu et al., 2003; Salihu et al., 2016). This method does not give the net effect of total interactions between various media components. Subsequently, screening of medium components for their significant effect on antibiotic production can be done using statistical factorial designs like Plackett Burman design (PBD) and Box Behnken Design (BBD). The Plackett-Burman design is a well-established and widely used statistical design technique for the screening of the medium components under shake flask conditions (Adinarayana et al., 2003). Later, the significant medium components can then be optimized using Response Surface Methodology (RSM)(Feng et al., 2011).

Statistical optimization allows quick screening of a large experimental runs and confirms the role of each of the parameters under study. For example, the nutritional requirements for maximum antimicrobial activity of *Streptomyces rimosus* AG-P1441 was optimized using statistical based experimental designs where glucose, corn starch and soybean meal shown significant effect on antimicrobial productivity (Ju et al., 2018). Besides medium components, the culture conditions can also be optimized using statistical methods. Influence of various culture parameters to improve the antibiotic production by *Amycolatopsis* AS9 was studied through RSM approaches with Central

Composite Rotatable Design (CCRD). From these experiments, pH, temperature, inoculum volume, and agitation speed were found to have a significant effect on the antimicrobial activity (Balachandar et al., 2019).

1.3.8. Combinatorial approaches

Combinatorial biosynthesis is a useful tool to improve the chemical diversity of natural products by means of altering scaffold backbone through variations in biosynthetic enzymes, functional groups and or other methods. It is an augmentation to the traditional drug development from microbes that provides a powerful platform for the diversity of natural products boosting the drug discovery process (Rizvi et al., 2019). Since 1980s, many natural products have been created through combinatorial approaches. Examples of commercial products derived from the combinatorial biosynthesis of compounds are PKS-I-derived erythromycin (Katz, 2009) and the NRPS-derived daptomycin (Baltz, 2014). The PKS-I and NRPS biosynthetic mechanisms are the reasons for a large fraction of commercially important natural products (Katz and Baltz, 2016). It should be noted that the commercialization of natural products of combinatorial biosynthesis are slow to develop. But, with the development of synthetic biology and improved DNA sequencing techniques will boost the commercial successes of combinatorial biosynthesis of natural products.

1.3.9. Nonculture-based approaches

Culture independent molecular approaches employ the existence of already known actinomycetes in different habitats (Xin et al., 2008). The molecular tools employing culture independent and metagenomic approaches are helpful in determining the uncultured microbial diversity and hence explore the biochemical

synthetic pathways of natural products (Figure 1.5). Sequence guided metagenomic investigations of different environments have proven to be useful in identifying new species or strains of microbes (Jensen et al., 2005). Metagenomic methods basically utilize the extraction of nucleic acids followed by the amplification of DNA or cDNA from RNA by polymerase chain reaction and with the subsequent analysis of the diversity of the amplified molecules. Furthermore, the amplified nucleic acids can be cloned and sequenced. The high quality sequences thus obtained can be compared with databases for the identification of new strains (Mahapatra et al., 2019). Currently, there is no single tool or technique to assess the actinomycetes diversity. Hence, a combination of techniques from molecular biology, microbiology, geochemistry, microsensors must be used to obtain a better understanding of actinomycetes diversity from the unexplored sources, that intern provide an important platform for obtaining biologically active compounds (Foulston, 2019).

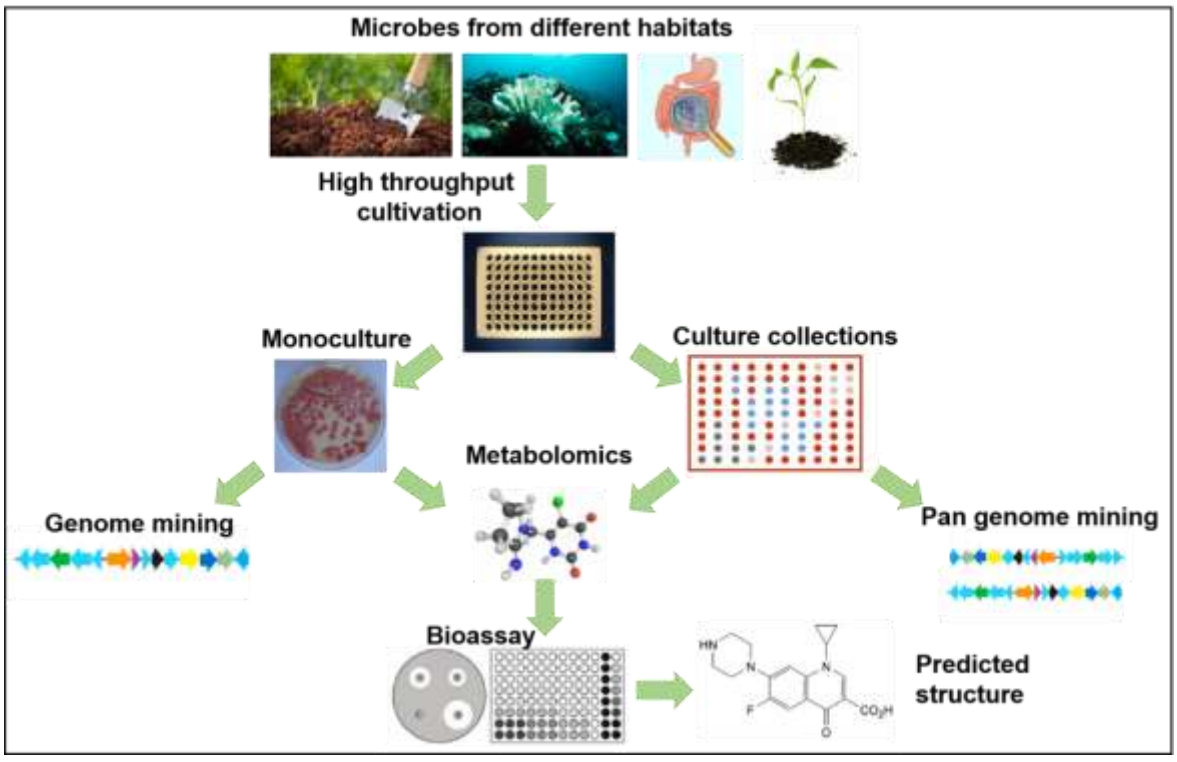

Figure 1.5: Non- culture strategies in the mining of novel natural products.

1.3.10. Actinomycetes as a plant friend: For sustainable agriculture practices

Biological control and plant growth promotion by plant beneficial microbes has been viewed as an alternative to the use of chemical pesticides and fertilizers. Prolonged indiscriminate use of toxic chemical fertilizers and pesticides could destroy the beneficial soil microbes thereby imbalancing the microecology. Soil biodiversity needs to be protected from these synthetic chemicals. Moreover, application of these chemicals reduces soil microbiota that is important in ecosystem sustenance and negatively impacts nutrient cycling, thereby affecting plant growth and crop yield. Application of PGPRs increase the agronomy efficiency by reducing the production cost and environment pollution (Kumar et al., 2018). Various microbial communities, *viz*., fungi, bacteria, actinomycetes and yeasts are used as inoculants and primarily they promote plant growth through nitrogen fixation, phosphate and potassium solubilization, exopolysaccharide secretion, biocontrol activity, organic matter decomposition, siderophores production etc. (Figure 1.6). These are naturally associated with plants and have a beneficial effect on plant growth by the alleviation of biotic and abiotic stresses were isolated and developed into biocontrol (BCA) and plant growth-promoting agents (PGPA). There are a large amount of research reports are available on Plant Disease Suppression (PDS) and Plant Growth Promotion (PGP) by a variety of microbes (Xiang et al., 2017; Liu et al., 2018; Chenniappan et al., 2019).

Most of actinomycete plant growth-promoting strains possess antibacterial or antifungal activity which was imminent during their screening as biocontrol agents (Zhao et al., 2012; Suarez Moreno et al., 2019). To substantiate this, many products such as Mycostop (*Streptomyces griseoviridis* K61), plantomycin (*Streptomyces griseus*),

Actinovate (*Streptomyces lydicus*) and Biomycin (*Streptomyces kasugaensis*) have been developed.

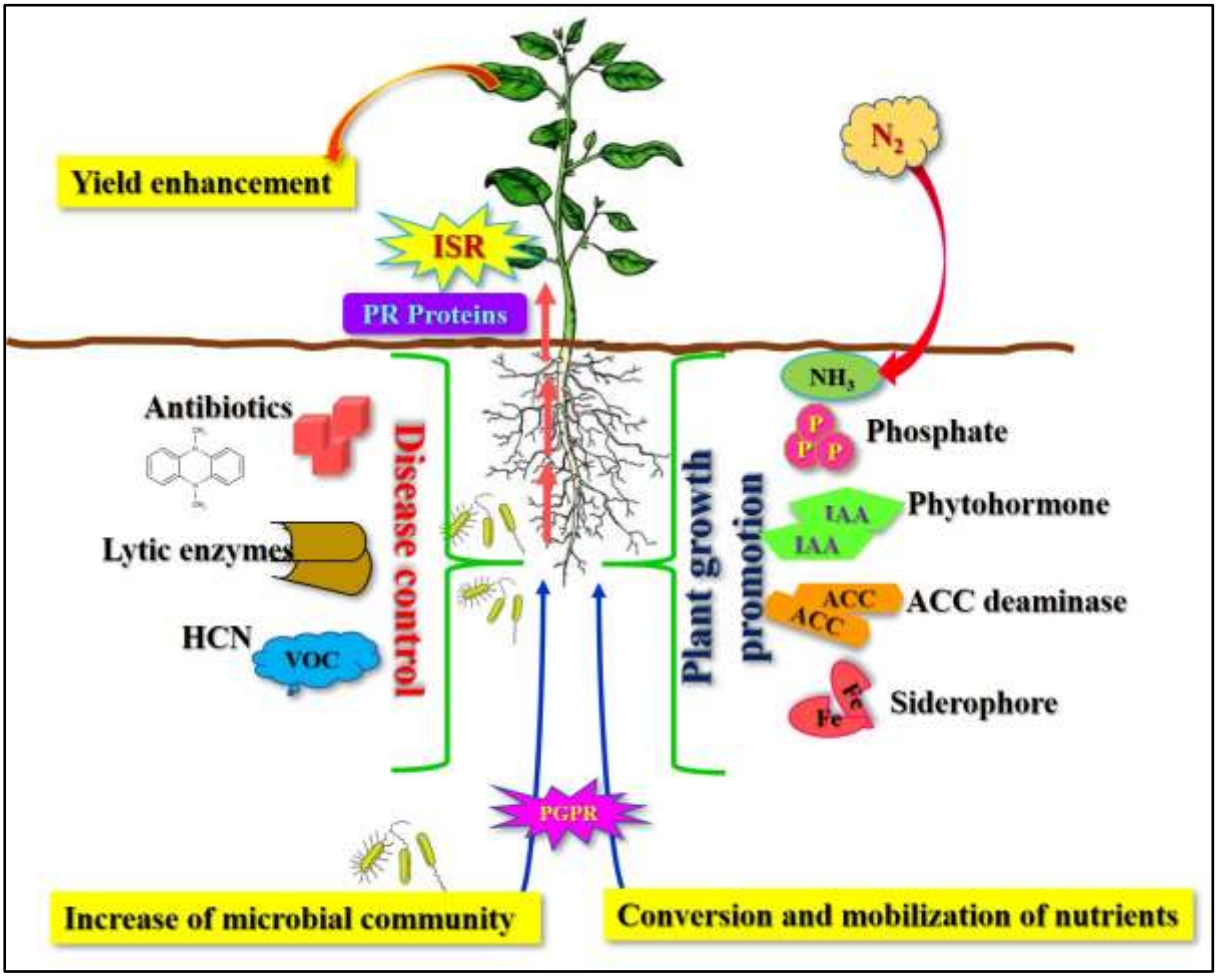

Figure 1.6: Effect of plant growth promoting rhizobacteria on plant growth and disease suppression.

1.3.11. Induction of plant defense by *Streptomyces*

Plants defend themselves from pathogen infections through a wide variety of local or systemic responses. The extent to which *Streptomyces* inhibit plant disease progression is poorly understood, but recent reports indicate that multiple mechanisms are involved this process. A *Streptomyces* strain elicited systemic defense response in oak tress upon pathogenic infestation. The plant defense response was due to induction

of the jasmonic acid or ethylene dependent pathway in addition to the salicylic acid-dependent pathway (Kurth et al., 2014). A rice endophytic *Streptomyces* strain induced defense response in paddy plants with H_2O_2 accumulation, defense-related enzymes activation, and elevated expression of salicylic acid (SA) and jasmonic acid (JA) pathways genes. These contribute to the resistance to pathogenic attack (Gao et al., 2018).

1.3.12. Management of diseases in mung bean

Mung bean or green gram (*Vigna radiata* L.) is one of the most important pulse crops belong to the family *Leguminosae*. Mung bean is an excellent source of high-quality protein. This leguminous crop has the capacity to fix-atmospheric nitrogen through symbiotic nitrogen fixation and used as green manure crop. It is grown in summer and kharif season in northern India and in southern India (Yadava, 1992). The major fungal diseases which infect the mung bean are root rot (*Macrophomina phaseolina* (Tassi) Goid), fusarium wilt (*F. oxysporum*), web blight (*Thanatephorus cucumeris*), powdery mildew (*Erysiphe polygoni* DC), Cercospora leaf spot (*Cercospora canescens* Ellis and Martin) and anthracnose (*Colletotrichum dematium* and *C. lindemuthianum*) (Grewal, 1988). Among them, *Meloidogyne incognita* and *F. oxysporum* poses a great problem to the cultivation of pulse crops by inflicting severe yield losses (Akhtar et al., 2005).

Various researchers have investigated the biological control of mung bean diseases such as root-rot disease and charcoal rot (Hussain et al., 1990; Ali Siddiqui et al., 2001; Adhilakshmi et al., 2014). Co-inoculation of PGPR strains, *Pseudomonas* and *Bradyrhizobium* significantly reduced the root rot disease in Mung bean plants (Sahu and Sindhu, 2011).

1.3.13. Actinomycetes as biocontrol agents in mung bean

Even though the biocontrol ability of actinomycetes were reported by many researchers, the management of diseases in mung bean by actinomycetes are limited. in an investigation, treatment with *Streptomyces* sp. recorded the highest mung bean yield in field trials as well as *Streptomyces* treated plants showed higher levels of activities of defense related enzymes (Adhilakshmi et al., 2014). Biofertilizer produced with *Streptomyces griseoflavus* and *Bradyrhizobium sp.* significantly improved plant growth, nodulation, nitrogen fixation, NPK uptake and seed yield in mung beans (Htwe et al., 2019). In another study, a strain GMKU 336 significantly increased mung bean plant elongation and biomass, chlorophyll content, leaf color, leaf area, adventitious roots formation and reduced the ethylene level under flooding conditions when compared to un-inoculated plants (Jaemsaeng et al., 2018).

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Chapter 2

Isolation, screening and identification of actinomycetes from different parts of Kerala soils

2.1. Introduction

More than one million natural compounds are available in the world, of which 5% are derived from microbes (Knight et al., 2003). Natural compounds from microbial origin are the major sources of current antibiotics exploited and many of microbial strains have made a tremendous contribution and impact in the field of drug discovery and development process (Demain and Sanchez, 2009). Due to lack of awareness among the common people, antibiotics are improperly used over a prolonged period, which lead to the development resistance in pathogenic microorganisms against many important diseases. These microorganisms acquire antibiotic resistance by horizontal gene transfer which incorporates drug resistant genes in their genomes. This phenomenon results in the development of various mechanisms to inactivate antibiotics (Wright, 2012). Hence, there is an urgent need for the discovery of bioactive metabolites against the emerging multidrug-resistant microbes which cause life-threatening diseases (Nasfi et al., 2018).

Soil harbors diverse ecological niches and its inhabitants produce different biologically active metabolites including anti-microbial molecules which possess clinical significance. Actinomycetes represent a major proportion of most of the forest soil microbiota (Radhakrishnan et al., 2010). They are filamentous Gram-positive bacteria which constitute one of the largest bacterial phyla with high G + C DNA. The potential of actinomycetes to produce various natural bioactive metabolites like antibiotics, antioxidants, enzyme inhibitors, immunomodifiers, pigments, plant growth promoting and herbicidal substances and other compounds of biotechnological interests make them a useful microorganism in biotechnology industry (Dholakiya et al., 2017). The importance of these organisms rely on the fact that more than 5000 compounds have

been reported from actinomycetes, first discovered in 1940s that contributed to the development of 90% of the commercial antibiotics being used for either research or clinical purposes (Baltz, 2008). Many members of actinomycetes are known producers of polyketides and non-ribosomal polyketide peptides by polyketide pathways (type-I and type-II) and non-ribosomal peptide synthetase pathways, respectively, which are the dominant pathways to produce secondary metabolites in this group of bacteria (Passari et al., 2015).

The present chapter aimed to investigate actinomycetes with antimicrobial potential in the soils of different parts of Kerala including microbiologically underexplored forest ecosystems located in the Western Ghats Biodiversity hotspot of Nelliyampathy area. We isolated soil actinomycetes from different soils, evaluated the potency of antimicrobial effect against different bacterial and fungal pathogens. Furthermore, the potent strains were identified based on molecular analysis. However, two strains which shown broad spectrum antimicrobial activities against human pathogenic bacteria, human and plant fungal pathogens were selected for further investigations.

2.2. Materials and Methods

2.2.1. Materials

2.2.1.1. Chemicals and reagents

Various media used for microbiological investigation were procured from Hi-Media Laboratories Pvt. Limited, Mumbai, India. The solvents used for extraction of bioactive compounds were purchased from Merck Life Sciences, Mumbai, India and

SDFCL, Mumbai, India. The antibiotics such as ampicillin, ciprofloxacin, fluconazole and kanamycin, which were used as standard positive control, were procured from Hi-Media.

2.2.1.2. Test bacterial pathogens

The bacterial pathogens used for antibacterial studies were *Bacillus cereus* MTCC 1305, *B. subtilis* MTCC 2756, *Mycobacterium smegmatis* MTCC 993, *Staphylococcus aureus* MTCC 902, *S. epidermidis* MTCC 435, *S. simulans* MTCC 3610, (all Gram positive) and *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425, *Pseudomonas aeruginosa* MTCC 2642, *Salmonella typhi* MTCC 3216, (all Gram negative). These are procured from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India. All the organisms were maintained on nutrient agar slants and kept at 4°C for the studies.

2.2.1.3. Test fungal pathogens

The fungal pathogens used for antifungal studies were *Aspergillus flavus* MTCC 183, *A. fumigatus* MTCC 3376, *A. niger* MTCC 2756, *A. tubingensis* MTCC 2425, *Colletotrichum gleosporiodes* MTCC 2151, *Fusarium oxysporum* MTCC 284, *Penicillium expansum* MTCC 2006, *Rhizoctonia solani* MTCC 4634, *Trichophyton rubrum* MTCC 296, *Alternaria solani* ITCC 5350, *Botrytis cinera* ITCC 6530, *Colletotrichum capsici* ITCC 6071, *Colletotrichum capsici* ITCC 6870, *Colletotrichum gleosporiodes* ITCC 6079, *Colletotrichum gleosporiodes* ITCC 6434, *Fusarium oxysporum f.sp.lycopersici* ITCC 1322, *Fusarium oxysporum f.sp.pisi* ITCC 4814, *Fusarium solani* ITCC 7075, *Candida albicans* MTCC 277, *C. glabrata* MTCC 3019, *C. parapsilosis* MTCC 998 and *C. tropicalis* MTCC 184. All the MTCC

designated fungal strains were procured from Microbial Type Culture Collection (MTCC) and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh and ITCC designated strains procured from the Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute, New Delhi, India. All the test pathogens were maintained on PDA agar slants and maintained at 4°C.

2.2.2. Methods

2.2.2.1. Site, sample collection

Twenty-five soil samples were collected from different parts of Kerala, included Nelliyampathy forest hills (10°32'20.2"N 76°41'37.1"E) of the Western Ghats region in Kerala, soils from Palakkad (11°4'1.61"N 76°25'17.76"E), Changanacherry (9°26'45.2"N 76°32'27.48"E), Kuttanadu (9°21'9.96"N 76°24'15.26"E), Thamaraserry (11°24'54.87"N 75°56'25.86"E), Thrissur (10°31'39.51"N 76°12'51.97"E) and Waynadu (11°42'11.54"N 76°5'0.24"E), The soil samples were collected from a depth of 5 to 10 cm after the removal of surface soil (4 cm) and transferred to sterile polythene bags and brought to the laboratory under sterile conditions for further studies.

2.2.2.2. Isolation of actinomycete strains and maintenance

The soil samples were dried in an oven (Equitron, Mumbai, India) at 45°C and processed within 36 h for the isolation of actinomycete strains (Rotich et al., 2017). For this, 10 g of soil samples were suspended in 90 ml sterile distilled water and shaken vigorously for 1 h in a shaking incubator (Lab Companion, Korea) at 160 rpm at 28±2°C. Samples were then allowed to settle and serial dilutions up to 10^{-3} were prepared and

aliquot of 100 µl from each dilution was spread evenly on actinomycete isolation agar (AIA) and Potato dextrose Agar (PDA) plates in triplicates and incubated at 28±2°C for 14 to 28 days. The emerging colonies with different morphological characters were selected and the purified strains were maintained on AIA. The viability of the strains were checked in AIA, PDA, ISP2 and ISP4, where PDA was the best, hence further storage and maintenance of the culture was done in PDA. The isolated strains were conserved at 4°C as well as in the presence of glycerol (50% v/v) at -20°C for a longer period.

2.2.2.3. Morphological characterization of actinomycetes

All actinomycete strains were grown on PDA medium for 21 days and the morphological features and colour of aerial and substrate mycelium were noted down at 7,14, and 21 days of incubation (Shirling and Gottlieb, 1966).

2.2.2.4. Screening of actinomycete strains for their antibacterial activities

2.2.2.4.1. Primary screening

The primary screening of actinomycete isolates was done by agar overlay technique (Alaadin et al., 2007). For this, from a 4 day old culture, a loopful of inoculum was spot inoculated on PDA plates and grown at 28±2°C for 7 days. Then the plates were covered with 0.6% of nutrient agar medium previously seeded individually with two test indicator bacterial pathogens (*E. coli* and *S. aureus*) to evaluate their antibacterial efficacy. The activity was recorded after 24 h of growth at 37°C and expressed as zone of inhibition (in mm).

2.2.2.4.2. Secondary screening

In the secondary screening, actinomycete strains which exhibited significant antibacterial activity against the two indicator organisms were further tested against eleven bacterial pathogens by agar overlay method as mentioned above, to check the broad-spectrum antibacterial properties. The strain which recorded best zone of inhibition was selected for broad-spectrum antifungal studies.

2.2.2.5. Identification of antagonistic isolates based on 16S rRNA gene sequencing

2.2.2.5.1. Genomic DNA Isolation

Genomic DNA was isolated from the culture using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. For this, 1 g actinomycete culture is taken in a microcentrifuge tube. To this, 180 µl of TI buffer and 25 µl of proteinase K was added and incubated at 56°C in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. Two hundred microlitres of B3 buffer was added and incubated at 70°C for 10 min. Two hundred and ten microlitres of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 min. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

2.2.2.5.2. Agarose Gel Electrophoresis for DNA quality and quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. One microlitre of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH 8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.2.2.5.3. PCR analysis

Polymerase Chain Reaction (PCR) contain 20 µl reaction volume composed of 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers (Table 2.1) and FTA disc as template. The PCR amplification was carried out in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using the following conditions: a pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 40 sec, extension at 72 °C for 60 sec and a repair extension at 72 °C for 7 min.

Table 2.1: Primers used in PCR Reaction

Target	Primer Name	Direction	Sequence (5' → 3')
16S rRNA	16S-UP-F	Forward	CGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG
	16S-UP-R	Reverse	CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT

2.2.2.5.4. Agarose Gel electrophoresis of PCR product

The PCR products was checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. One microliter of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 h, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.2.2.5.5. ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product was mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

2.2.2.5.6. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The PCR mix consisted of the following components: 10-20 ng of ExoSAP treated PCR product, 3.2 pM primer (either Forward or Reverse), 0.28 µl Sequencing Mix, 1.86 µl Reaction buffer. The mix was made up to 10µl with Sterile distilled water. The sequencing PCR temperature profile consisted of a 1st cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

2.2.2.5.7. Post Sequencing PCR Clean up

For this, Master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction and master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol were prepared. After that, 12µl of master mix I was added to each reaction containing 10µl of reaction contents and was properly mixed. 52 µl of master mix II was added to each reaction. The contents were mixed by inverting and incubated at room temperature for 30 minutes and spun at 14,000 rpm for 30 minutes. The supernatant was decanted and added 100 µl of 70% ethanol. This is spun at 14,000 rpm for 20 minutes and supernatant was decanted again and repeated 70% ethanol wash. Again, supernatant was decanted and air dried the pellet. The cleaned-up air-dried product was sequenced in ABI 3730 DNA Analyzer (Applied Biosystems).

2.2.2.5.8. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6 (Drummond et al., 2012).

2.2.2.5.9. Phylogenetic tree construction of selected strains

The high-quality 16S rRNA sequences were compared with other related species downloaded from the EzTaxon tool (Chun et al., 2000) and a phylogenetic tree was constructed with MEGA version 6. Sequences were aligned using the computer package ClustalW and were analyzed to determine the relationships between isolates by the neighbor-joining method Bootstrap values were generated using 1000 replicates.

2.2.2.6. Morphological, cultural and physiological characterization of selected strains

2.2.2.6.1. Morphological characterization of selected strains

Actinomycetes colonies were identified up to genus level based on the morphology of spore bearing hyphae with entire spore chain as described in Bergey's Manual of Systematic Bacteriology (Goodfellow et al., 2012). The selected strains were further characterized by colony characteristics and pigment production on various media viz. Nutrient agar (NA); Bennet's agar (BA); Czapek dextrose agar (CDA); Kustner's agar (KA); International Streptomyces Project (ISP) agar such as ISP1 (Tryptone yeast extract agar), ISP 2 (Malt extract agar), ISP 3 (Oat meal agar), ISP4 (Inorganic salt starch agar), ISP 5 (Glycerol asparagine agar), ISP 6 (Peptone yeast extract agar) and ISP 7

(Tyrosine agar); Potato dextrose agar (PDA) and Starch casein agar (SCA) (see annexure I). Colour formation was determined according to ISCC-NBS colour charts (Kelly and Judd, 1965).

2.2.2.6.2. Microscopic characterization of A30 and A32

2.2.2.6.2.1. Analysis of spores using scanning electron microscopy and light microscopy

Further morphological characteristics of selected strains were assessed by light microscopy and scanning electron microscopy (SEM). For this procedure, the cultures were directly grown on stubs and coated with a film of gold without using any fixatives and dehydrating procedures. For SEM analysis, sterilized circular aluminium stubs were inserted into the PDA plate at an angle of about 45°C and sterile coverslips were inserted at the same angle on the sample plate. The plates with stubs and coverslip were incubated at 28±2°C for 24 h to check any contamination during the handling procedure (Kumar et al., 2011). After 24 h, the isolates were then spread along the line where the surface of the stub met the medium, using an inoculating loop and then incubated at 28±2°C for 10 days. After incubation, the stubs were then carefully removed without disturbing the growth and the upper surface was coated under vacuum, with a film of gold for 15-20 minutes and viewed on the scanning electron microscope (Zeiss Evo 40 EP, Germany) at an accelerating voltage of 20 KV, to obtain secondary electron images. Similarly, the cover slip was also mounted on the glass slide having one drop of methylene blue (0.3 g in 10 ml distilled water) and fixed slides were observed under light microscope (Olympus CX41, Japan).

2.2.2.6.3. cultural characterization of selected strains

Biochemical tests viz. Gram staining, catalase, oxidase, hydrolysis of cellulose, gelatin, lipids, pectin, protein, starch and urea were done using the standard procedures. Catalase test was determined on the glass slide by adding a drop of 6% Hydrogen peroxide (Jones, 1949). Starch hydrolysis was done by the streaking across ISP 2 agar plates containing 1% starch using a loopful of spores and incubated for 4 days at 28±2 °C (Kokare et al., 2004). The plates were flooded with iodine solution and the clear zone was observed. Cellulose hydrolysis was done ISP 2 supplemented with Carboxy Methyl Cellulose (CMC, 1%) and incubated at 28±2°C for 4 days. At the end of incubation, the plates were flooded with 0.1% congo red and after 15 minutes washed with 0.1M NaCl solution (Andro et al., 1984). Pectin hydrolysis was determined by adding pectin (1%) in ISP2 medium and incubated for 4 days at 28±2°C. At the end of incubation, the plates were flooded with weak iodine solution and zone of clearance was recorded (Hankin et al., 1971). Casein hydrolysis was done according to a method described by (Chapman, 1952). Lipid hydrolysis was done on ISP 2 agar plates supplemented with 1% tributyrin and incubated for 4 days at 28±2°C. The transparent zone around the colonies indicates the lipid hydrolysis by the enzymes (Elwan et al., 1977). Urea hydrolysis was determined according to (Cappuccino and Sherman, 1992). Gelatin liquefaction of the strain was determined according to (Mossel and De Bruin, 1954).

For testing antibiotic susceptibility, strains were grown on ISP 2 slants (Waksman, 1961) at 28±2 °C for 7 days. Spore suspension was prepared by transferring spores from the mature culture to a 5 ml of sterile water and shaken vigorously. The suspension was

swabbed on to the ISP 2 agar plates and antibiotic discs containing known concentration was placed in medium. The plates were incubated for 4 days. Growth of strain on medium containing antibiotics discs was then compared with that on control medium without antibiotics. Cultural characteristics such as pH range (0.5 to 12) and temperature range (25 to 45 °C) were done by inoculating the strain in inorganic salt starch broth. The utilization of carbon sources was determined by growing the isolate in Basal liquid medium (BLM) supplemented with 1 % of various carbon sources (fructose, galactose, glycerol, lactose, maltose, mannitol, starch, sucrose, and xylose). BLM alone inoculated with the test strain served as a control. Nitrogen utilization of the strain was tested in BLM with 1 % of various nitrogen sources viz. ammonium chloride, ammonium sulphate, beef extract, bio-peptone, casein, malt extract, meat extract, meat infusion powder, meat peptone, peptone, potassium nitrate, soybean meal, urea and yeast extract. BLM supplemented with glucose served as a control. All the results were noted after 7 days of incubation.

2.2.2.7. Broad spectrum antimicrobial activities of selected strains

2.2.2.7.1. Antibacterial activity of live A30 and A32

The antibacterial activity of live A30 and A32 strains were confirmed through agar over lay technique and the zone of inhibition was recorded.

2.2.2.7.2. Antifungal activity of live A30 and A32

The dual culture technique according to Dileep Kumar (1998) was employed to find out the inhibitory activity of antagonistic strains against various filamentous fungi

(*F. oxysporum*, *R. solani* and *C. gloeosporioides*) on Potato Dextrose Agar (PDA) medium. For this, an actively growing mycelial disc (approx. 6 mm^2) was placed at one side of the Petri plate, 2 cm inside the periphery and a loopful the *Streptomyces* strain was streaked in a line on the opposite side at a distance of 5 cm from the mycelial disc. The plates were incubated at 28±2 °C and inhibition zone was measured as distance (in mm) between the respective antagonist strain and filamentous fungal pathogen after 7 days of incubation.

The agar overlay method as described by Rahalison et al. (1991) was employed for detecting the activity of *Streptomyces* strains against spore forming test fungi. For this, antagonistic strains were spot inoculated in PDA plates and incubated at 28±2 °C for 72h. Then the plates were overlaid with potato dextrose agar containing 1×10^6 spores of each fungus per ml. Zone of inhibition was measured after incubation at 28±2 °C for 7 days. For *Candida* sp. agar overlay method, as described above for the bacteria was adopted.

2.2.2.7.3. Antibacterial activity of A30 and A32 ethyl acetate extracts

2.2.2.7.3.1. Preparation of crude extract

The isolates designated as A30 and A32 were grown in submerged culture in 250 ml flasks containing 100 ml of ISP2 medium. A $2cm^2$ piece of agar from each seven-day-old culture grown on PDA was used to inoculate the flasks. These cultures were grown in an incubator shaker (Lab Companion, Korea) at 160 rpm, 28±2°C, for seven days. The resulting culture broths (approximately 100 ml) were separated from the mycelium by centrifugation at 8000 rpm (Remi C-24 plus, Mumbai) for 15 min. The cell free culture filtrate was extracted with an equal volume of ethyl acetate thrice. The ethyl acetate

extracts were combined, dried over anhydrous sodium sulfate, and concentrated using a rotary flash evaporator at 40°C to obtain the ethyl acetate extract.

2.2.2.7.3.2. Antibacterial activity (Agar disc diffusion assay)

Antibacterial activity of the ethyl acetate extracts was measured by agar disc diffusion assay (CLSI, 2012). For this, 0.1 ml containing 10^6-10^7 CFU/ml of test bacterial pathogen was swabbed into Mueller Hinton Agar (MHA) plates. The ethyl acetate extract (100 µg/ml) following microfiltration using a 0.22 µm syringe filter, were loaded on 6 mm sterile discs (Hi-media) and air dried. After that, the dried discs were placed on the MHA plate and incubated at 37 °C for 24 h to determine the diameter of zone of inhibition and expressed in millimetre. The experiment was performed in triplicates.

2.2.2.7.4. Antifungal activity of A30 and A32 ethyl acetate extracts

Antifungal activity of the ethyl acetate extracts was measured by agar disc diffusion assay (CLSI, 2012). For this, 0.1 ml spore suspension containing 10^3-10^4 CFU/ml of test fungal pathogen (filamentous and conidia forming fungi) was swabbed into PDA plates. In case of *Candida* sp., the stock suspension was adjusted to 10^5 to 10^6 cells/ml. The ethyl acetate extract (100 µg/ml) following microfiltration using a 0.22 µm syringe filter, were loaded on 6 mm sterile discs and air dried. After that, the dried discs were placed on the pathogen swabbed PDA plate and incubated at 28±2 °C for 48 h (for *Candida* sp.) and 7 days (for filamentous fungi) to determine the diameter of zone of inhibition and expressed in millimetre. The experiment was performed in triplicates.

2.2.2.8. Detection of antibiotic biosynthetic genes

Polyketide synthase (PKS) type I gene fragments were amplified using primer pairs: K1F 5′-TSA AGT CSA ACA TCC GBCA-3′ and M6R 5′-CGC AGG TTS CSG TAC CAG TA-3′. Non-ribosomal peptide synthetase (NRPS) gene fragments were amplified using degenerate primers: A3F 5′-GCS TAC SYS ATS TAC ACS TCS GG-3′ and A7R 5′-SAS GTC VCC SGT SGC GTA S-3′. Gene PKS-II was amplified using the degenerate primer: KSα 5′-TSG CST GCT TGG AYG CSA TC-3′ and KSβ 5′-TGG AAN CCG CCG AAB CCG CT-3′. PCR was carried out in 50 µl reaction volume containing 50 ng of genomic DNA, 20 pico moles of each primer (both forward and reverse), 1X Emerald Amp GT PCR Master Mix (Takara Bio Inc. Kusatsu, Shiga, Japan). PCR was carried out for 35 cycles in a Sure Cycler 8800 (Agilent Technologies, CA, USA) with initial denaturation at 94°C for 5 min, cyclic denaturation at 94°C for 1 min, annealing at 56 °C (for PKS I), 65 °C (for PKS II) and 63°C (for NRPS) for 1 min and extension at 72°C for 1 min with a final extension of 5 min at 72°C. For increasing specificity, the amplified products were visualized on 1.5 % (w/v) agarose gels prepared in 1×Tris-Borate-EDTA (TBE) buffer (Invitrogen) and stained with ethidium bromide. DNA banding was visualized, and images were acquired with a GelDoc EZ imager (Biorad, CA, USA).

2.3. Results

2.3.1. Site, sample collection

From seven sampling sites (Figure 2.1), twenty-five different soil samples were collected. All the samples were processed aseptically and different media were employed for actinomycete isolation.

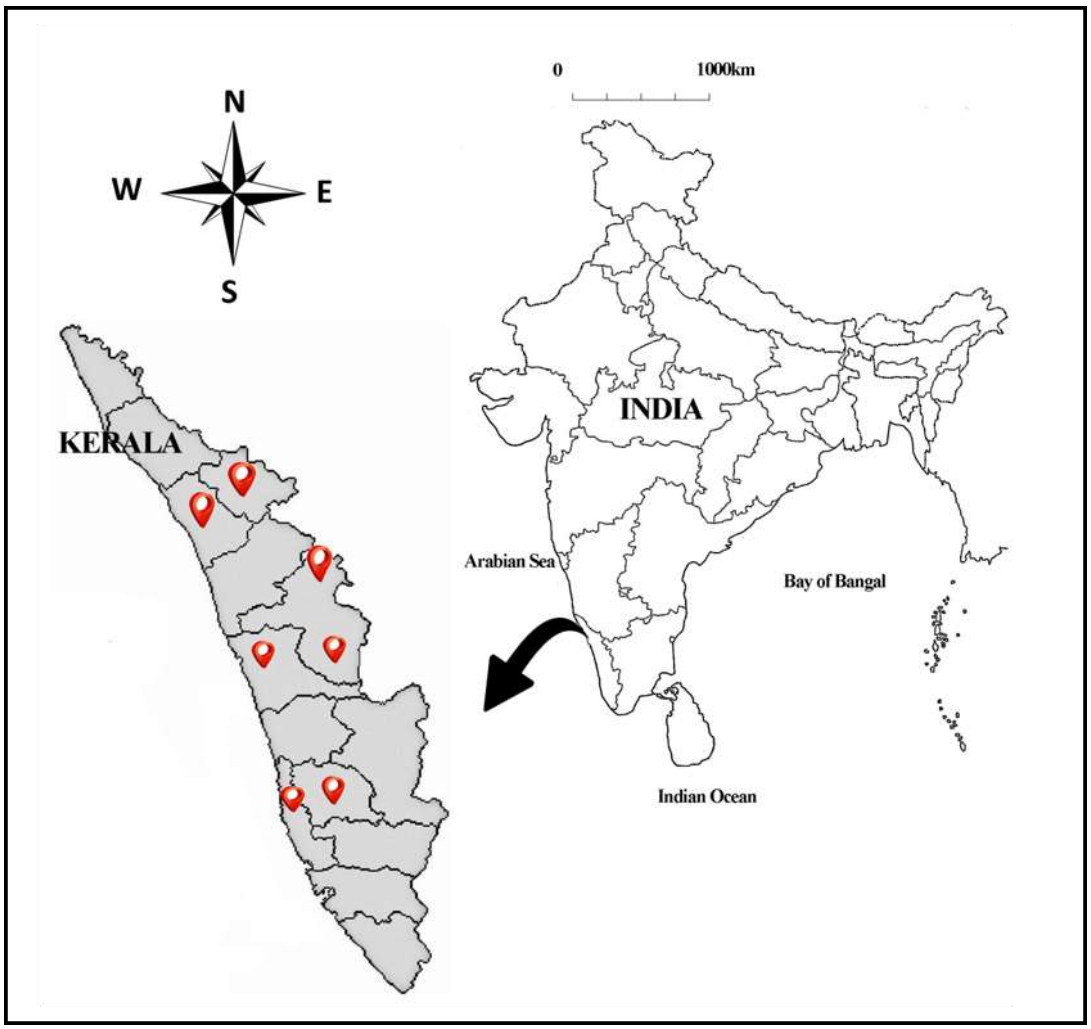

Figure 2.1: Location of sampling sites.

