Molecular Characterization and Evaluation of Antimicrobial potential of Actinobacteria Isolated from Lakes and Rivers of Mizoram

Thesis submitted in partial fulfilment of the requirement for the degree of
Doctor of Philosophy in Biotechnology

By

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Ph.D. Registration No: MZU/Ph. D. /711 of 22.05.2015

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2018

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CERTIFICATE

This is to certify that **Mr. Zothanpuia**, Ph.D. Scholar, Registration No. **MZU/Ph.D. /711 of 22.05.2015** has work on the Thesis entitled "**Molecular Characterization and Evaluation of Antimicrobial Potential of Actinobacteria Isolated from Lakes and Rivers of Mizoram**" under my supervision, for the award of the Degree of Doctor of Philosophy in the Department of Biotechnology, Mizoram University, Aizawl. He has fulfilled all criteria prescribed by the UGC (Minimum Standard and Procedure governing Ph.D. Regulations). He has fulfilled the mandatory publication (Publication enclosed) and completed Ph.D. course work.

This also certifies that the scholar has been admitted in the Department through an entrance test, followed by an interview as per clause 9(i) and (ii) of the UGC Regulation 2009.

Date: **Dr. Bhim Pratap Singh**

Declaration

I, **Zothanpuia,** hereby declare that the subject matter of this thesis entitled "**Molecular Characterization and Evaluation of Antimicrobial Potential of Actinobacteria Isolated from Lakes and Rivers of Mizoram"** is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Biotechnology.

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Acknowledgement

First and foremost, I am grateful to the almighty God, giving me good health and strength to undertake this research task and enabling me to its completion.

I would like to pay my deepest gratitude to my supervisor Dr. Bhim Pratap Singh for his guidance, support, help and providing all the required facilities to complete my research work. Words cannot express how grateful I am for his encouragement and patience.

I am also thankful to the Department of Biotechnology, New Delhi for the establishment of the DBT-BIF centre and DBT-State Biotech Hub in the Department. I would also like to specifically mention Prof. N. Senthil Kumar, Coordinator, DBT-BIF centre and DBT-State Biotech Hub for his encouragement and support throughout my Ph.D.

I am thankful to Dr. J. Bhattacharya, Head, Department of Biotechnology, Mizoram University for his kind cooperation and all the faculties and technical staff of the Department for their constant help and support.

Special thanks to;

Department of Science and Technology (DST), Government of India, New Delhi for providing the fellowship DST-YS (No:SERB/F/2501/2013-14) to complete my research works.

Ministry of Tribal Affairs (MOTA) for providing National fellowship for higher education of ST (NFST).

My labmates Dr. Ajit Kumar Passari, Vineet Kumar Mishra, Vincent Vineeth Leo, Lallawmsanga, Dhaneshwaree, Joanne Zote, Lalrokimi for their cooperation throughout the completion of my research.

My parents and all my family for their support, my beloved wife Rosie Lalmuanpuii and daughter Elena Lalthlamuanpuii, who are my source of inspiration.

Date: Zothanpuia

Abbreviations and Symbols

%	percentage
+ve	Positive
±	Plus or Minus
≤	Less than or equal to
° **C**	Degree Celsius
16S rRNA	16S-Ribosomal Ribonucleic Acid
AIA	Actinomycetes Isolation Agar
AIC	Akaike Information Criterion
ANOVA	Analysis Of Variance
BIC	Bayesian Information Criterion
BlAST	Basic Local Alignment Search Tool
bp	Base Pair
CCA	Colloidal Chitin Agar
CMCase	Carboxymethyl Cellulase
CYP	cytochrome P450 hydroxylase
d	Days
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNS	3,5-Dinitrosalicylic Acid
dNTPs	Deoxynucleotides
dTGD	dTDP-glucose-4, 6-dehydratase
e.g.	Exempli gratia: For example

ERIC	Enterobacterial Repetitive Intergenic Consensus
ESI-MS	Elecrospray ionisation-Mass Spectrometry
et al.	*et alii*: and others
etc.	et cetera: and other things
FEG-SEM	Field Emission Gun - Scanning Electron Microscopy
G+C	Guanine and Cytosine
GC-MS	Gas Chromatography-Mass Spectrometry
gyrB	Gyrase B-Subunit
h	Hour
Halo	Halogenase
ISP	International *Streptomyces* Project
ISP1	Tryptone Yeast Extract Broth
ISP2	Yeast Malt Extract Agar
ISP5	Glycerol Asparagine Agar
ISP7	Tyrosine agar
KA	Kuster's Agar
kb	Kilobyte
KS	Keto synthase
M	Molar
mg ml^{-1}	Milligram per Millilitre
MIB	Methylisoborneol
MIC	Minimum Inhibitory Concentration
mM	Milli Molar

mm	Millimeter
MTCC	Microbial Type Culture Collection
n	Number
NCBI	National Center of Biotechnology Information
ng	Nanogram
nm	Nanometre
NRPS	Non-ribosomal Peptide Synthetase
NJ	Neighbour-joining
ML	Maximum Likelihood
NA	Nutrient Agar
PCR	Polymerase Chain Reaction
pH	Negative Ion Of Hydrogen Ion Concentration
***phz*E**	aminodeoxyisochorismate synthase
PKS	Polyketide Synthase
PKV	Pikovskaya`S Medium
pmol	Pico-Mol
RNA	Ribonucleic Acid
rpoB	RNA Polymerase B- Subunit
SA	Streptomyces Agar
SCA	Starch Casein Agar
SCNA	Starch Casein Nutrient Agar
SD	Standard Deviation
sp.	Species

TAE	Tris Base, Acetic Acid and EDTA
TSA	Trypticase Soya Agar
TWYE	Tap Water Yeast Extract
U/ µl	Unit Per Micro Liter
UPLC	Ultra-Performance Liquid Chromatography
USA	United States of America
UV	Ultra-Violet
β	Beta
µM	Micromolar

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Chapter I

Introduction

1.1. General Introduction

Actinobacteria are Gram positive (+ve), aerobic, mostly filamentous and saprophytic unicellular prokaryotic microorganisms that constitute one of the largest taxonomic group and largest phyla within the kingdom/domain bacteria (Ludwig et al., 2012). Actinobacteria comprises of diverse morphological features from coccoid (e.g. *Micrococcus*), rod shape (e.g. *Arthrobacter*) to fragmenting hyphae (e.g. *Nocardiopsis*) forming branched mycelium (e.g. *Streptomyces*) (Goodfellow and Williams, 1983; Ventura et al., 2007) and many of them formed spores (Barka et al., 2016). They do not have distinct cell wall nevertheless producing non-septate and typically slender hyphae (Rosenberg, 2014). Actinobacteria were believed to be an intermediate group between bacteria and fungi, resembling the shape and morphology of fungus having aerial and substrate mycelium and were earlier called 'ray fungi'[which originates from Greek words ray (aktin) and fungi (mukēs)](Barka et al., 2016), while several of them are having bacterial like colonies (Waksman et al., 1940). Certain genera of actinobacteria reproduce by specialized structures called spores which generally were spiral and elongated and also exist as single cells or in chains of variable length and also help to survive under harsh and unfavourable conditions (Barka et al., 2016).

The morphological appearances, nature of colony and colour of the actinobacterial isolates remained so diverse on solid culture media and vary on different media (Zothanpuia et al., 2017). Certain actinobacterial colonies were found to be powdery while some of them were soft and sticky, attach firmly to agar surface in culture media (Passari et al., 2015). Several of them produce different pigments that are responsible for the colour of the substrate and aerial mycelia, the colour of

the aerial and substrate mycelium also vary among actinobacteria, the substrate mycelium helps in nutrients uptake and absorption for the growth and development of actinobacteria (Barka et al., 2016). The actinobacterial genomes are circular having approximately 3,300 coding sequences and are considered to have high guanine and cytosine (G+C) content in their genome. Among the actinobacteria, the G+C content ranges from around 50% in some genera like *Corynebacteria* to ≤ 70% in *Frankia* and *Streptomyces* (Ventura et al., 2007). They have been placed under the Phylum Actinobacteria, Class Actinobacteria, Sub-class Actinobacteridae, Family Actinomycetaceae, Order Actinomycetales. The Phylum consists of 5 Sub classes, 14 Sub-orders (Stackebrandt, 2000), 56 families and over 330 genera (Bae et al., 2016).

Actinobacteria are widely distributed in nature, largely isolated from aquatic systems generally from marine habitats and found to be more diverse in Oceans compared to fresh water (Goodfellow and Williams, 1983; Yuan et al., 2014). They represents more than 50% of the plankton in fresh water lakes and rivers, many of them are uncultivable and are also typical inhabitants of soil environments (Goodfellow and Williams. 1983), where they play an important role in the degradation of recalcitrant organic matter like lignocelluloses, chitin, etc. by the secretion of extracellular hydrolytic enzymes and serve important purposes in agriculture, recycle nutrients back into environment (Passari et al., 2015). They were also responsible for the production of secondary metabolites like geosmin and methylisoborneol (MIB) which gives earthy smell after the rain (Gerber and Lechevalier, 1965; Gerber 1983). They also play an important role in carbon cycle, however the exact ecological role and their influence in carbon cycle remains unknown (Kuznetsov, 1970). Several other secondary metabolites were also known

to be derived from actinobacteria and serve an important role in the production of clinically significant antibiotics (Sharma et al., 2016). Actinobacteria also inhabit inside the plant tissue (endophytic actinobacteria) that acts as a good bio-control agent to control fungal diseases, they play a vital role in plant biotechnology, enhancing the health of the host plants by supplying nutrition besides defending them, producing a variety of bioactive secondary metabolites for use in agriculture and pharmaceutical industry (Solans et al., 2011; Passari et al., 2015). Some genera of actinobacteria such as *Frankia* and *Streptomyces* served important drive for host plants due to their ability to fix nitrogen of non – leguminous plants and their capacity to inhibit phytopathogens (Hasegawa et al. 2006), other species including *Kocuria, Lefsonia, Rhodococcus, Saccaropolyspora,* etc. were also reported as endophytic actinobacteria (Passari et al., 2015). Actinobacteria are also found in association with lichens (Parrot et al., 2015).

Actinobacteria have been differentiated based on their ability to tolerate different conditions. There are mesophilic actinobacteria; having optimal growth at temperatures between 27° C and 30° C, majority of the actinobacteria are found to be mesophilic (Edwards et al., 1993). However, some actinobacteria like *Thermomonospora, Microbispora*, etc. were found to be thermophillic which can tolerate and grow at a temperature between 37-65° C and optimal growth temperature ranges from 50 to 60° C (Barka et al., 2016). Acidophilic actinobacteria (generally from terrestrial habitats) grow at pH range of 3-7 [optimum pH – 4.5.5] (Hagedorn, 1976). Actinobacterial genus *Streptacidiphilus* was found to be strictly acidophilic (Anandan et al., 2016). There are salt loving actinobacteria called halophilic actinobacteria commonly isolated from marine habitats, saline salts and

salt lakes. Extreme halophilic actinobacteria can grow in media containing 2.5-5.2 M salt concentration, several species of actinobacteria like *Micromonospora, Streptomyces* and *Rhodococcus* were largely isolated from saline habitat (Maldonado et al., 2005). Actinobacteria are also found to inhabit the gut system of different organisms where they were reported to play vital role in the uptake of nutrition, detoxification of certain toxic compounds, growth and protection against pathogens (Jami et al., 2015). The biosynthetic potential of *Streptomyces*, *Saccharomonospora*, *Micromonospora*, *Nocardiopsis*, *Arthrobacter*, *Kocuria*, *Microbacterium* and *Agromyces* from the gut microbiota of two freshwater fish species were also described (Jami et al., 2015).

Actinobacteria are the prevailing group of microbes responsible for the production of thousands of pharmaceutically and industrially beneficial biologically active secondary metabolites (Suthindhiran et al., 2013) and involved in the synthesis of thousands of valuable products such as enzymes (de Menezes et al., 2008; Chakraborty et al., 2015). A number of useful biologically active enzymes have been described from microbial origin such as cellulase which can degrade cellulose (Leo et al., 2016), amylase which are widely used in food, textiles and fermentation industries for degrading starch (Pandey et al., 2000) and lipases used widely in several industries like detergent, food, pharmaceutical, etc. (Schmid et al., 1998). Numerous other potential enzymes like proteases, L-asparaginases, catalases, chitinases and ureases were also described from different genera of actinobacteria (Sharmin et al., 2005; Dejong, 1972; Narayana et al., 2008; Saha et al., 2013; Latha and Dhanasekaran, 2013). Several isolates of actinobacteria were also reported for its potent probiotic properties (Das et al., 2006; You et al., 2007; Latha et al., 2016).

Moreover, probiotics derived from actinobacteria were further stated to enhance broiler production (Latha et al., 2016). There are bioactive secondary metabolites such as Anisomycin, bialaphos, phthoxazolin, hydantocidin, homoalanosin and herbicidines described for its herbicidal activity from actinobacteria (Yan, 1993). The larvicidal activities of extracellular secondary metabolites of actinobacteria such as tetranectin, avermectins, macrotetrolides, flavonoids, etc. were also reported (Vijayan and Balaraman 1991; Dhanasekaran et al., 2010; Rajesh et al., 2013). The flexibility of actinobacteria has contributed their use in other biotechnological applications such as phytohormones production, bioremediation of industrial wastes, biosurfactants/bioemulsifier (e.g. Cubicin), Vitamins (Rickes et al., 1948; Lichtman et al., 1949; Chakraborty et al., 2015). Pigments such as rhodomycin, actinomycin, granaticin, etc. were also described from several *Streptomyces* spp. (Anandan et al., 2016).

Actinobacteria represent an outstanding and remarkable source for the production of new bioactive secondary metabolites including antibiotics, antimicrobials, antitumor agent, antiparasitic, anticancer agents and enzymes (Stach et al., 2004; Goodfellow and Fiedler, 2010; Yuan et al., 2014). Secondary metabolites generally found in nature are the polyketides, terpenes, alkaloids, steroids and shikimic acid (Herbert, 1989). The phylum actinobacteria alone is accounting for the production of an approximately 75% of the total bioactive compounds comprising antibiotics and the remaining 25% were derived from fungi and other bacteria. Among different genera of actinobacteria, the genus *Stereptomyces* alone contributes more than 70% (Berdy, 2005; Das et al., 2006; Subramani and Aalbersberg, 2012). *Streptomyces* are still the main producers of

novel biologically active compounds with a wide range of activities including antimicrobial, anti-cancer agents and other pharmaceutically and biotechnologically useful compounds (Berdy, 2005; Kim et al., 2012; Saurav and Kannabiran, 2012; Wang et al., 2013; Rajan and Kannabiran, 2014; Ser et al., 2015). They are commonly distributed in various ecosystems like soils, fresh waters (rivers and lakes) and marine environments (Goodfellow and Williams, 1983).