2.3.2. Isolation of actinomycetes from soil samples

From the soil samples, 140 morphologically distinct actinomycete strains were obtained. Out of these, seventeen (12.14%) were isolated from soils collected from Changanacherry, eleven (7.85%) from Kuttanadu, thirty (21.42%) from Nelliyampathy, twelve (8.57%) from Palakkad, twenty seven (19.2%) from Thamarasserry, twenty (14.28%) from Thrissur and twenty three (16.42%) from Wayanadu area. The strains were stored in PDA slants for further investigations.

2.3.3. Morphological characterization of actinomycete strains

All the strains were grown on yeast malt agar media (ISP2) and shown typical morphology of actinomycetes. The colonies were appeared as slow growing (7-21 days), aerobic with aerial and substrate mycelium of various colours. In addition, all strains were Gram positive and fitted to the description of genus *Streptomyces* in Bergey's Manual of Systemic Bacteriology (Locci, 1989). The strains were then categorized into seven color series according to their fully developed aerial mycelium (Table 2.2). The white series strains were more predominant (57.14%) followed by yellow series (20%). Out of 140 strains, sixty-five strains (46.42%) possessed reverse side (substrate mycelium) pigmentation and forty-four strains (31.42%) produced melanoid pigments. Thirty-one strains (22.14%) were able to produce soluble pigments in yeast malt agar media.

2.3.4. Screening of actinomycete strains for their antibacterial activities

2.3.4.1. Primary screening

As a result of primary screening with two indicator microorganism such as *E. coli* and *S. aureus*, sixty-eight (48.57 %) actinomycete strains exhibited antibacterial activity against *S. aureus* while fifty six (40 %) shown activity against *E. coli.* Of these, sixteen (11.42 %) shown activity against both the test pathogens. The morphology of sixteen antagonistic strains in yeast malt media (ISP2) was illustrated in figure 2.2. Among sixteen strains, six (37.5 %) from Nelliyampathy area, four strains (25 %) from Palakkad region followed by Changanacherry, Kuttanadu and Thamarasserry (12.5 % each) regions (Table 2.3).

Table 2.2: Morphological characterization on ISP2 agar medium after 15 days of incubation at 28±2 °C.

	Colour series							
	White	**grey**	**yellow**	**red**	**blue**	**violet**	**Variable (pink, orange, brown)**	**Total**
No. of isolates	80	16	28	2	1	1	12	140
Pigment production								
Reverse side (substrate mycelium)	44	5	12	1	0	0	3	65
Melanoid (brown or black)	24	6	8	1	0	0	5	44
Soluble	12	5	8	0	1	1	4	31
Morphology of spore bearing hyphae								
Rectus (R)	12	5	6	1	0	0	7	31
Flexibilis (F)	16	8	4	1	1	1	2	33
Retinaculum apertum (RA)	2	1	1	0	0	0	2	6
Spirals (S)	5	1	1	0	0	0	1	8
Undetermined (U)	45	1	16	0	0	0	0	62

Figure 2.2: Plate picture of antagonistic strains in yeast malt (ISP2) agar medium.

Table 2.3: Primary screening of actinomycete isolates (by spot inoculation method in agar medium followed by agar overlay)

Sampling site (No. of samples)	**Total no. of strains**	**No. of strains against *S. aureus* [a]**	**No. of strains against *E. coli* [b]**	**No. of strains against both**	**Not active against either *S. aureus* or *E. coli***
Changanacherry (2)	17	5	6	2	6
Kuttanadu (3)	11	4	7	2	0
Nelliyampathy (6)	30	16	10	6	4
Palakkad (2)	12	6	6	4	0
Thamarasserry (4)	27	12	9	2	6
Thrissur (3)	20	12	8	0	0
Waynadu (5)	23	13	10	0	0
Total (25)	140	68	56	16	16

a, activity against Gram positive bacteria; b, activity against Gram negative bacteria

2.3.4.2. Secondary screening

All the sixteen strains which shown activity against both the indicator organisms in primary screening was taken for secondary screening process with eleven bacterial pathogens. Among them, A30 exhibited maximum zone of inhibition against all bacterial pathogens followed by A32. In case of A30, *S. epidermidis* was the most inhibited (59 mm) whereas *S. typhi* (29 mm) was the least inhibited. In case of A32, *P. mirabilis* was the most inhibited (46 mm) whereas *S. epidermidis* (25 mm) was the least inhibited. A32 was not shown any activities against *B. subtilis*, *M. smegmatis* and *S. typhi* (Table 2.4).

Table 2.4: Antagonistic activity of live strains through agar overlay method against human pathogenic bacteria

Strains	**Zone of inhibition (mm)**										
	BC	***BS***	***EC***	***KP***	***MS***	***PA***	***PM***	***ST***	***SA***	***SE***	***SS***
A2	0±0^{a}	0±0^{a}	22±1^{c}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	28±1.5^{d}	0±0^{a}	0±0^{a}
A3	0±0^{a}	0±0^{a}	15±0.57^{c}	0±0^{a}	21±0.57^{c}	0±0^{a}	0±0^{a}	0±0^{a}	22±1^{c}	26±1^{d}	0±0^{a}
A4	0±0^{a}	0±0^{a}	18±1^{c}	16±1.5^{c}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	26±0.57^{d}	30±1^{d}	0±0^{a}
A5	24±0^{d}	0±0^{a}	22±1^{c}	12±0^{b}	0±0^{a}	18±0^{c}	0±0^{a}	0±0^{a}	31±2^{d}	30±1.5^{d}	0±0^{a}
A14	0±0^{a}	0±0^{a}	18±1^{c}	0±0^{a}	13±0^{b}	0±0^{a}	0±0^{a}	0±0^{a}	13±1^{b}	0±0^{a}	0±0^{a}
A19	20±0^{c}	18±1^{c}	18±0.57^{c}	24±2^{d}	0±0^{a}	0±0^{a}	14±1^{b}	0±0^{a}	22±0.57^{c}	0±0^{a}	0±0^{a}
A21	0±0^{a}	0±0^{a}	12±0^{b}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	15±1^{c}	18±1^{c}	0±0^{a}
A23	22±1^{c}	0±0^{a}	28±0^{d}	0±0^{a}	0±0^{a}	0±0^{a}	22±0^{c}	0±0^{a}	26±1^{d}	28±2^{d}	30±0^{d}
A26	0±0^{a}	0±0^{a}	12±0.57^{b}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	12±0.57^{b}	16±1^{c}	0±0^{a}
A28	0±0^{a}	0±0^{a}	18±0^{c}	0±0^{a}	0±0^{a}	0±0^{a}	20±1.5^{c}	0±0^{a}	18±1^{c}	20±2^{c}	22±0.57^{c}
A29	0±0^{a}	0±0^{a}	12±1^{b}	12±0^{b}	0±0^{a}	0±0^{a}	0±0^{a}	10±1^{b}	11±0^{b}	0±0^{a}	0±0^{a}
A30	**41±1.15**^{e}	**38±0.57**^{e}	**42±2**^{e}	**43±1**^{e}	**32±2.5**^{d}	**49±1.15**^{e}	**53±1.15**^{f}	**29±1.15**^{d}	**49±1.15**^{e}	**59±1.15**^{f}	**54±1.73**^{f}
A32	**26±1.33**^{d}	**0±0**^{a}	**32±0.57**^{d}	**28±1**^{d}	**0±0**^{a}	**38±1**^{e}	**46±0.57**^{e}	**0±0**^{a}	**29±0.57**^{d}	**25±1.5**^{d}	**35±1**^{e}
A51	22±0.57^{c}	0±0^{a}	13±1^{b}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	18±1.5^{c}	20±1^{c}	28±2^{d}	14±0^{b}
A67	0±0^{a}	0±0^{a}	10±0.57^{b}	0±0^{a}	10±1^{b}	0±0^{a}	0±0^{a}	0±0^{a}	12±1^{b}	0±0^{a}	11±1^{b}
A68	22±1^{c}	0±0^{a}	23±0.33^{c}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	26±2^{d}	26±0.57^{d}	24±1^{c}
Control	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$. *BC: B. cereus, BS: B. subtilis, EC: E. coli, KP: K. pneumoniae, MS: M. smegmatis, PA: P. aeruginosa, PM: P. mirabilis, ST: S. typhi, SA: S. aureus, SE:S. epidermidis, SS:S. simulans.*

2.3.5. Identification of antagonistic isolates based on 16S rRNA gene sequencing

The strains which shown significant antagonistic activities against eleven bacterial pathogens were phylogenetically analyzed to obtain the insights into the relationship among the strains. High quality DNA were PCR amplified using 16S rRNA specific primers and resulted in 1.5kb product on gel electrophoresis (Figure 2.3) and sequenced. The sequences obtained were compared with the sequences in Ez taxon database and revealed that all antagonistic strains belong to the genera *Streptomyces*. The high-quality 16S rRNA partial sequence from the antagonistic strains were deposited in GenBank data library with assigned accession numbers (Table 2.5).

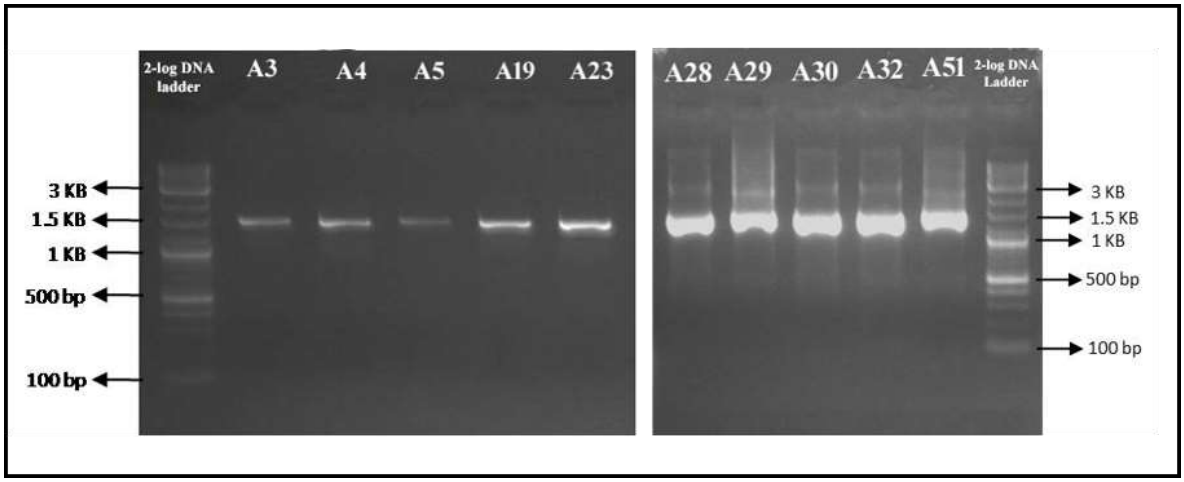

Figure 2.3: PCR amplified 16S rRNA product (1500bp) from antagonistic strains.

2.3.5.1. Phylogenetic tree construction of the selected strains

The high-quality sequences from the antagonistic strains were aligned using Clustal W software. The phylogenetic tree was drawn with the help of Mega 6.0 Version software using neighbor-joining method (Figure 2.4). Phylogenetic analysis showed that the strain A30 is identified as *Streptomyces nogalater* with 100 % similarity whereas A32 possess 100 % silmilarity with *Streptomyces luteosporeus*

NBRC 14657 (Table 2.5). The sequencing result of 16S rRNA region of antagonistic strains as follows:

>*Streptomyces nogalater* NIIST A30 (KX 190774.1)

GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATAC
CGGATACGAGTTTCACCGGCATCGGTGATTCTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGG
CCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGC
GACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
GCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAG
GCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTC
CACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCG
AAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATTAGAGATAGTGCCCCCCTTGTGGTCGG
TGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGG
GGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACA
CGTGCTACAATGGCCGGTACAAAGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGG
TCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA

>*Streptomyces luteosporeus* NIIST A32 (KX190775)

GGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATAC
CGGATATGACCTTCGAAGGCATCTTTGAAGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGG
CCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGC
GACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
GCGGCTTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAG
GCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTC
CACGTCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCG
AAGAACCTTACCAAGGCTTGACATACACCGGAAAGCGCTAGAGATAGTGCCCCCCTTGTGGTCGG
TGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGA
GCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCG
GGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAC
ACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCG
GTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGGAGTTGCTAGTAATCGCA

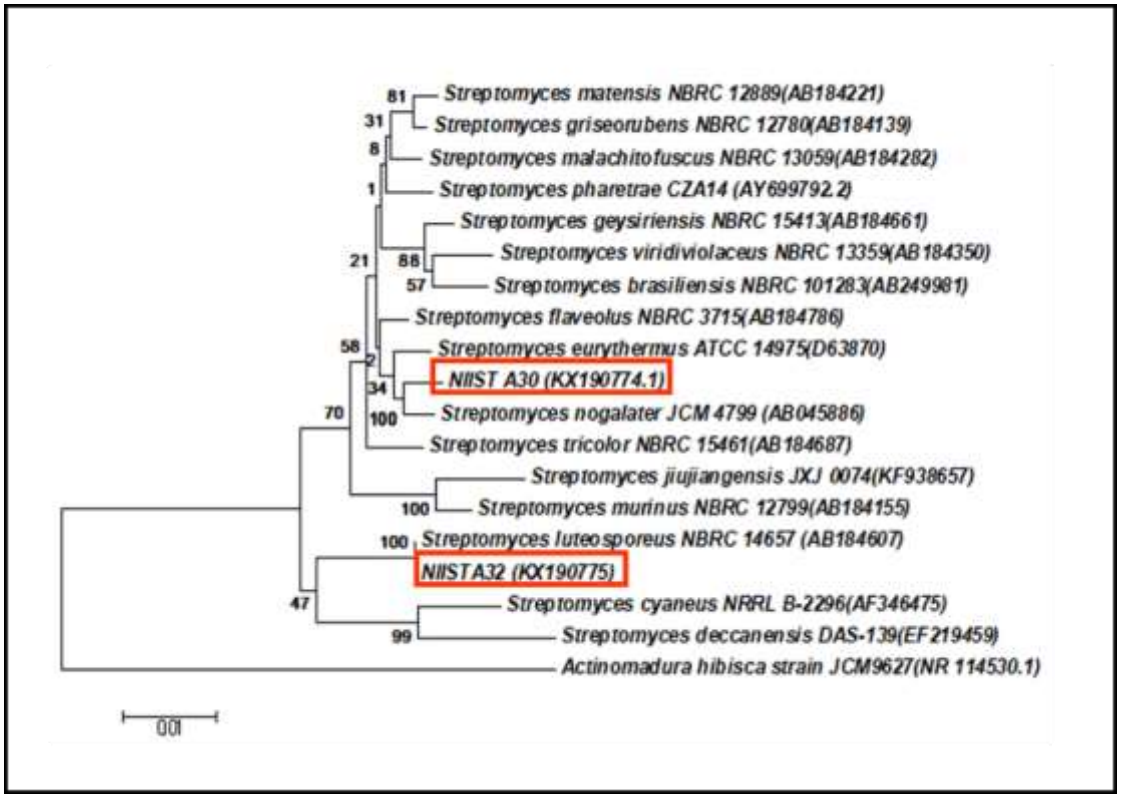

Figure 2.4: Neighbor-joining phylogenetic tree showing the evolutionary relationship of the strain A30 and A32 with its related species.

Table 2.5: Antagonistic strains identified through sequencing and phylogenetic analysis.

Strain no	Gene Bank accession number	Identity	Identification [a]	Collection site
NIIST A3	KT898051	100%	*Streptomyces hiroshimensis*	Thamarasserry
NIIST A4	KX190773.1	100%	*Streptomyces globosus*	Thamarasserry
NIIST A5	KM873339.1	99%	*Streptomyces* sp.	Changanacherry
NIIST A19	KT898049	100%	*Streptomyces griseoruber*	Kuttanadu
NIIST A23	KM873340.1	99%	*Streptomyces* sp.	Silent valley
NIIST A28	KT898050	100%	*Streptomyces lactacystinicus*	Silent valley
NIIST A29	KR375966	99%	*Streptomyces* sp.	Nelliyampathy
NIIST A30	**KX 190774.1**	**100%**	***Streptomyces nogalater***	**Nelliyampathy**
NIIST A32	**KX190775**	**100%**	***Streptomyces luteosporeus***	**Nelliyampathy**
NIIST A51	KT898048	100%	*Streptomyces misionensis*	Nelliyampathy

[a] the best hit through BLASTn analysis

Since A30 and A32 possess distinct activities against the bacterial pathogens, these organisms were studied in detail.

2.3.6. Morphological, cultural and physiological characterization of selected strains

2.3.6.1. Morphological characterization of A30 and A32

A30 produced grey coloured well-developed aerial mycelium and spores. The morphological characteristic of aerial mycelium was varied from media to media from white to grey (Table 2.6). The colour of the reverse side of the vegetative mycelium was brown. It exhibited the production of olive brown soluble pigment in different media such as PDA, ISP2, ISP5 and ISP7. The sporulating and pigment producing ability of the isolate was strongly dependent on the media composition (Figure 2.5).

On the other hand, strain designated as A32 produced a well-developed aerial and substrate mycelium with light pink coloured spores. The sporulation and pigment production ability of the strain was varied with media to media. The morphological appearance was also varied with media (Figure 2.5). The strain displayed abundant growth in BA, GSMB, ISP1, ISP 2, ISP3, ISP4, ISP6, NA, SA, and PDA and exhibited scanty growth in CDA. Soluble pigment production was found only in ISP6 medium (Table 2.7).

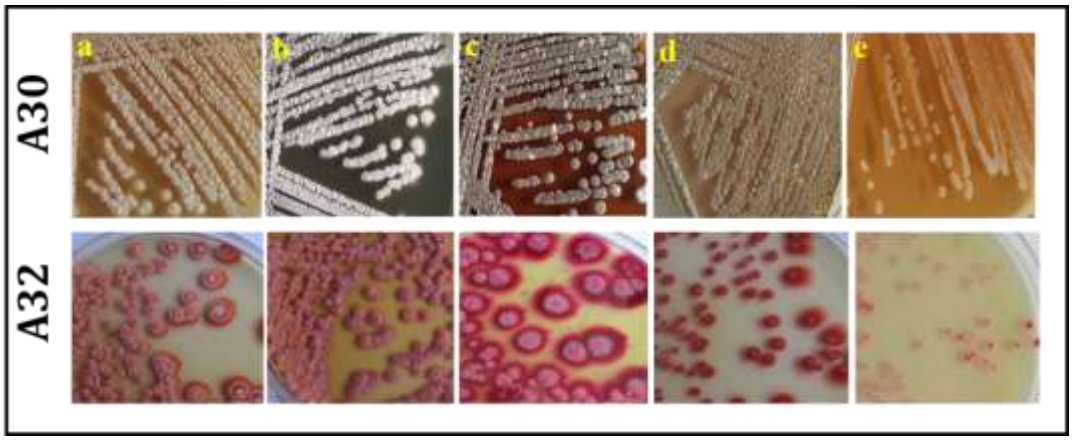

Figure 2.5: Morphology of A30 and A32 in various media (a, BA; b, ISP1; c, ISP2; d, ISP4 and e, NA).

Table 2.6: Morphological characteristics of A30 in different agar media.

Media	Growth	Colour of aerial mycelium	Colour of substrate mycelium	Pigmentation	Sporulation
AIA	+	White	Grey, not fully develop	Nil	Nil
BA	++	White	White to grey	Nil	Nil
CDA	+	Grey	Poorly develop	Nil	Nil
ISP 1	+	White	Not fully develop	Nil	Nil
ISP 2	+++	Grey	Grey	Olive brown	Good
ISP 3	+	Grey	Dark yellow	Deep yellow	Less
ISP 4	+++	Grey	Not fully develop	Deep brown	Good
ISP 5	++	Grey	Strong yellowish brown	Deep brown	Good
ISP 6	+	Grey	Strong yellowish	Moderate olive	Good
ISP 7	++	Grey	Moderate yellow	Dark yellow	Good
KA	++	White to grey	Deep yellowish brown	Deep brown	Less
NA	++	white	Not fully develop, white	Nil	Less
PDA	+++	White	Brown, well develop	Olive brown	Good
SA	+++	Grey	Grey	Olive brown	Good
SCA	++	Grey	Dark brown	Deep brown	Less

+++: Abundant growth; ++: Moderate growth; +: Scarce growth

Table 2.7: Morphological characteristics of A32 in different agar media

Media	Growth	Colour of aerial mycelium	Colour of substrate mycelium	Pigmentation	Sporulation
AIA	++	Vivid yellowish pink	Vivid yellowish pink	Nil	Nil
BA	+++	Vivid reddish orange	Vivid reddish orange	Moderate yellow	Vivid reddish orange
CDA	+	Moderate pink	Deep pink	Nil	Moderate pink
GSMB	+++	Vivid reddish orange	Vivid reddish orange	Nil	Light yellowish pink
ISP1	+++	Vivid reddish orange	Vivid reddish orange	Nil	Nil
ISP2	+++	Vivid reddish orange	Vivid reddish orange	Nil	Light yellowish pink
ISP3	+++	Vivid red	Vivid red	Nil	Deep yellowish pink
ISP4	+++	Strong pink	Vivid red	Nil	Nil
ISP5	++	Deep reddish orange	Deep reddish orange	Nil	Vivid red
ISP6	+++	Greyish yellowish pink	Strong reddish brown	Strong yellowish brown	Nil
ISP7	++	Deep reddish orange	Very deep red	Nil	Pale yellowish pink
NA	+++	Vivid reddish orange	Deep pink	Nil	Nil
PDA	+++	Vivid red	Vivid red	Nil	Light pink
SA	+++	Vivid red	Deep reddish orange	Nil	Vivid reddish orange
SCA	++	Deep yellowish pink	Deep yellowish pink	Nil	Nil

+++: Abundant growth; ++: Moderate growth; +: Scarce growth.

2.3.6.2. Microscopic characterization of A30 and A32

Microscopic observations showed that the strain A30 formed extensively branched, non- fragmented substrate and aerial mycelia. The spore chain morphology of the strain was straight and recti-flexible with smooth surface with less than 50 spores in a chain (Figure 2.6). Microscopic observation of A32 also formed branched, non-fragmented mycelium and the spore morphology couldn't be determined (Figure 2.7).

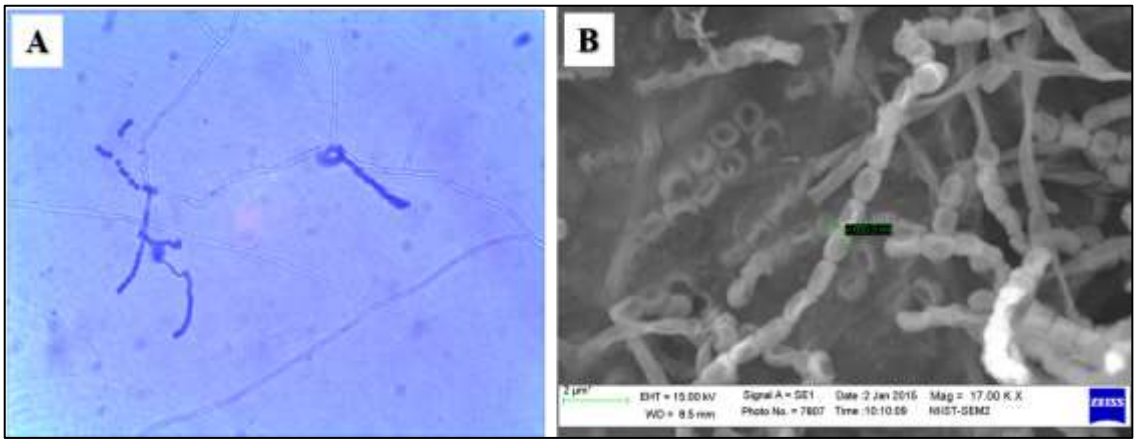

Figure 2.6: (A) Light microscopic and (B) scanning electron microscopic images of A30

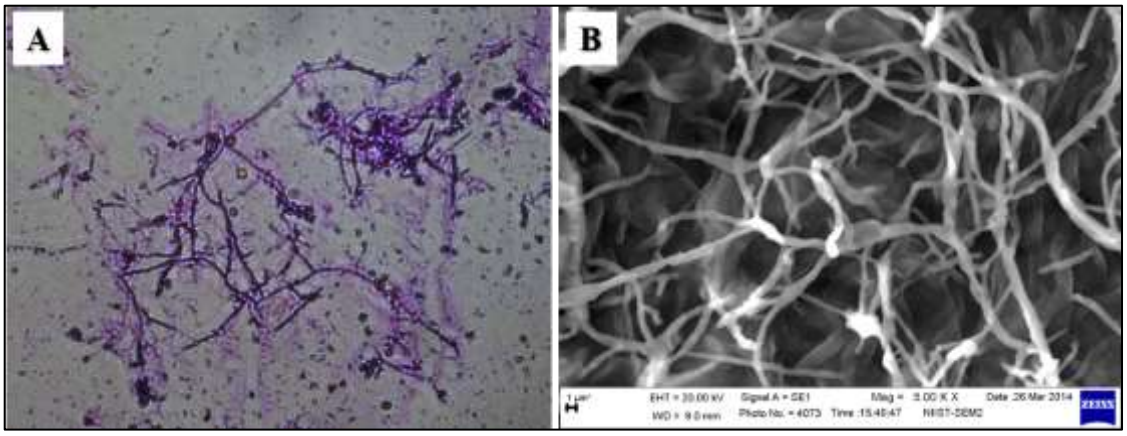

Figure 2.7: (A) Light microscopic and (B) scanning electron microscopic images of A32.

2.3.6.3. Cultural characterization of the selected strains

The strain A30 was Gram positive and aerobic with catalase and oxidase positive. A30 exhibited biochemical properties including hydrolytic activity of starch, urea, cellulose, protein and lipids. But it did not possess hydrolytic activity of pectin and gelatin. In carbohydrate utilization tests, A30 utilized and produced acids from various carbon sources except maltose. The strain was able to utilize various nitrogen sources except potassium nitrate (Table 2.8). Cultural characteristics of the strain showed that it tolerated pH ranges from 0.5 to 12 and was able to grow well at pH 2 to 9. It was also able to grow well at temperatures ranges from 25°C to 45°C and exhibited optimal growth at 3% (w/v) NaCl.

The strain A32 was Gram positive and aerobic with catalase, oxidase positive. It also possesses hydrolytic activity of starch, cellulose, protein. It was not shown any hydrolytic activity of pectin, lipid, urea and gelatin. A32 utilized various carbon sources except lactose, maltose and mannitol and nitrogen sources except ammonium chloride, ammonium sulphate and urea (Table 2.9). Cultural characteristics of the strain showed that it tolerated pH ranges from 2 to 12 and was able to grow well at pH 4 to 9. The isolate was also able to grow well at temperatures ranges from 25°C to 45°C. The salt tolerance of the strain showed that it was able to survive only at low NaCl concentrations (1 to 2%).

Table 2.8: Utilization of different carbon and nitrogen sources by A30

Carbon source	Growth	Nitrogen source	Growth
Fructose	++	Ammonium chloride	++
Galactose	+	Ammonium sulphate	+++
Glucose	+	Beef extract	++
Glycerol	++	Bio peptone	+
Lactose	+	Casein	+
Maltose	-	Malt extract	++
Mannitol	+	Meat infusion	+
Starch	+++	Meat peptone	++
Sucrose	+	Peptone	+++
Xylose	+	Potassium nitrate	-
		Sodium citrate	+
		Soybean meal	+++
		Urea	+
		Yeast extract	++

+++, excellent growth; **++,** moderate growth; **+**, poor growth; **-**, no growth.

Table 2.9: Utilization of different carbon and nitrogen sources by A32

Carbon source	Growth#	Nitrogen source	Growth#
Fructose	+	Ammonium chloride	-
Galactose	+	Ammonium sulphate	-
Glucose	+++	Beef extract	++
Glycerol	++	Biopeptone	++
Lactose	-	Casein	+++
Maltose	-	Malt extract	++
Mannitol	-	Meat infusion	++
Starch	++	Meat peptone	++
Sucrose	+	Peptone	+++
Xylose	+	Potassium nitrate	+
		Sodium citrate	+
		Soyabean meal	+++
		Urea	-
		Yeast extract	+++

+++, excellent growth; **++** moderate growth; **+**, poor growth; **-**, no growth.

2.3.6.4. Antibiotic sensitivity

Strain A30 showed antibiotic susceptibility against seven tested antibiotics such as doripenem, kanamycin, cinoxacin, nalidixic acid, minocycline, minonazole and methicillin. On the other hand, A32 exhibited sensitivity to doripenem, kanamycin, cinoxacin, nalidixic acid, minocycline, ciprofloxacin and methicillin (Table 2.10).

Table 2.10: Antibiotic susceptibility of selected strains A30 and A32

Antibiotics (Concentration in μg)	**A30**	**A32**
Doripenem (10)	S	S
Kanamycin (30)	S	S
Cinoxacin (100)	S	S
Nalidixic Acid (30)	S	S
Minocycline (30)	S	S
Minonazole (50)	S	R
Fluconazole (10)	R	R
Nafcilin (1)	R	R
Ketoconazole (10)	R	R
Nystatin (50)	R	R
Chloramphenicol (30)	R	R
Ciprofloxacin (5)	R	S
Carbenicillin (100)	R	R
Piperacillin (100)	R	R
Clindamycin (10)	R	R
Imipenem (10)	R	R
Methicillin (5)	S	S
Rifampicin (5)	R	R
Ampicillin (10)	R	R
Clotrimazole (10)	R	R
Amphotericin B (10)	R	R

S, sensitive; R, resistant.

2.3.7. Broad spectrum antimicrobial activities of selected strains

2.3.7.1. Antibacterial activity of live A30 and A32

A30 and A32 recorded significant zone of inhibition against both Gram positive and Gram negative human pathogens. A30 strain exhibited maximum zone of inhibition against *S. epidermidis* (59 mm) followed by *S. simulans* (54 mm) and *P. aeruginosa* (49 mm). In case of A32, the maximum inhibition was obtained against *P. mirabilis* (46mm) followed by *P. aeruginosa* (38 mm) and *E. coli* (35 mm). The antibacterial activities of both strains are illustrated in Table 2.11 and Figure 2.8.

Table 2.11: Zone of inhibition recorded by live A30 and A32 against various bacterial pathogens.

	Zone of Inhibition (mm)	
Pathogens	**A30**	**A32**
Gram positive bacteria		
B. cereus	41±1.5 [b]	26±1.33 [a]
B. subtilis	38±0.57 [b]	0
M. smegmatis	32±2.5 [a]	0
S. aureus	49±1.5 [c]	29±0.57 [b]
S. epidermidis	59±1.5 [e]	25±1.5 [a]
S. simulans	54±1 [d]	32±1 [c]
Gram negative bacteria		
E. coli	42±2 [b]	35±0.57 [c]
K. pneumoniae	43±1 [b]	28±1 [b]
P. aeruginosa	49±1.5 [c]	38±1 [d]
P. mirabilis	44±1.5 [b]	46±0.57 [e]
S. typhi	29±1.5 [a]	0
control	0	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

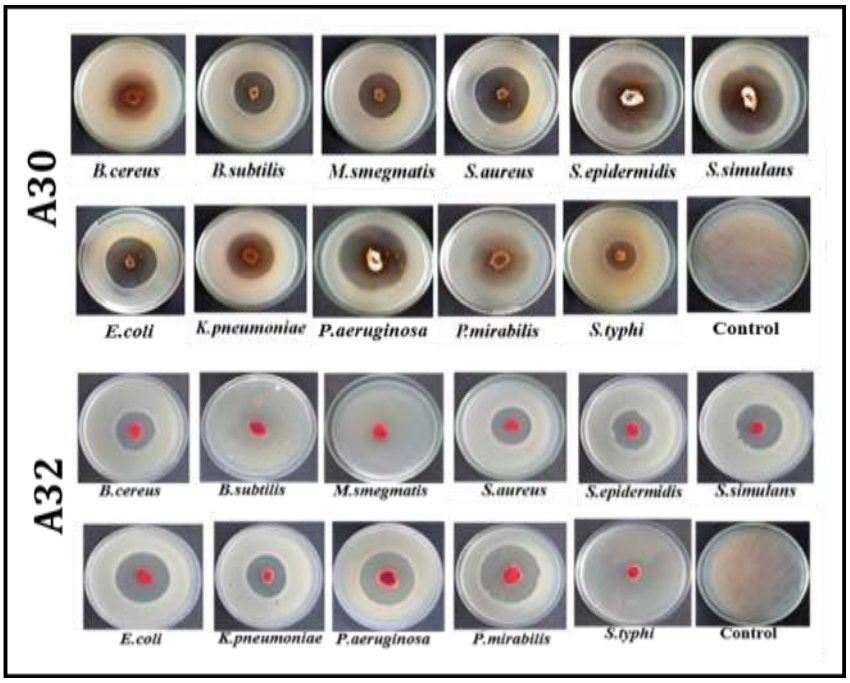

Figure 2.8: Broad spectrum antibacterial activities of live strains A30 and A32.

2.3.7.2. Antifungal activity of live A30 and A32

In vitro antagonism of fungal pathogens with live A30 shown that it significantly inhibited all the four *Candida* sp. tested. But there was no prominent inhibition observed for fungal phytopathogens. Among different *Candida* sp. tested, *C. tropicalis* (35 mm) was the most inhibited followed by *C. albicans* (30 mm) (Table 2.12 and Figure 2.9).

Table 2.12: Zone of inhibition recorded by live A30 against *Candida* sp.

	Zone of inhibition (mm)
Pathogens	**A30**
C. albicans	30±1.5 [b]
C. tropicalis	35±0.57 [c]
C. parapsilopsis	26±1 [a]
C. glabrata	25±0.33 [a]
control	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

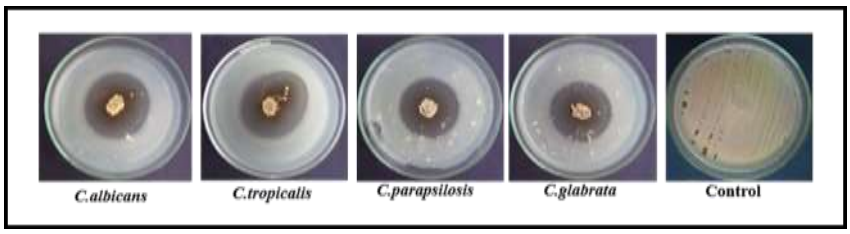

Figure 2.9: Antifungal activities of live A30 strain against *Candida* sp.

In vitro antagonism against fungal pathogens with live A32 showed inhibition against various plant pathogens. When tested against conidia forming fungi in agar overlay method, maximum zone of inhibition was exhibited for *A. niger* (48 mm) followed by *A. fumigatus* and *T. rubrum* (40 mm each). On the other hand, when tested against filamentous fungi in dual culture method, A32 exhibited a maximum zone of inhibition against *F. oxysporum* 4814 (20 mm) followed by *F.*

oxysporum 1322 (18 mm). Moreover, A32 was able to inhibit all the phytopathogens significantly (Table 2.13 and Figure 2.10).

Table 2.13: Zone of inhibition recorded by live A32 against various plant fungal pathogens

Pathogens	**Zone of inhibition (mm)**
	A32
Conidia forming fungi (Agar overlay)	
A. flavus	32±1.5 [d]
A. fumigatus	40±1.33 [e]
A. niger	48±2 [f]
A. tubingensis	31±0.57 [d]
P. expansum	35±1 [d]
T. rubrum	40±2 [e]
Filamentous fungi (dual culture)	
C. capsici 6071	15±0.33 [b]
C. capsici 6870	13±1 [a]
C. gleosporiodes 2151	12±0.57 [a]
C. gleosporiodes 6079	12±0.57 [a]
C. gleosporiodes 6434	14±0.57 [b]
F. oxysporum 1322	18±1 [c]
F. oxysporum 4814	20±1 [c]
F. oxysporum 584	12±0.57 [a]
F. solani	10±0.57 [a]
F. udum	14±0.33 [b]
R. solani	10±0.33 [a]
control	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

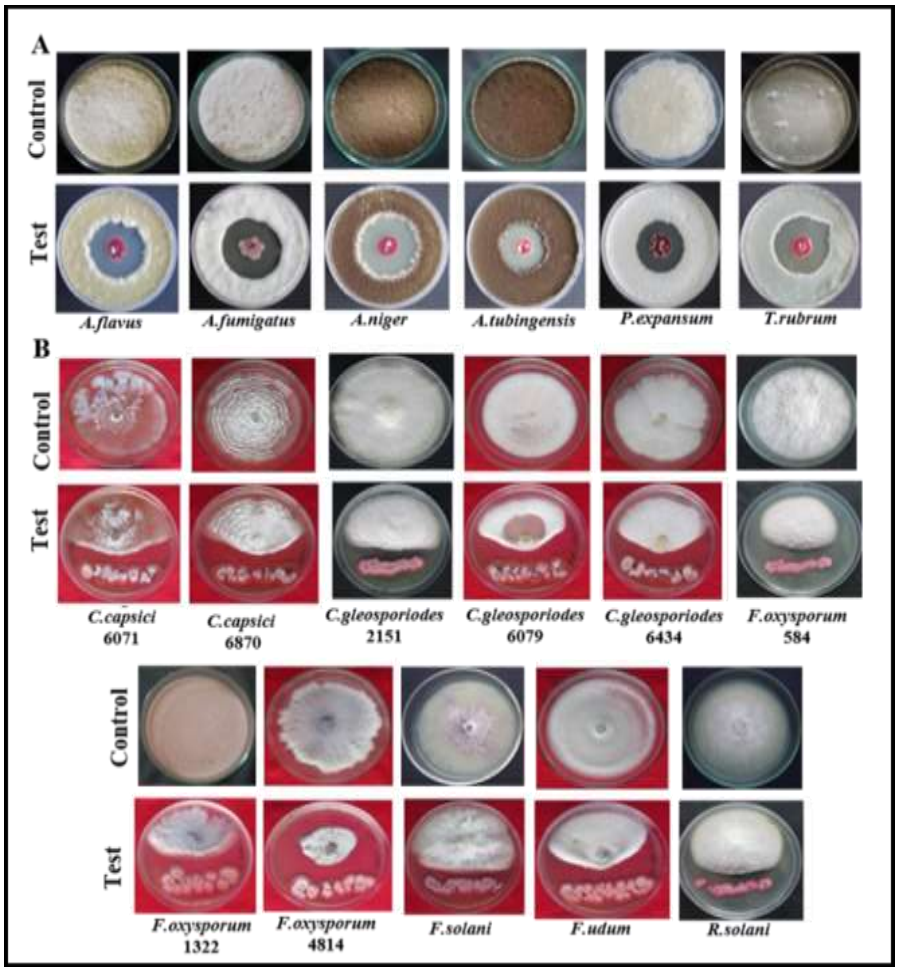

Figure 2.10: Broad spectrum antifungal activities of live A32. (A, agar overlay; B, dual culture).