The phylum actinobacteria is well known for its antibiotic production responsible for the production of two-third of the antibiotics commonly used in the present day which include several classes of antibiotics like aminoglycosides, ansamycins, anthracyclines, β-lactam and macrolides (Zothanpuia et al., 2018). Some of the most commonly used antibiotics derived from actinobacteria include Kanamycin, Streptomycin, Tetracyclines, Chloramphenicol, Neomycin, Erythromycin, Vancomycin, Rifamycin, Gentamicin, Daptomycin. Streptomycin, the first antibiotic derived from actinobacteria belongs to a class of aminoglycoside discovered by Waksman in 1943 from *Streptomyces griseus* which was used for the treatment of infections against *Mycobacterium tuberculosis* (Schatz et al., 2005). Tetracyclines, a sub-class of polyketide antibiotic having broad spectrum activity against bacterial infections was discovered in 1945 from the genus *Streptomyces* from which a number of other suitable antibiotics were derived (Mahajan and Balachandran, 2012). In 1949, an antibiotic Chloramphenicol was produced from *Streptomyces venezuelae* which later manufactured synthetically to fight different bacterial pathogens by inhibiting their protein synthesis. Later, several antibiotics were produced from actinobacteria such as Neomycin an aminoglycoside antibiotic derived from *Streptomyces fradiae,* Erythromycin a macrolide antibiotic from

Streptomyces erythreus, glycopeptide antibiotic Vancomycin from *Amycolatopsis orientalis*, an aminoglycoside antibiotic Gentamycin derived from *Micromonospora* and Daptomycin a lipopeptide antibiotic from *Streptomyces roseosporus* (Mahajan and Balachandran, 2012).

Although bio-prospecting of actinobacteria from different habitats have been carried out and a large number of actinobacteria were identified, described and screened for their biosynthetic potential, numerous habitats still remain unexplored. However, with the decline in the discovery of new drugs to fight the prevalence of antibiotic resistance, the search for new compounds needs to be lengthened. In recent years, there has been increasing information of the potential value of fresh water habitats which has been largely unexplored and could be a promising source for the isolation of pharmacologically useful secondary metabolites. The present study was planned with the following objectives:

1. Identification and phylogenetic analysis of actinobacteria by using 16S rRNA gene sequencing.
2. Antimicrobial activities of actinobacteria isolated from selected lakes and rivers of Mizoram.
3. Screening for the production of extracellular enzymes and detection of biosynthetic genes; non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKSII) from the potential isolates.

Chapter II

Review of Literature

Actinobacterial researches associated with the production of bioactive secondary metabolites have been reported from various habitats by different researchers worldwide and a number of antibiotics have been successfully determined, employing different techniques and methods. However, with the spread of antibiotic resistant pathogens in the environment, there has been a need to search for more antibiotics having different mechanisms of action globally to fight antibiotic resistant pathogens. Moreover, infectious diseases still remain the leading cause of death worldwide; therefore exploration of untapped habitats such as fresh water habitats is a promising challenge to discover new and novel bioactive compounds especially effective antibiotics from actinobacteria to serve the current needs. This chapter highlighted the research done so far on actinobacteria from different fresh water source accumulating different information from all around the globe such as the history of actinobacterial research associated with fresh water till date, the method and technique used for isolation, different isolates of actinobacteria obtained and the bioactive secondary metabolites reported from fresh water actinobacteria.

2.1 History of Freshwater actinobacterial research

The Phylum actinobacteria are found to be ubiquitous in nature, largely isolated from terrestrial habitats as well as aquatic system, mainly from marine ecosystem and are also commonly in fresh water habitats (Warnecke et al., 2004). In 1969, actinobacteria such as *Streptomyces* and *Micromonospora* were reported for the first time, isolated from fresh water stream and river of Blelham Tarn (Willoughby, 1971) which is a milestone in fresh water actinobacterial research. Few years later in 1976 and 1977, *Rhodococcus coprophilus* and other isolates of

actinobacteria such as *Micromonospora, Streptomyces, Nocardioform, Streptosporangium, Actinomadura, Actinoplanes, Dactyhsporangium, Microbispora* and *Thermomonospora* were reported from fresh water and agriculture habitats (Johnston and Cross 1976; Rowbotham and Cross, 1977). Cross (1981) described actinobacteria from fresh water habitats as a promising source for bioactive secondary metabolites (Goodfellow and Haynes, 1984). Two researchers from China, Jiang and Xu in the year 1966 isolated and identified six different genera of actinobacteria from lake water and sediment sample of Middle Plateau, Yunnan, China. The different genera of actinobacteria include *Streptomyces, Actinomadura, Microbispora, Micromonospora, Nocardia,* and *Saccharopolyspora* (Jiang and Xu, 1996). Wohl and Mc Arthur. (1998) also isolated nine different genera of actinobacteria *viz. Streptomyces, Nocardiodes, Pseudonocardia, Nocardia, Streptoalloteichus, Actinomadura Microbiospora, Micromonospora* and *Actinoplanes* from Fresh water macrophytes of Savannah river, South Carolina.

The interest of researchers on fresh water actinobacteria seems not much developed till the year 2000, which was clarified by the fact that literature concerning on fresh water actinobacteria was found to be very less as compared to other habitats like soil and marine ecosystem. Meanwhile in the past few years (2000-2017), the published literatures on actinobacterial research from fresh water have been increased up to certain extent, researchers focused more on the diversity and antimicrobial activity with the prevalence of antibiotic resistance among the pathogens (Deshmukh and Sridhar, 2002; Rifaat. 2003; Chou et al., 2008; de Menezes et al., 2008; Rifaat and El-Sayed. 2008; Sibanda et al., 2010; Cwala et al.,

2011; Sanasam et al., 2011; Rizvi et al., 2012; Gebreyohannes et al., 2013; Jami et al., 2015). The need to search for antimicrobial secondary metabolites from unexplored habitats have been increasing with a great demand on effective antibiotics as much of the habitats were explored in search for actinobacteria having biosynthetic potential. It has been emphasized that new group of microorganism from unexplored habits can serve as sources of novel antibiotics and other therapeutic agents to fight antibiotic resistance pathogens (Bull et al., 2000). According to the review on actinobacteriological research in India (Velho-Pereira and Kamat. 2013), various habitats have been screened for the study of actinobacteria, of which soil habitats have been largely surveyed followed by marine water, while fresh water have been neglected and most of which remains unexplored. Accordingly, fresh water habitats are becoming the area of great interest. Among the different genera of actinobacteria, *Micromonospora, Rhodococcus* and *Streptomyces* were found commonly in various fresh water habitats (Johnston and Cross, 1976; Rifaat and El-Sayed, 2008; Sibanda et al., 2010). There is also a possibility that certain actinobacteria from terrestrial habitats were washed into the rivers and streams whereas some of them were found to be indigenous (Cross, 1981).

The exploration of fresh water habitats and its associated actinobacteria in India has begun with the isolation of 170 isolates of actinobacteria from the sediments and leaf litter in west coastal fresh water stream of Karnataka, these isolates belongs to five different genera namely *Streptomyces, Micromonospora, Actinoplanetes, Therntoactirrotnyces* and *Nocardiopsis* (Deshmukh and Sridhar, 2002). A year later, 114 actinobacterial isolates, mostly *Streptomyces* spp. and

Micromonospora spp. were isolated from the longest rivers in the world, River Nile in Cairo, Egypt (Rifaat, 2003). A number of researches on fresh water actinobacteria have been found and continued to be reported worldwide, several of the isolated actinobacteria were found to be novel and serve an important purposes in agricultural and clinical field (Qu et al., 2018). Fresh water actinobacterial research continued to be reported from various habitats like Kaoshiung in Southern Taiwan (Chou et al., 2008), English Lake District- Esthwaite Water and Priest Pot (de Menezes et al., 2008), River Nile in Cairo (Rifaat and El-Sayed. 2008), Tyume River in eastern cape province of South Africa (Sibanda et al., 2010), Thadhasamudhram pond in Tamil Nadu, India (Radhika et al., 2011), Vembanadu lake in Kerela, India (George et al., 2011), University of Fort Hare in Alice, South Africa (Cwala et al., 2011), Nambul River in Manipur, India (Ningthoujam et al., 2011), Loktak Lake, in Manipur, India (Sanasam et al., 2011), River Godavari, India (Rizvi et al., 2012), Karimnagar in Andhra Pradesh, India (Mohan and Singarachraya 2012; Mohan et al., 2012), Lake Tana in Ethiopia (Gebreyohannes et al., 2013), Chahnimeh reservoirs in Zabol, Iran (Jami et al., 2015), Narowal, Punjab, Pakistan (Iqbal and Sajid. 2015), Pudukkottai, Tamilnadu, India (Saravanan et al., 2015), Lake Michigan, USA (Mullowney et al., 2015; Shaikh et al., 2015). Till date, this habitat remains an area of largely unexplored compared to other habitats for actinobacterial research.

2.2 *Streptomyces*: Antibiotic producer

Streptomyces is the most common genus among the Phylum actinobacteria and is one of the largest within the domain bacteria (Ludwig et al., 2012). The contribution of *Streptomyces* in various drugs and pharmaceutical industry was found

to be dominant among all the microorganisms (Ser et al., 2015). As described earlier that two third of the antibiotics in use were derived from actinobacteria, out of which more than 70% of the total antibiotics produced by actinobacteria were derived from the genus *Streptomyces* alone (Subramani and Aalbersberg, 2012). Among the different genera of actinobacteria, *Streptomyces* and *Micromonospora* were most commonly isolated from fresh water habitats. The genus *Micromonospora* was reported to be the most frequently isolated actinobacteria from freshwater habitat supported by Cross and Collins. (1966); Willoughby. (1971); Jiang and Xu. (1996); Rifaat and El-Sayed. (2008) while *Streptomyces* are generally the second most abundant which was also supported by Colmer and McCoy, (1943) and Willoughby, (1971). On the other hand, the studies based on literature survey of recent publications, *Streptomyces* were reported to be the most dominant genus among the phylum actinobacteria from fresh water habitats supported by a number of researchers (Wohl and McArthur, 1998; Deshmukh and Sridhar, 2002; Ningthoujam et al., 2011; Sanasam et al., 2011; Jami et al., 2015).

The morphology and physiology of *Streptomyces* were so diverse compared to other actinobacterial genera and were mostly spore bearing microbes which help them survive under unfavourable conditions (Goodfellow and Williams, 1983). They were largely responsible for the synthesis of various antibiotics and still continued to be the organisms of great interest for antibiotics production, so interminable screening for the bioactive secondary metabolites produced by this organism is necessary (Ser et al., 2015). An interest arises with the discovery of Streptothricin, the first antibiotic isolated from *Streptomyces* in 1942 and Streptomycin from the

same genus two years later (Berdy, 1974; Vandamme, 1984). Some other commonly used antibiotics such as Tetracyclines, Chloramphenicol, Neomycin, Erythromycin, Kanamycin, Daptomycin, etc. were all derived from the genus *Streptomyces*.

2.3 Rare Genera of Actinobacteria

The actinobacterial genera other than *Streptomyces* are called rare genera of actinobacteria and their isolation frequency using conventional methods were much lower than that of the *Streptomyces* genus (Passari et al., 2015). There has also been an attempt of isolating rare genera of actinobacteria worldwide and much efforts have been put forward by different scientists in search of potential actinobacteria from different habitats with the declined in the discovery of new *streptomyces* and the re-isolation of known compounds (Watve et al., 2001; Sharma et al., 2016). The rare actinobacteria encompassed of approximately 220 different genera till 2010 (Tiwari et al., 2012). Several researchers described the occurrence and isolation of rare genera of actinobacteria from different fresh water habitats: *Saccharopolyspora* and *Actinosynnema* were isolated from the Tyume River Eastern Cape Province of South Africa (Sibanda et al., 2010), *Nocardiopsis* and *Micromonospora* from Nambul River, Manipur, India (Ningthoujam et al., 2011), *Saccharomonospora, Micromonospora, Nocardiopsis, Arthrobacter, Kocuria, Microbacterium* and *Agromyces* from Chahnimeh reservoirs, Zabol, Iran (Jami et al., 2015), *Nocardia* and *Micromonospora* from Narowal, Punjab, Pakistan (Iqbal1 and Sajid. 2015), *Micromonospora sp* from Lake Michigan, USA (Mullowney et al., 2015). Ultramicrobacteria categorised as actinobacteria from five freshwater habitats in Europe and Asia was described (Hahn et al., 2015).

Few novel species of actinobacteria were also observed from fresh water habitats, *Nocardioides fonticola,* a novel species was isolated from fresh water spring located in Kaoshiung, Southern Taiwan (Chou et al., 2008). *Aurantimicrobium minutum,* a novel ultramicrobacterium of the family Microbacteriaceae was reported from river water (Nakai et al., 2015*).* Novel species *Saccharomonospora xiaoerkulensis* sp. was isolated from lake sediment (Li et al., 2016). A novel species of *Nocardioides taihuensis* was also isolated from fresh water lake sediment (Qu et al., 2017). A new taxa of actinobacteria from fresh water, *Longivirga aurantiaca* gen. nov., sp. was recently reported from the lake sediment of Taihu Lake, China (Qu et al., 2018).

A number of rare actinobacteria like *Actinomadura, Microbispora, Pseudonocardia, Streptoalloteichus, Therntoactirrotnyces, Actinopolyspora* and *Agromyces* were also reported by several researchers from different fresh water source (Silvey and Roach, 1953; Willoughby, 1971; Jiang and Xu, 1996; Wohl and McArthur, 1998; Deshmukh and Sridhar, 2002: Rifaat, 2003; Hahn and Pockl, 2005; de Menezes et al., 2008; Cwala et al., 2011; Sanasam et al., 2011; Mohan et al., 2012). Thus a continuous screening for the isolation of potential bioactive secondary metabolites from actinobacteria other than *Streptomyces* and exploration of the untapped bio-resources may further contribute crucial aspects in uplifting or improving the current scenario of antibiotic resistance.