2.3.7.3. Antibacterial activity of A30 and A32 ethyl acetate extracts

Ethyl acetate extract of A30 also recorded significant zone of inhibition against both Gram positive and Gram negative human pathogens (Table 2.14 and Figure 2.11). Here, A30 exhibited maximum zone of inhibition against *S. epidermidis* (22 mm) followed by *B. cereus* (20 mm) and *S. aureus* (19 mm). In case of A32, the maximum inhibition was obtained against *K. pneumoniae* (18 mm) followed by *P. aeruginosa* (16 mm) and *S. simulans* (14 mm).

Table 2.14: Zone of inhibition recorded by ethyl acetate extract of A30 and A32 against bacterial pathogens

	Zone of Inhibition (mm)	
Pathogens	**A30**	**A32**
Gram positive bacteria		
B. cereus	20±1.5 [c]	10±0.57[a]
B. subtilis	16±0.57 [a]	0
M. smegmatis	15±0.57 [a]	0
S. aureus	19±1.5 [b]	10±1 [a]
S. epidermidis	22±1.5 [c]	12±0.57 [a]
S. simulans	16±1 [a]	14±1 [b]
Gram negative bacteria		
E. coli	18±0.57 [b]	12±0.57 [a]
K. pneumoniae	18±1 [b]	18±2 [c]
P. aeruginosa	15±1.5 [a]	16±1 [b]
P. mirabilis	13±1.5 [a]	12±1.5 [a]
S. typhi	17±1.5 [b]	0
control	0	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

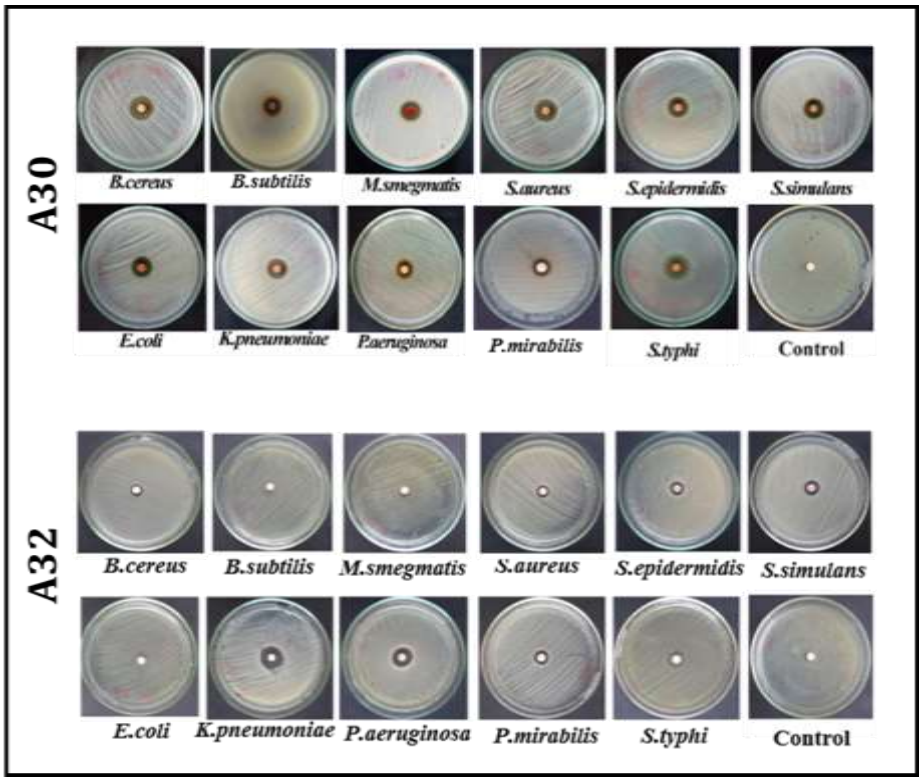

Figure 2.11: Broad spectrum antibacterial activities of ethyl acetate extracts of A30 and A32.

2.3.7.4. Antifungal activity of A30 and A32 ethyl acetate extracts

In vitro antagonism assay against fungal pathogens with ethyl acetate extract of A30 also inhibited *Candida* sp. significantly. There were less or no zone of inhibition obtained for phytopathogens (Table 2.15 and Figure 2.12). Among different *Candida* sp. tested, *C. glabrata* (26 mm) was the most inhibited followed by *C. tropicalis* (24 mm).

Table 2.15: Zone of inhibition recorded by ethyl acetate extract of A30 against fungal pathogens

Pathogens	Zone of inhibition (mm) A30
A. flavus	12±0.57 [b]
A. fumigatus	0
A. niger	15±0.33 [b]
A. tubingensis	8±0.33 [a]
C. tropicalis	24±1.5 [d]
C. albicans	20±1.5 [c]
C. capsici 6071	0
C. capsici 6870	0
C. glabrata	26±0.57 [d]
C. gleosporiodes 2151	0
C. gleosporiodes 6079	8±0.57 [a]
C. gleosporiodes 6434	8±0.33 [a]
C. parapsilosis	18±1.57 [c]
F. oxysporum 1322	13±1 [b]
F. oxysporum 4814	0
F. oxysporum 584	0
F. solani	0
F. udum	12±0.57 [b]
P. expansum	8±0.57 [a]
R. solani	0
T. rubrum	0
control	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

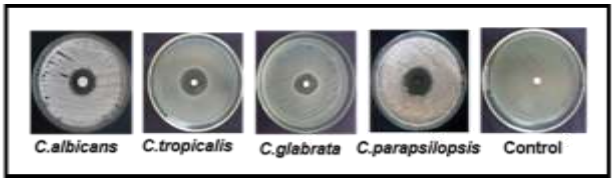

Figure 2.12: Anti-candidial activities of ethyl acetate extract of A30.

In vitro antagonism assay against fungal pathogens with ethyl acetate extracts of A32 showed significant inhibition against a broad spectrum plant pathogens (Table 2.16 and Figure 2.13). Maximum zone of inhibition was shown against *C. gleosporiodes* 2151 (34 mm) followed by *A. flavus* (32 mm) and *C. gleosporiodes* 6434 (22 mm).

Table 2.16: Zone of inhibition recorded by ethyl acetate extract of A32 against fungal pathogens

	Zone of inhibition (mm)
Pathogens	**A32**
A. flavus	32±0.33
A. fumigatus	18±0.57
A. niger	20±1
A. tubingensis	21±0.33
C. albicans	8±0.57
C. capsici 6071	20±0.57
C. capsici 6870	18±0.33
C. glabrata	0
C. gleosporiodes 2151	34±1
C. gleosporiodes 6079	20±0.33
C. gleosporiodes 6434	22±0.57
C. parapsilosis	0
C. tropicalis	0
F. oxysporum 4814	16±0.57
F. oxysporum 1322	18±0.57
F. oxysporum 584	15±0.33
F. solani	11±0.33
F. udum	15±0.33
P. expansum	13±0.57
R. solani	12±0.57
T. rubrum	14±0.57
Control	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

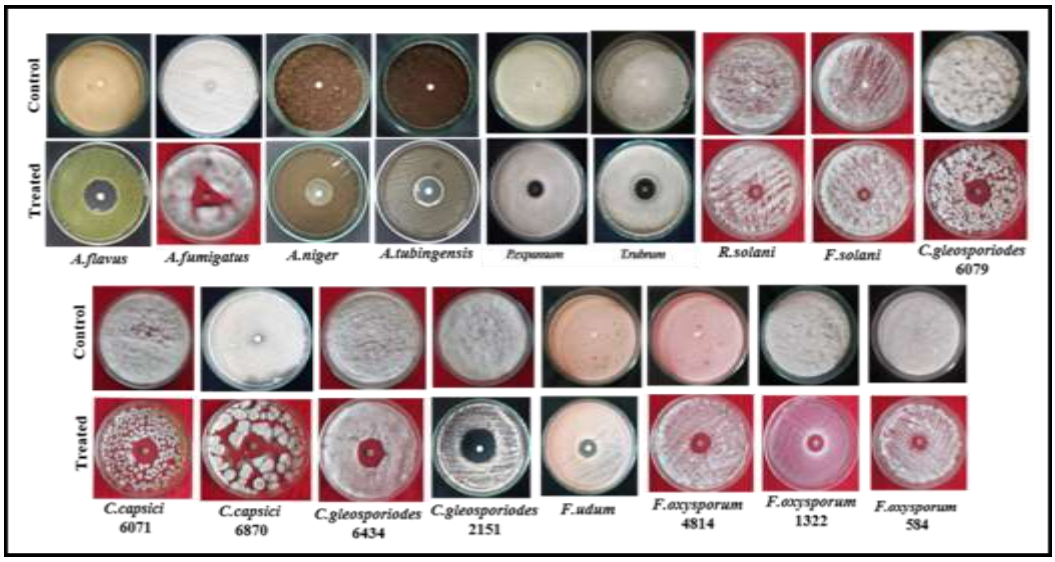

Figure 2.13: Broad spectrum antifungal activities of ethyl acetate extract of A32.

2.3.8. Detection of antibiotic biosynthetic genes

The antimicrobial activities of antagonistic strains were correlated with the presence of antibiotic synthetic genes. The strain, designated as A30 showed the presence of PKS I gene (figure 2.14), not PKS II and NRPS while strain A32 not shown the presence of any of these genes. PCR product of K1F and M6R primers resulted in a 776 bp length fragment of PKS I gene encoding type I PKS operon was submitted to GenBank (Nucleotide sequence with accession number- MN242813). At the gene level, it shared 85.12 to 94.98 % identity with polyketide synthase genes of other *Streptomyces* species. The sequence of PKS I gene from A30 is shown below:

GTGATGGCGTTGCGGCATGGTGTGTTGCCGAGGACGTTGCATGTGGAGGTGCCGTCGTCGCAT
GTGGACTGGTCCTCGGGTGCGGTGGAGGTGTTGACGCAGACACGGGCGTGGCCGGTGGTGGAG
CGTGCGCGCCGGGCGGCGGTCTCGGCGTTCGGGGTGAGCGGAACCAACGCACATGTCGTACTG
GAACAGGCGCCGTCACTGGAGGTGGAACCAGAGCCGACGCAGCCTGACCGAACCGTGCCGGTG
GTGGTGTCGGGTGCTTCGGCGACAGCTTTGGACGCGGGGTTGGGGCGTCTGGCCGGGCTGGTG
GAGTCCGATGGCGGGCTGGGTGTGGGCGAGGTCGGATGGTCTGCGGCGCATCGGTCGGTGTTC
GAGCATCGTGCGGTGGTGGTGGCGTCCGGACGTGAGGAACTGCTGGAGGGCCTGAAGGCCCTG
GTGGTGTCGGGTGTTGCCGGTTCGGTGGGTCGTTCGGTGTTGGTGTTTCCGGGTCAGGGGACG
CAGTGGGCGGGGATGGGTGCGGAGCTTTTGGAGTCTTCGCCGGTGTTCGCGGCGCGTATGGGG
GAGTGTGCTGCCGCGTTGGGCCGGTTTGTGGAGTGGGATCTGTTGGAGGTGGTTCGTTCGGGT
GAGGGGCTTGAGCGGGTGGATGTGGTGCAGCCGGTGACGTGGGCGGTGATGGTGTCACTGGCC

GAGGTGTGGGCCAGTGCGGGGGTGAGGCCGGATGCGGTTGTGGGCCATTCGCAAGGGGAGATC
GCTGCGGCCGTCGTGTCCGG

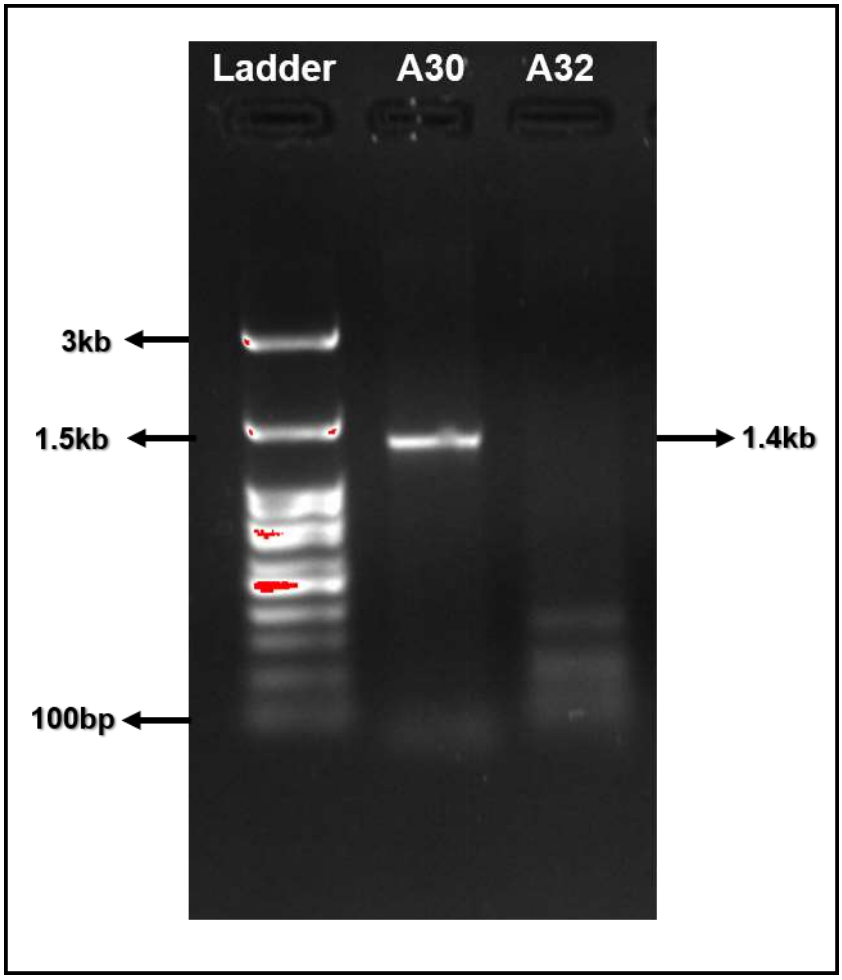

Figure 2.14: PCR based screening of PKS I gene from A30 and A32.

2.4. Discussion

Actinomycetes are filamentous, Gram-positive bacteria with high G+ C content in their genomes. A large percent of their genome is known for the production of bioactive metabolites. Besides that, there has been a decrease in the search for antimicrobial metabolites in recent years (Sharma et al., 2019). Therefore, the probability of finding new and efficient bioactive metabolites from actinobacteria can be increased by continuing the search in underexplored areas (Almalki, 2020). Among actinobacteria, *Streptomyces* species are the major producers of antimicrobial metabolites (Manteca and

Yagüe, 2019). In the present study, a total of 140 phenotypically different actinomycetes were isolated and all of them showed the resemblance to the genus *Streptomyces*. These strains were screened against human bacterial pathogens and out of them, 16 exhibited promising antagonistic properties. Phylogenetic position of the antagonistic strains suggested that the strains exhibited similarity to the genus *Streptomyces*. Selection of antagonistic strains needs screening of a large number of isolates. This is supported by an investigation by Hu et al. (2019) where two promising strains were obtained from 56 actinomycete strains isolated from a seashore of China. In another study, only one *Streptomyces* strain designated as RD-5 possessing broad spectrum antibacterial activities was obtained from 11 actinomycetes isolated from Gulf of Khambat (Dholakiya et al., 2017). Another investigation screened 172 actinobacterial isolates and 96 showed antimicrobial activity against at least one of the test microbial strains (Das et al., 2018). In the present study, we screened a total of 140 strains and out of them, 16 strains with antagonistic properties were obtained. This finding is similar to the previous antimicrobial studies with considerably high rate of recovery of actinobacteria with antibacterial and antifungal activities (Kitouni et al., 2005; Thakur et al., 2007).

The identification based on 16S rRNA gene revealed that all the antagonistic strains were belonging to the genus *Streptomyces*. This finding is congruent with earlier studies, which reported that the percentage of recovery of *Streptomyces* sp. with anti-microbial activity was higher than other actinobacterial genera (Das et al., 2018). As observed during *in vitro* experiments, 16 strains with antagonistic potential were recovered from 30 isolates from Nelliyampathy soils. Among them, two strains possess broad spectrum antimicrobial activities against both bacterial and fungal pathogens . 16S rRNA gene sequencing confirmed the identification of the strains as *S. nogalater* NIIST A30 and *S. luteosporeus* NIIST A32. The ethyl acetete extract from the strains also

revealed the ability of these strains to produce an array of a variety of antimicrobial metabolites that might be specifically antibacterial or broad spectrum in nature. These two strains were recoverd from Nelliyampathy forest ecosystems which are protected from human activities that may have contributed to the production of powerful metabolites. Recent studies indicated that the potential of various soil actinomycetes such as *Streptomyces* sp. as a potential resource of novel bioactive secondary metabolites (Sripreechasak and Athipornchai, 2019, Qi et al., 2019). In the present study, the promising strains were resistant to some of the antibiotics tested. The resistant pattern of the isolates towards the common antibiotics such as streptomycin was the ideal characteristic of *Streptomyces* species (Al-Ansari et al., 2019).

Streptomyces sp. are known for the production of polyketides and nonribosomal polyketide compounds, which have significant use in the medicinal community (Sharma et al., 2014). A PCR-based amplification of biosynthetic genes is a powerful tool for the prediction of actinobacteria with the potential ability of producing bioactive secondary metabolites (Qi et al., 2019). In the present study, one strain designated as A30 gave positive amplification of ketosynthase genes of PKS I gene cluster, but not PKS II or NRPS. This is probably because the degenerate primers used in the study might not be suitable for amplification of the NRPS gene. The other reason is the absence of NRPS gene cluster in both A30 and A32. It was supported by a study conducted by Qin et al. (2009) where the NRPS genes could not be detected in 34 strains of tested 46 actinomycetes.

Moreover, these genes and domains are known for the production of pigments and other bioactive secondary metabolites in *Streptomyces*. The PKS I gene encode multifunctional proteins with several active domains and are responsible for the assembly and modification of the polyketide carbon chain as well as the formation of complex macrolide polyketides (Ginolhac et al., 2004). Discovering a new drug or an

antimicrobial agent is a time consuming process and it also demands a good amount of economic support. This can be reduced by bioprospecting the presence and diversity of PKS and NRPS biosynthetic gene clusters among different bioactive *Streptomyces* sp. from different habitats (Das et al., 2018). Apart from contributing to the production of anti-microbial secondary metabolites, PKS genes also play a key role in making pathogenic actinobacteria resistant to host defence mechanisms. It is also reported that the PKS pathway is indispensable for the biosynthesis of fatty acids in some microbes (Donadio et al., 2007). For example, *Mycobacterium tuberculosis* and related mycobacteria species possess highly lipid-rich cell envelope containing a repertoire of lipidic polyketides, known as mycoketides, that provides resistance to host mechanisms of recognition and killing by functioning as an immunoprotective barrier (Parvez et al., 2018).

2.5. Conclusion

In this chapter, morphologically different 140 actinomycete strains were isolated from selected soil samples of Kerala. All strains were evaluated for their antibacterial efficacy against two indicator pathogenic bacteria such as *E. coli* and *S. aureus*. Among 140 strains tested, sixteen recorded potent antibacterial properties and were further screened against eleven bacterial pathogens. Of these, two strains designated as A30 and A32 (obtained from Nelliyampathy forest soil) identified based on 16SrRNA gene sequencing as *Streptomyces nogalater* NIIST A30 and *Streptomyces luteosporeus* NIIST A32 exhibited maximum inhibition against all the test pathogens. From antifungal experiments, A32 recorded significant antagonism against plant pathogenic bacteria while A30 had shown significant antagonism against human pathogenic fungi such as *Candida* sp. Moreover, the analysis of the antimicrobial biosynthetic genes (type I

polyketide synthase) showed that the antimicrobial activity of A30 was associated with the production of bioactive secondary metabolites. Since the possession of PKS I gene implies the possibility of production of small compounds with antimicrobial activities, the findings of this study revealed the antibiotic production from A30.

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Chapter 3

Optimization of fermentation conditions and media for enhanced antibacterial metabolite production from A30 and A32

3.1. Introduction

The production of secondary metabolites especially the bioactive compounds by microorganisms is highly dependent on the type of species and strains as well as their nutritional and cultural conditions (Rajeswari et al., 2015). Cultural conditions such as pH, temperature and incubation time can affect the production of secondary metabolites (Nguyen and Tran, 2018). Minor alterations in media composition can also affect the quality and quantity of metabolites produced along with the general metabolic profiles of the isolates. Therefore, the composition of a fermentation medium is a significant factor that influences the level of antimicrobial production by a microorganism (Jose et al., 2013).

The source and concentration of nutrients along with the cultural conditions are known to have mysterious effects on bioactive metabolite production in *Streptomyces* too, since the antibiotic production is not a static property (Grahovac et al., 2014). Moreover, the optimization of culture media is very complicated because any material that supports microbial growth can be a potential substrate (Yun et al., 2018). Therefore, designing an appropriate medium and culture conditions for cultivation has prime importance in improving the antibiotic yield. Empirical methods adopt single parameter per trial in which one factor is varied and keep all other factors constant (Nisha et al., 2011). This approach is laborious and time-consuming process involving many experimental trials to determine the optimum levels of all variables. In addition, it may not provide the correlation between different parameters and often fails to identify the variables that give an optimum response. A viable alternative for this is to develop a

strategy which involves statistical optimization that can resolve those issues related to conventional optimization (Xiong et al., 2008).

Response surface methodology (RSM), a collection of mathematical and statistical techniques for building empirical models, has been recognized as a fascinating approach for enhanced production of commercially important bioactive metabolites and enzymes. It has been successfully applied in various facets viz. improvement of biofuel and biomass production (Anvari et al., 2014), identifying the factors enhancing enzyme production as well as assessment of carbon mineralization from sewage sludges (Sevilla-Perea et al., 2015) and for the optimization of L-asparaginase production by *S. brollosae* NEAE-115 (El-Naggar et al., 2019). This RSM approach has been adopted to improve the production of antibacterial compounds in several *Streptomyces* species, including *Streptomyces* sp. HJC-D1 (Kong et al., 2014), *Streptomyces* sp. SY-BS5 (Souagui et al., 2015), *Streptomyces* sp. SYYLWHS-1-4 (Managamuri et al., 2017), *Streptomyces* sp. 1-14 (Yun et al., 2018), *S. chumphonensis* BDK01 (Manikandan et al., 2019) *Streptomyces* sp. GSBNT10 (Djinni et al., 2019). Hence, RSM strategies to maximize the yields of bioactive metabolites are inevitable.

Hence in the present study, best fermentation media for A30 and A32 strains were selected from eight test media. Fermentation conditions such as pH, temperature and incubation time were optimized though one-factor-at-a-time approach. RSM was preferred to optimize the media components in culture medium for maximum antibacterial metabolite production for strain A30. The interactive effect of different media components such as starch, KH_2PO_4 and $NH_4(SO_4)_2$ for enhanced antibacterial

metabolite production in A30 was investigated with the application of Plackett-Burman design (PBD) and Box-Behnken design (BBD) (Olia et al., 2020).

3.2. Materials and Methods

3.2.1. Materials

Various media used for microbiological investigation were procured from Hi-Media Laboratories Pvt. Limited, Mumbai, India. The solvents used for extraction of bioactive compounds were purchased from Merck Life Sciences, Mumbai, India and SDFCL, Mumbai, India. Normal cell lines such as L6 rat myoblast, H9c2 rat cardiac myoblast and RAW 264.7 murine macrophage were obtained from National Centre for Cell Sciences (NCCS), Pune, India.

3.2.2. Methods

3.2.2.1. Standardization of fermentation medium for maximum antibacterial metabolite production from two selected strains A30 and A32

The strains were cultivated in various media such as Glucose soybean meal broth (GSMB), Tryptone yeast extract broth (ISP1), Yeast malt broth (ISP2), Inorganic salt starch broth (ISP4), Kuster's broth (KB), Nutrient broth (NB), Sabouraud dextrose broth (SDB) and Starch casein broth (SCB) (see annexure I for media composition). All the media were inoculated with the strain A30 and incubated for 7 days at 28±2 °C. After fermentation, the culture broth was centrifuged (Remi cooling centrifuge, Mumbai, India) at 10,000 rpm for 15 min to obtain cell free culture filtrate. The culture filtrate was then extracted with an equal amount of ethyl acetate and agitated for 45 min. The solvent layer was concentrated using a rotary evaporator (Buchi, Switzerland)

at 40 °C and the crude extract was collected by rinsing with 5 ml of methanol, dried and kept at 4 °C for further studies.

3.2.2.2. Antibacterial study of the ethyl acetate extracts from different fermentation media

The antibacterial activity of the ethyl acetate extract from different fermentation media was examined using agar disc diffusion method (CLSI, 2012). The test bacteria were first inoculated into nutrient broth tubes and incubated at 37 °C for 24 h. Each of the inoculums were then adjusted to 0.5 McFarland turbidity standards and swabbed into Mueller-Hinton agar (MHA) plates. Six mm sterile filter paper discs (Whatman No.3, Hi-Media, India) impregnated with ethyl acetate extract (100 µg/ml) was placed on the swabbed MHA. A sterile disc with methanol alone served as the control. Plates were then incubated at 37 °C and the zone of inhibition was measured (in mm) after 24 h of incubation.

3.2.2.3. Standardization of fermentation conditions for enhanced antibacterial metabolite production from A30 and A32- One factor at a time method (OFAT)

3.2.2.3.1. Optimization of temperature

The effect of culture conditions on the production of enhanced antibacterial metabolite production was studied on ISP4 against *S. epidermidis* (Pathogenic bacteria which exhibited maximum activity in screening studies). The optimum temperature for the maximum antibacterial compound production was investigated on ISP4. Ten ml actinomycete suspension was introduced in 250 ml Erlenmeyer flasks containing 100

ml of broth and incubated at different temperatures *viz.* 25, 30, 35, 40 and 45 °C at pH 7.0. The antibacterial activity was assayed after 7 days by disk diffusion method as mentioned earlier.

3.2.2.3.2. Optimization of pH

The impact of pH on antibacterial metabolite production was studied at different pH, ranges from 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 and 10.5 at 35 °C and for 7 days. The pH was adjusted using 0.1N NaOH and 0.1N HCl.

3.2.2.3.3. Optimization of incubation period

The optimization of incubation period was also carried out by incubating for 3-15 days at the optimum pH and temperature. The antibacterial activity was monitored at every 24 h intervals by disc diffusion method.

3.2.2.4. Optimization of medium components in ISP4 for maximum production of antibacterial compounds by A30 through response surface methodology

3.2.2.4.1. Selection of media components using Plackett-Burman design (PBD)

The Plackett-Burman experimental design (Plackett and Burman, 1946) was chosen for initial evaluation of ISP4 medium components to short-list components having significant effect on antibacterial activity by A30 (Jubi et al., 2017). The factors considered were macronutrients in ISP4 medium such as starch, K_2HPO_4, $(NH_4)_2SO_4$, $CaCO_3$, $MgSO_4$ and NaCl, each were analyzed at two levels, low (-) and high (+). The micronutrients were taken as per standard medium and the total numbers of experiments as per the PBD were 13. The experiments were carried out in triplicates to

assess the consistency of results and the average antibacterial activity against *S. epidermidis* was determined as the response. The experimental design was developed using the statistical software package, MINITAB 17.

3.2.2.4.2. Optimization of significant components by response surface methodology (RSM)

Based on the preliminary Plackett-Burman analysis, according to the low *p*-values (< 0.05) and high confidence levels (>90 %), the significant media components were selected as the variables to test in the 15-run experiment of the Box-Behnken design (Box and Behnken, 1960). The variables were studied at low, middle and high concentration levels and were designated as −1, 0 and +1 (coded values), respectively. The MINITAB statistical (MINI-TAB 17) software was used to compute the results and generate response surface graphs. In this system, the regression analysis was performed to estimate the response function as a second order polynomial equation,

$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2$ (Wang et al., 2011).

Where, Y denotes the predicted response, β_0 is the intercept term, β_i is the linear coefficient, βij is the quadratic coefficient and β_{ii} is the interaction coefficient, and X_i X_j denotes independent variables under study. The statistical competence of the model was resolved through analysis of variance (ANOVA). The excellence of the polynomial model equation was concluded statistically through coefficient of determination (R^2) and adjusted R^2. Three-dimensional response surface plots were produced to elucidate the relationship between the responses and the experimental levels of each

independent variable. The optimum level of the variables for maximum antibacterial activity was resolved by response optimizer tool of the software.

3.2.2.5. Validation of statistical model and optimization through wet lab

The mathematical model and the optimization were experimentally validated by culturing A30 under non-optimized and optimized levels of variables at pH 7.0 and 35± 2 °C for 7 days. After incubation, the antimicrobial metabolite was extracted twice with equal volume of ethyl acetate and dried extract (350 mg/l) was suspended in 100 µl methanol and assayed as above for antibacterial activity.

3.2.2.6. Antioxidant activities of crude ethyl acetate extract (scavenging of free radicals)

3.2.2.6.1. DPPH free radical scavenging assay.

The antioxidant potential of crude ethyl acetate extract was determined by free radical DPPH (2, 2 -diphenyl -1-picrylhydrazyl) scavenging activity (Tan et al., 2015). A methanolic DPPH solution (0.016%) was mixed with serial dilutions (10-100 µg/ml) of crude extract and after 30 min, the absorbance was read at 515 nm. The radical scavenging activity was expressed as IC_{50} (µg/ml), (the dose required to cause a 50% inhibition). Vitamin C was used as the standard and the ability to scavenge the DPPH radical was calculated by the following formula:

DPPH radical scavenging activity (%) = $\frac{A0-A1}{A0} \times 100$

Where A_0 is the absorbance of the control at 30 min and A_1 is the absorbance of the sample at 30 min. The experiment was performed in triplicates.

3.2.2.6.2. ABTS free radical scavenging assay.

An ethanolic ABTS [2, 20-Azino-bis (3-ethyl- benzthiazoline-6-sulfonic acid)] solution was mixed with serial dilutions (10-100 µg/ml) of crude extract and after 5 min; the absorbance was recorded at 734 nm (Al-Duais et al., 2009). The radical scavenging activity was expressed as IC_{50} (µg/ml), (the dose required to cause a 50 % inhibition). Trolox was used as the standard. The ability to scavenge the ABTS radical was calculated by the following formula:

ABTS radical scavenging activity (%) = $\frac{A0-A1}{A0} \times 100$

Where A_0 is the absorbance of the control at 5 min and A_1 is the absorbance of the sample at 5 min. The experiment was performed in triplicates.

3.2.2.7. Effect of crude extract on normal cell viability

Viability of L6, H9c2 and RAW 264.7 were assessed by 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). For this, cells were seeded (1×10^4 cells/ well) in a 96-well microtiter plate. Subsequently, the cells were treated with various concentrations of ethyl acetate extracts of A30. After 24 h incubation, cells were washed and 100 µl of MTT (5 mg/ml), dissolved in Dulbecco's modified Eagle's medium (DMEM), was added to each well and incubated at 37°C in a CO_2 incubator. After 4 h incubation, DMSO was added to each well and the plate was kept on a shaker at 12 rpm for 45 min. The change in colour was monitored using a

microplate reader (Biotek, USA) at 570 nm. Results were expressed as percentage of cytotoxicity using the following formula:

$$\text{Percentage of toxicity} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

The experiment was performed in triplicates.

3.3. Results

3.3.1. Standardization of fermentation medium for maximum antibacterial production from two selected strains A30 and A32

Among the eight different fermentation media tested, A30 exhibited maximum metabolite production (180 mg/l) and antibacterial activity in starch containing inorganic salt starch broth (ISP4) followed by glucose soybean meal broth (GSMB), starch casein broth (SCB) and yeast malt broth (ISP2) (Table 3.1). Ethyl acetate extract from ISP4 broth inhibited Gram- positive pathogens significantly. Here, *S. epidermidis* (15±1.52 mm) was the best inhibited followed by *B. cereus* (13.6±0.57 mm).

Likewise, A32 exhibited maximum metabolite production (152 mg/l) and antibacterial activity in glucose soybean meal broth followed by yeast malt broth, starch casein broth and sabouraud dextrose broth (Table 3.2). Here, ethyl acetate extract from glucose soybean meal broth inhibited the Gram- negative pathogen, *K. pneumoniae* (26±2 mm) significantly, followed by *S. epidermidis* (24±1 mm) and *S. simulans* (23±0.57 mm).

Table 3.1: Antibacterial activity of crude extracts of A30 from different broths.

Test pathogens	**Zone of inhibition(mm)**							
	GSMB	ISP 1	ISP 2	**ISP 4**	KB	NB	SDB	SCB
B. cereus	9±0.57[a]	8±0.57[a]	7.6±0.57[a]	**13.6±0.57**[b]	11±0[b]	7±1[a]	8±0[a]	8±0.57[a]
B. subtilis	10±0[b]	10±0[b]	17.6±0.57[d]	**12±1**[b]	16±0	0	17±0	12.3±0.57[b]
E. coli	7.6±0.28[a]	7.5±0.5[a]	9±0[a]	**10±0**[b]	8±0.57[a]	9.3±0.57[a]	6±0[a]	10±0[b]
K. pneumoniae	6.5±0.5[a]	8.3±0.57[a]	9±1[a]	**12±0**[b]	11±0[b]	10.3±0.57[b]	7±0[a]	10.3±0.57[b]
M. smegmatis	11±1[b]	10±1[b]	10±1[b]	**11±0**[b]	11.3±0.28[b]	6±0[a]	6±0[a]	9.16±0.28[a]
P. aeruginosa	9.3±0.57[a]	0	7.6±0.57[a]	**10±1**[b]	6.8±0.28[a]	0	8±0.57[a]	9±1[a]
P. mirabilis	7.5±0[a]	0	8±0.57[a]	**11±0**[b]	10±0[b]	10±0[b]	11±0	10.3±0.28[b]
S. aureus	9.3±0.28[a]	10±0[b]	10±0[b]	**9±0.57**[a]	8±0.57[a]	7±0[a]	8±0[a]	10±0[b]
S. epidermidis	13.3±0.28[b]	12.3±0.28[b]	11±1[b]	**15±1.52**[c]	14±0.57[c]	12±0[b]	13.3±0.28[b]	14±0.57[c]
S. simulans	10±0[b]	11±0[b]	6±1[a]	**12±1**[b]	10±0[b]	12±0[b]	7±0[a]	8±0.57[a]
S. typhi	10±1[b]	5±0[a]	9±0[a]	**12±1**[b]	8±0[a]	10±0[b]	7.6±0.57[a]	5±0[a]

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

Table 3.2: Antibacterial activity of crude extracts of A32 from different broths.

Test pathogens	**Zone of inhibition(mm)**							
	GSMB	**ISP 1**	**ISP 2**	**ISP 4**	**KB**	**NB**	**SDB**	**SCB**
B. cereus	**12±0.57** b	10±0.57 b	20±1 e	0	8±1 a	0	8±1 a	0
B. subtilis	**21±0** e	9±0.57a	10±0.57b	10±0.57b	9±0 a	10±1 b	9±0 a	14±1.15 c
E. coli	**22±1** e	12±0 b	17±1.52 d	10±0.57 b	11±1 b	12±1 b	11±0 b	11±0.57 b
K. pneumoniae	**26±2** g	12±0.57 b	20±1 e	11±1.15 b	8±0 a	10±1 b	15±1 c	17±1 d
M. smegmatis	**22±0.57** e	14±1 c	15±2 c	12±1 b	7±1 a	8±1 a	8±1 a	19±1 e
P. aeruginosa	**15±1** c	0	13±1.52 b	0	8±1 a	0	13±1 b	0
P. mirabilis	**12±1** b	0	11±1 b	0	12±1 b	12±1 b	0	6±1 a
S. aureus	**22±1.52** e	10±1 b	14±1.15 c	11±0.57 b	8±1 a	10±1 b	12±1 b	11±1 b
S. epidermidis	**24±1** f	12±1 b	15±1 c	0	0	12±1 b	13±1 b	14±1.15 c
S. simulans	**23±0.57**ef	10±1 b	14±1.52 c	12±1 b	11±1 b	10±1 b	12±1 b	13±1 b
S. typhi	**11±1** b	14±1 c	17±1 d	0	0	0	10±1 b	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

3.3.2. Standardization of fermentation conditions for enhanced antibacterial metabolite production- One factor at a time method (OFAT)

A30 exhibited maximum antibacterial activity against *S. epidermidis* (recorded significant activity during initial standardization) at pH 7.0 (Figure 3.1a) and at a temperature of 35 °C (Figure 3.1b). Above and below these conditions, a decreased level of antibacterial activity was recorded by the test organism. The strain exhibited antibacterial activity from 3rd day onwards and attained maximum on 7th day. The antibacterial activity was declined for the rest of the period (Figure 3.1c).

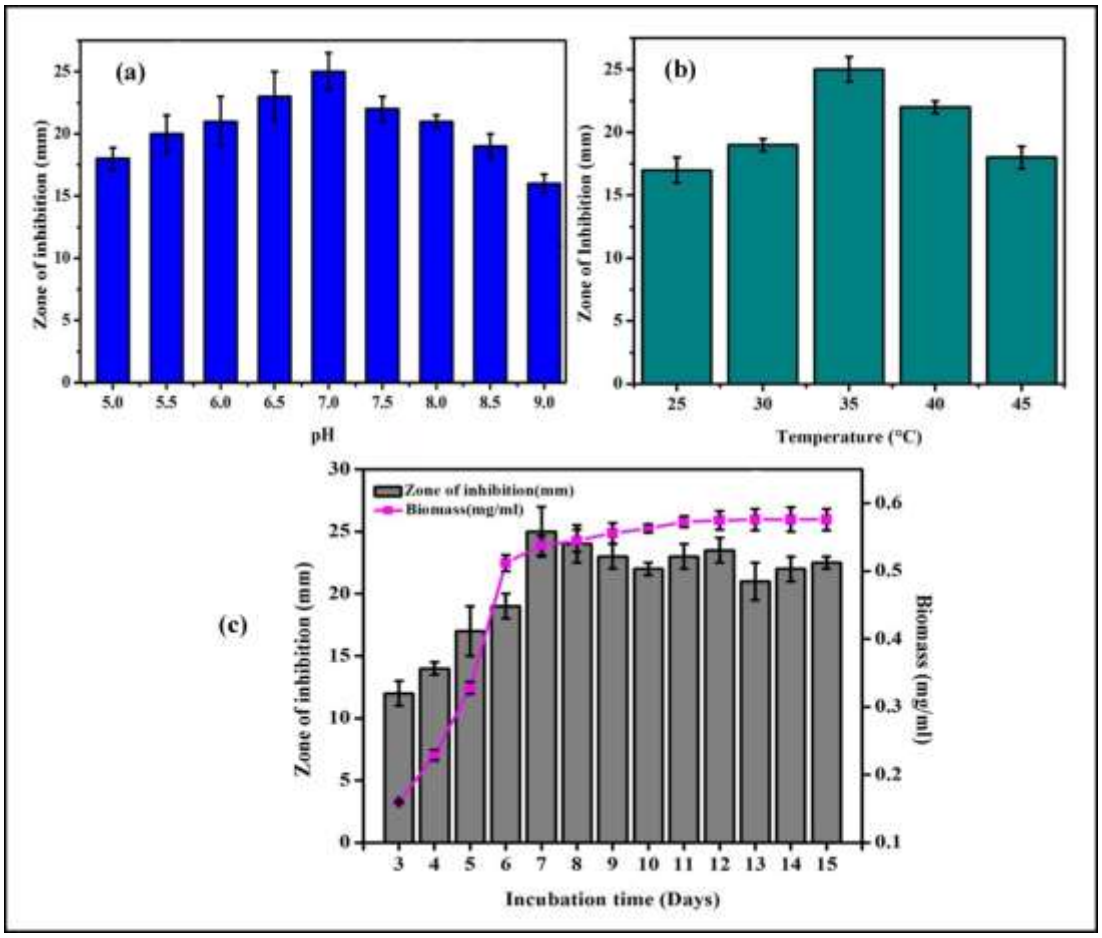

Figure 3.1: Antimicrobial activity of A30 in different pH (a), temperature (b) and incubation period (c).