2.4 Isolation of Fresh water actinobacteria

Isolation of actinobacteria from its wild habitats was very crucial, the method and technique employed largely depends on the successful isolation of actinobacteria

(Barka et al., 2016). Actinobacteria are very diverse as described earlier in the previous chapter, so selecting the right method can have a large influence on the successful isolation of actinobacteria. There are numerous actinobacteria which could not be isolated from their wild habitats using traditional cultivation method, where culture-independent method (Metagenomics approach) was successfully employed (Ghai et al., 2014; Iliev et al., 2017). It has been used in the study of actinobacterial diversity of a sediment sample from Xiaoerkule Lake (Guan et al., 2008). According to the literature survey on the exploration of different fresh water actinobacterial habitats, several methods were used which also depends on the interests of the researchers on the organisms expected and the type of work planned. Some of the most commonly used methods for the investigation of culturable fresh water actinobacteria were described (**Figure: 2.1**).

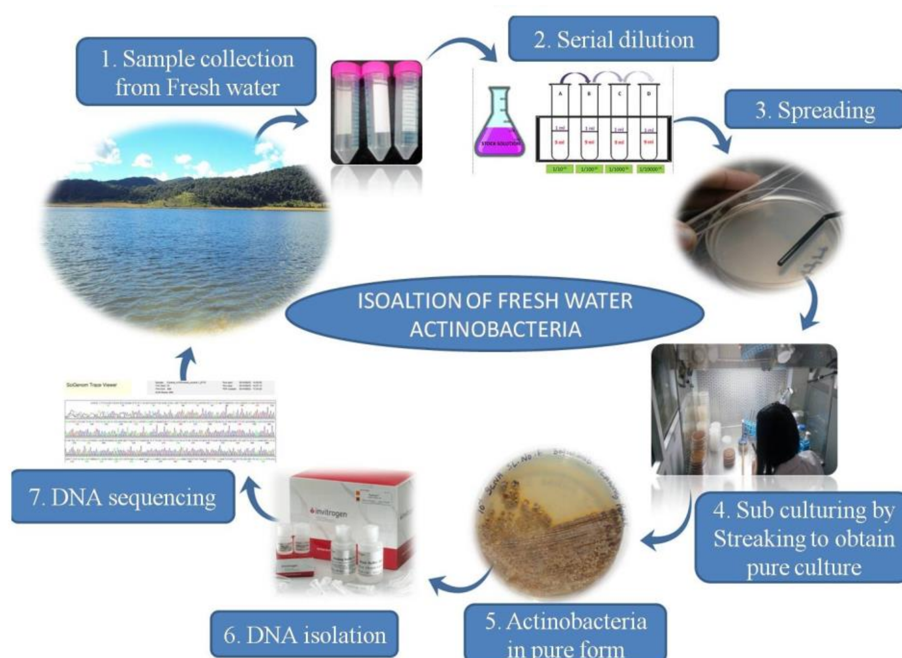

Figure 2.1: General pictorial depiction for the isolation of fresh water actinobacteria (Zothanpuia et al., 2018)

2.4.1 Pre-treatment of Fresh water samples

This is the initial steps for the selective isolation of actinobacteria, pre-treatment of the samples was successfully employed for different samples from different habitats such as marine sediments (Yuan et al., 2014). Fresh water ecosystem comprises of several samples which includes water, water sediments, phytoplankton, macrophytes, fish gut, etc. collected from different fresh water bodies like rivers, lakes, ponds and streams. Pre-treatment of the samples was generally used to facilitate the isolation of actinobacteria and to neglect unwanted organisms. Jayashree et al. (1991) employed five different pre-treatment methods for the isolation of actinobacteria from fresh water where dry heat at 100 ºC was found to be the most appropriate pre-treatment method since the number of fast growing bacteria and fungi were reduced which facilitates the growth of actinobacteria. Some of the most commonly pre-treatment methods used for the isolation of fresh water actinobacteria were described below:

2.4.1.1 Heat treatment

Heat treatment of the sample was the most commonly employed pre-treatment method for the isolation of fresh water actinobacteria: The temperature and period of treatment vary depend on the interests of the researchers. Heat treatment of the sediment sample was performed at 100º C (Jayashree et al., 1991; Wohl and McArthur, 1998), 55º C for 6 minutes (Jayashree et al., 1991; Rizvi et al., 2012). de Menezes et al. (2008) performed heat treatment at 65° C for 10 minutes for the selective isolation of *Micromonospora* and *Rhodococcus* which was in accordance with the findings of Rowbotham and Cross. (1977). Fresh water macrophyte samples

was pre-treated by heating at 60º C for 2 hours (Wohl and McArthur. 1998) while a slight heat was applied for the isolation of slow growing actinobacteria (Rifaat and El-Sayed. 2008; Cross, 1981). On the other hand, the sample was dried for a week prior to isolation (Williams et al., 1972; Ningthoujam et al., 2011; Saravanan et al., 2015).

2.4.1.2 Chemical treatment

Chemical treatment of the samples was uncommon as compared to heat treatment method and fewer reports were found for fresh water samples. Sanasam et al. (2011) performed chemical treatment by mixing the samples with 10% $CaCO_3$ for a week prior to plating. Other chemical treatment reported from fresh water sample includes mixing the sample with 10 ml phosphate-buffered saline (de Menezes et al., 2008) and 1% phenol for 10 minutes (Jayashree et al., 1991).

Based on the literature surveyed and the data obtained, it can be concluded that the used of pre-treatment method prior to isolation helps in minimising undesirable microorganisms such as fast growing bacteria and fungi which is certainly a problem for the microbiologist. So, it is obvious that pre-treatment of the samples help in selective isolation of actinobacteria.

Addition of antibiotics at the time of media preparation also helps in the elimination of unwanted microbes such as bacteria and fungi by inhibiting their growth in culture media. Certain antibiotics like cyclohexamide and nystatin were most commonly used in fresh water actinobacterial research (Ningthoujam et al., 2011). Cycloheximide and Nystatin were reported for the elimination of fungal growth at the culture media which were added prior to pouring of the media in petri-

plates, several researchers supported by selectively isolating different genera of actinobacteria (Ningthoujam et al., 2011; Mohan and Singarachraya, 2012; Rizvi et al., 2012). However, amphotericin B and polymixin B sulphate were used by Gebreyohannes et al. (2013) and Wohl and McArthur. (1998) respectively in their work for the selective isolation actinobacteria from fresh water sediments. Nalidixic acid was the most commonly used antibiotic for inhibiting fast growing bacteria (Rizvi et al., 2012).

2.4.2 Culture media for isolation of actinobacteria from fresh water

Selection of culture media for the isolation of actinobacteria is very important to achieve better yields and maximum diversities from the samples. Several nutritional media were reported for the investigation of fresh water actinobacteria, some of the most commonly used culture media were starch casein agar (SCA), actinomycetes isolation agar (AIA), streptomyces agar (SA), ISP medium no. 2 [yeast malt agar (YM agar)], ISP medium no. 5 (glycerol asparagine agar base), ISP medium no. 7 (tyrosine agar), chitin agar (CA), trypticase soya agar (TSA), colloidal chitin agar (CCA), kuster's agar (KA), water yeast extract agar (WYEA) and starch casein nitrate agar (SCNA) (Ningthoujam et al., 2011; Mohan and Singarachraya, 2012; Rizvi et al., 2012). The components of the media differ in their nutritional contents and the organisms' growth depends on the availability of the nutritional requirements, there is a possibility that actinobacteria might not be growing in every media, so maximum number of media should be utilised so as to obtain maximum diversities among the isolates. According to literature survey on fresh water actinobacteria, different researchers used different media and proposed different

media for the isolation of actinobacteria. A report was found where Kuster's medium was the best culture medium for the isolation actinobacteria while Bannet's agar completely failed to show any recovery of actinobacteria in the study of fresh water sediment samples (Jayashree et al., 1991). According to the present review, starch casein agar and actinomycetes isolation agar were the most commonly used media for the isolation of actinobacteria from fresh water source which were also recommended by several researchers as the best media for the isolation of fresh water actinobacteria (Deshmukh and Sridhar, 2002; Rifaat, 2003; Rizvi et al., 2012). For the isolation of actinobacteria, other than *Streptomyces* genus (rare actinobacteria), humic - vitamin B agar medium was suggested by Hayakawa and Nonomura. (1987); Rifaat and El-Sayed. (2008).

2.5 Serial dilution and spread plate technique

Subsequent to sampling and pre-treatment of the samples involves processing of the samples by serial dilution and spread plate technique, which is one of the most widely used techniques for the isolation of actinobacteria worldwide. It has also been used by several researchers for the isolation of fresh water actinobacteria (Jiang and Xu, 1996; Deshmukh and Sridhar, 2002; de Menezes et al., 2008; Sanasam et al., 2011; George et al., 2011; Iqbal1 and Sajid, 2015). This method involves diluting the samples up to several folds and the spread of the diluted samples on sterilised culture media. Proper sterilization is important and incubation temperature plays a crucial role for the appropriate growth of actinobacteria. Actinobacteria are mostly mesophilic having optimal growth at temperatures between 27° C and 30° C. However, some actinobacteria like *Thermomonospora, Microbispora*, etc. were

found to be thermophillic which can tolerate and grow at a temperature of 37-65° C and optimal temperature ranging from 50 to 60° C (Edwards et al., 1993; Barka et al., 2016). According to the present review, most of the fresh water actinobacteria are having an optimal growth temperature of 28º C (Radhika et al., 2011; Ningthoujam et al., 2011; Rizvi et al., 2012; Gebreyohannes et al., 2013). Most of the actinobacteria were observed with slow growth on culture media which normally takes 2 to 3 weeks for their maturation (Rifaat, 2003).

2.6 Morphological characterization of Fresh water actinobacteria

Actinobacteria showed different growth characteristics on culture media after few weeks of incubation and were generally used for characterization following the guidelines of International *Streptomyces* Project (ISP) described by Shirling and Gottlieb. (1966). Some of the characters of the pure colonies used by Scientist for characterization include colony colour and characteristics, colour of aerial and substrate mycelium, spore mass colour, production of diffusible pigment, utilization of carbon sources and spore chain morphology (Goodfellow and Haynes, 1984; Sanasam et al., 2011). The spore chain morphology were also diverse among the actinobacteria (**Figure 2.2**) which was mainly visualised and studied using Field Emission Gun-Scanning electron microscopy (FEG-SEM). It is widely used for analysing biological samples which were also reported to study the *in vitro* antimicrobial activity and enzymatic activity in various actinobacteriological research worldwide (de Menezes et al., 2008, Passari et al., 2015; 2016). Bergey's

Manual of Determinative Bacteriology (Bergey and Holt, 2000) was also used by various researchers for the identification of actinobacteria.

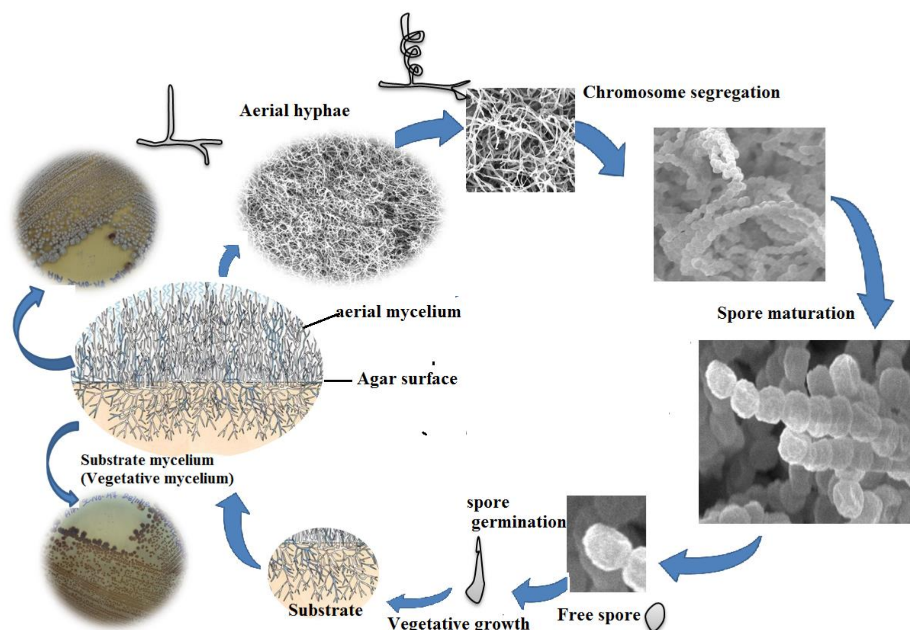

Figure 2.2: Schematic representation of the life cycle of sporulating actinobacteria

2.7 Molecular identification of actinobacteria

Molecular characterization involves the identification of organisms at their genetic level which is one of the most reliable and accepted level for characterization and identified organisms up to species level. The process involves DNA isolation from the purified isolates, gene amplification using Polymerase Chain Reaction (PCR) and sequencing of the amplified gene for identification.

2.7.1 Isolation of genomic DNA

Standardisation of the procedure for Genomic DNA isolation is crucial for the attainment of quality DNA. A number of procedures have been developed by different researchers globally and several companies came up with different isolation

kits which were commercially available. Some of the most commonly used Genomic DNA isolation kit used in fresh water actinobacterial research (based on literature survey) includes FastPrep kit (MP Biomedical, USA), PureLink® Genomic DNA Mini kit (Invitrogen™), DNA extraction kit (Mol Bio UltraClean) (de Menezes et al., 2008) and PowerSoil® DNA Isolation Kit (Shaikh et al., 2015).

2.7.2 Gene for identification

There are few genes which were used by several researchers for the molecular identification of actinobacteria; some of the genes used most frequently in fresh water actinobacterial research were described.

2.7.2.1 16S rRNA gene amplification

16S rRNA gene is a conserved region present in the ribosomes of all the bacterial genomes and is used widely for molecular identification of not only actinobacteria, but all the bacteria and not only from fresh water habitats but also from different habitats worldwide. Based on the present review, phylogenetic studies using 16S rRNA gene sequencing and defining their taxonomy have been found significantly in fresh water actinobacterial research (Yokota, 1997; de Menezes et al., 2008; Sanasam et al., 2011; Iqbal1 and Sajid, 2015; Jami et al., 2015). Some common 16SrRNA primers used in the study of actinobacteria from fresh water were given:

- Forward primer: PA (5’-AGAGTTTGATCCTGGCTCAG-3’) reverse primer: PH (5‘-AAGGAGGTGATCCAGCCGCA-3’).