On the other hand, A32 exhibited maximum antibacterial activity against *K. pneumoniae* (recorded significant activity during initial standardization) at pH 7.5 (Figure 3.2a) at a temperature of 30 °C (Figure 3.2b). Above and below these conditions, a decreased level of antibacterial activity was recorded by the organism. The strain exhibited antibacterial activity from 3rd day onwards which attained maximum on 10th day and declined slightly for the rest of the period (Figure 3.2c).

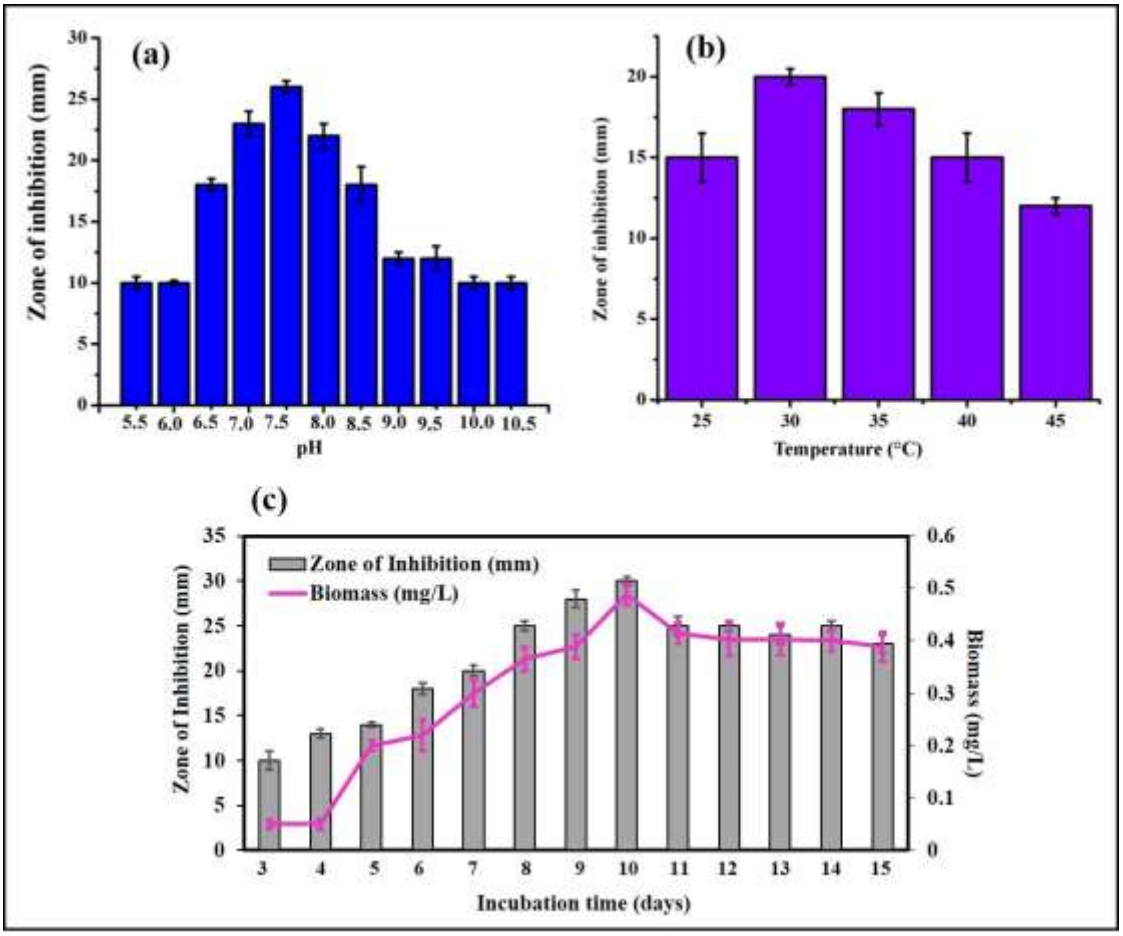

Figure 3.2: Antimicrobial activity of A32 in different pH (a), temperature (b) and incubation period (c).

Since A30 exhibited significant antimicrobial activity against human bacterial pathogens, the constituents in ISP4 (shown maximum activity in antibacterial experiments) was statistically optimized. The antibacterial experiments were done using *S. epidermidis* since it recorded maximum zone of inhibition during medium selection. However, the original glucose soybean meal broth was selected for the fermentation of A32 in further experiments.

3.3.3. Statistical optimization of media components for improved antibacterial production from A30 through response surface methodology

3.3.3.1. Screening of essential medium components using Placket-Burman design

Six media components were analyzed for their impact on antibacterial activity against *S. epidermidis* using Placket-Burman design with 13 experiment set up to observe significant media components (Tables 3.3 and 3.4). "-1" indicates the low value and "+1" indicates the high value of the media components (factors). Effect estimates and analysis of variables for antibacterial activity from experimental design was as shown in Table 3.5. Based on the low *p*-values and high confidence levels, three variables such as starch, $(NH_4)_2SO_4$ and K_2HPO_4 were determined to have a significant effect on the antibacterial compound production. Pareto chart (Figure 3.3) strongly confirm the fact that the most important factors influencing antibacterial compound production were starch, $(NH_4)_2SO_4$ and K_2HPO_4.

Table 3.3: Range of variables used for PBD.

Factor codes	Factors	Levels	
		-1 (g/l)	+1 (g/l)
X_1	Starch	7	13
X_2	K_2HPO_4	0.5	1.5
X_3	$MgSO_4.7H_2O$	0.5	1.5
X_4	NaCl	0.5	1.5
X_5	$(NH_4)_2SO_4$	1	3
X_6	$CaCO_3$	1	3

Table 3.4: Plackett-Burman experimental design.

Run Order	X_1	X_2	X_3	X_4	X_5	X_6	Antimicrobial activity against *S. epidermidis,* ZOI (mm)
1	7	0.5	0.5	0.5	1	1	12 ± 1
2	13	0.5	1.5	0.5	1	1	21 ± 0.5
3	7	0.5	0.5	1.5	3	3	17 ± 1.5
4	7	1.5	1.5	1.5	1	3	18 ±1
5	7	1.5	1.5	0.5	3	1	19 ± 0.5
6	13	1.5	0.5	1.5	3	1	25 ± 1
7	13	0.5	1.5	1.5	1	3	21 ± 0.5
8	13	1.5	1.5	0.5	3	3	24 ± 1.5
9	13	0.5	0.5	0.5	3	3	22 ± 1
10	10	1	1	1	2	2	20 ± 0.5
11	7	1.5	0.5	0.5	1	3	18 ± 2
12	7	0.5	1.5	1.5	3	1	17 ± 1.5
13	13	1.5	0.5	1.5	1	1	21 ± 1

ZOI, Zone of Inhibition.

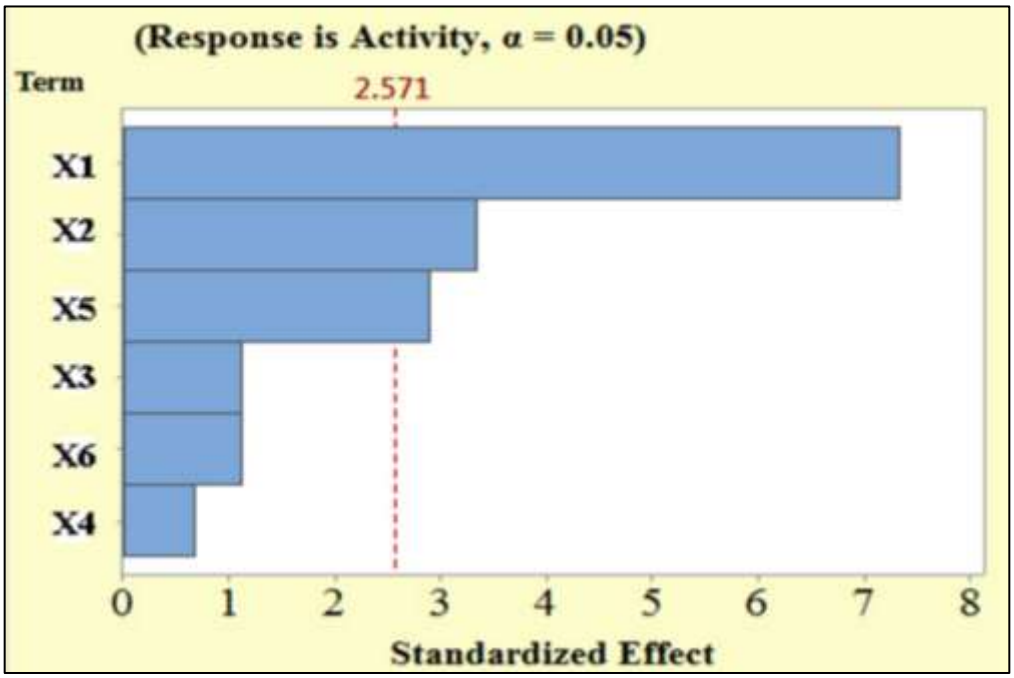

Figure 3.3: Pareto plot showing the effect of different variables (media components) on antibacterial metabolite production against *S. epidermidis*.

Table 3.5: Statistical analysis of effects of variables (media components) on antibacterial activity employing Plackett-Burman design.

Variables	Medium constituents	Effect	*t*-value	*p*- value *	Confidence level (%)	Standard error coefficient
X_1	Starch	1.833	7.34	**0.001**	**99.9**	0.125
X_2	K_2HPO_4	5.000	3.34	**0.021**	**97.9**	0.749
X_3	$MgSO_4.7H_2O$	1.667	1.11	0.317	68.3	0.749
X_4	NaCl	1.000	0.67	0.534	41.6	0.749
X_5	$(NH_4)_2SO_4$	2.167	2.89	**0.034**	**96.6**	0.375
X_6	$CaCO_3$	0.833	1.11	0.317	68.3	0.375

*: at confidence level of 95%, values ≤ 0.05 is acceptable.

3.3.3.2. Optimization of selected media components using Box-Behnken design

The variables which give a *p* - value less than or equal to 0.05 were chosen for next level of optimization by response surface methodology with Box-Behnken design. The significant media components such as starch, $(NH_4)_2SO_4$ and K_2HPO_4 were selected recorded response as antibacterial activity and the design matrix was as shown in Table 3.6.

Table 3.6: Box-Behnken response surface design.

Run order	Starch	$(NH_4)_2SO_4$	K_2HPO_4	ZOI in mm
1	15	5	1	26 ± 1.5
2	15	3	2	**30 ± 2**
3	20	3	1	15 ± 1
4	15	5	3	22 ± 1.5
5	20	5	2	18 ± 1
6	10	3	1	13 ± 1.5
7	15	1	3	27 ± 0.5
8	20	3	3	16 ± 1
9	15	3	2	**30 ± 1.5**
10	10	1	2	17 ± 0.5
11	15	1	1	23 ± 1
12	10	3	3	15 ± 1
13	20	1	2	16 ± 0.5
14	15	3	2	**30 ± 1.5**
15	10	5	2	14 ± 1

ZOI, Zone of Inhibition.

The regression equation coefficients were also determined and the data was fitted to a second-order polynomial equation. The RSM response regarding antibacterial activity is expressed as the following equation:

$$Y = - 98.56 + 13.575X_1 + 2.937X_5 + 18.12\ X_2 - 0.4700\ X_1X_1 - 0.5000\ X_5X_5 - 3.500X_2X_2 + 0.1250\ X_1X_5 - 0.0500X_1X_2 - 1.000\ X_5X_2.$$

Where Y is the antibacterial activity (zone of inhibition in mm) against *S. epidermidis* and X_1, X_2 and X_5 were starch, K_2HPO_4, and $(NH_4)_2SO_4$ respectively. The statistical significance of the fitted model was evaluated by ANOVA (Table 3.7). For the regression model, F = 252.07 and P <0.00, reaching the limit level, indicating that the regression equation was very good. Furthermore, the predicted R^2 of 96.48% is in reasonable agreement with the adjusted R^2 value. The adjusted coefficient of determination (R^2) was 0.9938 (99.38%), which further denotes the reliability of the current model. Therefore, the regression model was believed to reliably predict and analyze the antibacterial activity of the actinomycete strain A30.

Table 3.7: Analysis of variance of fitted quadratic model

Source	Degrees of freedom	Sum of Squares	Mean Sum of squares	F-Value	*P*-Value
Model	9	567.150	63.017	252.07	0.000
Error (Residual)	5	1.250	0.250		
Lack-of-Fit	3	1.250	0.417		
Pure Error	2	0.000	0.000		
Total	14	568.400			

Determination of co-efficient, R^2=0.9648; Adjusted determination of co-efficient, Adj R^2= 0.9938

3.3.3.3. Response surface or contour plots

By using the response surface 3D plots the interactions between the two factors and their optimum levels were studied. Figure 3.4, (a) and (b) shows the effect of starch and $(NH_4)_2SO_4$ on antibacterial activity. With a moderate concentration of starch and $(NH_4)_2SO_4$, the antibacterial activity increased and after that the activity decreased with a higher concentration of starch and $(NH_4)_2SO_4$. The same trend was observed in the effects of K_2HPO_4 and starch on antibacterial activity [Figure 3.4, (c) and (d)]. Figure 3.4 (e) and (f) shows the effect of K_2HPO_4 and $(NH_4)_2SO_4$ on antibacterial activity. Initially, the antibacterial activity increased with increasing concentration of both K_2HPO_4 and $(NH_4)_2SO_4$. Further addition of K_2HPO_4 and $(NH_4)_2SO_4$ leads to reduced antibacterial activity. The 3-Dimensional RSM plots clearly indicated that the maximum antibacterial activity should appear with a medium level of starch, K_2HPO_4 and $(NH_4)_2SO_4$. The 3D plots and their respective contour plots provided a visual interpretation of the interaction among the factors. With the help of numerical optimization, the quadratic model predicted that the optimal values of test factors viz, starch =14.97g/l, $(NH_4)_2SO_4$ = 2.89 g/l, and K_2HPO_4 = 2.07 g/l. Furthermore, the maximum antibacterial activity (zone of inhibition) achieved should be of 30 mm.

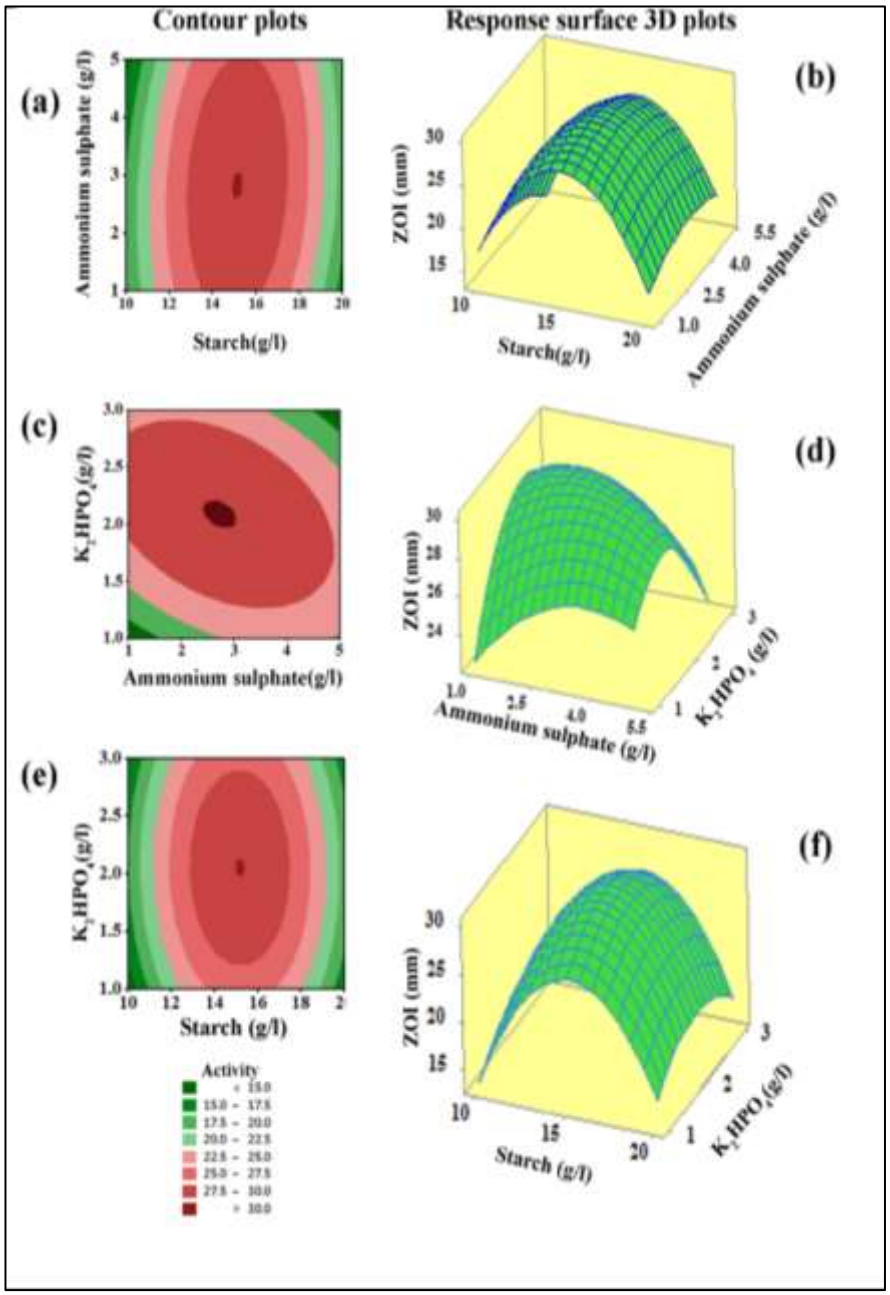

Figure 3.4: Response surface contour and 3D plots showing individual and interactive effects of variables on antibacterial activity of A30. (a) and (b) Effects of $(NH_4)_2SO_4$ and starch on antimicrobial activity (c) and (d) Effects of K_2HPO_4 and $(NH_4)_2SO_4$ on antimicrobial activity (e) and (f) Effects of K_2HPO_4 and starch on antimicrobial activity.

3.3.4. Experimental validation of optimization

The medium component parameters predicted from RSM was experimentally proven in triplicates. The antibacterial activity against *S. epidermidis* obtained experimentally was 28.5 ± 1.5 mm which was in close accordance with the predicted value of 30 mm (Figure 3.5). Therefore, the developed model is accurate and reliable for predicting the production of the antimicrobial compound by A30. The predicted results matched well with the experimental results, thus validating this RSM model with good correlation. The final optimized medium contained 14.97 g of soluble starch, 2.89 g of $(NH_4)_2SO_4$, 2.07 g of K_2HPO_4, 1 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 2 g of $CaCO_3$, 1 mg of $FeSO_4.7H_2O$, 1 mg of $MnCl_2.7H_2O$ and 1 mg of $ZnSO_4.7H_2O$ as the initial concentration per liter of distilled water.

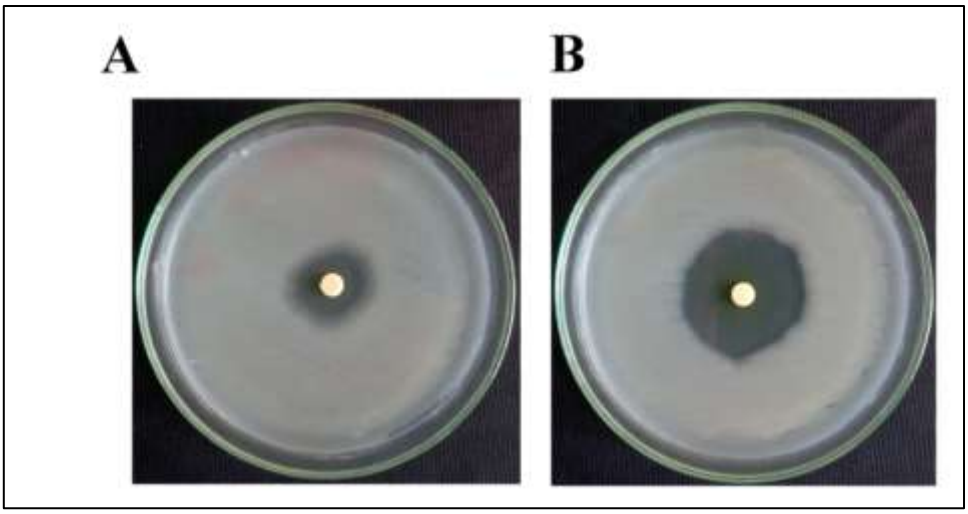

Figure 3.5: Antibacterial activity of control (A) and optimized (B) medium against *S. epidermidis.*

3.3.5. Antioxidant activities of crude ethyl acetate extract (Scavenging of free radicals)

A30 ethyl acetate extract exhibited significant dose dependent inhibition of DPPH activity, with a 50% inhibition (IC_{50}) at 30 µg/ml. The IC_{50} value for vitamin C was 295 µg/ml. ABTS scavenging activity demonstrates 50% inhibition (IC_{50}) at 20 µg/ml (Figure 3.6).

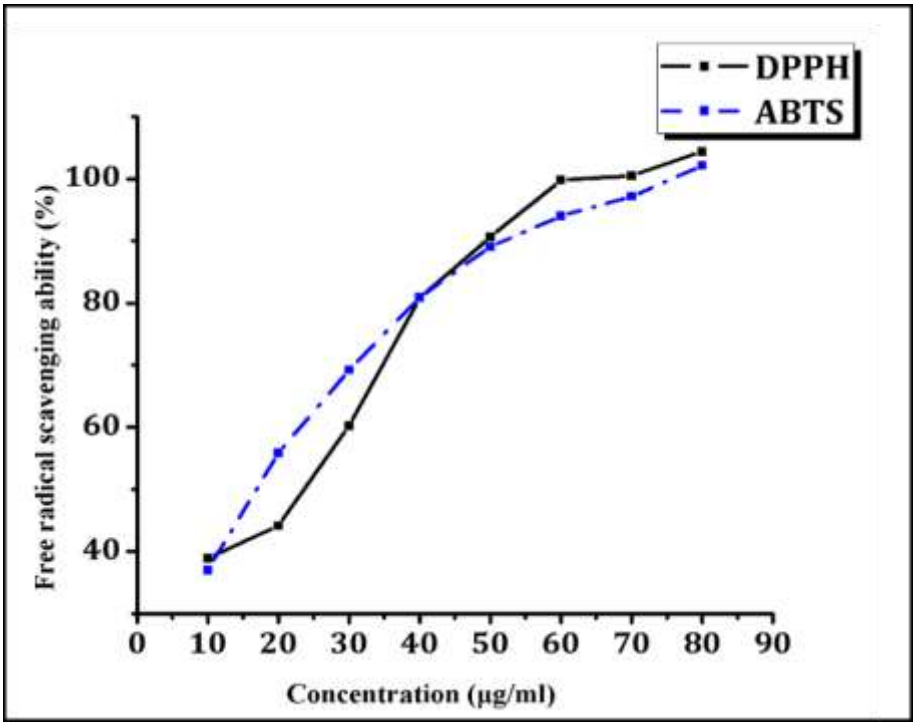

Figure 3.6: Free radical scavenging activity of ethyl acetate crude extract.

3.3.6. Effect of A30 ethyl acetate extract on normal cell viability

The concentration of ethyl acetate extract from A30 up to 50 µg/ml was found to be less than 20% toxic in all the cell lines for a period of 24 h (Figure 3.7). However, a concentration of 100 µg/ml and above was found to be toxic (40%) in RAW 264.7 cell

lines whereas in L6 and H9c2 cell lines the extract exhibited 20 and 23 percentages toxicity, respectively.

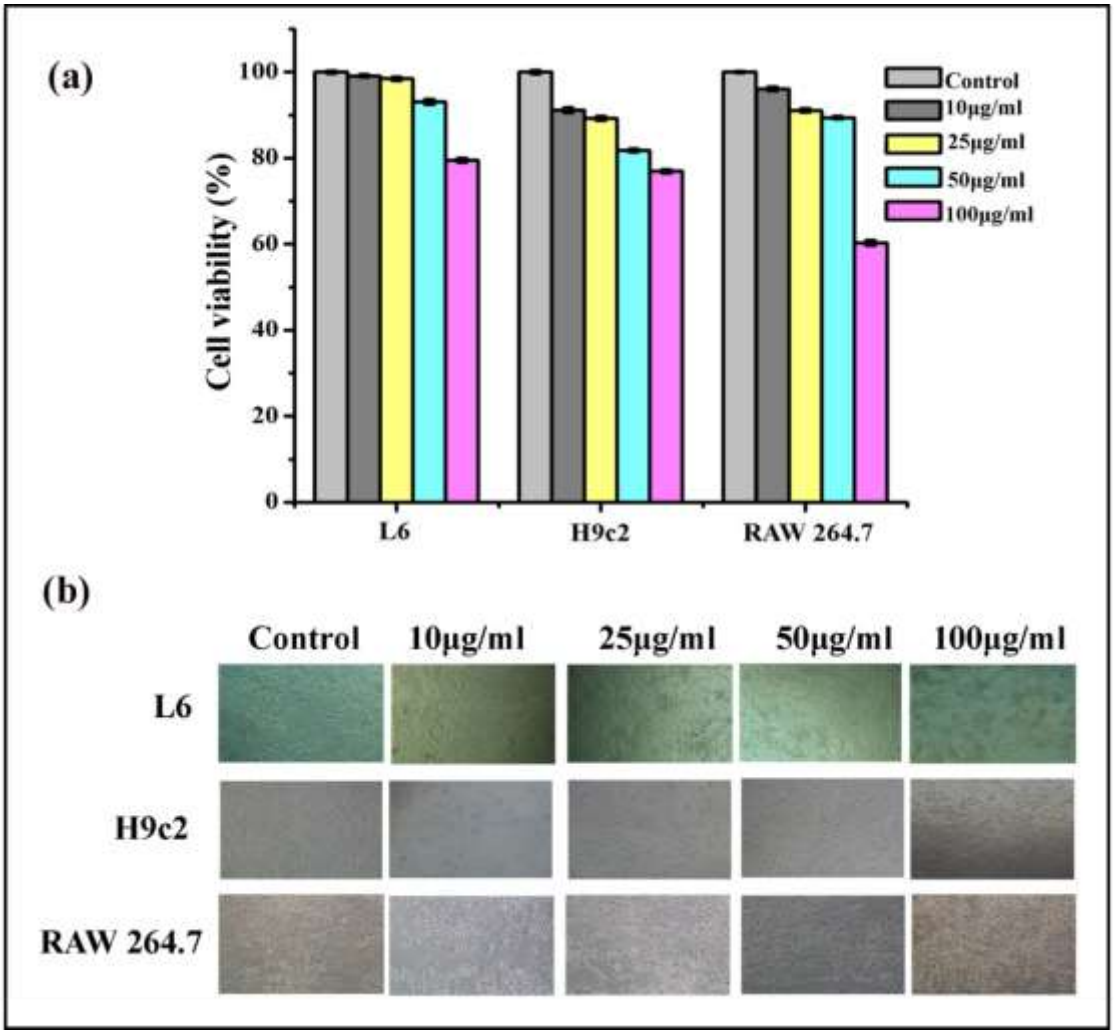

Figure 3.7: The effect of normal cell viability of ethyl acetate extract from A30. (a, MTT assay; b, phase contrast image).

3.4. Discussion

Most of the metabolites produced by *Streptomyces* are extracellular in nature, with potent antimicrobial activities (Singh et al., 2014). The enhanced production of secondary metabolites usually involves the choice of appropriate fermentation medium.

In this study, out of eight fermentation media tested, inorganic salts starch broth (ISP4) recorded maximum antibacterial activity for A30 which indicated that the strain was able to utilize starch for better antibacterial metabolite production. On the other hand, A32 preferred glucose soybean meal broth for maximum metabolite production against *K. pneumoniae*. It is known that the presence of carbon sources in fermentation media enhances the production of bioactive secondary metabolites from microbes. Our results are similar to other findings reported by Lotfy et al. (2019) where the presence of starch was significantly affected the production of antibacterial compound, di-(2-ethylhexyl) phthalate from *Bacillus subtilis* AD35. Chandrakar and Gupta (2019) reported that a high yield of actinomycin D and actinomycin X_{ob} was obtained from an endophytic *Streptomyces parvulus* when fermented in glucose soybean meal broth media. Another study reported the best enhanced antimicrobial metabolite production from a *Bacillus* species, when fructose was used as the carbon source among the water soluble carbon sources (Nishanth et al., 2013). This investigation also indicated that the synthesis of bioactive metabolites varies with the modification of fermentation media.

Carbon source act as the energy source for microbial metabolism, and the selection of suitable carbon source can increase the rate of microbial reproduction and metabolite production (Jose and Jebakumar, 2013). A source of carbon rapidly assimilable exerts a negative effect on biosynthesis (catabolite repression or ‘‘glucose effect”) (Li et al., 2015) which can be overcome by the use of complex carbon source like polysaccharides (starch, dextrins). A complex source of carbon metabolizable (starch) increased antifungal production by a *Streptomyces* sp. TKJ2 (Messis et al., 2014). Starch

was used as a carbon source in culture medium for improved antifungal production by *Streptomyces* sp. K03-0132 (Fukuda et al., 2005). In this study too, *Streptomyces nogalater* NIIST A30 was able to produce an enhanced level of broad spectrum antibacterial metabolites with starch as carbon source.

Cultural parameters also influence the production of bioactive secondary metabolites. The yield of antimicrobial substances actually determines the effect of antagonism (Duan et al., 2020). The yield and type of microbial metabolites are related to their fermentation conditions. The pH of the culture medium affects the ability of bacteria to absorb nutrients and produce bioactive substances. Fermentation temperature affects the growth of microorganisms and the production of secondary metabolites while the incubation time affects the production and activity of secondary metabolites (Pageni et al., 2014). Therefore, exploring suitable culture conditions for a microbe is important. In the present study, fermentation conditions such as pH, temperature and incubation time were optimized through conventional means by single factor at a time. A30 showed best growth and antimicrobial activity at pH 7.0, 35°C after an incubation period of 7 days. A32 showed best growth and antimicrobial activity at pH 7.0, 30 °C after an incubation period of 10 days. These results are somewhat similar to a report of maximum antimicrobial activity of pH 7.5 at 32 °C after 11 days with a rare actinomycete, *Nonomuraea* sp. JAJ18 (Jose and Jebakumar, 2014). From earlier reports, it is also evident that the maximum antibiotic production by *Streptomyce*s cultures required at least 96 h.

In this study, A30 was investigated for the scavenging abilities on DPPH and ABTS synthetic radicals. DPPH is a useful reagent to evaluate the free radical scavenging

ability of the hydrogen donating antioxidant, which can transfer hydrogen atoms or electrons to DPPH radicals. Compounds from A30 were able to reduce the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine. Hydroxyl radical is one of the reactive oxygen species generated in the body and removing hydroxyl radicals is important for antioxidant defence in living cell systems (Magalhães et al., 2008). On the other hand, ABTS radical cation which is blue in colour reacts towards antioxidants and converted to a colourless neutral form. Even though these methodologies present similarities, a considerable antioxidant capacity was observed, and ABTS radical scavenging was higher than DPPH (40% to 80% scavenging). A probable explanation for this phenomenon is the fact that ABTS is soluble in water as well as in organic solvents, allowing the antioxidant activity of hydrophilic and lipophilic compounds. The cytotoxicity in normal cell lines demonstrated the non-toxic effect of crude extract up to a concentration of 50 µg/ml, which indicates the non-toxic effect of compounds in the crude extract.

The antibiotic producing capability of *Streptomyces* sp. can be significantly affected by constituents of production medium. Previous studies on secondary metabolite production were conducted using conventional methods. However, this method frequently failed to measure the region of optimum response because the combined effect of parameters has not been considered. Statistical methods such as RSM have been used in various phases of fermentation optimization (Suganthi and Mohanasrinivasan, 2015). Ibrahim et al. (2019) was also reported RSM for the improved production of bacteriocin substance from *Lactobacillus plantarum*. In this study, the significant media components were statistically optimized for enhanced

antibacterial compound production against *S. epidermidis*. The compelling media components were screened and selected using Plackett-Burman design. Data obtained showed that the starch, K_2HPO_4 and $(NH_4)_2SO_4$ exerted positive effects rather than other components. The antibiotic production in *Streptomyces* sp. RUPA-08PR was markedly increased in medium supplemented with K_2HPO_4 (Ripa et al., 2009). Another investigation reported the importance of optimal concentration of $(NH_4)_2SO_4$ in antibiotic production by *Streptomyces* viridochromogenes (Zhu et al., 2007). In the present study, media components such as starch, $(NH_4)_2SO_4$ and K_2HPO_4 in moderate amount facilitated maximum metabolite production by A30.

The statistical significance of the model was checked by *p*-value and co-efficient of determination (R^2). In the current work, the R^2 value was found to be 0.9648 which means that the model can explain 96.48% of total variations. The closer the R^2 to 1.0, the stronger the model and better it predicts the response. The predicted results matched well with the experimental results obtained using the optimal conditions, which validating the RSM models with good correlation. The model suggested that the starch, $(NH_4)_2SO_4$ and K_2HPO_4 affected the metabolite production from A30. The response surface and 2D contour plots obtained with Box-Behnken design illustrated the interactive effects of two independent variables while maintaining the third variable at a fixed level. In the present study, after optimization through RSM, the antibacterial activity has been notably increased when compared to unoptimized medium. Antibacterial activity against *S. epidermidis* in terms of zone of inhibition was increased from 15±1.5 to 28±1.5 mm. An 86.66% increase from modified media strongly suggests that the quantity of media components affects antibacterial

metabolite production in *S. epidermidis*. Hence, the current statistical experimental design was found to be accurate in optimizing the significant media components.

3.5. Conclusion

In this chapter, we investigated the best fermentation medium and conditions such as pH, temperature and incubation time for better antibacterial production from the strains A30 and A32. Standardization of fermentation conditions for the strains A30 and A32 was optimized using OFAT method. Here, optimum conditions for A30 was found to be pH 7.0, temperature 35 °C and incubation for 7 days. However, the optimum conditions for A32 was found to be pH 7.0, temperature 30 °C and incubation for 10 days. Fermentation media standardization exhibited that ISP4 and GSMB was best for antibacterial production for A30 and A32 respectively. The significant media components for maximum metabolite production of A30 was optimized through response surface methodology employing Plackett-Burman and Box-Behnken designs. The media optimization for A30 by statistical approaches resulted in a maximum antibacterial activity of 28.15±1.5 mm which is an 86.66% increase in comparison with that obtained in the control broth. Thus, the statistical trial using RSM for optimization of medium compounds by *S. nogalater* NIIST A30 was validated to be a potent and useful tool. The ethyl acetate extract from strain A30 exhibited antioxidant properties with IC_{50} value of 30 µg/ml and had no cytotoxicity towards L6, H9c2 and RAW 264.7 cell lines up to a concentration of 50 µg/ml.

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Chapter 4

Exploitation of A30 for medicinal applications

4.1. Introduction

Antifungal resistance is a predominant concern in the field of medicine nowadays because of the overuse and misuse of antibiotics (Ventola, 2015). This problem has initiated the search for new molecules for such type of pathogens which can be administered alone or as synergistic agents in combined therapies together with the currently used antibiotics (Ferreira et al., 2018). Resistance to antimicrobial agents is an important survival mechanism by pathogens that further favour the infection process. In many drug resistant pathogens, some of the molecular and biochemical mechanisms of resistance involve the inactivation or modification of drugs by enzymes, presence of efflux pumps, alterations in membrane permeability, modification of drug targets (Figure 4.1) and other structural changes to barriers and antibiotic targets (Li et al., 2015). The tolerance that some pathogens develop against the current antimicrobial drugs leads to acute, persistent, chronic and relapsing human infections.

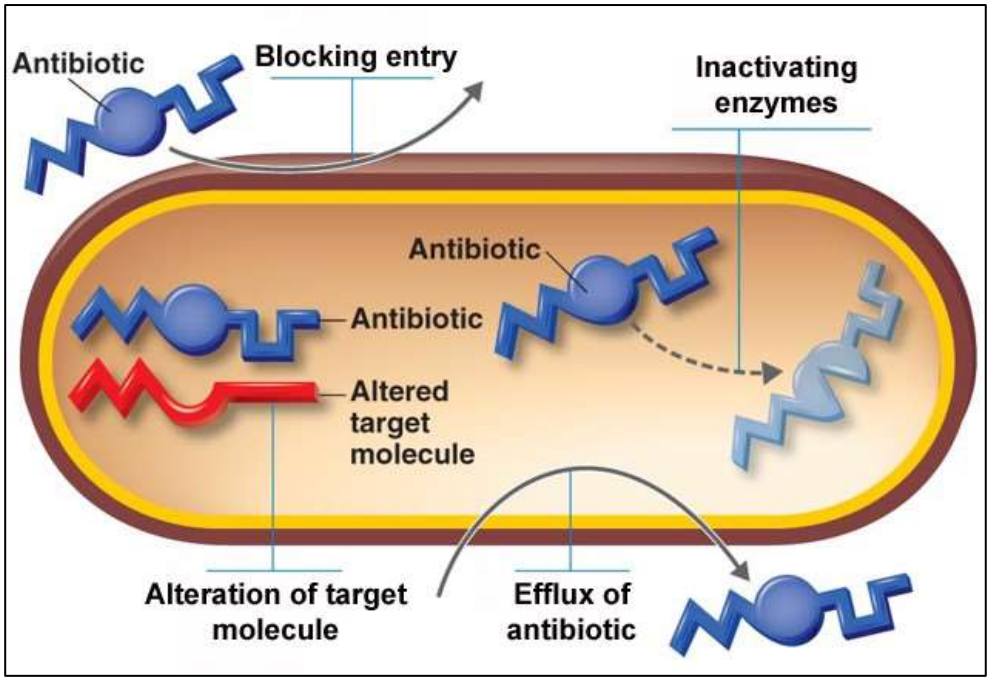

Figure 4.1: Mechanisms of antibiotic resistance in multi-drug resistant pathogens.

The major human fungal pathogens belong to the genus *Candida* can thrive in anatomically distinct sites within the host. Therefore, they can cause opportunistic infections collectively called "Candidiasis" in immunocompromised individuals. Among the infections, approximately 90% are caused by *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata* and *C. krusei* (Prasad et al., 2019). Candida strains resistant to diverse antifungals such as azoles, echinocandins and polyenes, have been reported (Spampinato and Leonardi, 2013). The mechanism of drug resistant action involves the decrease of intracellular concentration of drugs by the action of efflux pumps, metabolic bypasses and alterations in drug targets (Sanglard, 2016). Sometimes, the formation of biofilms can also contribute to this (Taff et al., 2013).

Natural products and their derivatives are the main source of our antibiotic drugs (Wright, 2017). Compounds from microbial sources comprise a diversity of bioactive molecules that have been evaluated as potential innovative therapeutic agents, playing an extremely important role in the discovery and development of new drugs (Bérdy, 2012). Bioactive metabolites from *Streptomyces* species contribute to diverse biological activities including antimicrobial action (Ilic et al., 2007). Since actinomycetes from various habitats are capable of producing a large number of antifungal antibiotics, they may be the treasure troves for pharmaceutical as well as agrochemical compounds (Gandotra et al., 2012).

Currently, only three structural classes of antifungal drugs such as azoles, polyenes and echinocandins are marketed to be used in *Candida* infections. Out of these, polyenes and echinocandins are naturally derived and produced by microorganisms (Zida et al., 2017). Roemer et al. (2011) through *C. albicans* fitness test screening proved

that target-specific inhibitors identified from fungi and actinomycete were very diverse. Therefore, it is necessary to expand the natural products screening diversity. With this point of view, the antifungal potential of active fraction designated as FC5 from the antagonistic strain A30 against *C. albicans* and *C. tropicalis* was investigated. The antimicrobial activities, killing kinetics and the detailed analysis of the mechanism of antifungal action of FC5 on *C. albicans* and *C. tropicalis* was also examined in this study.

4.2. Materials and Methods

4.2.1. Materials

Various media used for microbiological investigation were procured from Hi-Media Laboratories Pvt. Limited, Mumbai, India. The solvents used for extraction of bioactive compounds were procured from Merck Life Sciences, Mumbai, India and SDFCL, Mumbai, India.

4.2.2. Methods

4.2.2.1. Solvent selection for large scale extraction

Three different solvents such as ethyl acetate, chloroform and acetone were selected for large scale extraction of bioactive metabolites from A30. The extracts were then checked for antimicrobial activity and the solvent extract which exhibited significant antimicrobial activity was selected for large scale extraction.

4.2.2.2. Large scale fermentation of bioactive metabolites

A30 strain was mass cultured for the production of its bioactive secondary metabolites. For this, a loop full of actinomycete culture of 36 h was inoculated into

ISP2 broth. The flasks were incubated at 28± 2 °C for 72 h on a rotary shaker. Subsequently, these were transferred to fermentation medium (RSM optimized media) and incubated at 28± 2 °C for 7 days in a 20 litre fermenter. The working volume was 15 litres and the impeller was run at 160 rpm. The aeration was maintained at 1 vvm of air. The foam was controlled by intermittent addition of 1% antifoam SE-15 (Sigma Aldrich). After fermentation, the media was filtered and the culture filtrate was extracted three times with ethyl acetate (v/v) in the ratio 1:1. The extract was concentrated on a rotary evaporator (Heidolph, Germany) set at 42 °C and 240 mbar and the crude extract (2.5 g) was kept at -20 °C for further use.

4.2.2.3. Auto biographic test

The ethyl acetate crude extract was evaluated for antibacterial activity through autobiographic agar overlay method (Valgas et al., 2007). For this, the developed TLC plate was air dried and encased in a sterile Petri plate and overlaid with potato dextrose agar medium containing 0.6 % agar inoculated with test fungal suspension. The plates were then incubated at 37 °C and zone of inhibition around the spots were observed after 48 h.

4.2.2.4. Bioactivity guided fragmentation of ethyl acetate extract

The crude extract (2.5 g) from A30 was fractionated by silica gel column chromatography eluted with a gradient of Hexane/Ethyl acetate (v/v, 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100) and Ethyl acetate /Methanol (v/v, 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and

0:100) to give seven fractions (FC1-FC10). The fractions were concentrated and dissolved in methanol and checked for purity.

4.2.2.5. Biological activities of metabolites from A30

4.2.2.5.1. Antimicrobial activity of active fractions

Antibacterial activity of column fractions was measured by agar disc diffusion assay (CLSI, 2012). For this, 0.1 ml containing 10^6-10^7 CFU/ml of test bacterial pathogen was swabbed into Mueller Hinton Agar (MHA) plates. The active fractions (100 µg/ml) were loaded on 6 mm sterile discs (Hi-media) and air dried. After that, the dried discs were placed on the MHA plate and incubated at 37 °C for 24 h. Likewise, the antifungal activity of the active fractions were recorded. For this, the stock suspension of *Candida* sp. was adjusted to 10^5 to 10^6 cells/ml. The fractions were loaded on 6 mm sterile discs and air dried. After that, the dried discs were placed on the pathogen swabbed PDA plate and incubated at 28±2 °C for 48 h to determine the diameter of zone of inhibition and expressed in millimetre. The experiments were performed in triplicates.

4.2.2.5.2. Determination of Minimum inhibitory concentration (MIC)

The active fraction designated as FC5 was selected for further studies. The susceptibility of FC5 was measured by microdilution method (CLSI, 2012) in 96 well plates. For this, a single colony of *Candida* sp. was grown in PDA was inoculated in RPMI 1640 medium (Sigma Aldrich) and incubated at 28±2 °C for 24 h. The cells were then harvested by centrifugation at 8000 rpm for 10 min and the pellets were resuspended

in phosphate buffered saline (PBS, pH 7.4). The cell suspension was adjusted to 10^6 CFU/ml and treated with two-fold dilution starting from 2.5 mg/ml. Simultaneously, RPMI broth was added to all wells and a blank well and negative control wells without FC5 were also maintained in each experiment. Fluconazole was used as a positive control. After incubation at 28±2 °C for 48 h, the results were recorded. The MICs were determined as the lowest concentration of the FC5 that inhibited the growth of bacteria by ≥90 % when compared to control. All experiments were performed in triplicates. MBC values were determined by removing 100 µl of cell suspension from the well demonstrating no visible growth in MIC experiment and inoculating in fresh PDA plates and incubated at 28±2 °C for 48 h. MBC was determined as a lowest concentration of FC5 that kills 99.9 % of the cells after 24 h incubation (48 h for *Candida* sp.) at 28±2 °C. The MBC is determined with the wells whose concentrations are greater than MIC. Each experiment was done in triplicates.

4.2.2.6. Mechanism of antifungal action of FC5

4.2.2.6.1. Time kill assay

Both *C. albicans* and *C. tropicalis* were adjusted to 10^6 CFU/ml before starting the experiment. The cells were inoculated in RPMI 1640 medium containing fraction FC5 in various concentrations (1/2 MIC, MIC and 2 MIC) and incubated at 28±2 °C for different intervals (0, 2, 4, 6, 8, 10, 12 and 24 h). During each time intervals, 100 µl of aliquot was transferred and serially diluted in PBS and plated on PDA plates. The time kill curve was plotted as log CFU/ml versus time in hour for each time point (Kumar et al., 2015).

RPMI medium without any treatment was served as negative control. Fluconazole was used as a positive control. All the experiments were done in triplicates.

4.2.2.6.2. Electron microscopic studies of *Candida* sp.

The *Candida* cells were observed by Scanning electron microscopy (SEM) after fraction, FC5 treatment according to Qiang et al. (2019). For this, cell suspension of 10^6 CFU/ml was incubated with MIC concentration of FC5 at 28±2 °C for 24 h. After incubation, the cells were harvested by centrifugation and washed two times in PBS. The cells were then suspended in 2% (v/v) gluteraldehyde solution at 4 °C for 12 h. The samples were washed three times in PBS for 5 min each. The cells were post fixed using 1% (w/v) osmium tetroxide at 4 °C for 1 h and washed thrice with PBS. The samples were further dehydrated in graded ethanol series (30, 50, 70, 80, 95 and 100 %) for 5 min each. The cell suspensions were diluted and few drops were drop casted on aluminium foil attached to conductive carbon tape placed over the aluminium stubs and dried in air flow. The samples were further sputtered with gold to avoid charging and analyzed at 10 kV. Images were taken using the scanning electron microscope (Zeiss Evo 40 EP, Germany).

4.2.2.6.3. Determination of reactive oxygen species (ROS) production

The endogenous ROS levels of *C. albicans* and *C. tropicalis* were measured by fluorometric assay using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Tian et al., 2017). Briefly, the cell suspension was adjusted to 10^6 CFU/ml and exposed to FC5 of MIC concentration. The negative and positive (fluconazole) controls were used following the same procedure. After incubating the cells with the FC5 at 28±2 °C for 2 h,

10 µM DCFH-DA was added to the mixture for 30 min at 28±2 °C in dark. After incubation, cells were then harvested, washed, and resuspended in PBS buffer (pH 7.4). The fluorescence intensities (excitation 485 nm and emission 540 nm) of the suspensions were measured with multimode reader (Synergy 4 Biotek multiple reader, USA) and the images were taken with a florescence microscope (Pathway 855, BD Bioscience, USA).

4.2.2.6.4. Measurement of membrane permeabilization

For the analysis of the permeabilization of yeast membrane after FC5 treatment, both *C. albicans* and *C. tropicalis* cells of 10^6 CFU/ml were first harvested and suspended in RPMI 1640 medium. Cells were incubated at 28±2°C at 160 rpm with 5 µg/ml propidium iodide (PI) and FC5 at MIC values, for 60 min (Maurya et al., 2011). After incubation, cells were harvested by centrifugation and suspended in phosphate buffer saline PBS. Untreated cells were served as negative control and fluconazole was taken as a positive control. Images were taken using a florescence microscope (Pathway 855, BD Bioscience, USA).

4.2.2.6.5. Determination of DNA and nuclear damage

The damage caused by FC5 in both *C. albicans* and *C. tropicalis* cells was analyzed by fluorescence microscopy using DAPI staining (Tian et al., 2017). Nuclear condensation and fragmentation were analyzed by DAPI staining. For this, both cell suspensions were adjusted to 10^6 CFU/ml and treated for 2 h with FC5 at MIC concentration. The cells were harvested, washed, resuspended in PBS buffer, and

incubated with 1 µg/ml DAPI in the dark for 20 min. The stained cells were observed with a fluorescence microscope (Pathway 855, BD Bioscience, USA).

4.2.2.7. GC -MS analysis of A30 ethyl acetate extract

Gas chromatography–mass spectrometry analysis (GC-MS) was performed to profile the possible bioactive compounds present in A30 extract. The detection involved the use of the Thermo Scientific Trace 1310 GC-MS-MS equipped with TSQ 8000 Mass Selective Detector (MS), TG-5MS (5% methyl siloxane) capillary column of dimensions 30.0 m × 0.25 mm × 0.25 µm and helium as carrier gas at 1 ml/min with automated injector (AIAS 1310). For the initial 5 min, the column temperature was maintained at 40°C. The run was initiated with increasing temperature of 10°C/min until 200°C and followed by maintaining the temperature isothermally for 5 min. The MS was operated at 70 eV. The scan range was 45-600 with electronic ionization (EI) in split mode. By comparing to the mass spectral data available in National Institute of Standards and Technology (NIST library), the detected compounds in the extract were identified.

4.3. Results

4.3.1. Selection of solvent for large scale extraction

Among the three solvent systems tested, ethyl acetate exhibited maximum antimicrobial activity against a broad spectrum of pathogens followed by chloroform and acetone (Figure 4.2 and Table 4.1). Hence, ethyl acetate was chosen for large scale extraction.

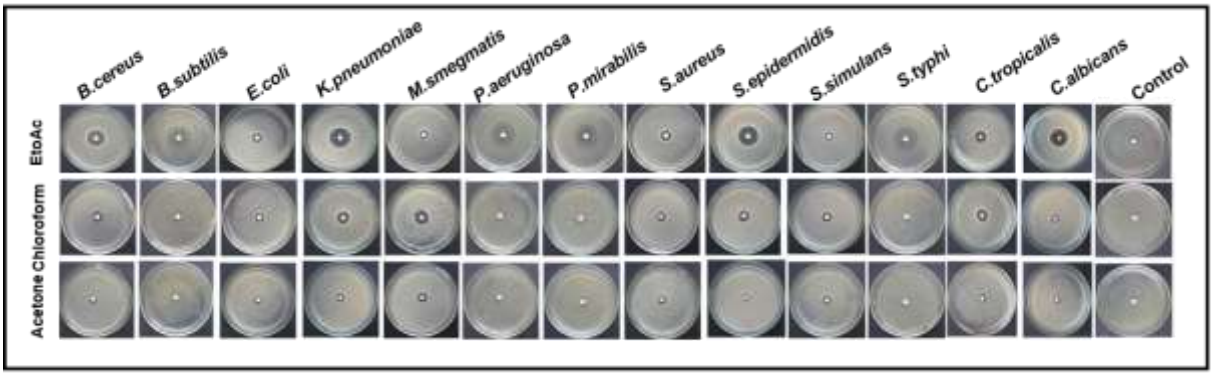

Figure 4.2: Antimicrobial activity of various solvent extracts from A30.

Table 4.1: Zone of inhibition recorded by ethyl acetate extract of A30 and A32 against bacterial pathogens.

	Zone of Inhibition (mm)		
Pathogens	**Ethyl acetate**	**chloroform**	**Acetone**
Gram positive bacteria			
B. cereus	20±1.5 ^{cd}	10±0.57 ^{a}	6±0.57 ^{a}
B. subtilis	26±0.57 ^{d}	0	0
M. smegmatis	8±0.57 ^{a}	14±1 ^{b}	8±1 ^{a}
S. aureus	10±1.5 ^{a}	8±1 ^{a}	7±0.33 ^{a}
S. epidermidis	24±1.5 ^{d}	12±0.57 ^{b}	7±0.57 ^{a}
S. simulans	7±1 ^{a}	10±1 ^{a}	8±0.33 ^{a}
Gram negative bacteria			
E. coli	18±0.57 ^{c}	6±0.57 ^{a}	7±1 ^{a}
K. pneumoniae	18±1 ^{c}	10±2 ^{a}	6±0 ^{a}
P. aeruginosa	15±1.5 ^{b}	0	0
P. mirabilis	13±1.5 ^{b}	0	0
S. typhi	28±1.5 ^{e}	0	0
***Candida* sp.**			
C. albicans	27±0.57 ^{e}	8±1 ^{a}	7±0.57 ^{a}
C. tropicalis	12±1 ^{b}	12±0.33 ^{b}	7±0.57 ^{a}
Control	0	0	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

4.3.2. Large scale fermentation of bioactive metabolites

The strain A30 had highest biomass and metabolite production in optimized medium of pH 7.0 at 35 °C with 1 % seed inoculum for 7 days of incubation. From 15 litre fermentation broth, Brown colored, sticky ethyl acetate crude extract (2.5 g) was obtained.

4.3.3. Bioautography in thin layer chromatography

The TLC bioautography proved the antibacterial activity of compounds against *C. albicans* with reference to compound 1 (Rf 0.65) and compound 2 (Rf 0.3). Compound 2 was exhibited prominent antibacterial activity (13±0.57 mm) against the pathogen as compared to compound 1 (5±0.57 mm, Figure 4.3).

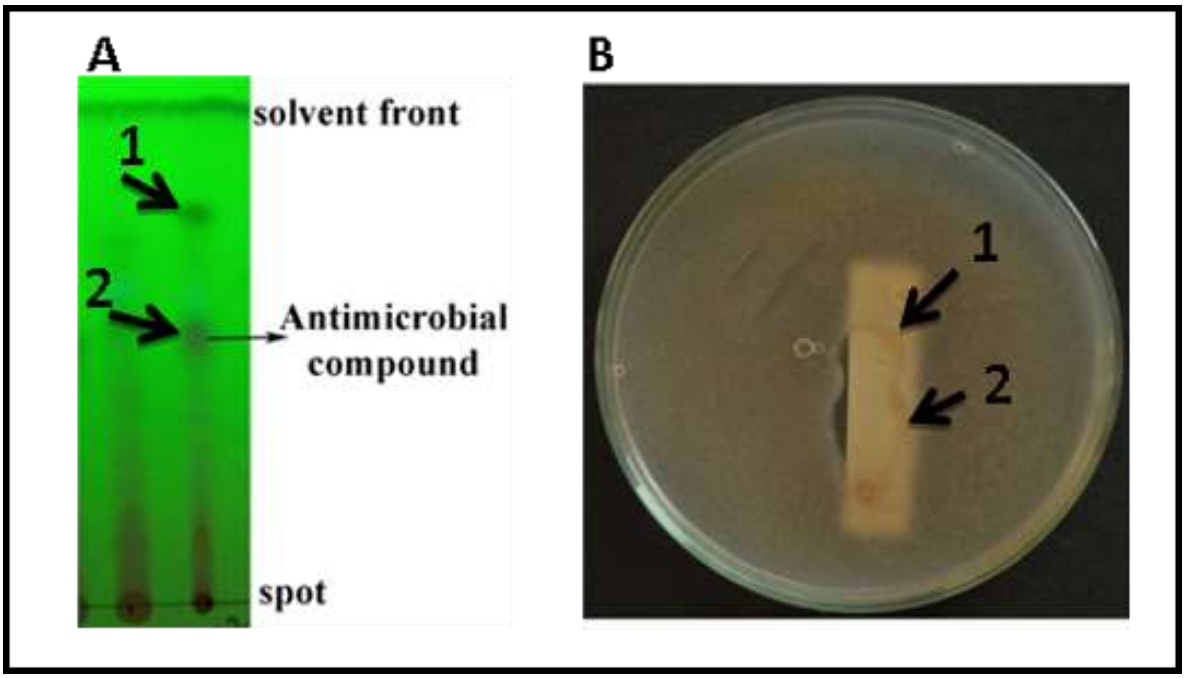

Figure 4.3: (A) TLC chromatogram and (B) TLC-Bioautography of bioactive metabolites from A30.

4.3.4. Bioactivity guided fragmentation of ethyl acetate extract

The ethyl acetate extract on silica gel column chromatography provided ten fragments and are named as FC1 (89.2 mg), FC2 (31 mg), FC3 (96 mg), FC4 (140.6 mg), FC5 (236 mg), FC6 (81.6 mg), FC7 (112 mg), FC8 (85 mg), FC9 (65 mg) and FC10 (186 mg).

4.3.5. Biological activities

4.3.5.1. Antimicrobial activities

After determination of antifungal activity against *C. albicans* and *C. tropicalis* (Figure 4.4 and Table 4.2), the most active fraction was found to be FC5.

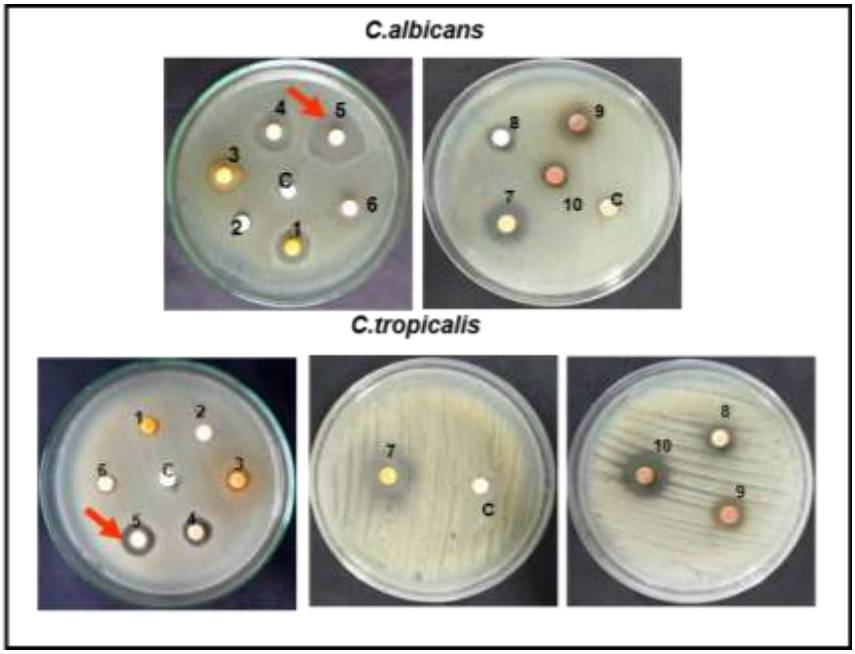

Figure 4.4: Antimicrobial activity exhibited by fractions against *C. albicans* and *C. tropicalis*.

Table 4.2: Zone of inhibition exhibited by fractions from A30 against *Candida* sp.

	Zone of Inhibition (mm)	
Fractions	***C. albicans***	***C. tropicalis***
FC1	13±0.33 [b]	7±0.57[a]
FC2	7±0.57 [a]	0
FC3	10±0.57 [b]	7±0.33 [a]
FC4	13±0.33 [b]	14±1 [bc]
FC5	22±0.57 [d]	15±0.57 [c]
FC6	0	7±1 [a]
FC7	13±0 [b]	15±0 [c]
FC8	8±1 [a]	9±0 [a]
FC9	12±0.57 [b]	12±0.33 [b]
FC10	8±0.33 [a]	15±0.57 [c]
Control	0	0

Values are expressed as mean± SD. Means with same letter are not significantly different at $p<0.05$.

4.3.5.2. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of this active fraction against human bacterial and yeast pathogens indicated that the MIC values are comparable to standard antibiotics. The MIC concentrations of *C. albicans* and *C. tropicalis* was found be 78.12 and 156.25 µg/ml respectively while that of *C. glabrata* and *C. parapsilosis* was found to be 312.5 and 625 µg/ml respectively (Table 4.3).

Table 4.3: Minimum Inhibitory Concentration (MIC) values shown by active fraction FC5.

Pathogens	**Minimum Inhibitory Concentration (µg/ml)**			
	FC5	Fluconazole	Ciprofloxacin	Kanamycin
C. albicans	78.12	25	ND	ND
C. tropicalis	156.25	50	ND	ND
C. glabrata	312.50	50	ND	ND
C. parapsilosis	625	25	ND	ND
E. coli	37.06	ND	4	8
K. pneumoniae	78.12	ND	4	20
S. aureus	37.06	ND	3	8
M. smegmatis	19.53	ND	3	16
P. aeruginosa	37.06	ND	2	4
S. typhi	78.12	ND	8	16

Values are average of three readings; ND, Not determined. (Fluconazole, an antifungal compound while ciprofloxacin and kanamycin are antibacterial).

4.3.5.3. Determination of Minimum Bactericidal Concentration (MBC)

The MBC concentrations of *C. albicans* and *C. tropicalis* was found be 156.25 and 312.50 µg/ml respectively while that of *C. glabrata* and *C. parapsilosis* was found to be 312.5 and 625 µg/ml respectively (Table 4.4).

Table 4.4: Minimum Bactericidal Concentration (MBC) values exhibited by active fraction FC5.

Pathogens	**Minimum Bactericidal Concentration (µg/ml)**			
	FC5	Fluconazole	Ciprofloxacin	Kanamycin
C. albicans	156.25	50	ND	ND
C. tropicalis	312.50	100	ND	ND
C. glabrata	312.50	100	ND	ND
C. parapsilosis	625	50	ND	ND
E. coli	78.12	ND	8	16
K. pneumoniae	78.12	ND	8	40
S. aureus	78.12	ND	6	16
M. smegmatis	37.06	ND	6	32
P. aeruginosa	78.12	ND	4	8
S. typhi	78.12	ND	16	32

Values are average of three readings; ND, Not determined. (Fluconazole, an antifungal compound while ciprofloxacin and kanamycin are antibacterial).

4.3.6. Mechanism of action of FC5

4.3.6.1. Time -kill kinetics of *Candida* sp.

In the time kill assay, the log CFUs of the *C. albicans* and *C. tropicalis* cells were rapidly reduced after treatment with FC5 at three concentrations (1/2MIC, 1 MIC and 2 MIC). However, 2 MIC concentration of FC5 was able to kill *C. albicans* and *C. tropicalis*

cells in 24 h and the killing kinetics of FC5 were compared with fluconazole (MIC 40µg/ml), a well-known potent antifungal agent. It was observed that fluconazole was unable to completely kill *C. albicans* cells even after 24h. In comparison to fluconazole, the active fraction showed complete killing of *C. albicans.* In case of *C. tropicalis*, both FC5 (2 MIC) and fluconazole followed the same pattern of killing kinetics for 24h (Figure 4.5).

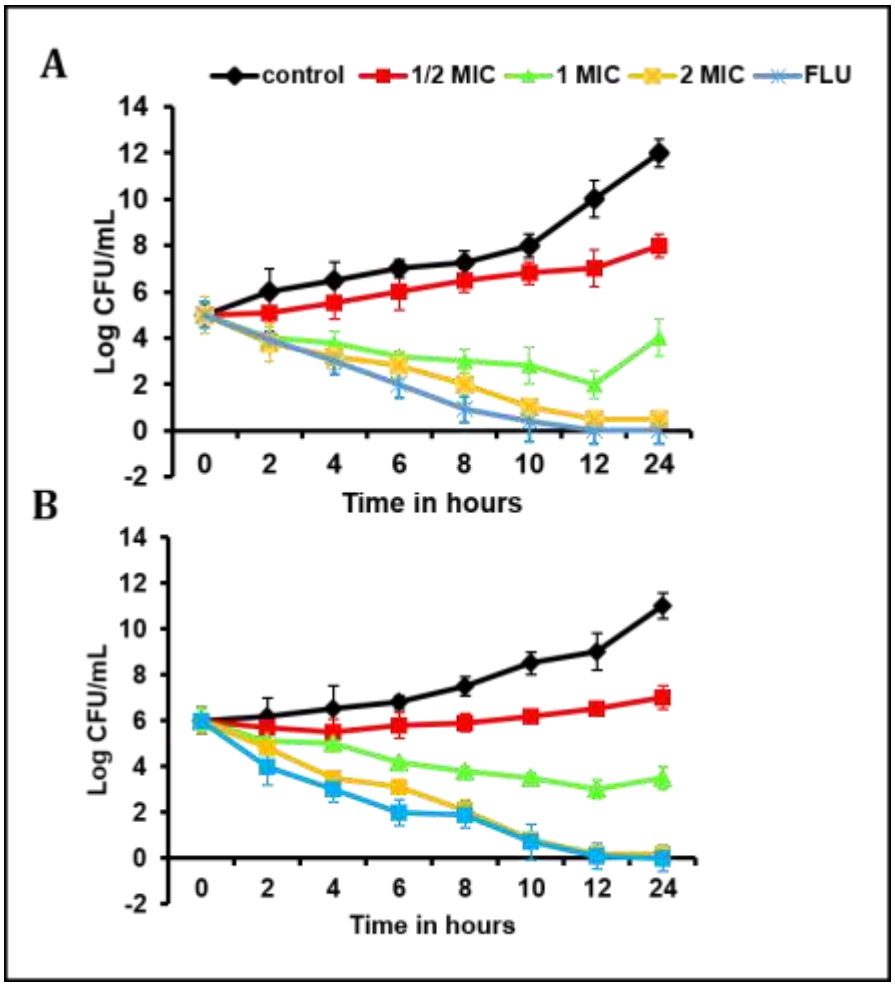

Figure 4.5: Time kill kinetics of *C. albicans* (A) and *C. tropicalis* (B) by FC5 and fluconazole at their three concentrations (1/2 MIC, MIC and 2 MIC).

4.3.6.2. *Candida* cell lysis observed with scanning electron microscopy

To understand the mode of action, we looked for morphological changes in both *Candida* cells by scanning electron microscopy (SEM). Cells treated with FC5 at their respective MIC values for 24 h showed wrinkling of the cell surface, compared with the smooth surface of untreated cells. The FC5 induced breakage in the cell wall was also clearly visible (Figure 4.6).

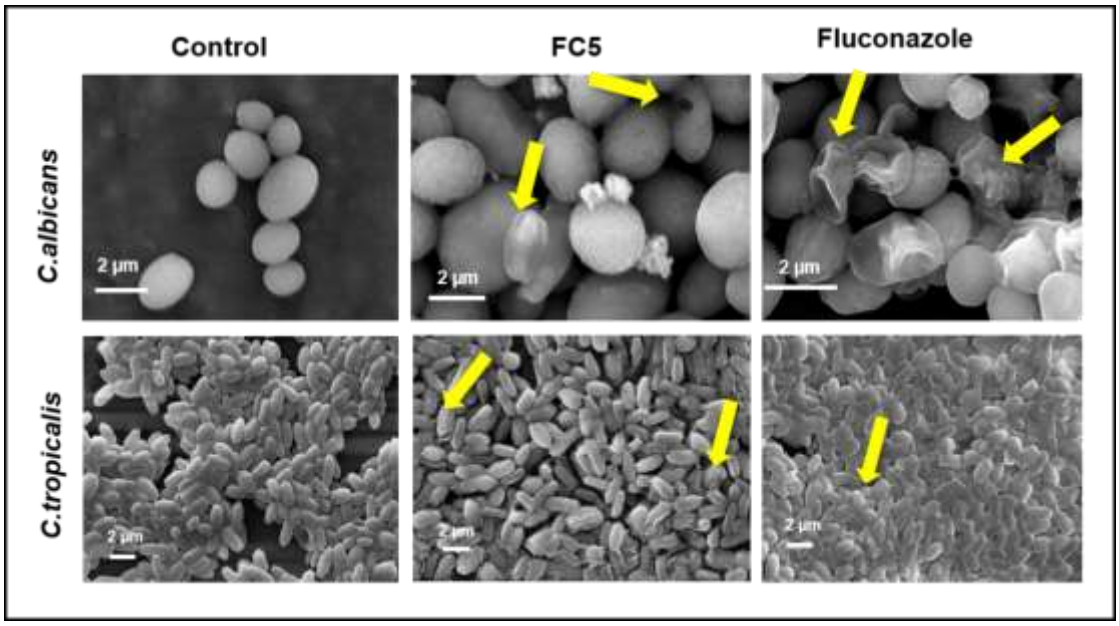

Figure 4.6: Scanning electron micrographs showing *C. albicans* and *C. tropicalis* cell damage by FC5 and fluconazole.

4.3.6.3. Generation of Reactive Oxygen Species (ROS) by FC5

In case of *C. albicans*, compared with the control, which did not have any treatment, at an MIC concentration of FC5, the proportion of fluorescent cells increased gradually in a time dependent manner up to 60 min. The ROS detection demonstrated

that FC5 at 78.12 µg/ml and fluconazole at 25 µg/ml had resulted in a significant increase in ROS (Figure 4.7).

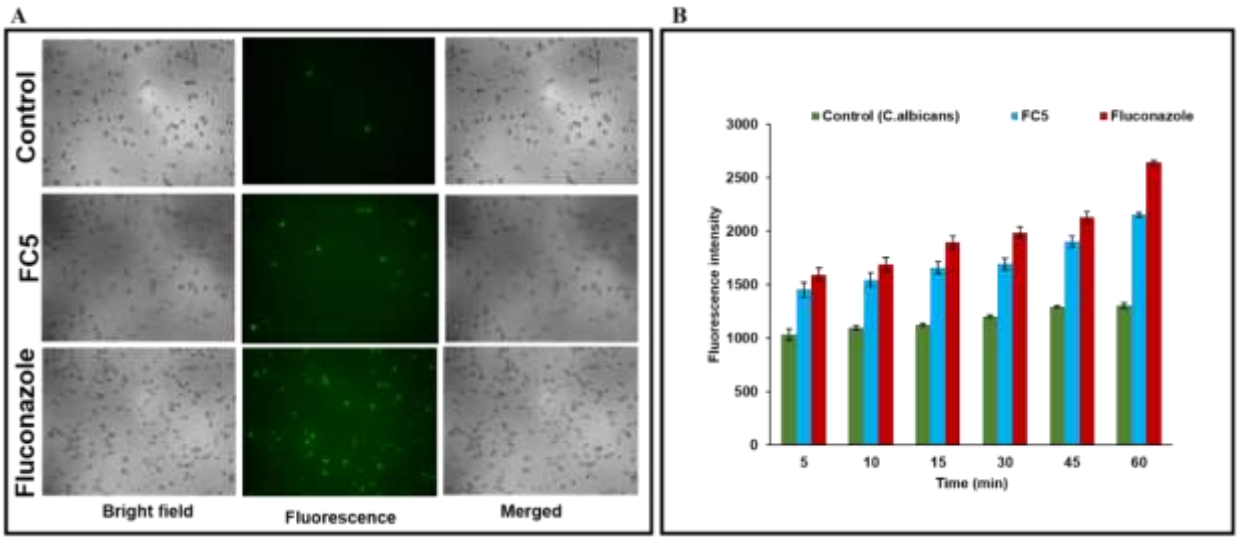

Figure 4.7: ROS levels in the presence of FC5 in *C. albicans* cells.

In case of *C. tropicalis*, compared with the control, which did not have any treatment, at an MIC concentration of FC5, the proportion of fluorescent cells increased gradually in a time dependent manner up to 60min. The ROS detection demonstrated that FC5 at 78.12 µg/ml and fluconazole at 25 µg/ml had resulted in a significant increase in ROS (Figure 4.8).

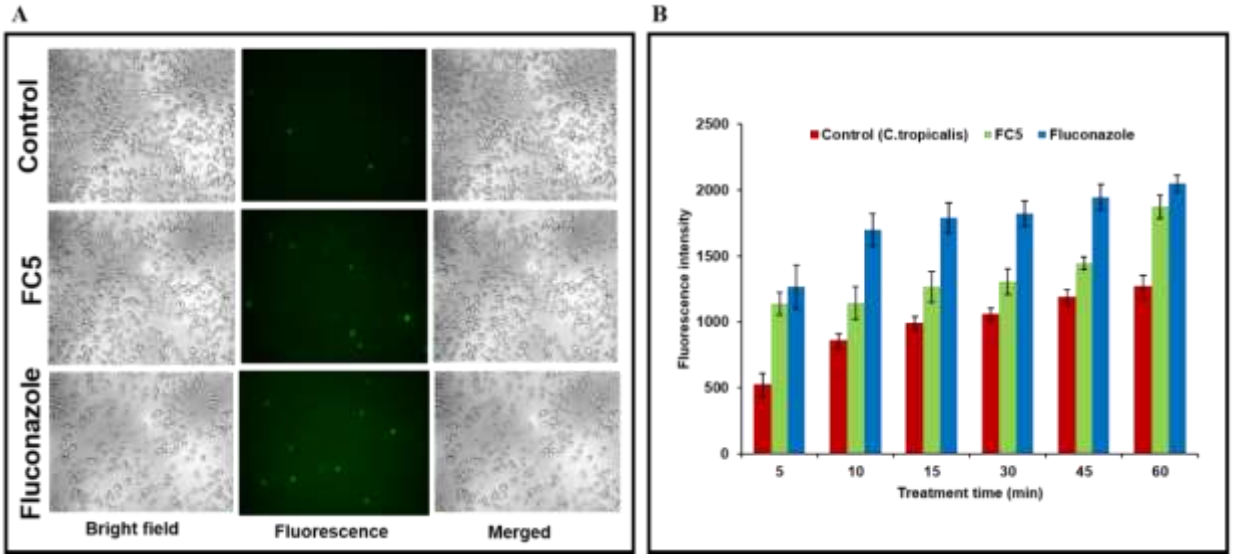

Figure 4.8: ROS levels in the presence of FC5 in C. *tropicalis* cells.

4.3.6.4. Propidium Iodide (PI) uptake assay- *Candida* membrane permeabilization

Incubation of FC5 with *Candida* cells resulted in PI uptake by the cells as monitored by fluorescent microscopy (Figure 4.9 and 4.10). Here, a time dependent increase in the PI associated fluorescence was observed. FC5 and fluconazole treated *Candida* cells showed red fluorescence after 60 min of incubation. PI fluorescence was considerably low when *Candida* cells were incubated with PI alone.

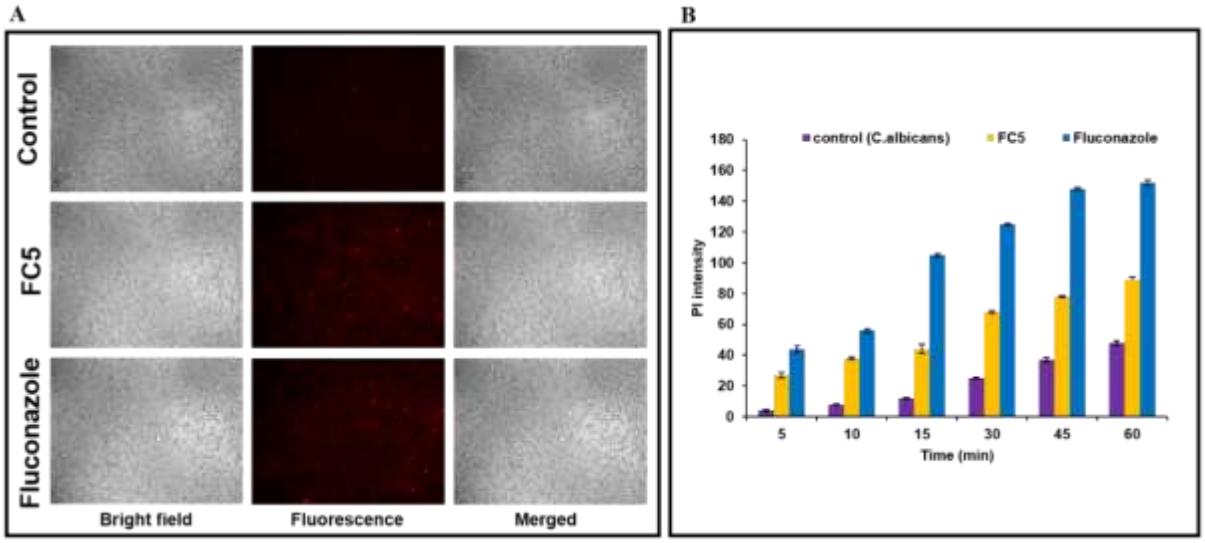

Figure 4.9: Confocal microscopy analysis of membrane permeabilization assay by PI uptake in *C. albicans* cells.