- Forward primer: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') reverse primer: 1100R (5'-GGGTTGCGCTCGTTG-3') (Ningthoujam et al., 2011).
- Forward primer: 357F (5'- CTCCTACGGGAGGCAGCAG-3') reverse primer: 1492R (5'-GGTTACCTTGTTACGACTT-3') (Ningthoujam et al., 2011).
- Forward primer: 8F (5-AGA GTT TGA TCC TGG CTC AG-3) reverse primer: 1492R (5-GGT TAC CTT GTT ACG ACT T-3) (Jami et al., 2015).

2.7.2.2 Gyrase B gene

The used of gyrase B gene as molecular marker for the identification of fresh water actinobacteria was very rare compared to 16S rRNA gene. The comparison of these two markers (16S rRNA gene and gyrase B gene) were done by de Menezes et al. (2008) in the study of actinobacteria (*Micromonospora*) from fresh water and concluded that gyrase B gene is a better phylogenetic marker than 16S rRNA gene by using gyrB universal primers (UP1TL 5'-CAy GCn GGn GGn AAr TTy GA-3'; positions 295–314 and UP2rTL 5'-TCn ACr TCn GCr TCn GTC AT-3'; positions 1486–1505). Separation of actinobacteria at the species level studied in the genus *Micromonospora* resulted that gyrase B gene showed better discriminatory pattern than 16S rRNA gene which was supported by the findings of Kasai et al. (2000). The discriminatory pattern of ribosomal RNA between closely related species/genera was found to be little unreliable which can even create uncertainty, so the use of other molecular markers such as *rpoB, gyrase B, ssgB,* etc. in addition to ribosomal RNA

gene will further help to discriminate better between closely related species/genera (Girard et al., 2013).

DNA fingerprinting of actinobacteria was also performed using different degenerate primers as reported by several researcher from different habitats (Versalovic et al., 1991; Rademaker et al., 2000). In this review, a study was found where degenerate primer BOX-A1R (5-CTA CGG CAA GGC GAC GCT GAC G-3) was effectively used in the study of Fresh water fish gut micro-biota (Jami et al., 2015). Based on the present study, other molecular markers were not much found to be used for fresh water actinobacteriological research.

2.8 Biotechnological implications of actinobacteria

Actinobacteria are one of the most valuable microbes on earth, the largest antibiotic producers accounting for more than 50% of the commonly used antibiotics and other pharmaceutically useful bioactive secondary metabolites comprising of different chemical composition (Goodfellow and Fiedler, 2010; Subramani and Aalbersberg, 2012; Yuan et al., 2014). Antibiotics discovered from actinobacteria include peptides, polyketides, polyesters, beta-lactams, chloramphenicol, macrolide, nucleosides, polyenes, tetracycline, aminoglycosides, glycopeptides, anthracyclines etc. (Barka et al., 2016) **(Figure 2.3**).

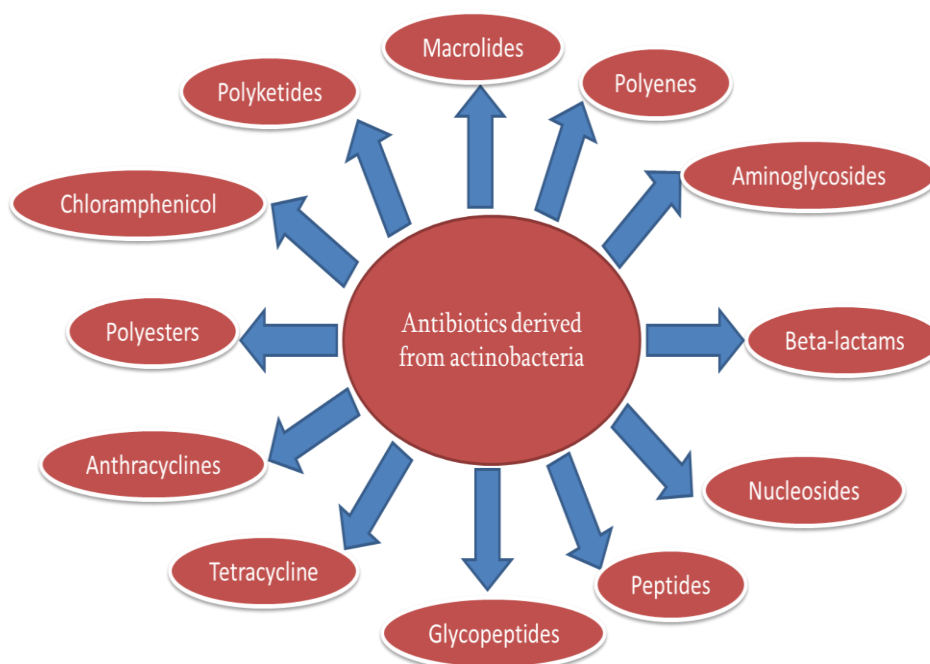

Figure 2.3: Common antibiotic compounds produced from actinobacteria

An antibiotic actinomycin, was the first purified antibiotic from actinobacteria (*Streptomyces antibioticus*) discovered in 1940 by Selman Waksman reported for its anti-cancer activity, which opens new prospect for actinobacteriological research in search of antibiotics to fight against pathogens (Waksman, 1940). Later many other biologically active secondary metabolite compounds having antibacterial, antifungal, immuno-modifiers, enzyme inhibitors and antitumour activities were also reported from various habitats worldwide (Stach et al., 2004; Goodfellow and Fiedler, 2010) **(Figure 2.4)**. They played great role in pharmaceutical and agricultural field, they were also reported as a good plant growth promoters (Tanaka and omura, 1990; Waksman and Lechevelier, 1962).

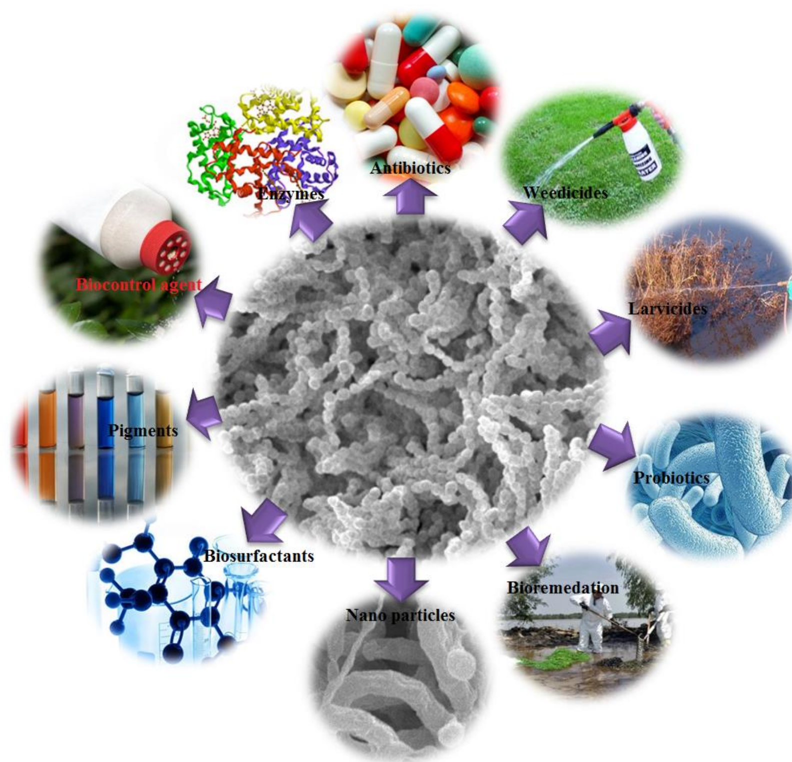

Figure 2.4: Actinobacteria as antibiotics, enzymes, biocontrol agent, pigments, biosurfactants, nano-particles, bioremediation, probiotics, larvicide, and weedicides

2.8.1 Antimicrobial potential of Fresh water actinobacteria

Actinobacteria have been reported for its potential as antimicrobials to fight different bacterial pathogens (de Menezes et al, 2008; Gebreyohannes et al., 2013). In this review, a number of antimicrobial potential of actinobacteria from different fresh water habitats have been found worldwide. Gebreyohannes et al. (2013) reported that actinobacteria from fresh water Lake Tana appears to have great potential as a source of antibacterial compounds. *Streptomyces* was the most common actinobacteria isolated from fresh water environments as described earlier and are also the most common antimicrobial isolates. The antifungal activity of *Streptomyces* and *Micrmonospora* were reported from Nile River in Egypt (Rifaat,

2003). *Streptomyces* sp. strain AZ-NIOFD1 and other *Streptomyces* species having broad - spectrum antimicrobial activity was also described from River Nile (Atta et al., 2009) and Krishna river, India (Ellaiah et al., 2002). The antimicrobial activity of rare genera of actinobacteria were also reported by several researchers worldwide, Cwala et al. (2011) isolated rare actinobacteria; *Actinopolyspora* strain TR 008 from fresh water of the Eastern Cape Province, South Africa having antimicrobial potential against Gram positive and Gram negative bacteria. Sibanda et al. (2010) also described the antibacterial activity of actinobacteria belonging to *Sachharopolyspora* and *Actinosynemma* from Tyume River, South Africa. Potential mesophilic and thermophilic isolates of actinobacteria were also reported from the fresh water stream in West Coast of India (Deshmukh and Sridhar, 2002). Several other studies on the potential aspects of different fresh water actinobacteria were also described by many researchers globally which clarified that more exploration of fresh water habitat will uplift the present scenario of antibiotic crisis due to the spread of antibiotic resistance pathogens.

2.8.2 Enzymatic activity of Fresh water actinobacteria

Literature regarding the enzymatic profiling of fresh water actinobacteria was found to be very less, however few reports were reviewed in the present study. *Micromonospora* isolates of actinobacteria recovered from water column, sediment and cellulose baits of fresh water English lake were reported for their potential degradation of cellulose without the requirement of additional nutrients (de Menezes et al., 2008). Amylase, protease, urease, cellulase and lipase activity of fresh water actinobacteria were also described (Mohan and Charya, 2012). Researchers should

emphasise more on the enzymatic potential of fresh water actinobacteria since a number of reports have been found for their ability to degrade recalcitrant organic matters as mentioned earlier.

2.8.3 Bioactive secondary metabolites from Fresh water actinobacteria

Microbial secondary metabolites are the antibiotic factories which were largely reported from different organisms, different habitats all around the world (Yuan et al., 2014; Jami et al., 2015). Actinobacteria are the dominant contributor among the microbes for secondary metabolites production, reported largely from terrestrial and marine habitats. The metabolites produced by actinobacteria were so diverse and offers strong area of research in antibiotic discovery. Based on the present review, fresh water habitats have been less explored in search of actinobacterial secondary metabolites which remained neglected and most of the studies were limited to antimicrobial screening and diversity. The first report of actinobacterial secondary metabolites from fresh water was found in 2015, the metabolites include diazaquinomycins H and J (DAQH and DAQJ) which were reported for its inhibitory activity against *Mycobacterium tuberculosis* (Mullowney et al., 2015). Deuteromethylactin B, an eight membered lactone secondary metabolite was also reported for its antitumor activity from fresh water derived actinobacteria, *Streptomyces* spp. (Shaikh et al 2015). The potential antibacterial secondary metabolites nactins (polynactin) were also produced by *Streptomyces* sp. strain 156A isolated from Lake Baikal, the largest fresh water lake in the world (Tatyana et al., 2016). To best of our knowledge these are the only Fresh water derived actinobacterial secondary metabolites reported in literature till date.

2.9 Biosynthetic (secondary metabolite) genes from Fresh water actinobacteria

The genes associated with the production of bioactive secondary metabolite have been reported in various genera of actinobacteria, several reports have been found from fresh water actinobacteria as well, which includes polyketide synthases gene type I and type II, the KS gene encodes ketosynthase enzyme which is part of modular polyketides (Khosla, 1997), nonribosomal peptide synthetases (NRPSs) genes involved in the synthesis of peptide compounds, aminodeoxyisochorismate synthase (*phz*E) gene; involves in the synthesis of enzyme responsible for phenazine pathways, dTDP-glucose-4, 6-dehydratase (dTGD) gene; the enzyme involves in 6-deoxyhexoses glycosylation pathway, halogenase (Halo) gene; the enzyme of halogenation pathway and cytochrome P450 hydroxylase (CYP) gene; the enzyme for polyene polyketide biosynthesis (Ayuso-Sacido and Genilloud, 2005; Yuan et al., 2014). The study of the correlation between biosynthetic genes and its secondary metabolites was performed using polymerase chain reaction (PCR). Detection and amplification of biosynthetic genes using PCR and identification was important for evaluating the chemical constituents and biosynthetic potential of actinobacteria (Minowa et al., 2007). Several reports have been found on the amplification and detection of biosynthetic genes from fresh water habitats (Jami et al., 2015).

Chapter III

Distribution and Abundance of Actinobacteria in Fresh water Lakes and Rivers of Mizoram and their Phylogenetic Affiliation

3.1 Introduction

Actinobacteria are ubiquitous in nature, they are typical inhabitants of soil environments (soil dwellers) where majority of the actinobacteria were found to be isolated (Goodfellow and Williams. 1983). They are also found commonly in freshwater ecosystems (Johnston and Cross, 1976; Warnecke et al., 2004; Rifaat and El-Sayed; 2008; Sibanda et al 2010). The fresh water ecosystem includes water, sediments, planktons, sponges, fishes, crab, etc. and among them, actinobacteria are most commonly isolated from water and sediment samples (Jami et al., 2015). The interests in the bio-prospection of potential actinobacteria begins with the discovery of the first antibiotic (actinomycin) from actinobacteria in 1940, which further resulted in the isolation of many useful bioactive secondary metabolite compounds especially antibiotics and become an important source for discovery of new and novel drug compounds (Trujillo 2008; Passari et al., 2017). Various habitats continued to be explored by several researchers and a number of useful compounds have been expansively reported from different ecosystems (such as soil, fresh water, marine, plants and different extreme environments) worldwide (Goodfellow and Williams, 1983; Passari et al., 2015; Maldonado et al., 2005) and have been examined for their prospective contributions in agricultural and pharmaceutical industry (Lam, 2006; Kavitha et al., 2010; Wang et al., 2013; Passari et al., 2016; Sharma et al., 2016; Wang et al., 2017).

However, with the intensification in reports on the isolation and identification of actonbacteria from various sources, there has been a significant decline in the rate of discovery of novel actinobacteria in recent years (Alvan et al., 2011: Zotchev,

2012) and there has been an increasing isolation of known organisms besides re-isolation of known compounds. Moreover, the requirements of new and novel antibiotics having unique mechanisms of actions are increasing with the spread on antibiotic resistant pathogens (Sharma et al., 2016). Hence, referring to this emergence, the investigation of potential actinobacteria from untapped habitats is a significant approach for discovering novel antibiotic compounds to meet the current needs (Lazzarini et al., 2001; Poulsen et al., 2011). Bio-prospection studies on the phylum actinobacteria mainly focused on terrestrial and marine ecosystems and few studies have been found from freshwater ecosystems (Sibanda et al., 2010).

Mizoram is one of the seven states of Northeast India, and is a large bio-prospecting area identified as the Indo-Burma mega-biodiversity hotspot by Conservation International, the area is well known for its rich biodiversity and unexplored biological resources (Myers et al., 2000). All the lakes and rivers of Mizoram are fresh water (Zothanpuia et al., 2015). There are several reports that have examined the diversity of actinobacteria in freshwater worldwide (Sibanda et al., 2010; Radhika et al., 2011: Saravanan et al., 2015) and numerous genera of actinobacteria were observed, but few studies have reported on their biosynthetic potential. Therefore, it will be of great significance to characterize the various actinobacterial microorganisms from unexplored freshwater sediments. In this study, we intended to isolate actinobacterial cultures, to study the distribution and abundance in different fresh water systems and phylogenetically identify from freshwater sediments of selected lakes and rivers of Mizoram, India. To our knowledge, no studies have reported on the diversity of culturable Actinobacteria

from Fresh water sediments of Mizoram. The findings may be a preliminary stage for improving our understanding of the actinobacterial microbial population and are significant for the biotechnological exploitation.

3.2 Materials and Methods

3.2.1 Sediment Sampling

Water sediment samples were collected from four rivers [Tlawng river (24º 52´N; 92º 36´E), Tuirial river (24º 21'N 92º 53′), Tuichang river (23°33' N, 93°06' E), Murlen stream (23° 37′ N 93° 18′ E)] and two lakes [Tamdil Lake (23º 44´N; 92º 57´ E), Rihdil (23^0 20' N, 93^0 23' E)] (**Figure 3.1**) of Mizoram Northeast India (**Figure 3.2**). Samples were collected randomly from five different stations of each rivers and lake with an average of 2-5 metre depth with the help of local swimmers. The labelled samples were placed in sterile screwed capped tubes (50 mL) and transported to Molecular Microbiology and systematic Laboratory, Mizoram University and were processed immediately for the isolation of actinobacteria.

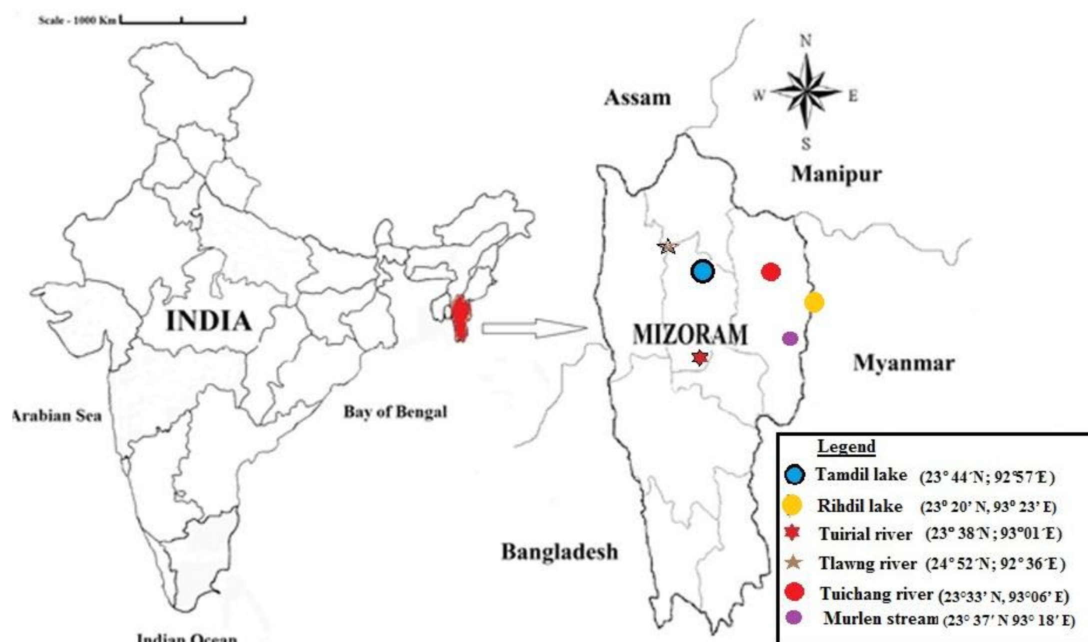

Figure 3.1: Map showing the location of the sampling site of various rivers and lakes (Mizoram, Northeast India)

Figure 3.2: Selected study site (fresh water lakes and rivers) of Mizoram.

3.2.2 Isolation and Enumeration of Fresh water Actinobacteria

The collected samples were subjected to physical pre-treatment method (pre-treatment of the samples was performed by heating the sediments in water bath at 55° C for 6 minutes) in order to hinder the growth of fast growing bacteria and favouring the growth of actinobacteria (Yuan et al., 2014). Isolation of actinobacteria was done by serial dilution method and spread plate technique. Serial dilution was performed by preparing stock solution of the sample with 1 mL of water sediment (water + sediment suspension) and 9 ml of sterile distilled water in a test tube and agitated for ten minutes. The suspension was serially diluted by transferring 1ml of aliquots to a series of test tubes; each containing 9ml of sterile distilled water to prepare the final volume of 10^{-1}, 10^{-2} and 10^{-3}, and spread over the surface of selected nutritional media. Seven selective media: starch casein agar (SCA), yeast extract malt extract Agar (ISP2), Actinomycetes isolation agar (AIA), Streptomyces agar (SA), glycerol aspargine agar (ISP5), tyrosine agar (ISP7) and tap water yeast extract agar (TWYE) were used for the isolation of actinobacteria **(Table 3.1)** which were supplemented with nalidixic acid (30 mg ml^{-1}) and cyclohexamide (30 mg ml^{-1}) to inhibit the growth of Gram negative bacteria and fungi respectively. The plates were incubated at 28±1° C for 7 - 30 days and the colonies were observed periodically. The pure cultures were obtained after two to three successive sub-culturing to fresh isolation media and it was stored in their respective slants at 4° C and 30% glycerol at -80° C for preservation.

Table 3.1: Composition of various nutritional media used for isolation of fresh water actinobacteria

Media	Composition	Ingredients Gms/Litre
Actinomycete Isolation Agar (AIA)	Litre Sodium caseinate	2.0
	L-Asparagine	0.1
	Sodium propionate	4.0
	Dipotassium phosphate Magnesium	0.5
	sulphate	0.1
	Ferrous sulphate	0.001
	Agar	15.0
Starch Casein Agar (SCA)	Casein powder	1
	soluble starch	1
	Starch	10
ISP Medium No. 2 (Yeast Extract Malt Extract Agar)	yeast extract	4
	malt extract	10
	dextrose	4
	agar	15
ISP Medium No. 5 (Glycerol Asparagine Agar Base)	L-Asparagine	1
	Dipotassium phosphate	1
	Trace salt solution (ml)	1
	*(Ferrous sulphate heptahydrate, Manganese chloride tetrahydrate, Zinc sulphate heptahydrate (0.001 each)	20
	Agar	
ISP Medium No. 7 (Tyrosine Agar)	L-Asparagine	1
	L-Tyrosine	0.5
	Dipotassium phosphate	0.5
	Magnesium sulphate $7H_2O$	0.5
	Sodium chloride	0.5
	Trace salt solution (ml)	1
	*(Ferrous sulphate, 7H2O 1.360mg Copper chloride, 2H2O 0.027mg Cobalt chloride, 6H2O 0.040mg Sodium molybdate, 2H2O 0.025mg Zinc chloride 0.020mg Boric acid 2.850mg Manganese chloride, 4H2O	

	1.800mg Sodium tartarate 1.770mg)	20
	Agar	
Streptomyces agar (SA)	Malt extract	10
	Yeast extract	4
	Dextrose	4
	Calcium carbonate	2
	Agar	12
Tap water yeast extract medium (TWYE)	Yeast extract	0.25
	Dipotassium hydrogen phosphate	0.50
	Agar	18

3.2.3 Preliminary Identification of Actinobacterial isolates

Pure culture of the isolates were identified based on their morphological and cultural characteristics following International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966), the nature of the colony, color of aerial and substrate mycelium, production of diffusible pigments and utilization of carbon source were studied (Goodfellow and Haynes, 1984). The spore chain morphology of the isolates was studied using emission gun-scanning electron microscopy (FEG-SEM). The mycelium structures were observed using phase contrast microscope (Olympus) and the organisms were identified according to *Bergey's Manual of Determinative Bacteriology* 9th edition (Bergey et al., 2000).

3.2.4 Molecular Identification of actinobacteria

Genomic DNA of the selected isolates was isolated using DNA extraction kit (Invitrogen, USA) according to the manufacturer's protocol, DNA quantity was checked by measuring optical density (OD) at 260/280 nm. 16S rRNA gene was amplified by using universal bacterial primers PA (5'-AGAGTTTGATCCTGGCTCAG-3') as forward primer and PH (5'-

AAGGAGGTGATCCAGCCGCA-3') as reverse primer (Weisburg et al., 1991). PCR reaction was carried out in a total volume of 25 µl, consisting 1.0 µl of genomic DNA (50 ng), 0.2µl of each primer (10 pmol), 2.0 µl of deoxynucleotide triphosphates (2.5 mM each), 2.5 µl of 1X PCR buffer, 0.2 µl of Taq DNA polymerase (1U/ µl) and 15.9 µl MilliQ grade water. PCR was performed on Veriti thermal cycler (Applied Biosystem, Singapore) under following conditions: initial denaturation at 95 ^{0}C (4 min), followed by 30 cycles of denaturation at 94 ^{0}C (30 sec), annealing at 57.5 ^{0}C (40 sec) and extension at 72 ^{0}C (1.3 min) with a final extension step at 72 ^{0}C (10 min). A negative control reaction mixture without DNA template of actinobacteria was also included with each set of PCR reactions. The amplified PCR product was checked by using 1.2 % agarose gel electrophoresis using TAE buffer and analyzed under UV light and documented using a Bio-rad Gel Doc XR^{+} system (Hercules, CA, USA). The PCR products of 16S rRNA gene was purified by quick PCR purification kit (Invitrogen) and sequenced commercially at SciGenome Pvt. Ltd. Kochin, India.

3.2.5 Phylogenetic Analysis of Actinobacteria

Sequences were compared for similarity percentage with the reference strains of actinobacteria from National Centre for Biotechnology Information (NCBI) genomic database, using BLASTn search available at http://www.ncbi.nlm.nih.gov/blast website. Type strains with highest similarity percentage were retrieved from EzTaxon database (Kim et al., 2012) and multiple sequence alignment was performed using Clustal W software packaged in MEGA 6.0 software available at http://www.ebi.ac.uk/clustalw (Thompson et al., 1997). The

evolutionary models were selected based on the lowest Bayesian Information Criterion (BIC) scores and highest Akaike Information Criterion (AIC) values using MEGA 6.0 (Saitou and Nei, 1987; Tamura et al., 2013). Phylogenetic analysis was performed using MEGA 6 software using maximum-likelihood method and using Tamura Nei parameters algorithm taking *E. coli* as out-group (Tamura et al., 2011). The significance of the branching order was determined by bootstrap analysis of 1000 alternative trees. The obtained nucleotide sequences of the 16S rRNA gene fragments were deposited and accession numbers were acquired. Trees were viewed and edited by using program Fig Tree 1.3.1.

3.3 Results

3.3.1 Isolation of Actinobacteria

A total of 115 isolates of actinobacteria were isolated from the sediment samples of six different fresh water systems of Mizoram, Northeast India **(Table 3.2)**. All the obtained isolates were purified by subsequent streaking and identified based on morphological characteristics after two to three weeks of incubation on different media. Most of the isolates showed specific morphological characters such as slow growth (generally starts growing from 3rd to 7th day onwards), colour of mycelia from yellow, brownish white to blackish white, while some isolates formed brown and purple coloured pigments on the media. Certain isolates were powdery, while some were hard, soft and sticky in nature (**Figure 3.3**). Microscopically, the Gram positive isolates were selected based on Gram staining (**Figure 3.4**) and the field emission gun-scanning electron microscopy (FEG-SEM) analysis revealed that

majority of the aerial mycelia of actinobacterial isolates produced long and spiral spore chains (**Figure 3.5**).