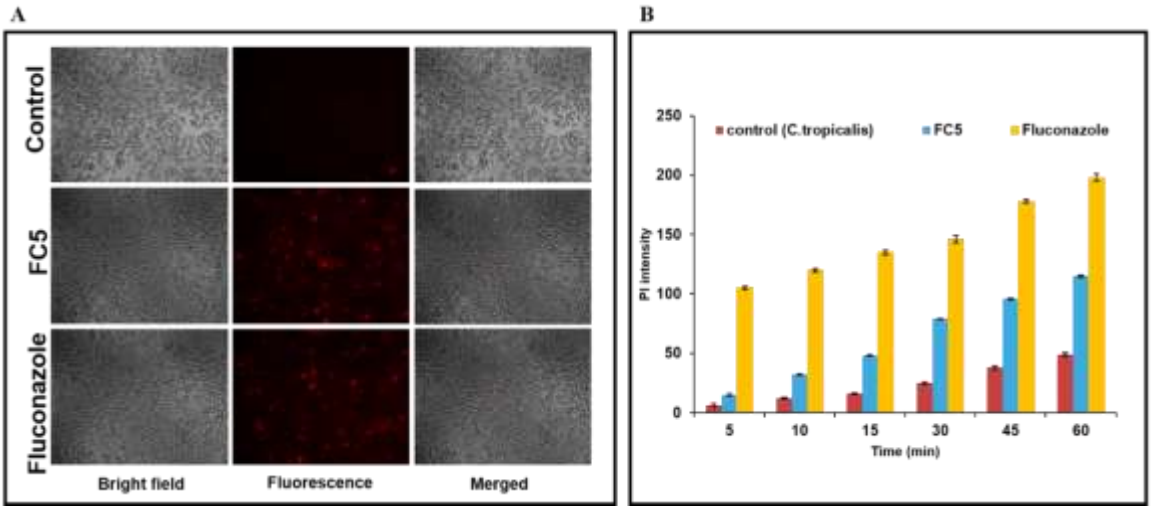

Figure 4.10: Confocal microscopy analysis of membrane permeabilization assay by PI uptake in *C. tropicalis* cells.

4.3.6.5. Treatment with FC5 produces morphological change in the nuclei

Nuclear condensation and fragmentation were analyzed using a DAPI stain, which is a cell-permeable fluorescent dye. *C. albicans* and *C. tropicalis* cells that were treated with MIC concentrations of FC5 were stained with DAPI and showed a more concentrated fluorescence intensity in single cells compared with untreated cells (Figure 4.11). These results showed that the treatment of *Candida* cells with FC5 caused nuclear morphologic changes. Treatment with FC5 induced 9 % and 3.7 % increase in nuclear fragmentation compared to treatment with fluconazole alone.

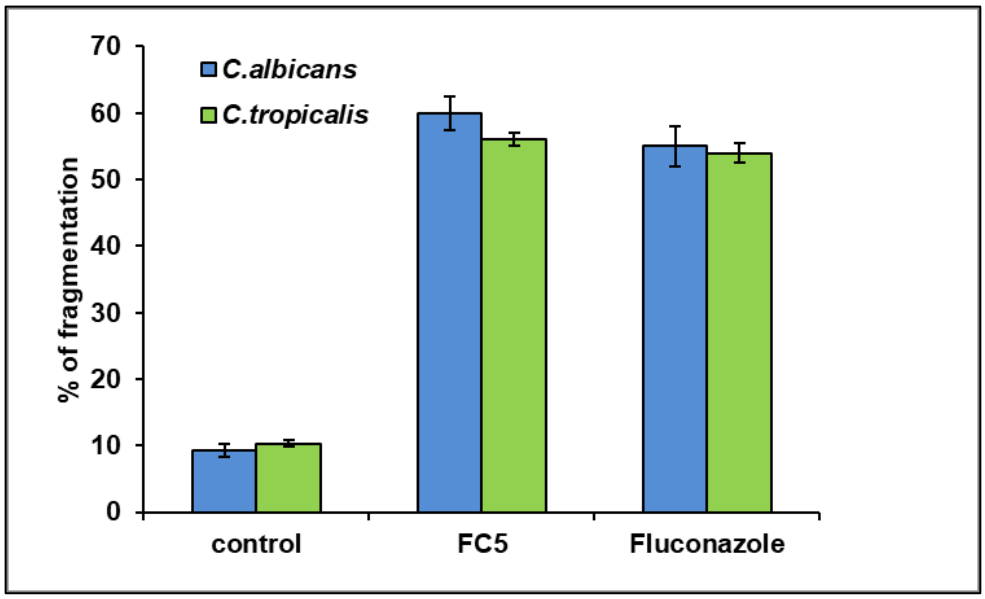

Figure 4.11: Percentage of nuclear fragmentation exhibited by FC5 in *C. albicans* and *C. tropicalis*.

4.3.7. GC-MS identification of active principles in A30

The GC-MS results revealed 7 major and 10 minor compounds present in A30 extract (Table 4.5). The spectral data and the chemical structures of major compounds are illustrated in figure 4.12 and figure 4.13.

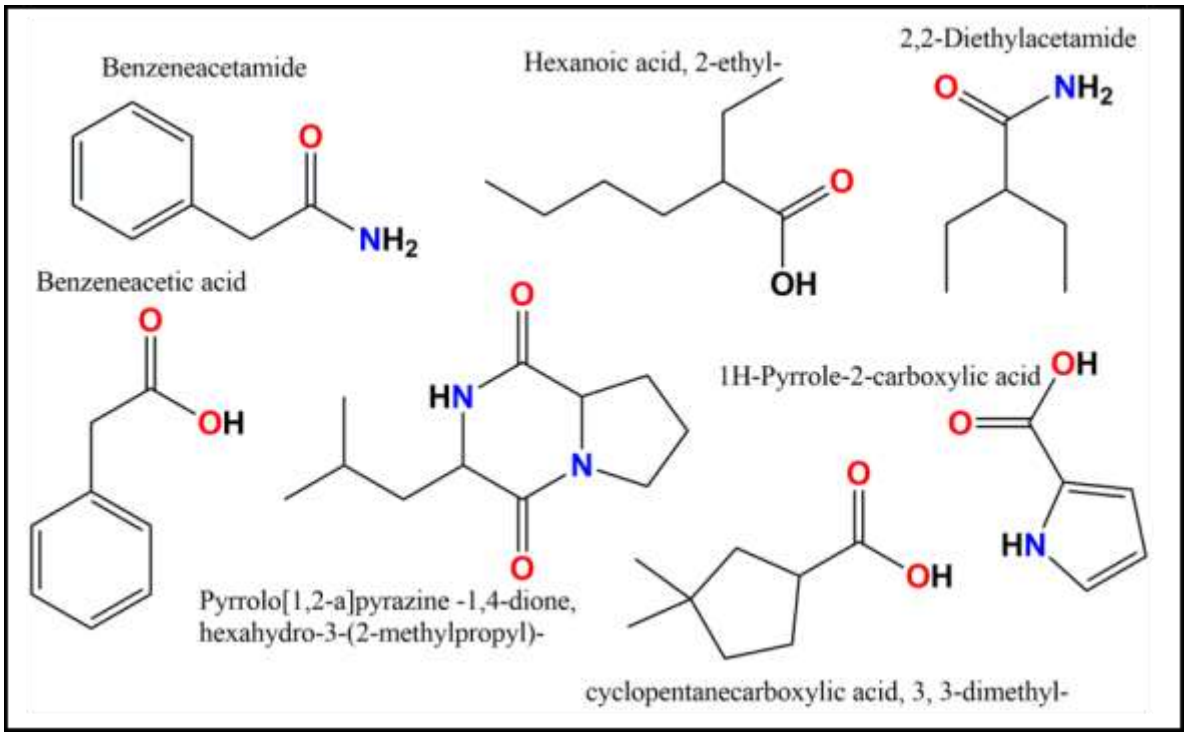

Figure 4.12: Major chemical constituents detected in A30 extract

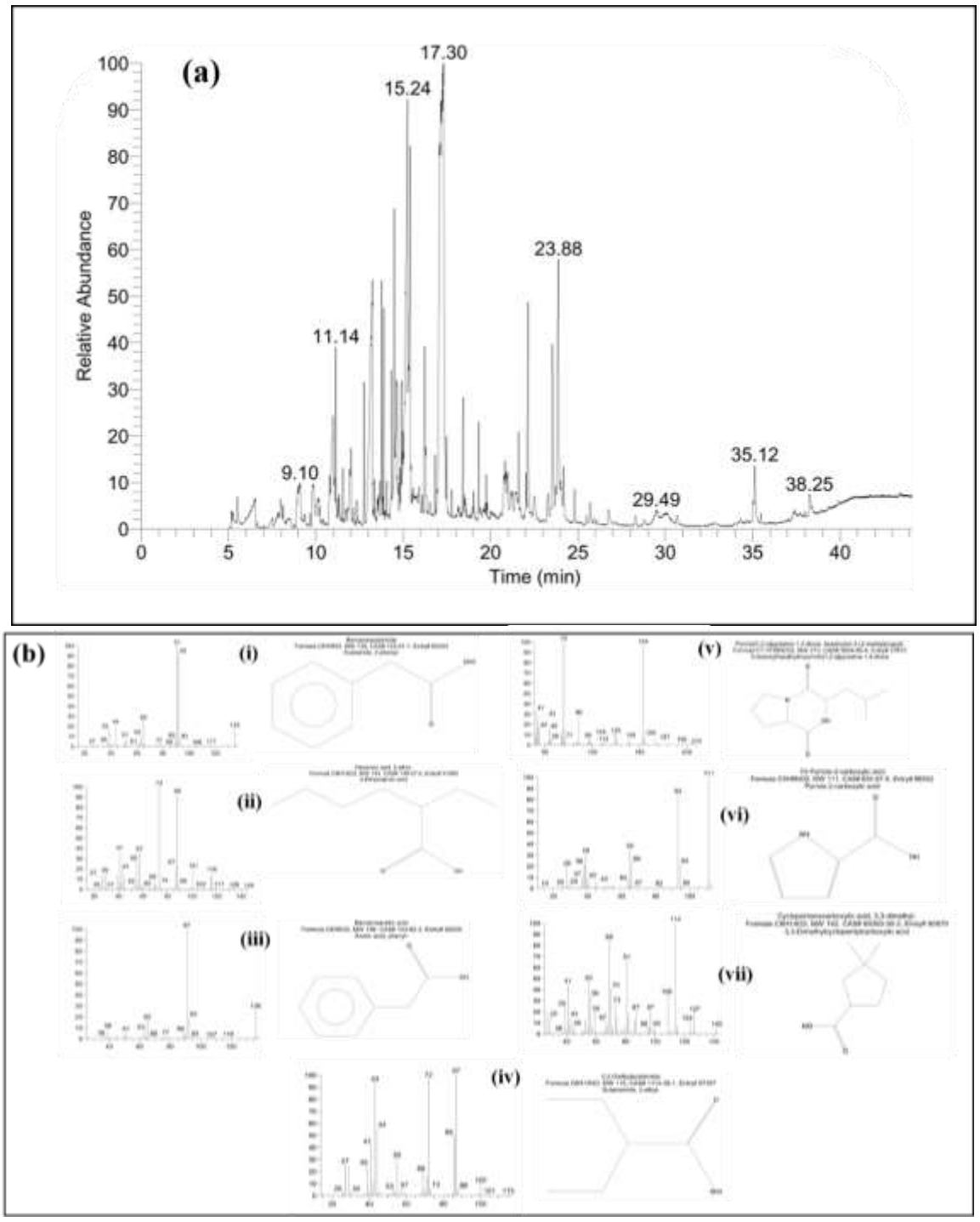

Figure 4.13: GC-MS chromatogram (a) and individual peaks (b) of major compounds detected in A30 (i, Benzeneacetamide; ii, Hexanoic acid, 2-ethyl; iii, Benzene acetic acid; iv, 2,2- Diethylacetamide; v, Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- ; vi, 1H-Pyrrole-2-carboxylic acid and vii, Cyclopentanecarboxylic acid, 3,3 dimethyl-).

Table 4.5: Chemical constituents detected in A30 ethyl acetate extract.

RT	Compounds detected	Area (%)	Activity reported	References
Major compounds (2% and above)				
13.21	Hexanoic acid, 2-ethyl	5.59	Antifungal	Boukaew et al., 2013
14.49	Cyclopentanecarboxylic acid, 3,3 dimethyl-	2.36	Antioxidant, cytotoxicity	Narendhran et al., 2014
14.63	1H-Pyrrole-2-carboxylic acid	2.76	Antimicrobial	Nguyen et al. 2015; Hassan et al. 2016
15.23	Benzeneacetic acid	5.13	Antimicrobial	Al-Dhabi et al., 2016
15.39	2,2-Diethylacetamide	4.4	This study	
17.29	Benzeneacetamide	12.91	Antimicrobial, antitumor	Balachandran et al. 2012
23.87	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	4.24	Antimicrobial, antioxidative, antitumor	Siddharth and Vittal 2018
Minor compounds (0.9- 1.9%)				
9.10	Propanamide, 2-methyl-	1.05	This study	
9.81	Butanamide	1.45	Antimicrobial	Sultan et al., 2002
10.98	Methanamine, N-butylidene-, N-oxide	1.81	No activity	Shubhrasekhar et al., 2013
11.14	3,6-Heptanedione	1.02	No activity	Sriram and Sarvamangala, 2018
12.00	Hexanoic acid, 4-methyl-	1.47	Antifungal	Boukaew et al., 2013
12.75	Phenylethyl Alcohol	1.09	Antimicrobial, antioxidant	Lei et al., 2016
13.77	5-(Hydroxymethyl)-3,3-dimethyldihydro-2(3H)-furanone	1.9	This study	
18.42	Hexanoic acid, 2-ethyl-, heptyl ester	1.02	This study	
20.83	Tetradecanoic acid	1.01	This study	
35.12	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-	0.95	Antifungal	Kannabiran, 2016

4.4. Discussion

New-generation antifungal drugs are urgently required to treat fungal infections especially in case of *Candida* sp. because the currently available clinically prescribed drugs are associated with low efficacy, high toxicity, and drug resistance (Sangamwar et al., 2008). There are five major classes of systemic antifungal compounds are currently in clinical use viz. the polyene antibiotics, the azole derivatives, allylamines, thiocarbamates and the fluoropyrimidines (Groll and Gastine, 2020). Microorganisms have served as the source for the majority of the drugs of current use (Mohanta et al., 2019). Among them, actinomycetes are the unparalleled source of bioactive metabolites and they continue to play a key role in drug discovery and development by providing natural bioactive molecules with pharmaceutical potential and novel platforms for synthetic modification (Jakubiec-Krzesniak et al., 2018). In the present study, we investigated the antifungal activity of A30 isolated from Nelliyampathy forest soils of Western Ghats area. The result of bioautography indicates the presence of two bioactive compounds. Bioactivity guided fragmentation provided an active fraction from A30 and a lower MIC value was obtained for *C. albicans* (78.12 µg/ml) followed by *C. tropicalis* (156.25 µg/ml). MIC value of 50 µg/ml was obtained for fluconazole, a positive control used in this investigation.

Time-kill measurements provide an understanding of the extent of antifungal action of the FC5 which is generally made over 24 h (Pfaller et al., 2004). In this context, FC5 was fungicidal against the two *Candida* sp. in a dose-dependent manner across the

three concentrations tested. The maximum reduction of growth in *Candida* was recorded between 10 and 24 h of treatment. Significant reduction of *C. albicans* cells was observed for 2×MIC concentration of FC5 at 24 h. This concentration reduced 99.99 % of *C. albicans* cells. In case of *C. tropicalis*, significant reduction was again observed for 2×MIC concentration of FC5 at 12 h which resulted in 100% inhibition of cells. These results are consistent with previous observations where a concentration of MIC and above can be fungicidal (Khan et al., 2019; Sanguinetti et al., 2019).

Evaluation of anti-candidal action of the active fraction of strain A30 by SEM may be helpful to understand the cell damage mechanism. The changes in morphology of yeast cells after treatment with FC5 are also consistent with findings of other researchers (Nurkanto and Julistiono, 2014; Dias et al., 2020). The shrinkage of cells was clearly observed in the electron microscopic images which may be due to loss of cytosolic volume, which is mainly observed in case of many antibiotics (Kumar et al., 2014). These findings support that the strain A30 possess strong anti-candidal activity and killed pathogenic yeast due to considerable morphological changes.

Moreover, it is necessary to investigate the mechanisms behind fungal cell death to identify the manner of cell death. In recent years, several studies have reported that Reactive Oxygen Species (ROS) are sufficient as a primary cell death regulator of the apoptotic pathways in yeast which is regarded as one of the early changes (Tian et al., 2017; Postigo et al., 2019). The accumulation of ROS can cause oxidative damage to the macromolecules, which results in DNA damage, DNA strand breaks, and mitochondrial damage in cells (Haque et al., 2019). We also analyzed the induction of ROS in *C.*

albicans and *C. tropicalis* cells that were treated with FC5. To confirm intracellular Reactive Oxygen Species (ROS) in *Candida* cells, the ROS generation in these cells was monitored using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) in fluorescent microscopy. A green fluorescence, resulting from oxidation of dye DCFH-DA was observed indicating the presence of ROS. The DCFH-DA assay results indicated that FC5 triggered a noticeable generation of ROS in both cells. Excessive ROS is a major cause of apoptosis because it disrupts the balance of intracellular ROS, which leads to oxidative stress (Ding et al., 2016; Jia et al., 2019).

In the sequence of examining the mechanism of action, FC5 treated *Candida* cells revealed contracted cell morphology indicating harmed cell membrane that are able to release the intracellular components into the surrounding medium, which prompted us to study the FC5 effect on membrane integrity. To confirm such inference, *Candida* sp. treated with FC5 was exposed to DNA binding dye PI, which emits red fluorescence (Lei et al., 2019). In this context, FC5 was able to permeabilize both *C. albicans* and *C. tropicalis* cell membrane within 5 min. Intracellular localization of FC5 in a time dependent manner was demonstrated by the gradual increase in the fluorescence for both pathogens. These findings are in agreement with the previous reports, wherein the cell membrane disturbance was observed after treatment with sertraline against *Candida* sp. (Gowri et al., 2020).

Nuclear condensation and fragmentation are also considered to be late apoptotic responses that result from the accumulation of ROS, which could lead to apoptosis through the shrinkage of the cell (Hwang et al., 2012). In our investigation, nuclear

damage was analyzed by fluorescence microscopy using DAPI staining and the percentage of fragmentation was more compared to fluconazole. The GC-MS analysis of chemical constituents in A30 extract revealed the presence of benzeneacetamide (12%), Hexanoic acid, 2-ethyl (5.59%) and benzeneacetic acid (5.13%) previously reported for antimicrobial activities. Benzeneacetic acid showed antimicrobial activity against infectious human pathogens like *S. aureus* and *E. coli* (Kim et al., 2004). The extract also consisted of pyrrolizidines [pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)], which are natural heterocyclic compounds known to exhibit antimicrobial activity (Das et al., 2018). The results of this investigation indicate that FC5 is a potent candidacidal candidate for the treatment of infections cause by *C. albicans* and *C. tropicalis* after successful isolation of bioactive compound/s.

4.5. Conclusion

From the experiments, it is concluded that ethyl acetate extract of A30 harbour some compounds with antimicrobial activities as detected in GC-MS analysis. The bioactivity guided fractionation of ethyl acetate extract from the strain yielded an active fraction FC5 with antifungal properties against *Candida* sp. FC5 showed a minimum inhibitory concentration of 78.12 and 156.25 µg/ml for *C. albicans* and *C. tropicalis* respectively. Structural analysis of FC5 treated *Candida* cells revealed several malformations. The dye PI uptake assay was used to demonstrate FC5 induced cell membrane permeabilization. Time-kill kinetics, PI uptake assay, intracellular accumulation of reactive oxygen species (ROS) and nuclear damage suggested that FC5 have multiple detrimental effects on target pathogen which ultimately result in cell wall disruption and killing. Therefore, the active compounds present in FC5 will be a good

template for further design and development as antifungal agents against Candidiasis.

4.6. References

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Chapter 5

Modulation of plant growth and induction of systemic resistance against fusarial wilt in Mung bean by A32

5.1. Introduction

Mung bean [*Vigna radiata* (L.) R. Wilczek] is an important annual legume crop with short duration (70-90 days), under the family *Fabaceae* that has attracted attention because of its high nutritional value. It is the third most important pulse crop after chickpea and pigeon pea in India (Nair et al., 2012) with more than 50 % of world production and exporters around the globe (http://www.spagricon.com.au/ pulses.html). India has an annual production of 1.5-2.0 tonnes of mung bean from 3-4 million hectares (2014-2015) with an average productivity of 0.5t ha^{-1} (Jadhav et al., 2016). According to annual report of Ministry of agriculture and farmers welfare, Rajasthan contributes 39.06 % of total mung bean production followed by Madya Pradesh (11.55 %) and Maharashtra (8.57 %) in the country. Despite of its higher production, mung bean varieties are vulnerable to both biotic and abiotic stresses. Among the biotic stresses, diseases caused by fungi are responsible for the reduction of major crop yield (Nair et al., 2019). Dry root rot and wilt are the major soil-borne diseases that affect plant vascular regions, since the pathogen affects the plant during all growth stages and subsequently causes significant yield loss up to 40-60 % depending upon the crop variety (Pandey et al., 2018). Wilt disease caused by *Fusarium oxysporum* usually leads to severe economic damage worldwide in this crop. Currently, there are no satisfactory control methods available to prevent this disease. But this disease can be managed to an extent by various cultural practices and the usage of chemical fungicides. However, the chemical method usually has some adverse environmental effects, in particular, toxic effects in foods and the growing resistance of pathogens to chemicals. Hence, there is demand for new methods to supplement the existing disease management

programmes to achieve better disease control. The integration of biocontrol agents (BCAs) is an important strategy in crop protection involving variety of disease suppression mechanisms contributed to the trust that this strategy is more effective than chemical applications (Shahid and Khan, 2019; Khan et al., 2019). Many rhizospheric as well as endophytic bacteria such as *Bacillus*, *Pseudomonas*, *Trichoderma* and actinomycetes have been used to control various plant diseases. These are also used for the growth promotion and yield enhancement in many crops (Glick, 2012; Volpiano et al., 2019).

Actinomycetes, especially *Streptomyces* is a Gram-positive, aerobic, filamentous bacteria with high G+C content are being used as biocontrol agents against soil-borne diseases of plants. They are reported to inhibit many phytopathogens such as *Fusarium oxysporum* (Faheem et al., 2015), *Colletotrichum gleosporiodes* (Liotti et al., 2019), *Rhizoctonia solani* (Wu et al., 2019) and *Alternaria alternata* (Verma et al., 2011). Some of the potent strains are already developed as bioformulations such as Actinovate®AG , Mycostop®, Kasumin™, Biomycin and omycin in many countries including India (Palaniyandi et al., 2013). Antagonistic activity of these BCAs mainly due to the production of antifungal metabolites, volatile compounds and cell wall degrading enzymes such as chitinase and β-1,3- glucanase. Apart from these activities, *Streptomyces* also provide induction of systemic resistance in host plants and this important aspect is poorly studied in *Streptomyces sp.* antagonistic to *Fusarium* species (Bubici, 2018). Instead of disease control, many *Streptomyces* strains are also reported for plant growth promotion and yield enhancement in agricultural plants due to their ability of phosphate solubilization, siderophore production and phytohormones production. However, more

strains are needed to be identified and developed as bioformulations from under explored areas such as Western Ghats region of India.

In this scenario, a soil actinomycete designated as A32 isolated from Western Ghats forest region of Kerala and identified as *Streptomyces luteosporeus* based on 16S rRNA sequence analysis is shown to possess distinctive inhibitory activity against *Fusarium oxysporum* ITCC 4814. A32 has imparted certain abnormalities in pathogen hyphae. The activities of defense related enzymes such as Phenylalanine Ammonia Lyase (PAL), Peroxidase (POD) and Polyphenol Oxidase (PPO) along with pathogenesis related protein such as β-1, 3-glucanase in mung bean leaves were dramatically increased after treatment with A32. The total phenol contents and the level of chlorophyll a, chlorophyll b and total chlorophyll were also increased in plants treated with A32. In short, the strain A32 and its metabolites can effectively suppress *Fusarium oxysporum* and trigger induced resistance in mung bean against fusarial wilt.

5.2. Materials and Methods

5.2.1. Materials

5.2.1.1. Site

The present study was carried out under the agro-climatic condition of Thiruvananthapuram, Kerala, India, at the Agro-Processing and Technology Division of CSIR-NIIST during the year 2015 to 2018. The experimental site was situated between 8.4725 °N latitude and 76.9895 °E longitude at an elevation of 4.9 m above mean sea level.

Hot tropical climate and rain during monsoon seasons are the common features of this region.

5.2.1.2. Soil and Seeds

The experiments were conducted on sandy loam soil with pH 6.8, total nitrogen of 0.896 per cent, total phosphorus of 0.215 percent and total potassium of 0.474 percent and having no previous history of any pesticide or synthetic agrochemical application. Mung bean seeds used in this investigation was purchased from a local market in Thiruvananthapuram, Kerala.

5.2.2. Methods

5.2.2.1. Poisoned food technique

The antifungal activity of the ethyl acetate extract of A32 was tested against four plant pathogenic fungi such as *A. fumigatus*, *C. gleosporiodes*, *F. oxysporum* and *R. solani* using the poisoned food technique (Ibrahim et al., 2017). For this, 62.5 µl was taken from a 40 mg/ml stock solution aseptically and added to 10 ml molten potato dextrose agar to obtain a concentration of 250 µg/ml. The solution was mixed thoroughly and added into sterilized Petri plate and after that, 6 mm mycelial disc of each test fungus was inoculated at the center of each Petri plate. The plates were incubated at 28 ± 2 °C and diameter of fungal colony was measured in mm at an interval of 24 h till the control plates attained the full growth. The media mixed with 250 µl of methanol served as the control growth plate. The experiment was done in triplicates. Percentage inhibition of mycelia growth of the test fungi by the extracts was calculated relative to the mycelia growth of the test fungi on the control plates.

5.2.2.2. Effect of A32 ethyl acetate extract on Fusarial wilt fungi

To investigate the alteration of membrane permeabilization under A32 treatment, a cell membrane impermeable fluorescent dye propidium iodide (PI) was used (Xu et al., 2019). For this, spore suspension of *F. oxysporum* (10^5 CFU /ml) was incubated with 5 % (v/v) A32 culture broth or without (control) for 48 h**.** The emerged hyphae were washed, resuspended in phosphate- buffered saline (PBS) and stained with PI (5 µg/ml) at 37 °C for 10 min under dark conditions. Fluorescence was further detected using a fluorescence microscope (BD Biosciences, pathway 855, USA).

5.2.2.3. Stability of culture broth of A32

For checking the stability of culture broth, the strain was cultured in GSMB broth and kept at 4 °C for a five months storage time. The inhibition test was performed at every month to test its antagonistic activity against the test fungus.

5.2.2.4. Determination of plant growth promotion and biocontrol traits

Phosphate solubilization efficiency of strain A32 was qualitatively determined by inoculating the isolate in pikovskaya agar (Pikovskaya, 1948) and expressed as Ssolubilizing Efficiency (% SE). Indole Acetic Acid (IAA) production was quantitatively determined by measuring the absorbance at 530 nm using a UV-visible spectrophotometer and estimated using IAA standard curve (Gordon and Weber, 1951). Siderophore production was determined qualitatively as per the universal assay by inoculating the strain on Chrome Azurol S (CAS) agar plates (Schwyn and Neilands, 1987). For antifungal volatile compound assay, a paired Petri dish technique was used (Solanki et al., 2015) and expressed as the percentage fungal growth inhibition.

Qualitative estimation of HCN production was performed by Picrate assay (Castric, 1975) by inoculating the strain in ISP 2 agar medium amended with glycine. The ammonia production was determined according to the standard protocols (Cappuccino and Sherman, 2005).

5.2.2.5. Growth promotion and yield studies under nursery conditions

The experiment had three set of replications with twenty-five plants in each treatment and repeated thrice for recording the data. The mung bean seeds were planted in plastic pots filled with a mixture of garden soil and Farmyard Manure (FYM) in the ratio of 3:1. The experiments were done in the following segments *viz.* i) *F. oxysporum* ITCC 4814 (Pathogen alone, P) ii) A32 (actinomycete A32 alone, F) iii) *F. oxysporum* + A32 (P+F) and iv) control (C). The experiments were given A32 grown in GSMB broth (seven days old, adjusted to have 1.0 X 10^8 CFU/ml, a homogenised suspension) and fungal pathogen (well homogenized mycelial solution grown in Potato dextrose broth for 10 days) added 25 ml each per plant (Morang et al., 2018). The experiments were done in triplicates and the data were recorded in an interval of five days up to 30 days. Healthy condition of the plants was maintained by periodic weeding, light forking and irrigation when required. After a month, plants were again treated twice with the same doze of respective pathogen and/or bacterial strain as per the treatment specifications at an interval of 15 days and five plants were maintained in each pot. The effect of growth promotion in various treatments such as shoot height, root length, fresh weight and dry weight were determined on 30 Days After Treatment (DAT). The yield parameters, such as the number of pods per plant, number of seeds per pod, and seed yield were

determined up to 90 days. Total chlorophyll content was determined according to Arnon (1949) by monitoring the absorbance at 645 and 663 nm. This experiment was conducted during consecutive years from December 2016 to February 2017 and from December 2018 to February 2019.

5.2.2.6. Induction of systemic resistance in mung bean plants with strain A32 under split root conditions

The ability of the strain for induction of systemic resistance was evaluated under split root conditions (Dutta et al., 2008) with some modifications. The mung bean seeds were surface sterilized (Dileep Kumar et al., 2001) and applied in plastic pots (22×18 cm) filled with soil and FYM in the ratio 3:1. Seven days old mung bean seedlings with identical sizes were selected for split root experiment. For this, 9×7.5 cm diameter plastic pots were filled with soil mixture was used. Roots of seven days old mung bean seedlings were split into two halves and the main root was cut using a sterile scalpel. Single seedling was transferred to the split root set up with half of the root system grown in each pot below. The upper pot is used as a support for the plant specially to protect its upper part. The set up includes the following treatments.

1. Control (C): both halves of the root system were untreated (control: control).

2. Pathogen treatment (P): both halves of the root system were treated with pathogen (pathogen: pathogen).

3. Pathogen + A32 (P+F): half of the root system was treated with A32 strain and the other half treated with pathogen to assess systemic effects of A32 on the Fusarium wilt infection (pathogen: A32).

4. A32 treatment (F): Both halves of the root system were inoculated with A32 strain (A32: A32).

Bacterization was performed by watering the plants with 10 ml of A32 cell suspension (10^8 CFU/ml) twice in a month at an interval of 15 days in treatments 3 and 4. Pathogen treatments were applied with a spore suspension (5×10^4 CFU /ml) of *F. oxysporum* at an interval of 15 days.

5.2.2.6.1. Determination of pathogenesis related compounds and enzymes

The total phenol content was determined (Bray and Thorpe, 1954) and expressed as mg/100 gm tissue weight using catechol standard curve. Pathogenesis-related enzyme, β-1,3-glucanase was determined (Liang et al., 1995) by adding laminarin and enzyme extract absorbance was monitored at 550 nm in a UV-spectrophotometer.

5.2.2.6.2. Determination of defense related enzymes

For the determination of defense related enzymes *viz*. L-phenylalanine ammonia lyase (PAL), Peroxidase (POD) and Polyphenol oxidase (PPO), the enzyme extracts were prepared as per the protocols according to Mahadevan and Sridhar (1986). PAL activity was studied according to Sadasivam and Manickam (1991) and recorded as micromole trans-cinnamic acid formed per 100 g fresh weight per min. POD activity was determined according to Thimmaiah (1999) and calculated as units/min/100 g fresh weight of

sample considering one unit of enzyme as an increase in OD by 1.0 under standard conditions. The PPO activity was determined according to the method of Sadasivam and Manickam (1991) and calculated in units per 100 g fresh weight.

5.2.2.6.3. Determination of disease assessment, A32 and pathogen counts

After 30 DAT, mung bean plants were evaluated for yellowing or necrosis symptoms of wilt disease. For this 20 plants were sampled from each treatment and the disease severity was recorded on a 0-4 visual scale of the leaves, according to (Zhao et al., 2012) in which 0 = plants with no symptoms, 1 = wilting of >25 % , 2 = wilting of 25-50 %, 3 = wilting of 50-75 %, and 4 = wilting of 75-100 %. Disease index (%) was calculated as follows:

$$\text{Disease index (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of plants observed}} \times 100$$

To monitor the presence of A32 in mung bean rhizosphere soil, the plants were carefully uprooted after 30 days and 1 g soil associated with roots were taken and serially diluted in sterile distilled water and plated in ISP2 media supplemented with nalidixic acid (20 µg/ml) to inhibit other soil bacteria. After incubation of seven days, the emerging colonies were counted. Likewise, the fungal counts were determined by plating in potato dextrose agar plates (Suarez Moreno et al., 2019).

5.2.2.7. Root colonization studies

To determine the ability of A32 for mung bean plant colonization, the roots were examined through microscopy. The mung bean roots were cut into pieces 2 cm long and fixed in 2.5 % glutaraldehyde in phosphate buffer at 4 °C for 12 h. After incubation, the

samples were washed with phosphate buffered saline and post-fixed in 2% osmium tetroxide for 2 h and dehydrated using different concentrations of ethanol. The dehydrated samples were dried and transferred onto aluminium stubs with double-stick adhesive carbon tape. The samples were then treated with standard protocols as described in Chapter 2 and the presence of *Streptomyces* on root surfaces were monitored (Gopalakrishnan et al., 2015).

5.2.2.8. GC -MS analysis of A32 culture crude extract

Gas chromatography–mass spectrometry analysis (GC-MS) was performed to profile the possible bioactive compounds present in A32 extract. The experiment was performed as described in Chapter 4.

5.2.2.9. Toxicity studies of A32 culture filtrate in L6 skeletal muscle cells

The cytotoxicity of A32 culture filtrate was determined according to Scudiero et al. (1988) as described in Chapter 3.

5.2.2.10. Statistical analysis

The data were subjected to one- way Analysis of Variance (ANOVA) using SPSS (version 20.0; IBM SPSS). Data for plant growth study was presented as mean± standard deviation. Statistical significance was evaluated using DMRT (Duncan's Multiple Range Test) and a $p< 0.05$ was considered to demonstrate a significant difference.

5.3. Results

5.3.1. Poisoned food technique

Maximum percentage of radial growth inhibition was obtained by *A. fumigatus* (75 %) while a minimum percentage was exhibited by *C. gleosporiodes* (52 %). The radial mycelial inhibition percentage for *F. oxysporum* 1322 and *F. oxysporum* 4814 were 65 and 60 % respectively (Figure 5.1).

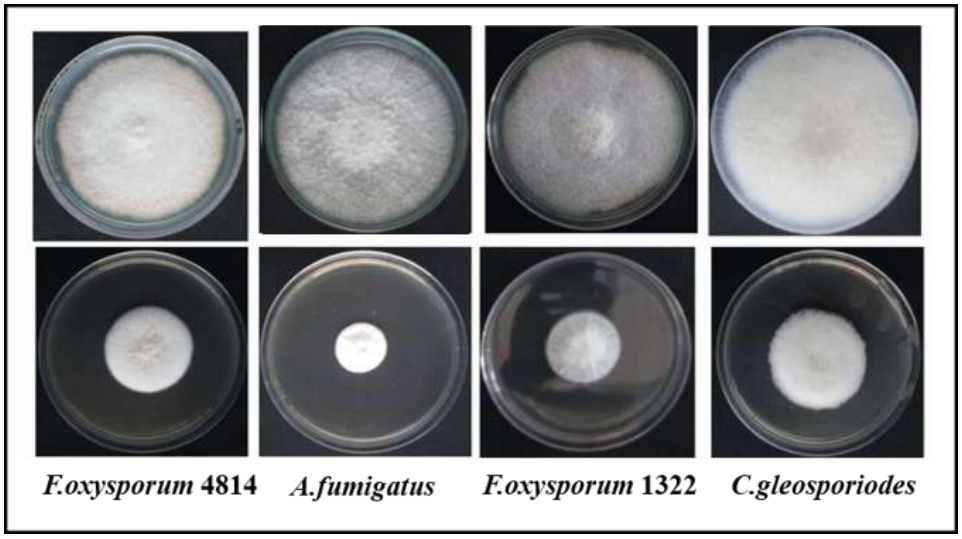

Figure 5.1: Mycelial radial growth inhibition in presence of A32 crude extract.

5.3.2. Effect of A 32 crude extract on fusarial wilt fungus

From antifungal experiments, it is observed that the hyphae did not develop massive growth on plates with A32 crude extracts. Abnormal hyphae were seen in treated plates where their cytoplasm was uneven in distribution and shrinkage and cellular leakage was also observed. The increase in PI fluorescence implicated that the membrane disruption leading to cell impermeable dye intake integrity could be due to the direct action of A32 (Figure 5.2a and 5.2b).

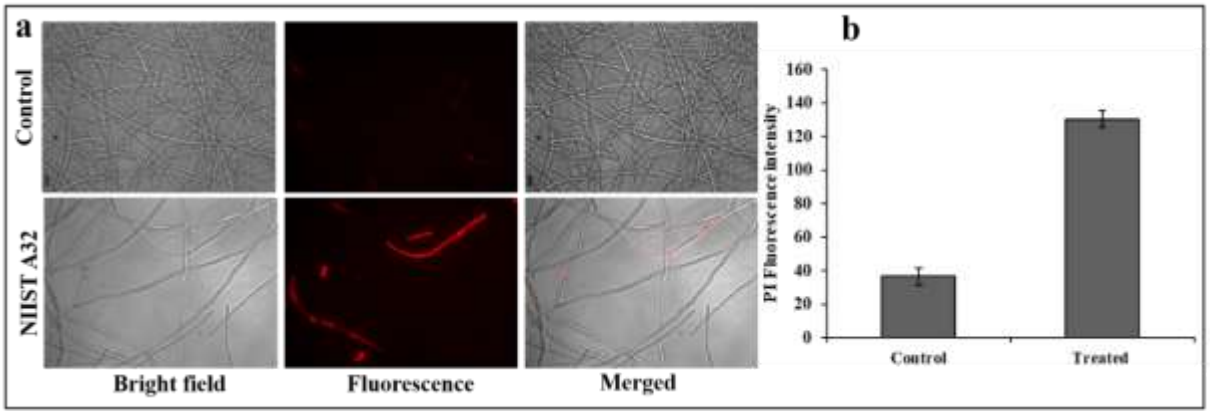

Figure 5.2: (a) Fluorescence microscopic image of control and A32 treated *F. oxysporum* stained with propidium iodide (b) PI fluorescence intensity of both control and A32 treated samples.

5.3.3. Stability of culture filtrate

While storage under refrigerated conditions, the culture filtrate of A32 retained its 80 % inhibitory activity towards *F. oxysporum* up to 3 months. After three months, the antifungal activity was started to reduce to 72 % and 68 % in 4th and 5th month respectively (Figure 5.3).

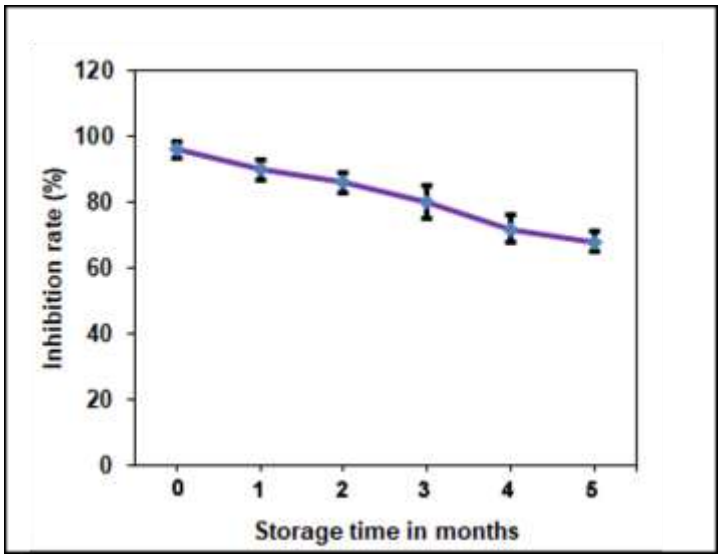

Figure 5.3: Antifungal effect of A32 fermented broth under storage time**.**

5.3.4. Determination of plant growth promotion and biocontrol traits

In vitro tests suggest that A32 could produce plant growth promoting traits such as IAA, phosphate solubilization, siderophores, volatiles, HCN, ammonia and extracellular enzymes. With colorimetric method, IAA production was estimated as 84.92 µg/ml. The phosphate solubilization efficiency was calculated as 68.94±0.91 %. The strain was positive for siderophores by producing orange colour around the colony. A32 also shown brown colour in filter paper in HCN production test and the volatiles from the strain inhibited mycelium of *F. oxysporum* by 55.55 % (Figure 5.4). The strain exhibited positive for cellulase and amylase, but not chitinase and protease.