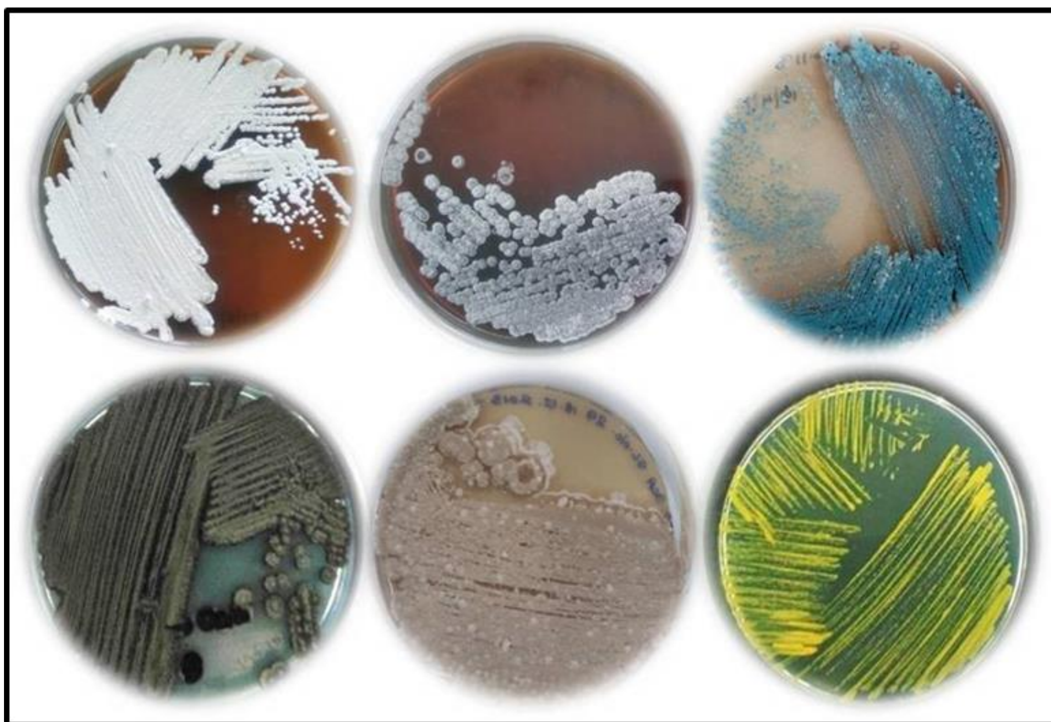

Figure 3.3: Morphological appearances of some of the isolated actinobacteria in solid media

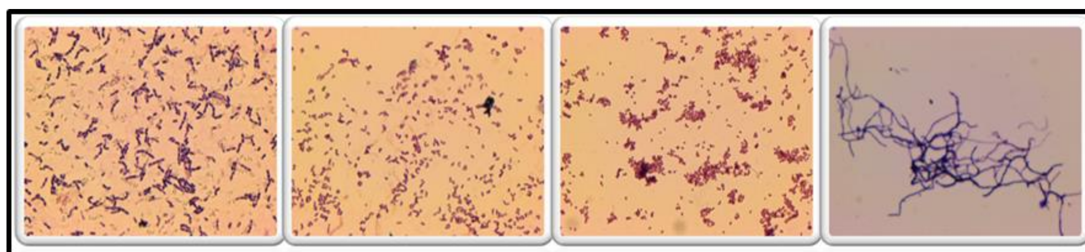

Figure 3.4: Gram staining characteristics of some actinobacterial isolates

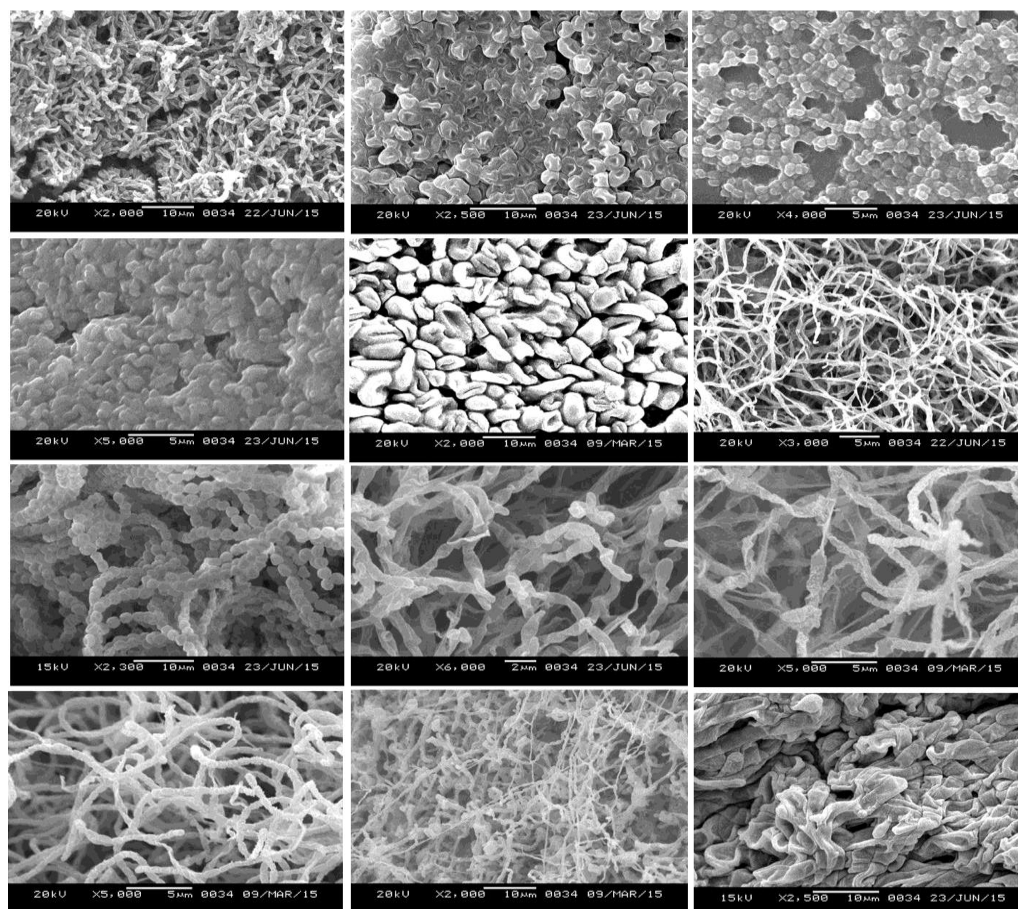

Figure 3.5: Spore chain morphology of actinobacteria isolates under Field Emission Gun-Scanning electron microscope

Table 3.2: Morphological and microscopic characteristics of 115 actinobacterial isolates.

S. No	Organisms and isolate no.	Colony nature	Aerial mycelium	substrate mycelium	Pigmentation
1	*Promicromonospora* sp. (DST8)	Sticky	Yellow	Yellow	-
2	*Streptomyces violascens* (DST10)	Soft	Light yellow	Light yellow	-
3	*Streptomyces* sp. (DST12)	Hard	White	Light yellow	-
4	*Streptomyces* sp. (DST13)	Hard	White	Brown	+
5	*Streptomyces cyaneofuscatus* (DST15)	Soft	White	Light brown	+

6	*Streptomyces* sp. (DST16)	Hard	brown	Brown	+
7	*Streptomyces* sp. (DST17)	Hard	White	Purple	+
8	*Streptomyces* sp. (DST18)	Hard	White	brown	-
9	*Streptomyces* sp. (DST19)	Hard	White	brown	-
10	*Nocardiopsis* sp. (DST21)	Hard	White	brown	+
11	*Prauserella* sp. (DST22)	Hard	White	Pale yellow	-
12	*Streptomyces* sp. (DST23)	Hard	White	Pale yellow	-
13	*Streptomyces parvus* (DST24)	Hard	White	light yellow	-
14	*Streptomyces* sp. (DST25)	Powdery	Brown	brown	+
15	*Kocuria* sp. (DST27)	Soft	White	yellow	-
16	*Streptomyces intermedius* (DST29)	Hard	Black	Pale yellow	-
17	*Streptomyces cellulosae* (DST28)	Hard	White	brown	-
18	*Streptomyces flavogriseus* (DST30)	Hard	White	brown	-
19	*Saccharopolyspora* sp. (DST31)	Soft	White	White	-
20	*Nocardiopsis* (DST32)	Hard	White	Pale yellow	+
21	*Streptomyces* sp. (DST35)	Hard	White	yellow	-
21	*Rhodococus* sp. (DST38)	Hard	Light brown	Light yellow	-
23	*Kocuria palustris* (DST43)	Hard	White	brown	-
24	*Streptomyces pactum* (DST44)	Hard	Pale yellow	yellow	-
25	*Nocardiopsis* sp. (DST46)	Hard	Light brown	Dark brown	+
26	*Streptomyces koyangenesis* (DST48)	Soft	White	Pale yellow	-
27	*Micrococcus luteus* (DST49)	Soft	White	yellow	-

28	*Streptomyces* sp. (DST50)	Soft	yellow	yellow	-
29	*Rhodococcus* sp. (DST51)	Soft	White	brown	-
30	*Streptomyces flavogriseus* (DST52)	Soft	White	Light yellow	-
31	*Streptomyces griseoplanus* (DST53)	Hard	White	Pale yellow	-
32	*Streptomyces* sp. (DST54)	Hard	White	Pale yellow	-
33	*Streptomyces* sp. (DST56)	Hard	White	White	-
34	*Streptomyces cyaneofuscatus* (DST57)	Hard	White	Light yellow	-
35	*Streptomyces somaliensis* (DST58)	Hard	White	brown	+
36	*Streptomyces cyaneofuscatus* (DST59)	Hard	White	Dark brown	-
37	*Streptomyces* sp. (DST60)	Hard	Off white	Dark brown	-
38	*Amycolatopsis* sp. (DST61)	Hard	Off white	Dark brown	-
39	*Streptomyces* sp. (DST62)	Hard	White	Pale yellow	+
40	*Streptomyces* sp. (DST63)	Hard	Off white	Dark brown	-
41	*Streptomyces cyaneofuscatus* (DST64)	Hard	White	Pale yellow	-
42	*Streptomyces lavendulae* (DST65)	Hard	Pale yellow	Pale yellow	-
43	*Streptomyces olivaceus* (DST66)	Hard	Black	black	-
44	*Streptomyces griseoplanus* (DST67)	Soft	White	Pale yellow	-
45	*Streptomyces violarus* (DST68)	Hard	White	black	+
46	*Streptomyces* sp. (DST69)	Soft	Red	Red	-
47	*Streptomyces* rubiginosohelvolus	Hard	Off white	Brown	-

	(DST70)				
48	*Streptomyces albidoflavus* (DST71)	Soft	White	White	-
49	*Streptomyces rubiginosohelvolus* (DST72)	Hard	Dark brown	brown	-
50	*Streptomyces atratus* (DST73)	Sticky	White	yellow	-
51	*Streptomyces atroolivaceus* (DST74)	Hard	White	yellow	-
52	*Streptomyces koyangenesis* (DST75)	Soft	White	yellow	-
53	*Streptomyces qancidicus* (DST76)	Soft	Yellow	Yellow	-
54	*Streptomyces* sp. (DST86)	Soft	White	white	-
55	*Micrococcus luteus* (DST87)	Soft	White	yellow	-
56	*Micrococcus* sp. (DST88)	Soft	White	Brown	-
57	*Saccharopolyspora* sp. (DST89)	Hard	Black	Black	-
58	*Nocardiopsis* (DST95)	Hard	Off white	brown	+
59	*Streptomyces albidoflavus* (DST96)	Sticky	White	yellow	+
60	*Saccharopolyspora* sp. (DST97)	Soft	White	Pale yellow	+
61	*Saccharopolyspora* sp. (DST98)	Hard	Pale yellow	Pale yellow	+
62	*Streptomyces cyaneofuscatus* (DST99)	Hard	Off white	yellow	+
63	*Streptomyces albidoflavus* (DST100)	Soft	White	Pale yellow	+
64	*Streptomyces albidoflavus* (DST102)	Soft	White	White	-
65	*Streptomyces cyaneofuscatus* (DST103)	Hard	White	White	+

66	*Streptomyces* sp. (DST104)	Powdery	White	Purple	-
67	*Nocardiopsis* (DST105)	Hard	White	Purple	-
68	*Streptomyces atroolivaceus* (DST106)	Hard	White	light brown	-
69	*Streptomyces* sp. (DST116)	Soft	blackish brown	blackish brown	-
70	*Nocardiopsis* sp. (DST117)	Hard	blackish brown	Black	-
71	*Streptomyces griseus* (DST118)	Hard	White	light brown	-
72	*Streptomyces* sp. (DST119)	Hard	White	pale yellow	-
73	*Streptomyces fulvissimus* (DST120)	Powdery	White	pale yellow	-
74	*Streptomyces caviscabies* (DST121)	Hard	White	light brown	-
75	*Streptomyces albidoflavus* (DST122)	Powdery	dark grey	Yellow	-
76	*Streptomyces* sp. (DST123)	Powdery	White	pale yellow	-
77	*Brevibacterium epidermidis* (DST124)	Hard	White	light brown	-
78	*Streptomyces albidoflavus* (DST125)	Powdery	White	White	-
79	*Rhodococcus* sp. (DST126)	Hard	dark brown	light brown	-
80	*Micrococcus luteus* (DST127)	Powdery	White	pale yellow	-
81	*Streptomyces vellosus* (DST128)	Soft	Brown	Brown	-
82	*Streptomyces thermocarboxydus* (DST129)	Hard	White	creamish white	-
83	*Streptomyces lavendulae* (DST130)	Soft	pale yellow	pale yellow	-
84	*Streptomyces violascens* (DST130)	Soft	Black	Black	-
85	*Streptomyces* sp. (DST 142)	Powdery	White	pale yellow	-
86	*Brevibacterium* sp.	Powdery	White	pale yellow	-

	(DST143)				
87	*Rhodococcus* sp. (DST144)	Powdery	Grey	Green	-
88	*Nocardiopsis* sp. (DST 145)	Powdery	White	White	-
89	*Arthrobacter* sp. (DST 146)	Powdery	White	pale yellow	-
90	*Leucobacter* sp. (DST 147)	Hard	Black	Black	-
91	*Streptomyces* sp. (DST 148)	Hard	White	White	-
92	*Brachybacterium* sp. (DST 149)	Soft	Black	Black	-
93	*Nocardiopsis sp.* (DST150)	Hard	White	Brown	+
94	*Arthrobacter* sp. (DST151)	Powdery	White	pale yellow	-
95	*Corynebacterium* sp. (DST152)	Hard	Black	Black	-
96	*Brevibacterium* sp. (DST153)	Powdery	White	light brown	-
97	*Leucobacter* sp. (DST154)	Hard	White	pale yellow	-
98	*Corynebacterium* sp. (DST155)	Powdery	White	light brown	-
99	*Brachybacterium* sp. (DST156)	Hard	White	Brown	-
100	*Tsukamurella* sp. (DST157)	Hard	brownish white	yellowish brown	-
101	*Arthrobacter sp.* (DST158)	Hard	White	pale yellow	-
102	*Arthrobacter* sp. (DST159)	Hard	White	pale yellow	-
103	*Corynebacterium* sp. (DST160)	Powdery	White	White	-
104	*Arthrobacter* sp. (DST161)	Powdery	brownish white	Brown	+
105	*Arthrobacter* sp. (DST162)	Soft	White	White	-
106	*Arthrobacter* sp. (DST163)	Hard	Black	Black	-
107	*Arthrobacter* sp. (DST164)	Hard	Black	Black	-
108	*Brachybacterium* sp. (DST165)	Powdery	brownish white	pale yellow	-
109	*Kocuria* sp.	Powdery	White	White	-

	(DST166)				
110	*Brachybacterium* sp. (DST167)	Powdery	White	White	-
111	*Corynebacterium* sp. (DST168)	Powdery	White	Brown	-
112	*Brevibacterium* sp. (DST 169)	Soft	Brown	Brown	-
113	*Micrococcus* sp. (DST 170)	Powdery	Black	Blue	-
114	*Zhihengliuella* sp. (DST 171)	Hard	White	Yellow	-
115	*Rhodococcus* sp. (DST 172)	Soft	White	pale yellow	-

3.3.2 Molecular Characterization of fresh water actinobacteria

Molecular characterization of actinobacteria involves the isolation of genomic DNA from the organisms, amplification of 16S rRNA gene and sequencing of the gene which were then subjected to bioinformatics analysis.