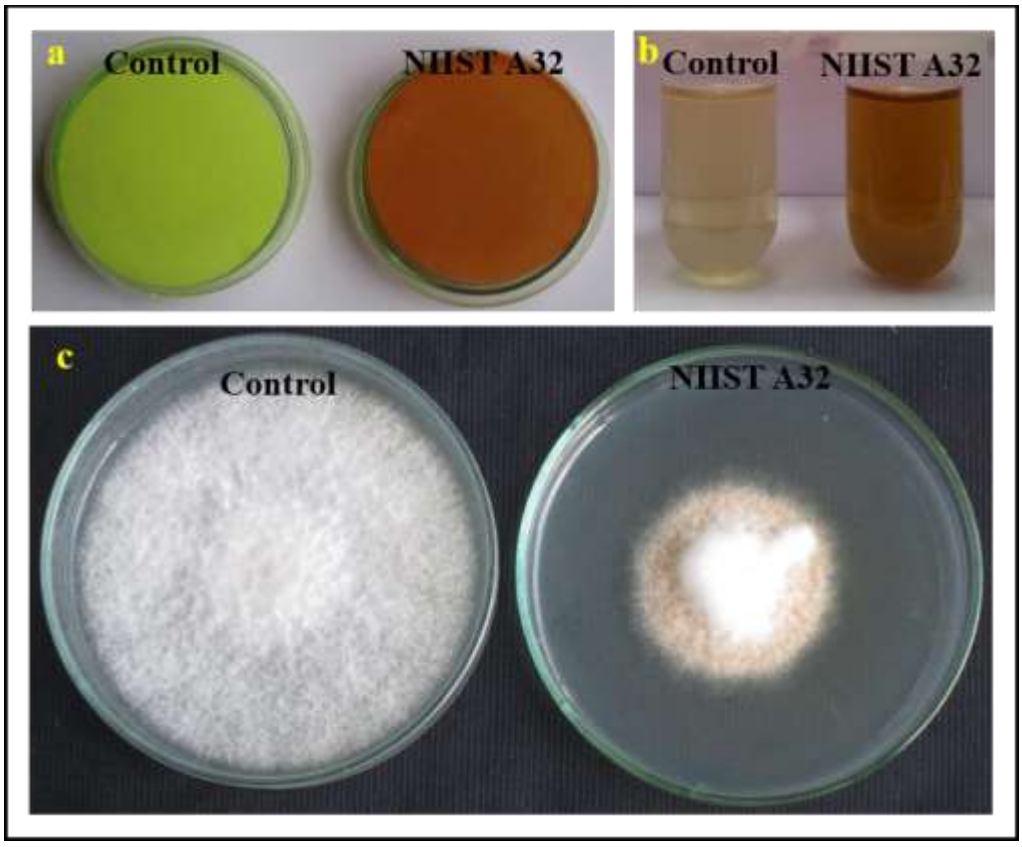

Figure 5.4: Plant growth promoting traits exhibited by A32; a, HCN production; b, ammonia production and c, *F. oxysporum* ITCC 4814 mycelial inhibition by volatiles**.**

5.3.5. Effect of A32 on mung bean growth promotion and yield

5.3.5.1. Percentage germination

Difference in percentage of germination was observed in various treatments. All seeds were germinated in control pots. The germination percentage in F, P+F and P treatments were 95, 80 and 40 respectively (Figure 5.5).

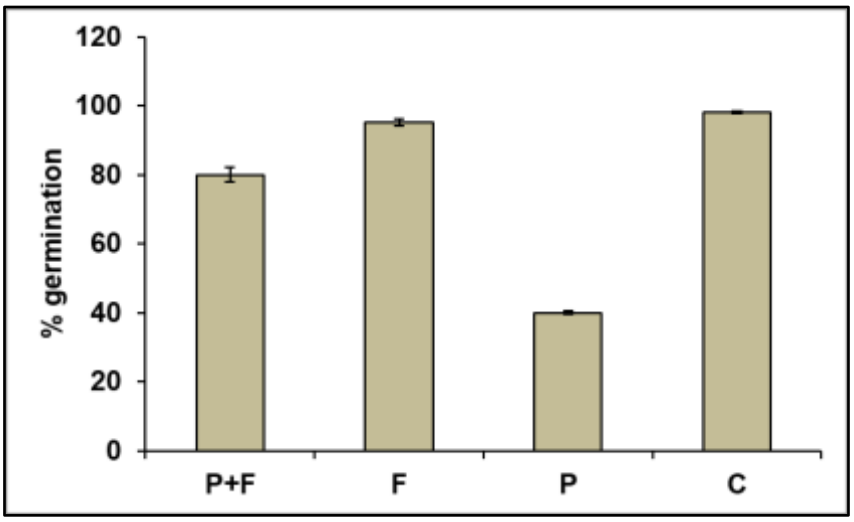

Figure 5.5: Percentage of gemination of mung bean seeds in different treatments.

The effect of various treatments in mung bean plants is depicted in figure 5.6.

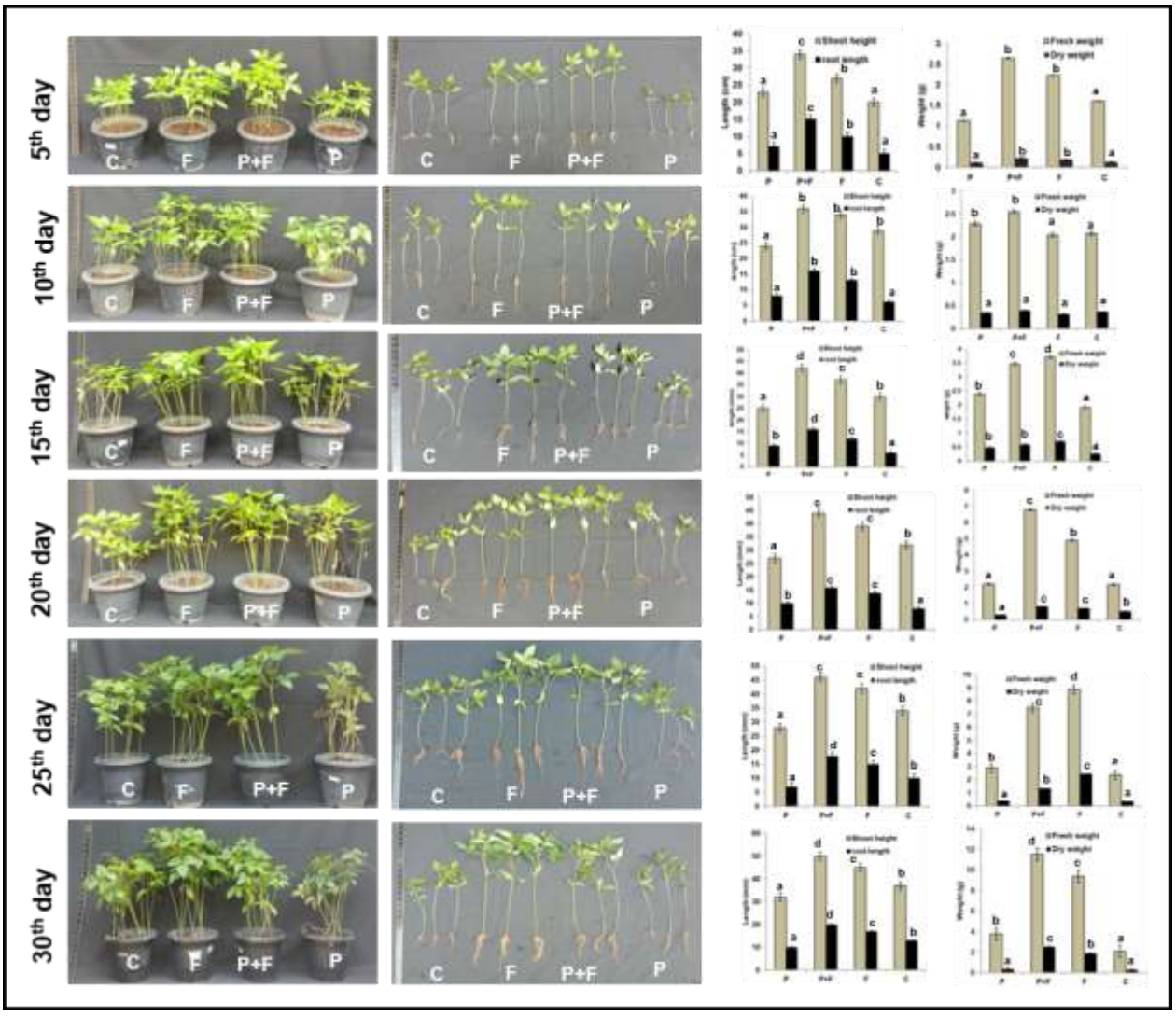

Figure 5.6: Effect of different treatments on plant growth promotion in mung bean plants (P, pathogen alone; P+F, pathogen +A32; F, A32 alone and C, Control**.** Alphabets with same letters are not significantly different at $p < 0.05$)

5.3.5.2. Shoot height and root length

As on 30 DAT, maximum shoot height was exhibited by P+F (48.85 cm) treatment followed by F (46.42 cm) which was 65.59 and 57.35 percent increase over the control Likewise, in shoot height maximum root length as on 30 DAT was recorded with P+F treatment (17.42 cm) followed by A32 alone (15.83 cm). The maximum per cent

increment of treatments over the control was recorded by P+F (50.56 %) followed by F (37.42 %) treatment as depicted in Figure 5.7a.

5.3.5.3. Fresh and dry weight

Maximum fresh weight among the treatment was recorded by P+F (11.59 g) followed by A32 alone (6.38 g). The percent increase was recorded highest for P+F treatment (305.24 %) followed by F (123.07 %). The maximal dry weight of the treated plants was recorded by P+F (2.17 g) and is succeeded by F (1.24 g) on 30 DAT. The treatment with P+F recorded the best increase of dry weight (442.5 %) which was followed by F (211.25 %) treatment (Figure 5.7b).

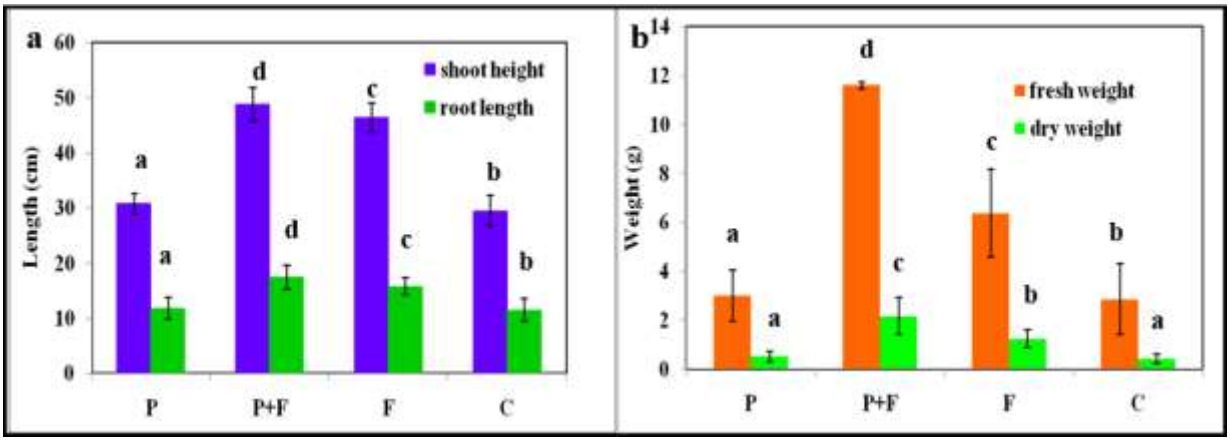

Figure 5.7: Effect of various treatments on growth promotion in mung bean plants (a, shoot height and root length; b, fresh weight and dry weight).

5.3.5.4. Chlorophyll content

An enhanced level of chlorophyll a, chlorophyll b and total chlorophyll was recorded in the P+F treatment and F treatment over the pathogen alone (P) (Table 5.1). Application of A32 was recorded the maximum increment of 4.25, 1.53 and 5.78 mg/g respectively for chlorophyll a, chlorophyll b and total chlorophyll followed by P+F

treatment (2.71, 2.29 and 5.00 mg/g). From this table, the treatment P+F was found to exhibit 192.76, 78.24 and126.17 percent increase respectively for chlorophyll a, chlorophyll b and total chlorophyll when compared to pathogen alone.

Table 5.1: Chlorophyll content on 30 DAT.

Treatment	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophyll (mg/g)
P	0.93±0.08^{a}	1.29±0.027^{b}	2.21±0.032^{a}
P+F	2.71±0.08^{c} (192.76)	2.29±0.06^{d} (78.24)	5.00±0.010^{b} (126.17)
F	4.25±0.104^{d} (359.38)	1.54±0.041^{c} (19.3)	5.79±0.063^{c} (161.63)
C	1.97±0.02^{b}	0.62±0.018^{a}	2.18±0.415^{a}

Data within parentheses are percentage of increase compared to pathogen alone. Values are expressed as mean± standard deviation. Mean values with the same letters in each column are not significantly different at $p < 0.05$ (Duncan's Multiple Range test).

5.3.5.5. Number of pods and seed yield

An enhancement in the number of pods over the pathogen is evident from the Table 5.2. The number of pods per pot, seeds per pod, seed weight and seed yield per pot were increased significantly over the pathogen alone treated plants.

Table 5.2: Effect of A32 treatment on yield.

	Number of pods pot^{-1}	Seeds per pod	100 seed weight (g)	seed yield per pot (g)
P	20±1.69^{a}	3.5±0.223^{a}	4.95±0.32^{a}	5.35±0.32^{a}
P+F	28.5±0.89^{b} (42.50)	4.67±0.21^{b} (33.42)	6.2±0.17^{b} (31.31)	7±0.29^{c} (30.84)
F	26.67±0.61^{b} (33.35)	4.67±0.42^{b} (33.42)	6±0.26^{b} (21.21)	8.21±0.16^{d} (53.45)
C	19.33±0.49^{a}	3.67±0.33^{a}	5.87±0.24^{b}	6.23±0.21^{b}

Data within parentheses are percentage of increase compared to pathogen alone.
Values are expressed as mean± standard deviation.
Mean values with the same letters in each column are not significantly different at $p < 0.05$ (Duncan's Multiple Range test).

5.3.6. Induction of systemic resistance under spit-root conditions

Efficiency of A32 to suppress fusarial wilt of mung bean through induction of systemic resistance (ISR) was also determined using split-root system (Figure 5.8). In the disease control treatment (seedlings inoculated only with pathogen), a high disease incidence (70.06 %) was recorded on mung bean plants on 30 DAT. The typical wilt symptoms such as yellowing symptoms, reduced growth and vigour on wilted plants were observed. The disease severity index and population count of both A32 and pathogen are given in Table 5.3. The disease severity index was reduced to 26.66 % in P+F treatment which is 61.96 % lower than pathogen alone. The average number of A32 present in the rhizoplane soil in P+F and F treatment was estimated as 9×10^8 and 36×10^8 respectively.

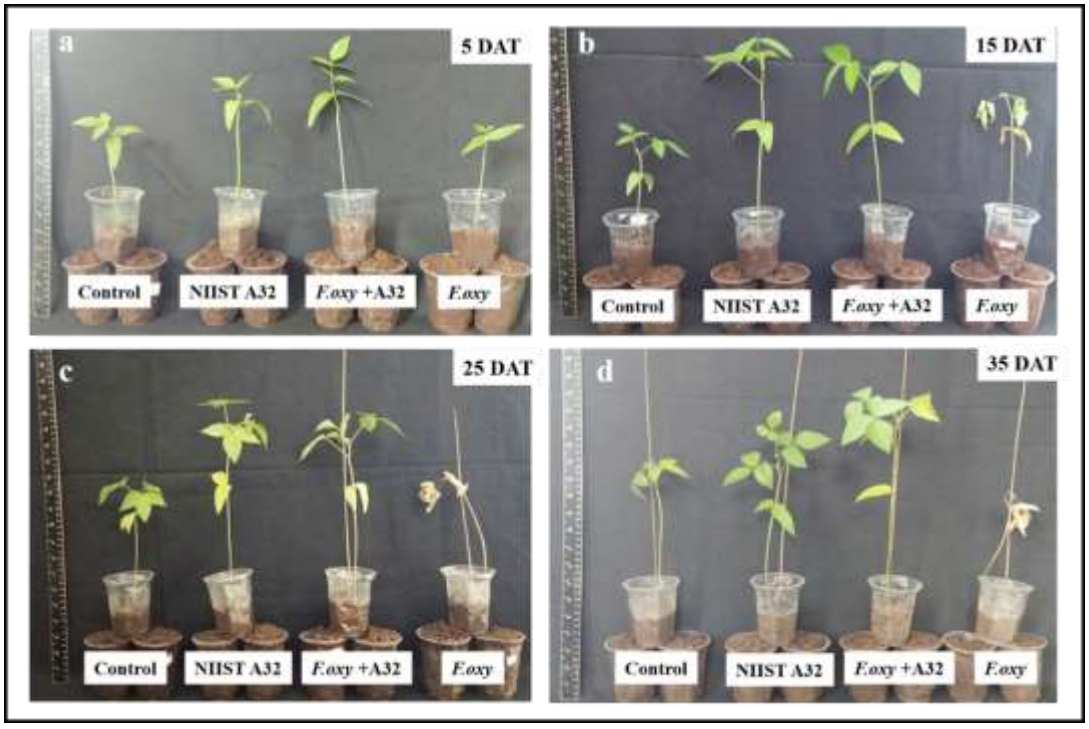

Figure 5.8: Split root experiments showing reduction in disease incidence in mung bean plants.

Table 5.3: Disease severity index (DSI) and A32, pathogen counts after 30 days

Treatments	Disease severity index (%)	Strain A32 counts in rhizosphere soil (10^8CFU/g)	Pathogen counts in rhizosphere soil (10^3CFU/g)
P	70.06^c	0^a	426^c
P+F	26.66^b (61.91)	9^b	98^b
F	0^a	36^c	0^a
C	0^a	0^a	0^a

Alphabets indicate significant differences between treatments (P≥0.05) from Duncan's multiple range tests.

Data within parentheses is percentage of reduction over pathogen alone.

Values are from three different experiments.

5.3.6.1. Pathogenesis-related enzymes and compounds:

5.3.6.1.1. β-1,3 glucanase

Total activity of β-1,3 glucanase enzyme was found to have increased in all the treatments with the progress of time (Figure 5.9a). It is clear from the data obtained that the highest activity of this enzyme was found in P+F (1716.21 µM) followed by control (1479.27 µM) and F (1464.86 µM) on 30 DAT. From the figure, on 25 DAT, the enzyme activity was almost same and drastically decreased in pathogen alone treatment on 30 DAT. In case of F treatment, the enzyme activity was increased up to 25 DAT (1497.29 µM) and the enzyme level was decreased on 30 DAT (1464.86 µM).

5.3.6.1.2. Total phenolic content

The treatments P+F and F exhibited a higher level phenolics content up to 30 DAT were 18.31 and 19.2 mg respectively. The lower level was exhibited by control (15.22 mg) and pathogen alone treatment (7.61 mg) on 30 DAT. In case of pathogen alone treatment after 5 DAT, the total phenolic content was reduced up to 30 DAT (Figure 5.9b).

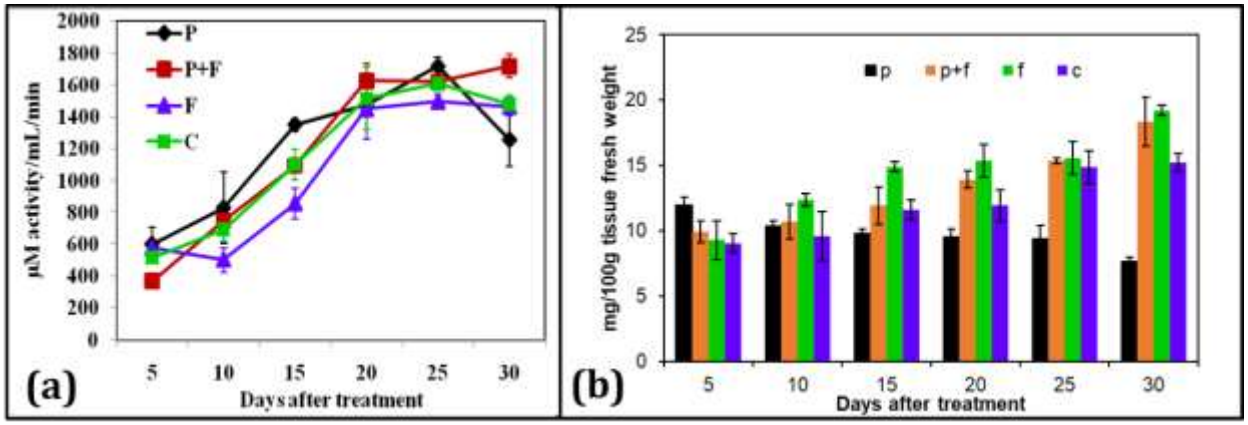

Figure 5.9: Effect of various treatments on pathogenesis related enzymes and compounds (a, β-1,3 glucanase and b, total phenol content)

5.3.6.2. Determination of defense related enzymes

5.3.6.2.1. PAL

The activity of PAL in plants with various treatments in different days up to 30 DAT are as given in Figure 5.10a. From this figure it is evident that highest activity of PAL was noted in P+F treated plants (22.42 units) whereas, lowest activity was recorded in control plants (7.48 units) as on 30 DAT. In case of pathogen treated plants, level of enzyme activity was increased rapidly up to 20 DAT (16.88 units) and after that a fall in

its level was noted. The activity of enzyme in plants treated with F and P+F was found to be increased in all the days of observation.

5.3.6.2.2. POD

Here too, the total activity of POD in plants treated with only pathogen showed an increase up to 25 DAT (0.215 units) and then exhibited a decrease in rest of the period (Figure 5.10b). Highest activity of peroxidase enzyme was observed in treatment with P+F (0.351 units) followed by F (0.275 units) on 30 DAT. A steady increase in the activity of enzyme was recorded in case of plants treated with the bacterial strain throughout the period of observation. It is evident from the figure that the total activity of POD in plants treated with P+F was higher when compared to pathogen alone.

5.3.6.2.3. PPO

Total activity of PPO enzyme was found to have increased in both P+F and F treatments with the progress of time (Figure 5.10c). It is clear from the data obtained that the highest activity of this enzyme was found in P+F (3900 units) followed by F (3750 units) and control (3023 units) treatments on 30 DAT. From the figure, maximum PPO activity was recorded with pathogen alone treatment up to 20DATwith a slight reduction on 25 and 30 DAT. The treatments P+F and F exhibited the same pattern of increase for the entire duration with maximum activity on 30 DAT.

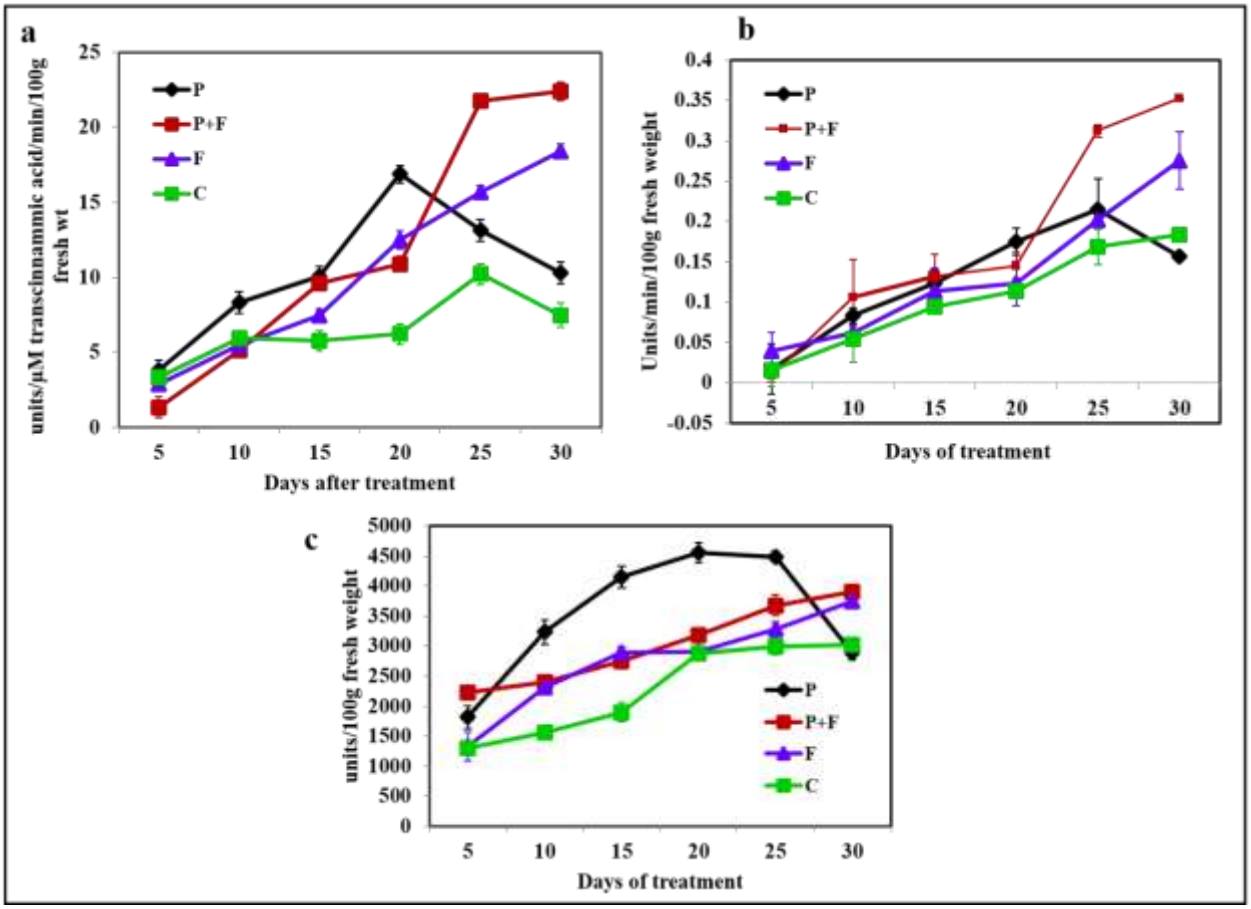

Figure 5.10: Effect of different treatment on the induction of defense related enzymes (a, PAL; b, POD and c, PPO).

5.3.7. Root colonization experiments

The microscopic images of mung bean plant roots clearly establish the colonization ability of A32 in plant roots (Figure 5.11).

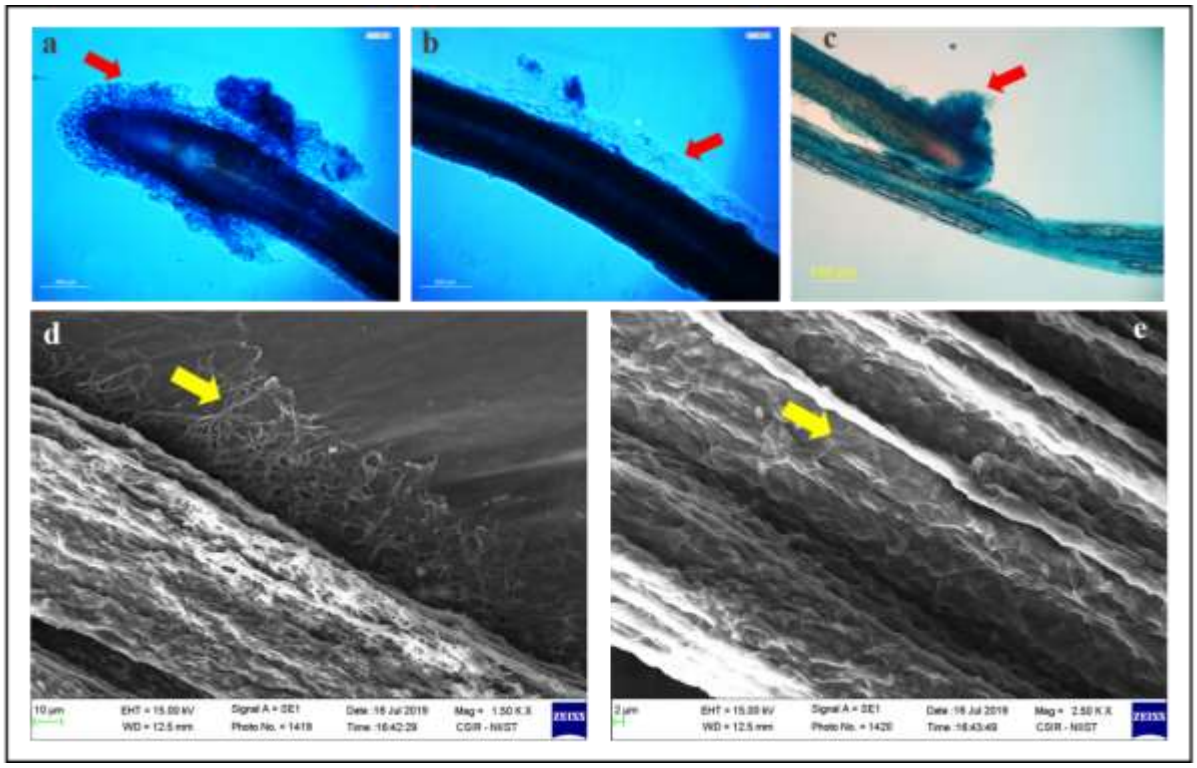

Figure 5.11: Root colonization of A32 in mung bean plant roots. Arrow marks indicate mycelial structures of A32 (a-c, Light microscopic images of roots; d-e, scanning electron microscopic images).

5.3.8. GC -MS analysis of A32 culture crude extract

The GC-MS results revealed 6 major and 13 minor compounds present in A32 extract (Table 5.4). The spectral data and chemical structures of the major compounds are illustrated in figure 5.12 and figure 5.13.

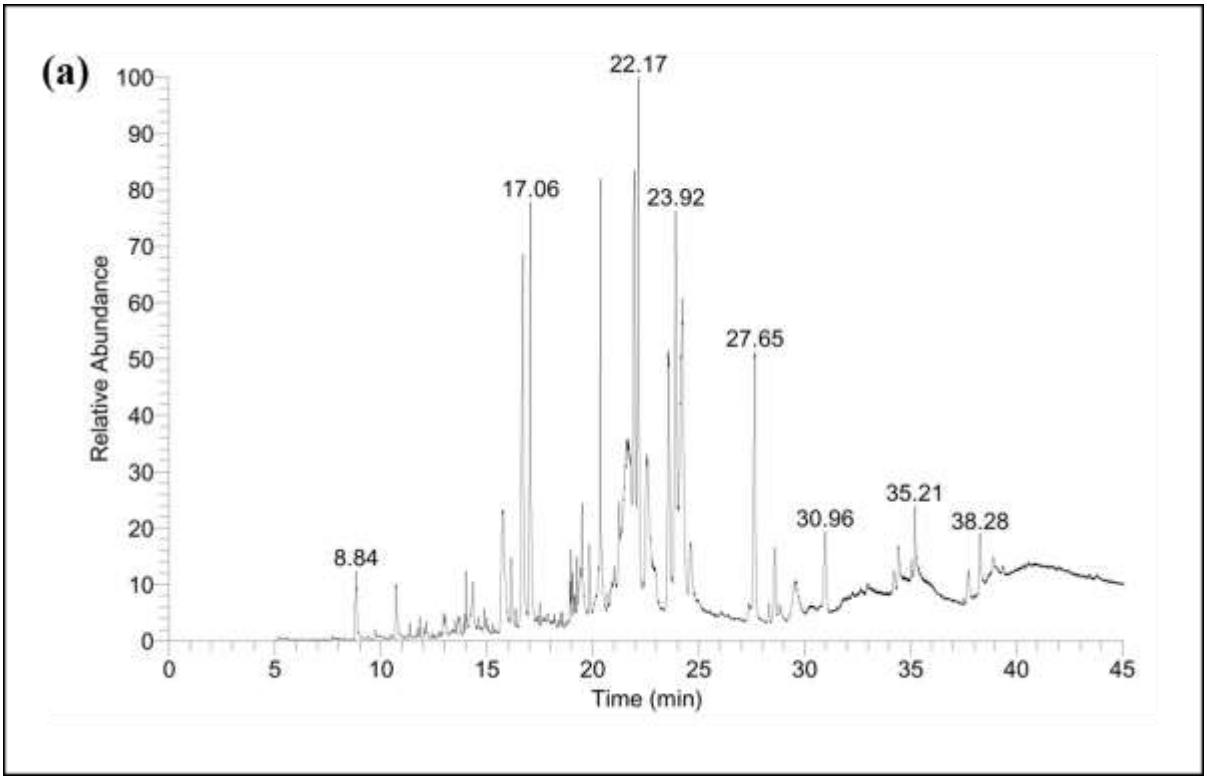

Figure 5.12: (a) GC-MS chromatogram and (b) individual peaks of major compounds detected in A32 (i, pyrrole 2-carboxamide; ii, Benzeneacetamide; iii, o-veratramide; iv, 4a-Hydroxy-6,8-dimethyl-2,8-dihydro-4ah-pyrimido[5,4-E][1,2,4]triazine-5,7 -dione; v, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-; and vi, 1,4-Dimethoxy-6,7,8,9-tetrahydro-5-benzocycloheptenone).

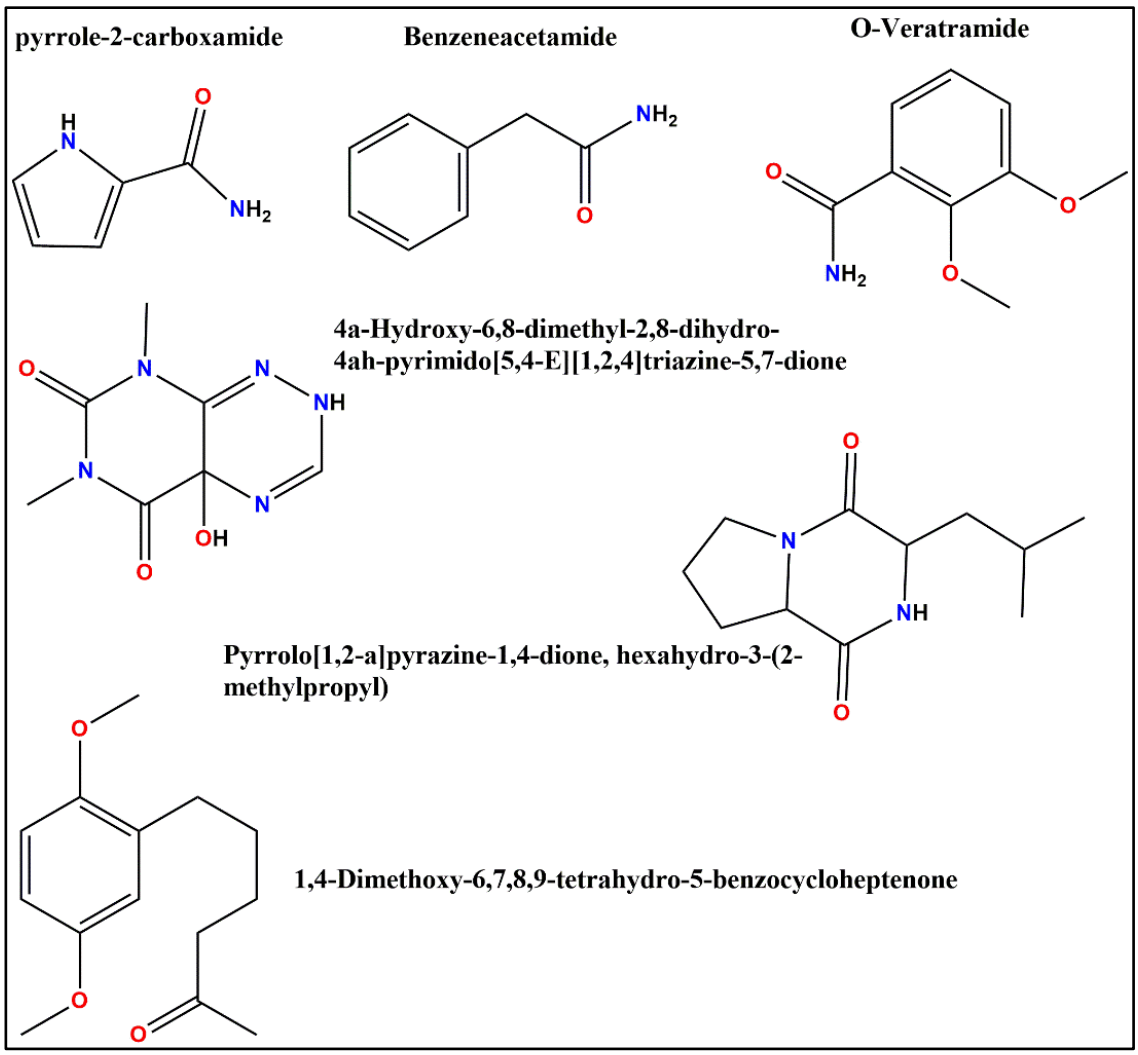

Figure 5.13: Major chemical constituents detected in A32 extract.

Table 5.4: Chemical constituents detected in A32 extract.

RT	Compounds detected in A32 extract	Area (%)	Activity reported	Reference
Major compounds (3% and above)				
15.76	Pyrrole-2-carboxamide	3.49	No activity reported	Chen et al., 2015
17.06	Benzeneacetamide	6.60	Antimicrobial, antitumor	Balachandran et al., 2012
20.37	o-Veratramide	4.52	Antibacterial	Al-Salman, 2019
21.95	4a-Hydroxy-6,8-dimethyl-2,8-dihydro-4ah-pyrimido[5,4-E][1,2,4]triazine-5,7 -dione	6.56	No activity reported	This study
23.91	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	7.50	Antimicrobial, antioxidative, antitumor	Siddharth and Vittal 2018
27.65	1,4-Dimethoxy-6,7,8,9-tetrahydro-5-benzocycloheptenone	5.74	No activity reported	This study
Minor compounds (0.45- 2%)				
14.03	2-Methyl-3-methoxy-4H-pyran-4-one	0.45	No activity reported	This study
14.34	1H-Pyrrole-2-carboxylic acid	1.14	Antimicrobial	Nguyen et al., 2015; Hassan et al., 2016
16.15	Benzamide	1.20	Antimicrobial, antioxidative	Yang et al., 2015
18.97	Dodecanoic acid	0.71	Antibacterial	Kiran et al., 2014
19.50	2,3-Dimethoxybenzoic acid	1.37	No activity reported	This study
19.84	Octahydropyrano[3,2-b]pyridin-6-one	0.89	No activity reported	This study
24.62	1H-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl	1.31	No activity reported	This study
28.59	2,5-di-tert-Butyl-1,4-dimethoxybenzene	1.40	No activity reported	This study
29.55	2,5-Piperazinedione,3,6-bis(2-methylpropyl)-	0.80	Antimicrobial	Al-Dhabi et al., 2016
30.97	6,7,8,9-Tetrahydro-1,2,3-trimethoxy-9-methyl-5H-benzocycloheptene	1.84	No activity reported	This study
34.43	Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'- (phenylmethyl)-, (5'à,10à)-	0.78	No activity reported	This study
37.72	Isoxazolo[2,3-b][1,2]benzoxazine-2-carboxylic acid, decahydro-4-phenyl-8a-[(trimethylsily l)oxy]-, methyl ester	0.69	No activity reported	This study
38.27	2-Acetylamino-3-phenylpropionicacid, 1-carbamoylethyl ester	1.04	No activity reported	This study

5.3.9. Toxicity studies of A32 culture filtrate in L6 skeletal muscle cells

The toxicity studies reveal that the fermentation broth from A32 has no effect on cytotoxicity in L6 rat skeletal muscle cells (Figure 5. 14). The cell viability was more than 99% up to a concentration of 50 µg/ml (Table 5.5).

Table 5.5: Viability of L6 cells of A32 culture filtrate

Concentration of culture filtrate (µg/ml)	L6 Cell viability (%)
2	152.78
5	109.18
10	104.22
20	109.07
50	99.89

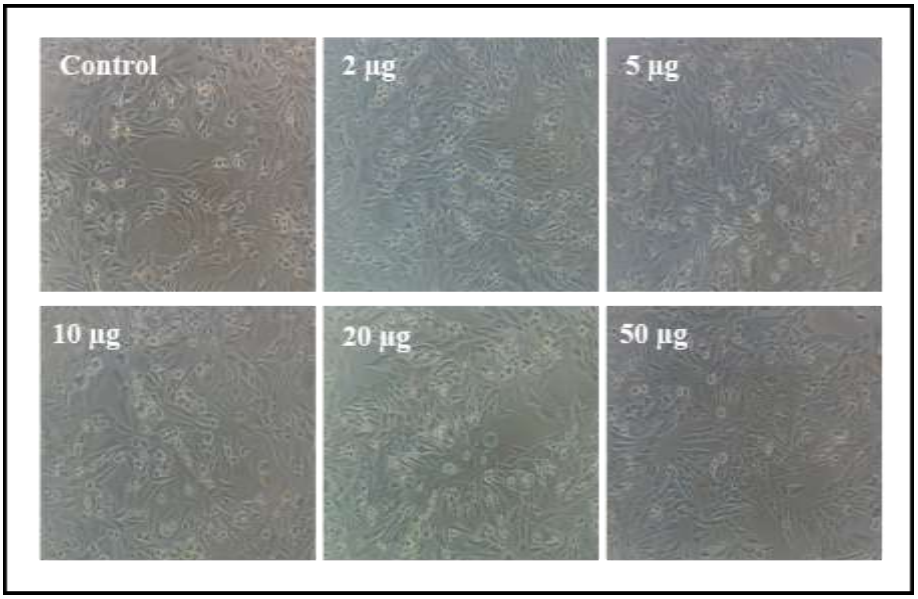

Figure 5.14: L6 cell images treated at various concentrations of A32 extract.

5.4. Discussion

Sustainable agriculture practices seek alternative approaches for current chemical usage in the agricultural fields to alleviate the toxic effect of chemicals. The usage of these chemical compounds drastically affects not only humans and animals, but also generate resistance in target organisms after a prolonged usage. In this regard, usage of native microorganisms has proven an efficient system for plant growth promotion and disease control in agricultural fields. Such beneficial microorganisms have been reported for their plant growth promotion and biocontrol potential, but we still need to investigate the effect of these organisms against the plant diseases. The plant growth promotion properties of many PGPR including *Azotobacter*, *Azosprillum, Bacillus, Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia, Streptomyces* and *Variovorax* have been well reported (Glick, 2012; Hassan et al., 2019; Goswami and Suresh, 2020).

Actinomycetes were studied for their applicability in the areas of plant growth promotion, plant interaction and biocontrol. They could be potential targets as biocontrol agents (BCA) since they have many properties that control diseases, increase nutrient supply and enhance growth of plants (Palaniyandi et al., 2013; Newitt et al., 2019). These are mesophilic and widespread in natural environments and continue to be extensively screened for their potential for producing useful compounds. Ultimately, this approach may be combined with others to prevent losses and damages caused by plant diseases.

Mung bean legume yield in India have been reduced due to infection with fungal pathogens in both seed or seedling stage, out of which fusarial wilt caused by *F. oxysporum* has increased substantially in recent years and yield losses of up to 80% were

reported in the susceptible mung bean cultivars (Pandey et al., 2018). The most common approach to reduce the disease incidence is the application of fungicides. But improper usage of fungicides associated with fungicide resistance has been reported from several countries (Price et al., 2015). Therefore, biological method of disease control which is ecologically safe and effective at low doses would be beneficial. Among actinobacteria, *Streptomyces* spp. were widely reported for their potential to control many phytopathogens including *Fusarium* sp. (Abbasi et al., 2019; Anusha et al., 2019). However, we do not have an efficient biocontrol agent to reduce the fusarial wilt disease in mung bean.

In the present investigation, we aimed to isolate a *Streptomyces* strain NIIST A32 having antagonistic potential to many bacterial and fungal pathogens from Nelliyampathy forest hills, an unexploited area of natural sources in Western Ghats region of Kerala. Generally, underexplored areas harbour many potential strains and those can be exploited for agricultural use. The development of biocontrol agents against various plant diseases needs screening of large number of isolates. The strain was selected from 140 actinomycete isolates through *in vitro* plating technique from Nelliyampathy forest hills. The second-round screening includes the assessment of the antagonist for plant growth and biocontrol traits. Finally, the selected candidate was used for preventing the disease under nursery conditions.

Biocontrol agent selection is not easy since many screening methods must be applied before application in the fields (Rooney et al., 2019). First of all, the bio-formulation should not be toxic to humans and/or to the environment (Vurukonda et al.,

2018). In our study, the crude metabolite from A32 had no toxic effect on the normal cells tested under *in vitro* conditions. Moreover, the organisms in the bio-formulation must retain their viability and efficiency for a prolonged time. This study presents a *Streptomyces* strain viable for five months and retained its antagonistic activity against the test fungus. PI has been reported to be a useful fluorescent dye for staining membrane impermeable nucleic acid. Examination of PI uptake by *F. oxysporum* showed that the fungal cells treated with A32 culture filtrate displayed an increase in the red fluorescence level when compared to that of the untreated cells. Xu et al. (2019) also found that the antagonistic activity of S. *hygroscopicus* OsiSh-2 attributed to the damage of cell wall integrity of rice blast pathogen, *M. oryzae*. In our investigation, fungal inhibitory activity obtained through poisoned food technique also substantiate the antagonistic activity of crude metabolite from the strain. The antifungal results indicated that the strain could be used as biocontrol agents against different plant diseases.

Many researchers have demonstrated significant biocontrol activity of antimicrobial actinomycetes against *F. oxysporum* (Cuesta et al., 2012; Wang et al., 2016). A study conducted by Zhao et al. (2018) described the suppression of watermelon Fusarium wilt disease and plant growth promoting ability of a rhizobacterium, *B. amyloliquefaciens* JDF 35. In another investigation, treatment with *S. albospinus* CT205 enriched bio-organic fertilizer significantly inhibited *Fusarium* wilt in cucumber plants (Wang et al., 2016). The plant fresh weights in the actinobacterial treatment were higher than those of the organic fertilizer treatments, demonstrating that bio-organic fertilizer with *S. albospinus* CT205 not only reduced *Fusarium* wilt but also promoted plant growth. A similar condition was observed in our results with the biocontrol strain A32. The mung

bean plants treated with A32 strain exhibited significant increase in plant growth parameters such as shoot height, root length, fresh and dry weights than that of untreated plants. There could be many reasons for this phenomenon; for example, the actinobacterial strain may produce biologically active substances, such as amino acids and peptides, that stimulate the growth of plants (Zhang et al., 2008). The plant growth promoting traits such as IAA production and phosphate solubilization were also observed in the strain. The yield assessment in mung bean plants have also demonstrated that the strain was able to increase the seed yield per pot, number of pods per plant and seeds per pod in treated plants. This is also in agreement with (Gopalakrishnan et al., 2015) where the plant growth promoting *Streptomyces* spp. significantly increased total grain yield in chick pea plants.

Successful plant-microbe interaction is essential for better plant growth promotion and disease control activities. This is dependent on the bacterial population in the soil and rhizosphere competence (Lakshmipathi et al., 2019). Interestingly, A32 was also able to colonize on the surface of plant roots, and hence, the PGP effects along with the colonization studies affirm the potential candidature of the strain. Field experiments with this isolate may be required to determine if they can colonize the roots under natural conditions.

The biocontrol agents also contribute to defense mechanisms such as induction of systemic resistance in host plants. These defense mechanisms can cause notable changes in plants leading to resistance against pathogen (Kannojia et al., 2019). The induced resistance against plant pathogens using *Streptomyces* spp. biocontrol agents could be an eco-friendly and promising strategy. In an investigation, the induced resistance

against *Magnaporthe oryzae* in rice seedling by a biocontrol agent *Streptomyces* JD211 was evaluated and reported significant increase in the activities of different enzymes such as catalase, phenylalanine ammonia-lyase (PAL) and β-1,3-glucanase in presence of the strain (Shao et al., 2018). Similarly, in our study, mung bean leaves under A32 treatment exhibited higher enzyme activities of PAL, POD, PPO and β -1,3-glucanase than those under control, indicating that the increased activity of defense enzymes is the plant's response to fungal infection. Naz et al. (2018) also suggests that the induced resistance prior to challenge infection elevates the level of some defense compounds and sensitizes the plants to rapidly produce some compounds after infection and thereby, provide protection against the disease. These results suggest that the initiation of induced systemic resistance in plants correlates with the increased activity of defense related enzymes and pathogenesis related proteins (PR). Moreover, the ISR may be dependent on the accumulation of phenolic compounds and phytoalexins. In our study, the total phenolic contents were higher in plants treated with A32 compared to pathogen and uninoculated controls. Similar results were obtained for many other studies on biocontrol (Bekkar et al., 2018). A good biocontrol agent should not possess any toxicity to humans and other organisms. Toxicity studies in the present investigation depicts the safe use of the culture filtrate from the strain since it didn't possess any toxicity in L6 skeletal muscle cells.

Attempts on identification of antimicrobial compound is convenient in developing biocontrol agents and the genus *Streptomyces* is notable for their ability to produce bioactive compounds especially antibiotics. Actinomycetes are used in the production of a diverse array of antibiotics including aminoglycosides, macrolides, β-lactams, peptides,

polyenes, polyethers and tetracyclines (Subramani and Aalbersberg, 2012). GC-MS play a major role in the natural product discovery from bacteria including *Streptomyces* sp. (Zheng et al., 2019). In this study, 6 major and 13 minor compounds were detected in A32 crude extract through GC-MS analysis. Among them, Pyrrolo [1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- was found to have 7.5 % followed by Benzeneacetamide (6.6 %), 4a-Hydroxy-6,8-dimethyl-2,8-dihydro- 4ah-pyrimido [5,4-E] [1,2,4]triazine-5,7 -dione (6.56 %), 1,4-Dimethoxy-6,7,8,9-tetrahydro-5-benzocycloheptenone (5.74 %), o-Veratramide (4.52 %) and Pyrrole-2-carboxamide (3.49 %). Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- was previously reported for antimicrobial and antioxidant activities (Tan et al., 2015; Awla et al., 2016; Siddharth and Vittal, 2018). Benzeneacetamide was previously detected in *Methylobacterium* sp. found to have antimicrobial and cytotoxic activities as reported by Balachandran et al. (2012). *o*-Veratramide, also known as 2, 3-dimethoxy benzamide along with other volatile compounds produced by *B. atrophaeus* CAB-1 was found to inhibit *Botrytis cinerea* (Zhang et al., 2013). There were no literature reports for the presence of 4a-Hydroxy-6,8-dimethyl-2,8-dihydro- 4ah-pyrimido [5,4-E][1,2,4] triazine-5,7 -dione and 1,4-Dimethoxy-6,7,8,9- tetrahydro-5- benzocycloheptenone in *Streptomyces* sp.

Furthermore, out of thirteen minor compounds, only three were reported to have biological activities. 1H-Pyrrole-2-carboxylic acid (PCA) produced by *S. griseus* shown antagonism against *Phytophthora capsici* H7602 as investigated by Nguyen et al. (2015). Benzamides from *Streptomyces* sp. has been suggested to have antimicrobial, antioxidant and cytotoxic activities (Shaaban et al., 2012; Yang et al., 2015). Lastly, 2,5-

Piperazinedione, 3,6-bis(2-methylpropyl)- has been detected in an antimicrobial *Streptomyces* sp. Al-Dhabi-1 isolated from a hot spring (Al-Dhabi et al., 2016). According to GC-MS analysis, most of the detected compounds are well recognized for their antimicrobial activity. Hence, we propose that a combined action of these compounds might be one reason behind the biocontrol activity of the strain. It is also noted that the metabolites with no functional annotation in the GC-MS spectrum may also contribute to antifungal activity and need to be elucidated in further studies**.** From the present investigation, a schematic model for antifungal action exhibited by A32 was constructed which include production of antibiotics, siderophores, phytohormones and elicitors for induction of systemic resistance as depicted in figure 5.15.

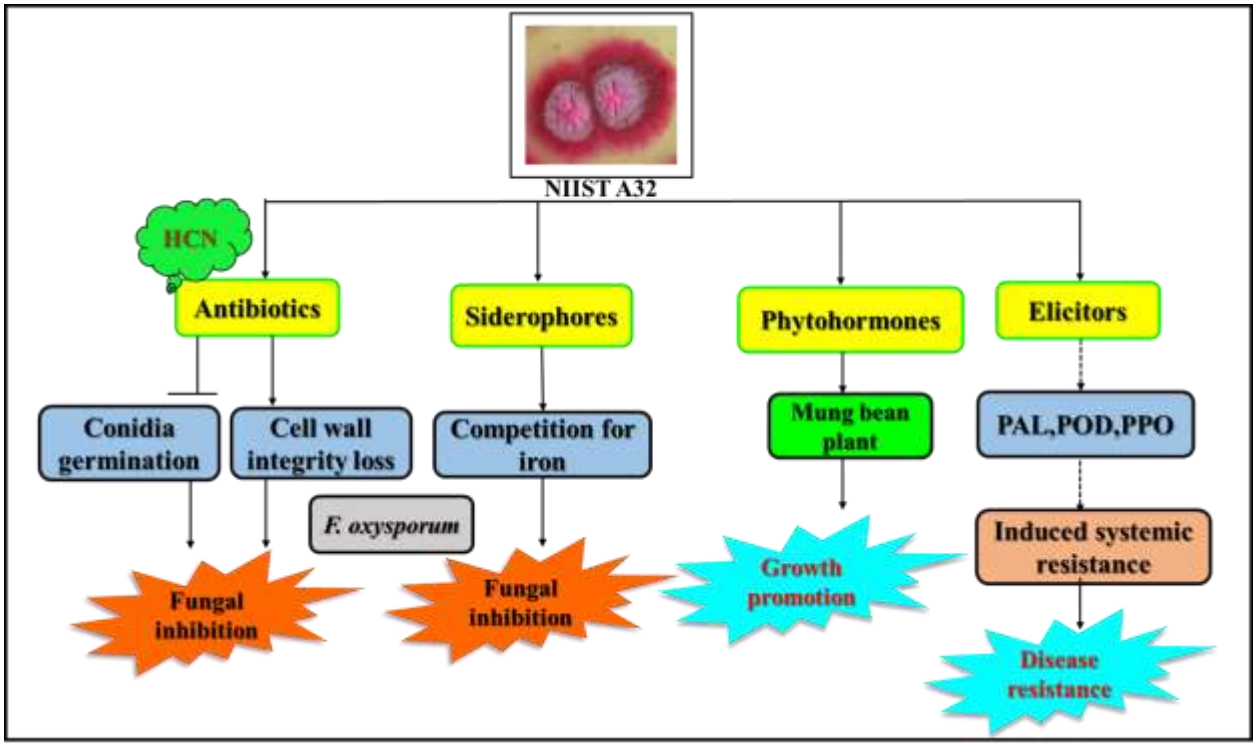

Figure 5.15: Possible mode of action of A32 in plant growth promotion and induction of systemic resistance in mung bean.

5.5. Conclusion

In conclusion, *S. luteosporeus* NIIST A32 possess plant growth promotion traits both *in vitro* and *in vivo*. The ethyl acetate extract was recorded significant inhibition against *F. oxysporum* ITCC 4814. The increased fluorescence in propidium iodide stained fungal cells followed by microscopic analysis revealed the loss of membrane integrity in A32 treated fungal cells. The GC-MS analysis of crude extract shown six major compounds (with an area of 3 to 8 %): Pyrrole-2-carboxamide, Benzeneacetamide, o-Veratramide, 4a- Hydroxy-6,8-dimethyl-2,8-dihydro-4ah-pyrimidom [5,4-E] [1,2,4] triazine-5,7 -dione, Pyrrolo [1,2-a] pyrazine-1,4-dione,hexahydro-3- (2-methylpropyl)- and 1,4-Dimethoxy-6,7,8,9-tetrahydro-5-benzocycloheptenone and thirteen minor compounds (with an area of 0.50 to 3%). It is also noted that the combined action of the detected antifungal compounds could be responsible for this antagonism. Pot experiments showed an increased growth percentage of mung bean in terms of shoot height (57.35 cm), root length (37.42 cm), fresh (123.07 g) and dry weights (211.25 g), total chlorophyll (161.63 mg/g) and total phenol content (26.14 mg) in A32 treated plants compared to control. Application of A32 exhibited increased yield percentage of mung bean plants in terms of number of pods per pot (37.97), seeds per pod (27.24) and seed yield per pot (31.78 g) compared to untreated plants. The strain was able to reduce wilt disease severity by 61.94 % compared to pathogen alone under nursery conditions. Studies of induction of systemic resistance in mung bean plants shown an increased level of the activities of defense related enzymes *viz*. phenyl alanine ammonia lyase, peroxidase and polyphenyl oxidase as well as the pathogenesis related compounds such as phenol content in plants treated with A32. Scanning electron microscopic images of the plant roots confirmed the

successful colonization of the strain in the mung bean roots. The stability studies exhibited that of culture filtrate from the strain retained more than 60 % inhibitory activity against the fungal pathogen up to five months. Cytotoxicity studies in rat skeletal L6 muscle cells confirmed the non-toxicity of A32 culture filtrate up to a concentration of 50 µg/ml. Hence, studies under nursery conditions demonstrated that metabolites of the strain possess biocontrol properties against fusarial wilt in mung bean and reveals the biofertilizer application of this strain after field studies.

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Chapter 6

Summary and Conclusion

Due to the high frequency of multidrug resistant pathogens in the world, there has been increasing demand for searching effective antibiotic molecules from soil actinomycetes from diversified ecological niches. Majority of bacterial natural products discovered so far came from organisms that inhabit in the soil. Since soil itself is a highly porous mixture of minerals and organic matter, this plays host to a rich and diverse community of bacteria. Actinomycetes, a group of bacteria has many applications in the field of microbial biotechnology. Since they were successfully employed by many researchers for the mining of bioactive metabolites as well as in sustainable agricultural practices, hence the present investigation was carried out. Here, the randomly selected soil samples from seven different sites of Kerala were used for the isolation of actinomycetes. The samples were collected from undisturbed areas and having no previous history of fertilizer applications and human interference. One hundred and forty strains were isolated from twenty-five soil samples through the traditional plating technique. All of them possess the resemblance to the genera *Streptomyces* based on morphological characterization. This thesis mainly describes the experiments and observations related to the antagonistic nature of actinomycete strains. The following could be summarized as the major results of the present research:

All strains were evaluated for their antibacterial efficacy against two indicator human pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus*. Among them, sixteen strains recorded potent antibacterial properties and were further screened against eleven bacterial pathogens. Of these, two strains designated as A30 and A32 identified based on 16SrRNA gene sequencing as *Streptomyces nogalater* NIIST

A30 and *Streptomyces luteosporeus* NIIST A32 exhibited maximum inhibition against all the bacterial pathogens. These two strains were obtained from Nelliyampathy forest soil of Kerala region. From antifungal experiments, A32 recorded significant antagonism against plant pathogenic bacteria so that its efficacy was exploited for agricultural applications. On the other hand, A30 had shown significant zone of inhibition against human pathogenic fungi such as *Candida albicans* (30±1.5 mm) and *C. tropicalis* (35±0.57 mm), so that its efficacy was studied for medicinal use. Analysis of the antimicrobial biosynthetic type I polyketide synthase gene showed that the antimicrobial activity of A30 was associated with the production of bioactive secondary metabolites.

The next challenge was to investigate the best fermentation medium and fermentation conditions such as pH, temperature and incubation time for maximum metabolite production from the strains A30 and A32. Standardization of fermentation conditions for the strains A30 and A32 was optimized using One Factor at a Time method. Here, optimum conditions for A30 was found to be pH 7.0, temperature 35 °C and incubation for 7 days. However, the optimum conditions for A32 was found to be pH 7.0, temperature 30 °C and incubation for 10 days. Standardization of various fermentation media provided that ISP4 and GSMB was best media for antibacterial production for A30 and A32 respectively. The significant media components for maximum metabolite production of A30 was optimized through response surface methodology employing Plackett-Burman and Box-Behnken designs. From Plackett-Burman result analysis, media components such as starch, $(NH_4)_2SO_4$ and K_2HPO_4 were found to have a significant effect on maximum metabolite production. Furthermore,

optimization using Box-Behnken response surface design confirms the statistical significance of the fitted model. This optimization resulted in a maximum antibacterial activity of 28.15±1.5 mm which is an 86.66 % increase in comparison with that obtained in the control broth. Thus, the statistical trial using RSM for optimization of medium compounds by *Streptomyces nogalater* NIIST A30 was validated to be a potent and useful tool. The ethyl acetate extract from strain A30 exhibited antioxidant properties with IC_{50} value of 30 µg/ml and had no cytotoxicity towards L6, H9c2 and RAW 264.7 cell lines up to a concentration of 50 µg/ ml.

Further experiments concluded that ethyl acetate extract of A30 harbour some compounds with antimicrobial activities when analysed by GC-MS. This revealed the presence of benzeneacetamide (12%), Hexanoic acid, 2-ethyl (5.59%) and benzeneacetic acid (5.13%) previously reported for antimicrobial activities. The bioactivity guided fractionation of ethyl acetate extract from the strain yielded an active fraction FC5 with antifungal properties against *Candida* sp. FC5 showed a minimum inhibitory concentration of 78.12 and 156.25 µg/ml for *C. albicans* and *C. tropicalis* respectively. Structural analysis of FC5 treated *Candida* cells revealed several malformations. The dye PI uptake assay was used to demonstrate FC5 induced cell membrane permeabilization. Time-kill kinetics, PI uptake assay, intracellular accumulation of reactive oxygen species (ROS) and nuclear damage suggested that FC5 have multiple detrimental effects on target pathogen which ultimately result in cell wall disruption and killing. Therefore, the active compounds present in FC5 will be a good template for further design and development as antifungal agents against *Candida* sp.

Moreover, *S. luteosporeus* NIIST A32 possess plant growth promotion traits both *in vitro* and *in vivo*. The ethyl acetate extract was recorded significant inhibition against *F. oxysporum*. The increased fluorescence in propidium iodide stained fungal cells followed by microscopic analysis revealed the loss of membrane integrity in A32 treated fungal cells. The GC-MS analysis of crude extract shown six major compounds (with an area of 3 to 8 %): Pyrrole-2-carboxamide, Benzeneacetamide, o-Veratramide, 4a- Hydroxy-6,8-dimethyl-2,8-dihydro-4ah-pyrimidom [5,4-E] [1,2,4] triazine-5,7 -dione, Pyrrolo [1,2-a] pyrazine-1,4-dione,hexahydro-3- (2-methylpropyl)- and 1,4-Dimethoxy-6,7,8,9-tetrahydro-5-benzocycloheptenone and thirteen minor compounds (with an area of 0.50 to 3%). It is also noted that the combined action of the detected antifungal compounds could be responsible for this antagonism. Pot experiments showed an increased growth percentage of mung bean in terms of shoot height (57.35 cm), root length (37.42 cm), fresh (123.07 g) and dry weights (211.25 g), total chlorophyll (161.63 mg/g) and total phenol content (26.14 mg per 100 g) in A32 treated plants compared to control. Application of A32 exhibited increased yield percentage of mung bean plants in terms of number of pods per pot (37.97), seeds per pod (27.24) and seed yield per pot (31.78 g) compared to untreated plants. The strain was able to reduce wilt disease severity by 61.94% compared to pathogen alone under nursery conditions. Studies of induction of systemic resistance in mung bean plants shown an increased level of the activities of defense related enzymes *viz.* phenyl alanine ammonia lyase, peroxidase and polyphenyl oxidase as well as the pathogenesis related compounds such as phenol content in plants treated with A32. Scanning electron microscopic images of the plant roots confirmed the successful colonization of the

strain in the mung bean roots. The stability studies exhibited that of culture filtrate from the strain retained more than 60 % inhibitory activity against the fungal pathogen up to five months. Cytotoxicity studies in rat skeletal L6 muscle cells confirmed the non-toxicity of A32 culture filtrate up to a concentration of 50 µg/ml.

Results of the present investigation showed that the strains A30 and A32, isolated from Nelliyampathy forest soils, an unexplored biodiversity hot spot possessed promising antimicrobial activities. This again proved that actinomycetes are good sources of unique natural bioactive metabolites. However, further characterization of active compounds from A30 are needed for their optimum utilization toward the medicinal purposes. In future, this may lead the way towards large scale profitable production of antimicrobials and bioactive compounds. Furthermore, our studies under nursery conditions demonstrated that metabolites of the A32 strain possess biocontrol properties against fusarial wilt in mung bean and reveals the biofertilizer application of this strain in near future agriculture after successful field trials.

Annexures

Composition of various microbiological media

1. Actinomycete Isolation Agar (AIA)

Sodium caseinate	- 2.0g
L- Asparagine	- 0.1g
Sodium propionate	- 4.0g
K_2HPO_4	- 0.5g
$MgSO_4.7H_2O$	- 0.1g
$FeSO_4.7H_2O$	- 0.001g
Distilled water	- 1 litre
Agar	- 15.0 g

pH is 8.1 ± 0.2

2. Basal Liquid Medium (BLM)

$NaNO_3$	- 2.0g
K_2HPO_4	- 1.0g
$MgSO_4.7H_2O$	- 0.5g
KCl	- 0.5g
$FeSO_4.7H_2O$	- 0.01g
Distilled water	- 1 litre

3. Bennet's Agar (BA)

Yeast extract	- 1.0g
Hiveg extract	- 1.0g
Hiveg hydrolysate	- 2.0g
Dextrose	-10.0g
Agar	- 15.0g
Distilled water	- 1litre

pH is 7.3 ± 0.2

4. Czapek Dox Broth (CDB)

Sucrose	- 30.0g
Sodium nitrate	- 2.0g
K_2HPO_4	- 1.0g
$MgSO_4.7H_2O$	- 0.5g
KCl	- 0.5g
$FeSO_4.7H_2O$	- 0.01g
Distilled water	- 1 litre

pH is 7.3 ± 0.2

Czapek Dox Agar – CDB supplemented with 20g Agar.

5. Glucose Soybean Meal Broth (GSMB)
   Glucose - 10.0g
   Soyabean meal - 10.0g
   Sodium chloride - 10.0g
   Calcium carbonate - 1.0g
   Distilled water - 1 litre
   pH is 7.5± 0.2

6. Glycerol Asparagine media (ISP5)
   2 – Asparagine - 1.0g
   K_2HPO_4 - 1.0g
   Glycerol - 1.0 ml
   Trace salt solution - 1.0 ml
   Trace salt solution contains,
   $FeSO_4$. $7H_2O$ - 0.001g
   Manganous chloride - 0.001g
   Zinc sulphate - 0.001g
   Distilled water - 1 litre
   pH is 7.4 ± 0.2.
   ISP5 Agar – ISP5 broth supplemented with 20g Agar.

7. Yeast Malt Broth (ISP 2)
   Yeast extract - 4.0g
   Malt extract - 10.0g
   Dextrose - 4.0g
   Distilled water - 1 litre
   pH is 6.2 ± 0.2
   ISP2 Agar – ISP2 Broth supplemented with 20g Agar.

8. Inorganic Salts Starch Agar (ISP 4)
   Starch (soluble) - 10.0g
   K_2HPO_4 - 1.0g
   $MgSO_4.7H_2O$ - 1.0g
   Sodium chloride - 1.0g
   Ammonium sulphate - 2.0g
   Calcium carbonate - 2.0g
   $FeSO_4$. $7H_2O$ - 0.001g
   Manganous chloride.$7H_2O$ - 0.001g

Zinc sulphate. $7H_2O$ - 0.001g
Distilled water - 1 litre
ISP 4 Agar – the ISP4 broth supplemented with 20g Agar.
pH is 7.2± 0.2

9. Kenknight and Munaier's Agar (KM)
   Dextrose - 1.0g
   $K(HPO_4)_2$ - 0.10g
   Sodium nitrate - 0.10g
   $MgSO_4.7H_2O$ - 0.10g
   KCl - 0.10g
   Distilled water - 1 litre
   Agar - 15.0g
   pH is 7.2± 0.2

10. Kuster's Broth (KB)
    Glycerol - 1.0g
    Casein - 0.3g
    KNO_3 - 2.0g
    K_2HPO_4 - 2.0g
    Soluble starch - 0.5g
    Asparagine - 0.1g
    $FeSO_4. 7H_2O$ - 0.01g
    Calcium carbonate - 0.02g
    $MgSO_4.7H_2O$ - 0.05g
    Distilled water - 1 litre
    pH is 7.0 ± 0.1
    Kuster's Agar – KB supplemented with 20g Agar.

11. Mueller Hinton Agar (MHA)
    Beef infusion - 300.0g
    Casein acid hydrolysate - 17.5g
    Starch - 1.5g
    Agar - 15.0g
    Distilled water - 1 litre
    pH is 7.3 ± 0.1

12. Nutrient broth (NB)
    Peptone - 5.0g
    Beef extract - 1.5g

Yeast extract - 1.5g
Sodium chloride - 5.0g
Distilled water - 1 litre.
Nutrient agar – Nutrient broth supplemented with 20g Agar.
pH is 7.3 ± 0.1

13. Potato Dextrose Broth (PDB)
    Potato Infusion from - 200.0g
    Dextrose - 20.0g
    Distilled water -1 litre
    Potato dextrose agar – PDB supplemented with20g Agar.
    pH is 5.6 ± 0.2

14. Sabouraud's Dextrose Broth (SDB)
    Dextrose - 20.0g
    Meat peptone - 10.0g
    Distilled water - 1 litre
    pH is 5.6 ± 0.2
    Sabouraud's Dextrose agar – SDB supplemented with20g Agar

15. Starch casein Broth (SCB)
    Starch - 10.0g
    K_2HPO_4 - 2.0g
    KNO_3 - 2.0g
    Casein - 0.3g
    $MgSO_4.7H_2O$ - 0.05g
    $CaCO_3$ - 0.02g
    $FeSO_4.\ 7H_2O$ - 0.01g
    Distilled water - 1 litre
    pH is 7.2 ± 0.1
    Starch casein Agar – SCB supplemented with 20g Agar.

16. Tryptone yeast extract broth (ISP1)
    Casein enzymatic hydrolysate - 5.0g
    Yeast extract - 3.0g
    Distilled water - 1 litre
    pH is 7.0 ± 0.2
    ISP1 Agar – ISP1 supplemented with 20 g Agar.

17. Oat meal Agar (ISP 3)

Oat meal	- 20.0g
Agar	- 15.0 g
Ferric sulphate heptahydrate	- 0.001 g
Manganese chloride tetrahydrate	- 0.001 g
Zinc sulphate heptahydrate	- 0.001g
Distilled water	- 1 litre

pH is 7.2 ±0.2

18. Glycerol Asparagine Agar (ISP 5)

L-Asparagine	- 1.0g
Dipotassium phosphate	- 1.0 g
Trace salt solution	- 1.0 ml
Agar	- 15.0 g
Distilled water	- 1 litre

Trace salt solution (1 ml)

Ferric sulphate heptahydrate	- 0.001 g
Manganese chloride tetrahydrate	- 0.001 g
Zinc sulphate heptahydrate	- 0.001g
Glycerol	- 1.0ml

pH is adjusted to 7.4 ± 0.2

19. Peptone yeast extract Iron Agar (ISP 6)

Peptic digest of Animal tissue	- 15.0g
Protease extract	- 1.0g
Yeast extract	- 1.0g
Ferric ammonium citrate	- 0.5g
Dipotassium phosphate	- 1.0g
Sodium thiosulphate	- 0.08g
Agar	- 15.0g
Distilled water	-1 litre

pH is adjusted to 6.7 ± 0.2

20. Tyrosine Agar (ISP 7)

L –Asparagine	- 1.0 g
L – Tyrosine	- 0.5 g
Dipotassium phosphate	- 0.5g
Magnesium sulphate	- 0.5 g
NaCl	- 0.5 g
Trace salt solution	- 1.0 ml
Agar	- 15.0g

Distilled water - 1 litre
pH is adjusted to 7.3 ± 0.1

Trace salt solution
Ferrous Sulphate.$7H_2O$ - 1.36 g
Copper chloride .$2H_2O$ - 0.027 mg
Cobalt chloride. $2H_2O$ - 0.04 g
Sodium molebdate. $2H_2O$ - 0.025 g
Zinc chloride - 0.02 g
Boric acid - 2.85 ml
Manganese chloride tetrahydrate - 1.8 mg
Sodium tartrate - 1.77 mg

21. Phosphate Buffered Saline (PBS)

Sodium chloride - 7.650g
Disodium phosphate -0.724g
Dipotassium hydrogen phosphate -0.210g
pH is adjusted to 7.2 ± 0.1

List of Publications

1. **Jubi J.**, Gopika V K., Drissya T., Dileep Kumar B.S. Endophytic bacterial strains induced systemic resistance in agriculturally important crop plants. In Ajay K & Radhakrishnan EK (Eds.) *Microbial endophytes: Functional biology and applications.,* 2020, Elsevier, USA (pp. 75-105).
2. Dileep Kumar B.S., **Jubi J.** Plant growth promoting rhizobacteria (PGPR) as a biological tool for augmenting productivity and disease control in agriculturally important crop plants. *Journal of Spices and Aromatic Crops* 2019, *28* (2), 77-95 (Equally contributed).
3. **Jubi J.**, Reshma U.R., Syama H.P., Jayamurthy P., Dileep Kumar B.S. Enhanced antibacterial metabolite production through the application of statistical methodologies by a *Streptomyces nogalater* NIIST A30 isolated from Western Ghats forest soil. *PLoS one* 2017, *12*(4), p.e0175919.
4. Arindam M., Rakesh M., Vijayakumar, **Jubi J.**, Dileep Kumar B.S., Ayyapanpillai Ajayaghosh. Supramolecular surface charge regulation in ionic covalent organic nanosheets for reversible exfoliation and controlled bacterial growth. *Angewandte Chemie* 2019, Doi: 10.1002/ange.201912363.
5. Chandrasekhar C., Jaice R., Mohini M. K., Sunil V., **Jubi J.,** Dileep Kumar B. S., Jayanta H., Lankalapalli R.S. Expedient synthesis of Indolo [2, 3-b] quinolines, Chromeno [2, 3-b] indoles, and 3-Alkenyl-oxindoles from 3, 3′-Di-indolylmethanes and Evaluation of Their Antibiotic Activity against Methicillin-Resistant *Staphylococcus aureus*. *ACS Omega* 2017, *2*(8), 5187-5195.
6. Reshma M.V., **Jubi J.**, Syamnath V.L., Habeeba V,P., Dileep Kumar B.S., Lankalapalli R.S. First report on isolation of 2,3,4-trihydroxy-5-methylacetophenone from palmyra palm (*Borassus flabellifer* Linn.) Syrup, its antioxidant and antimicrobial properties. *Food Chemistry* 2017, *228*, 491-496.
7. Emrin G., Nishanth K.S., **Jubi J.**, Bhaskara B., Lankalapalli, R.S., Morang P., Dileep Kumar B. S. Characterization of the bioactive metabolites from a plant growth-promoting rhizobacteria and their exploitation as antimicrobial and plant growth promoting agents. *Applied Biochemistry and Biotechnology* 2015, *176*(2), 529-546.

8. Nishanth K.S., Sreerag R.S., Rajesh L., **Jubi J.,** Dileep Kumar, B. S. Bala N. Protolichesterinic Acid: A prominent broad-spectrum antimicrobial compound from the Lichen *Usnea albopunctata*. *International Journal of Antibiotics* 2014, Article ID 302182.
9. **Jubi J.,** Dileep Kumar B.S. Interaction of *Streptomyces luteosporeus* NIIST A32 with mung bean (*Vigna radiata* L.) for crop management and induction of systemic resistance against wilt disease by *Fusarium oxysporum* (Manuscript to be communicated)

List of academic conferences/workshops

1. **Jubi J.,** Dileep Kumar B.S. Investigation of broad-spectrum antimicrobial, antioxidative potential of *Streptomyces nogalater* NIIST A30 isolated form Nelliyampathy forest soils of Western Ghats region. **Oral Presentation** *at the International seminar on Life Sciences for Sustainable Development: Issues and Challenges organized by University College, Trivandrum, Kerala, October 03-05, 2019.*
2. **Jubi J.**, Hima H., Dileep Kumar B.S. Induction of systemic resistance against Fusarial wilt in Mung bean through a plant growth promoting *Streptomyces luteosporeus* NIIST A32 and its metabolites**. Poster presentation** *at the National seminar on Frontiers in Biotechnology-Molecular, Epigenetic and Genomic Research Platforms in Healthcare and Food Security organized by Inter University Centre for Genomics and Gene Technology, University of Kerala, Thiruvananthapuram during March 1-3, 2017.*
3. **Jubi J.,** Dileep Kumar B.S. Antimicrobial and biocontrol potential of *Streptomyces luteosporeus* NIIST A32 isolated from forest soil. **Oral Presentation** *at 29th Kerala Science Congress held at Mar Thoma College, Thiruvalla, Kerala during January 28-30, 2017.*
4. Attended **National Workshop** on 'Soil to sequences', *organized by Microbial Type Culture Collection of CSIR- Institute of Microbial Technology (IMTECH), Chandigarh during March 02-12, 2015*.