3.3.2.1 Genomic DNA isolation of actinobacterial isolates

Genomic DNA of the isolated organisms was extracted and the quality and quantity was checked on 0.8% agarose gel. A single band of genomic DNA was visible when visualized under UV light and documented using a Bio-rad Gel Doc XR+ system (Hercules, CA, USA). The isolated DNA for each isolates was of good quality and the result obtained was shown below **(Figure 3.6**). On comparison with the molecular marker, the DNA concentration (ratio of 260/280 nm) for various samples was found to range between 20-100 ng/ul.

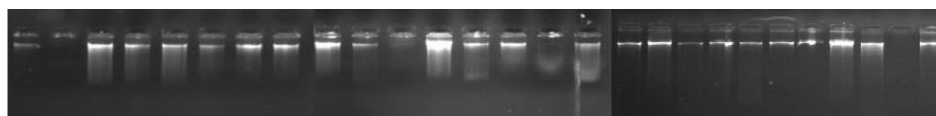

Figure 3.6: Genomic DNA bands of actinobacterial isolates under UV light, documented using a Bio-rad Gel Doc XR+ system

3.3.2.2 PCR amplification of 16S rRNA gene

All the isolates were subjected to amplification of 16S rRNA gene using Applied Biosystems thermal cycler polymerase chain reaction. The isolated DNA used for each reaction was 50 ng/ul and the PCR product was run on 1.2% agarose gel with low range DNA ruler plus (100 bp to 3 kb) as molecular markers. For all the isolates a single amplicon of 1500 bp was amplified **(Figure 3.7)** and sequencing of the amplified region was done commercially. Once the sequences were received, it was analysed by using bioinformatics tools before submitting to NCBI Genebank. After properly checked, the sequences were submitted to NCBI GenBank and accession numbers were given as KM243384, KM405296 - KM405298, KM405300 - KM405304, KM405306 - KM405307, KM405310, KM406395, KM406397, KM406398, KR703473 - KR703475, KR857285, KR857286, KR857288, KR857290 - KR857296, KR857298 - KR857318, KT232313 - KT232316, KT429605 - KT429610, KT429612, KT429614 - KT429616, KY077681, MF536299 - MF536302 **(Table 3.3).**

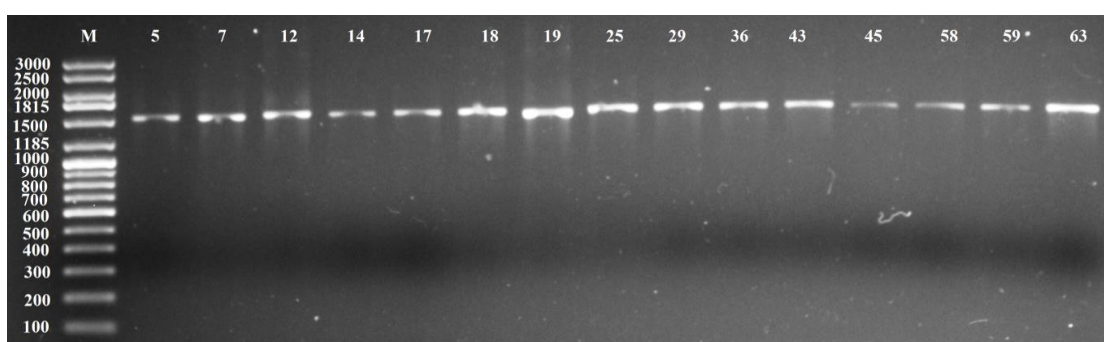

Figure 3.7: PCR amplification of 16S rRNA gene of some isolates. **M:** Low range DNA; Numbers indicates different isolates.

All 115 isolates were molecularly confirmed using 16SrRNA gene sequencing with similarity percentage ranging from 97% - 100%, All the isolates were divided into 16 different genera (**Figure 3.8**) of which *Streptomyces* constitutes the dominant genus comprises 55% (n=63) of the total isolates followed by *Nocardiopsis* (7%; n=8), *Arthrobacter* (7%; n=8), *Rhodococcus* (4%; n=5), *Micrococcus* (4%; n=5), *Saccharopolyspora* (3%; n=4), *Brevibacterium* (3%; n=4), *Brachybacterium* (3%; n=4), *Corynebacterium* (3%; n=4), *Kocuria* (3%; n=3), *Leucobacter* (2%; n=2), *Promicromonospora* (1%; n=1), *Prauserella* (1%; n=1), *Amycolatopsis* (1%; n=1), *Tsukamurella* (1%; n=1) and *Zhihengliuella* (1%; n=1).

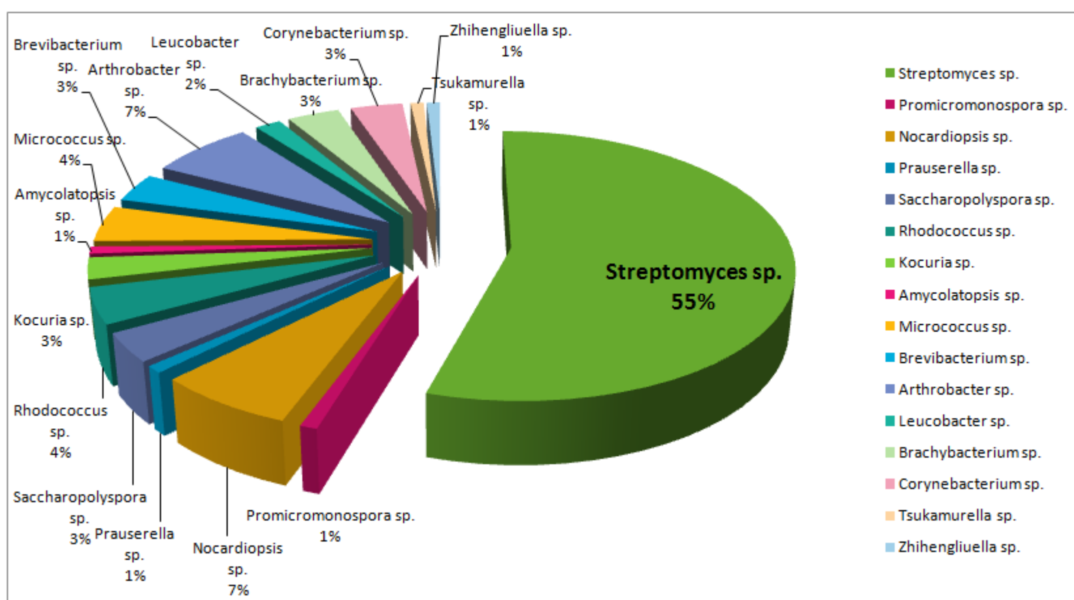

Figure 3.8: Percentage frequency of 16 actinobacterial genera isolated from fresh water sediment samples.

Table 3.3. Isolated actinobacterial isolates with their closest relative species retrieved from NCBI Genbank database and their accession number

S. No	Isolate name and isolate no.	Closest relative species in BLAST and accession no.	% Similarity	Source	Gene bank Accession number
1	*Promicromonospora* sp. (DST8)	*Promicromonospora* sp. (KC 550232)	99%	Tamdil	KM243384
2	*Streptomyces violascens* (DST10)	*Streptomyces violascens* (HQ23834)	99%	Tlawng	KM405296
3	*Streptomyces* sp. (DST12)	*Streptomyces* sp. (KJ494303)	99%	Tamdil	KM405297
4	*Streptomyces* sp. (DST13)	*Streptomyces* sp. (FJ490543)	100%	Tamdil	KM405298
5	*Streptomyces cyaneofuscatus* (DST15)	*Streptomyces cyaneofuscatus* (HG965215)	99%	Tamdil	KM405300
6	*Streptomyces* sp. (DST16)	*Streptomyces* sp. *(KJ889223)*	99%	Tlawng	KM405301
7	*Streptomyces* sp. (DST17)	*Streptomyces* sp. (KF770900)	99%	Tlawng	KM405302
8	*Streptomyces* sp. (DST18)	*Streptomyces* sp. (EF577245)	99%	Tamdil	KM405303
9	*Streptomyces* sp. (DST19)	*Streptomyces* sp. (EF577245)	99%	Tamdil	KM405304
10	*Nocardiopsis* sp. (DST21)	*Nocardiopsis* sp. (KJ470127)	99%	Tamdil	KM405306
11	*Prauserella* sp. (DST22)	*Prauserella* sp. (KJ574161)	99%	Tamdil	KM405307
12	*Streptomyces* sp. (DST23)	*Streptomyces* sp. (JQ812090)	99%	Tuicha ng river	KM405308

13	*Streptomyces parvus* (DST24)	*Streptomyces parvus* (KJ623765)	99%	Tuichang river	KM405309
14	*Streptomyces* sp. (DST25)	*Streptomyces* sp. (FJ492849)	99%	Tamdil	KM405310
15	*Kocuria* sp. (DST27)	*Kocuria* sp. (JF951262)	98%	Murlen stream	KM405312
16	*Streptomyces intermedius* (DST29)	*Streptomyces intermedius* (KJ571077)	100%	Murlen stream	KM406396
17	*Streptomyces cellulosae* (DST28)	*Streptomyces cellulosae* (KC429591)	99%	Tamdil	KM406395
18	*Streptomyces flavogriseus* (DST30)	*Streptomyces flavogriseus* (KC990785)	100%	Tamdil	KM406397
19	*Saccharopolyspora* sp. (DST31)	*Saccharopolyspora* sp. (GUT23669)	98%	Tamdil	KM406398
20	*Nocardiopsis* (DST32)	*Nocardiopsis* (KF543090)	99%	Tamdil	KR703473
21	*Streptomyces* sp. (DST35)	*Streptomyces* sp. (KM507719)	98%	Tamdil	KR703474
21	*Rhodococus* sp. (DST38)	*Rhodococus* sp. (KP128889)	99%	Tamdil	KR703475
23	*Kocuria palustris* (DST43)	*Kocuria palustris* (KP324952)	99%	Tamdil	KR857285
24	*Streptomyces pactum* (DST44)	*Streptomyces pactum* (KP324952)	100%	Tamdil	KR857286
25	*Nocardiopsis* sp. (DST46)	*Nocardiopsis* sp. (KP768309)	99%	Tamdil	KR857288
26	*Streptomyces koyangenesis* (DST48)	*Streptomyces koyangenesis* (KM678242)	100%	Tamdil	KR857290

27	*Micrococcus luteus* (DST49)	*Micrococcus luteus* (JF303043)	99%	Tamdil	KR857291
28	*Streptomyces* sp. (DST50)	*Streptomyces* sp. (KM979603)	99%	Tamdil	KR857292
29	*Rhodococcus* sp. (DST51)	*Rhodococcus* sp. (KP128889)	100%	Tamdil	KR857293
30	*Streptomyces flavogriseus* (DST52)	*Streptomyces flavogriseus* (GU166435)	99%	Tamdil	KR857294
31	*Streptomyces griseoplanus* (DST53)	*Streptomyces griseoplanus* (Hq238386)	99%	Tamdil	KR857295
32	*Streptomyces* sp. (DST54)	*Streptomyces* sp. (GU550579)	99%	Tamdil	KR857296
33	*Streptomyces* sp. (DST56)	*Streptomyces* sp. (JQ838074)	99%	Tamdil	KR857298
34	*Streptomyces cyaneofuscatus* (DST57)	*Streptomyces cyaneofuscatus* (LN824215)	99%	Tamdil	KR857299
35	*Streptomyces somaliensis* (DST58)	*Streptomyces somaliensis* (KF973287)	99%	Tamdil	KR857300
36	*Streptomyces cyaneofuscatus* (DST59)	*Streptomyces cyaneofuscatus* (LN824215)	99%	Tamdil	KR857301
37	*Streptomyces* sp. (DST60)	*Streptomyces* sp. (KM242419)	99%	Tamdil	KR857302
38	*Amycolatopsis* sp. (DST61)	*Amycolatopsis* sp. (AF466096)	99%	Tlawng	KR857303
39	*Streptomyces* sp. (DST62)	*Streptomyces* sp. (KM979603)	99%	Tlawng	KR857304

40	*Streptomyces* sp. (DST63)	*Streptomyces* sp. (KJ494330)	99%	Tlawng	KR857305
41	*Streptomyces cyaneofuscatus* (DST64)	*Streptomyces cyaneofuscatus* (LN824215)	99%	Tlawng	KR857306
42	*Streptomyces lavendulae* (DST65)	*Streptomyces lavendulae* (KC626003)	99%	Tlawng	KR857307
43	*Streptomyces olivaceus* (DST66)	*Streptomyces olivaceus* (AB184793)	99%	Tlawng	KR857308
44	*Streptomyces griseoplanus* (DST67)	*Streptomyces griseoplanus* (HQ238386)	99%	Tlawng	KR857309
45	*Streptomyces violarus* (DST68)	*Streptomyces violarus* (NR041116)	99%	Tlawng	KR857310
46	*Streptomyces* sp. (DST69)	*Streptomyces* sp. (GU550579)	99%	Tlawng	KR857311
47	*Streptomyces* rubiginosohelvolus (DST70)	*Streptomyces* rubiginosohelvolus (KJ632658)	99%	Tlawng	KR857312
48	*Streptomyces albidoflavus* (DST71)	*Streptomyces albidoflavus* (KP122209)	99%	Tlawng	KR857313
49	*Streptomyces rubiginosohelvolus* (DST72)	*Streptomyces rubiginosohelvolus* (KJ632658)	100%	Tlawng	KR857314
50	*Streptomyces atratus* (DST73)	*Streptomyces atratus* (KC462521)	99%	Tlawng	KR857315
51	*Streptomyces atroolivaceus* (DST74)	*Streptomyces atroolivaceus* (HQ831417)	100%	Tlawng	KR857316

52	*Streptomyces koyangenesis* (DST75)	*Streptomyces koyangenesis* (KM678242)	99%	Tlawng	KR857317
53	*Streptomyces qancidicus* (DST76)	*Streptomyces qancidicus* (KP792994)	99%	Tlawng	KR857318
54	*Streptomyces* sp. (DST86)	*Streptomyces* sp. (KT232313)	99%	Tuirial	KT232313
55	*Micrococcus luteus* (DST87)	*Micrococcus luteus* (KM37857)	99%	Tuirial	KT232314
56	*Micrococcus* sp. (DST88)	*Micrococcus* sp. (LN846826)	99%	Tuirial	KT232315
57	*Saccharopolyspora* sp. (DST89)	*Saccharopolyspora* sp. (JQ885595)	99%	Tuirial	KT232316
58	*Nocardiopsis* (DST95)	*Nocardiopsis* (KF270095)	99%	Tuirial	KT429605
59	*Streptomyces albidoflavus* (DST96)	*Streptomyces albidoflavus* (KT385695)	99%	Tuirial	KT429606
60	*Saccharopolyspora* sp. (DST97)	*Saccharopolyspora* sp. (GU723669)	98%	Tuirial	KT429607
61	*Saccharopolyspora* sp. (DST98)	*Saccharopolyspora* sp. (GU723669).	98%	Tuirial	KT429608
62	*Streptomyces cyaneofuscatus* (DST99)	*Streptomyces cyaneofuscatus* (KR857299)	99%	Tuirial	KT429609
63	*Streptomyces albidoflavus* (DST100)	*Streptomyces albidoflavus* (KT385695)	99%	Tuirial	KT429610
64	*Streptomyces albidoflavus*	*Streptomyces albidoflavus*	99%	Tuirial	KT429612

	(DST102)	(KT385695)			
65	*Streptomyces cyaneofuscatus* (DST103)	*Streptomyces cyaneofuscatus* (JOEM01000050)	99%	Tamdil	KT429613
66	*Streptomyces* sp. (DST104)	*Streptomyces* sp. (HM036677)	99%	Tuirial	KT429614
67	*Nocardiopsis* (DST105)	*Nocardiopsis* (KT025849)	100%	Tuirial	KT429615
68	*Streptomyces atroolivaceus* (DST106)	*Streptomyces atroolivaceus* (KX130875)	99%	Tuirial	KT429616
69	*Streptomyces* sp. (DST116)	*Streptomyces* sp. (KM368927)	99%	Tuirial	KY077681
70	*Nocardiopsis* sp. (DST117)	*Nocardiopsis* sp. (KX502849)	99%	Tuirial	MF536299
71	*Streptomyces griseus* (DST118)	*Streptomyces griseus* (YIM130689)	100%	Tuirial	MF536300
72	*Streptomyces* sp. (DST119)	*Streptomyces* sp. (EU734615)	99%	Tuirial	MF536301
73	*Streptomyces fulvissimus* (DST120)	*Streptomyces fulvissimus* (KX714721)	100%	Tuirial	MF536302
74	*Streptomyces caviscabies* (DST121)	*Streptomyces caviscabies* (KU158268)	100%	Murlen stream	MF536303
75	*Streptomyces albidoflavus* (DST122)	*Streptomyces albidoflavus* (KU981092)	100%	Murlen stream	MF536304
76	*Streptomyces* sp. (DST123)	*Streptomyces* sp. (KU507509)	100%	Murlen stream	MF536305
77	*Brevibacterium epidermidis*	*Brevibacterium epidermidis*	98%	Murlen stream	MF536306

	(DST124)	(KP337607)			
78	*Streptomyces albidoflavus* (DST125)	*Streptomyces albidoflavus* (KU981092)	100%	Murlen stream	MF536307
79	*Rhodococcus* sp. (DST126)	*Rhodococcus* sp. (LC011659)	99%	Tuichang river	MF536308
80	*Micrococcus luteus* (DST127)	*Micrococcus luteus* (KT307978)	99%	Tuichang river	MF536309
81	*Streptomyces vellosus* (DST128)	*Streptomyces vellosus* (KT438921)	99%	Tuichang river	MF536310
82	*Streptomyces thermocarboxydus* (DST129)	*Streptomyces thermocarboxydus*	100%	Tuichang river	MF536311
83	*Streptomyces lavendulae* (DST130)	*Streptomyces lavendulae* (KX698040)	100%	Tuichang river	MF536312
84	*Streptomyces violascens* (DST130)	*Streptomyces violascens* (KU158256)	100%	Tuichang river	MF536313
85	*Streptomyces* sp. (DST 142)	*Streptomyces* sp. (JQ838120)	99%	Rihdil	MG711553
86	*Brevibacterium* sp. (DST143)	*Brevibacterium* sp. (KM507608)	99%	Rihdil	MG711554
87	*Rhodococcus* sp. (DST144)	*Rhodococcus* sp. (FN357284)	99%	Rihdil	MG711555
88	*Nocardiopsis* sp. (DST 145)	*Nocardiopsis* sp. (KU382690)	99%	Rihdil	MG711556
89	*Arthrobacter* sp. (DST 146)	*Arthrobacter* sp. (KR906432)	99%	Rihdil	MG711557
90	*Leucobacter* sp. (DST 147)	*Leucobacter* sp. (KX289462)	99%	Rihdil	MG711558
91	*Streptomyces* sp.	*Streptomyces* sp.	99%	Rihdil	MG711559

	(DST 148)	(KP99845)			
92	*Brachybacterium* sp. (DST 149)	*Brachybacterium* sp. (FJ357630)	99%	Rihdil	MG711560
93	*Nocardiopsis* sp. (DST150)	*Nocardiopsis* sp. (JN942114)	97%	Rihdil	MG711561
94	*Arthrobacter* sp. (DST151)	*Arthrobacter* sp. (KM362724)	99%	Rihdil	MG711562
95	*Corynebacterium* sp. (DST152)	*Corynebacterium* sp. (CP009251)	97%	Rihdil	MG711563
96	*Brevibacterium* sp. (DST153)	*Brevibacterium* sp. (AM398220)	99%	Rihdil	MG711564
97	*Leucobacter* sp. (DST154)	*Leucobacter* sp. (KJ461710)	99%	Rihdil	MG711565
98	*Corynebacterium* sp. (DST155)	*Corynebacterium* sp. (KF428998)	99%	Rihdil	MG711566
99	*Brachybacterium* sp. (DST156)	*Brachybacterium* sp. (KF875581)	100%	Rihdil	MG711567
100	*Tsukamurella* sp. (DST157)	*Tsukamurella* sp. (JN695025)	99%	Rihdil	MG711568
101	*Arthrobacter* sp. (DST158)	*Arthrobacter* sp. (KJ619485)	97%	Rihdil	MG711569
102	*Arthrobacter* sp. (DST159)	*Arthrobacter* sp. (KJ642536)	97%	Rihdil	MG711570
103	*Corynebacterium* sp. (DST160)	*Corynebacterium* sp. (KF428998)	100%	Rihdil	MG711571
104	*Arthrobacter* sp. (DST161)	*Arthrobacter* sp. (KT724297)	99%	Rihdil	MG711572
105	*Arthrobacter* sp. (DST162)	*Arthrobacter* sp. (KJ619485)	99%	Rihdil	MG711573
106	*Arthrobacter* sp.	*Arthrobacter* sp.	99%	Rihdil	MG711574

	(DST163)	(KR906430)			
107	*Arthrobacter* sp. (DST164)	*Arthrobacter* sp. (KM035405)	99%	Rihdil	MG711575
108	*Brachybacterium* sp. (DST165)	*Brachybacterium* sp. (KP027820)	98%	Rihdil	MG711576
109	*Kocuria* sp. (DST166)	*Kocuria* sp. (GU318369)	99%	Rihdil	MG711577
110	*Brachybacterium* sp. (DST167)	*Brachybacterium* sp. (DQ399745)	99%	Rihdil	MG711578
111	*Corynebacterium* sp. (DST168)	*Corynebacterium* sp. (KF428998)	100%	Rihdil	MG711579
112	*Brevibacterium* sp. (DST 169)	*Brevibacterium* sp. (DQ347560)	98%	Rihdil	MG711580
113	*Micrococcus* sp. (DST 170)	*Micrococcus* sp. (KJ685828)	97%	Rihdil	MG711581
114	*Zhihengliuella* sp. (DST 171)	*Zhihengliuella* sp. (JF274915)	99%	Rihdil	MG711582
115	*Rhodococcus* sp. (DST 172)	*Rhodococcus* sp. (GU143683)	99%	Rihdil	MG711583

3.3.3 Abundance and Distribution of Actinobacteria

Six water bodies (2 lakes and 4 rivers) of Mizoram were selected for the isolation of actinobacteria. Maximum diversities were observed at Rihdil Lake where 31 isolates belonging to 12 different genera (*Streptomyces, Nocardiopsis, Rhodococcus, Kocuria, Micrococcus, Breviacterium, Arthrobacter, Leucobacter, Brachybacterium, Corynebacterium, Tsukamurella* and *Zhihengliuella*) were obtained followed by Tamdil Lake where 31 isolates belonging to eight different genera (*Streptomyces, Promicromonospora, Nocardiopsis, Rhodococcus, Kocuria*

and *Micrococcus*) were obtained. Nineteen isolates belonging to three genera of actinobacteria (*Nocardiopsis, Micrococcus* and *Streptomyces*) were found in Tuirial River whereas nineteen isolates under the genera *Streptomyces* and *Amycolatopsis* were found in Tlawng River. Nine isolates of actinobacteria belonging to *Brevibacterium* and *Streptomyces* genera were obtained from Tuichang River and six isolates belonging to *Streptomyces* and *Micrococcus* were obtained from Murlen Stream. The genus *Streptomyces* represent the most versatile organisms among the isolated actinobacteria that were observed in the sediment samples of the entire selected study site (**Figure 3.9**).

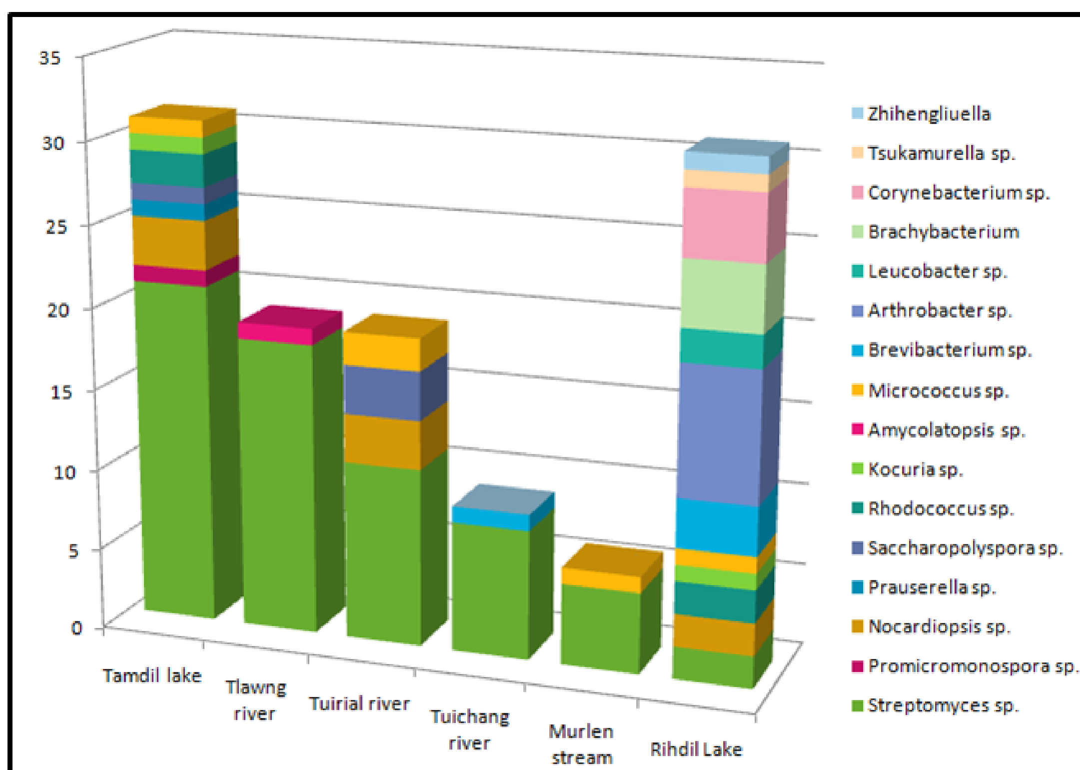

Figure 3.9: Distribution of actinobacteria in six fresh water systems of Mizoram

Seven different nutritional media were used to obtain 115 isolates of actinobacteria. Among the media employed for isolation, Starch casein agar (SCA) yields maximum number of isolates which accounts for 43% of the total isolates, followed by Actinomycetes isolation agar (29%), tyrosine agar medium (10%), glycerol aspargine agar (7%), Streptomyces agar (6%), tap water yeast extract agar (3%) and yeast extract malt extract Agar (2%) (**Figure 3.10**).

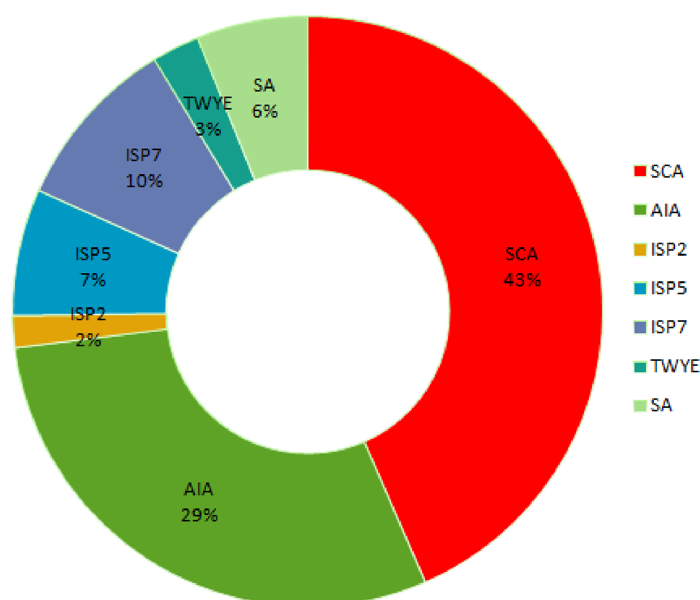

Figure 3.10: Nutritional media used for the isolation of fresh water actinobacteria

3.3.4 Phylogenetic analysis

Phylogenetic tree was constructed for 115 isolates of actionobacteria having *Escherichia coli* as an out-group. The tree was constructed using Maximum-likelihood (ML) and Tamura Nei (TN93+G) parameter with lowest Bayesian Information Criterion (BIC) values (22482) and highest Akaike Information Criterion (AICs) value (20275) (**Figure 3.11**). An estimated value of transition/transversion bias (R) is 1.03. The topology of the tree generated differentiated the isolates into five major clades. All the genus of *Streptomyces*

formed a major clade I with a bootstrap support value of 98%. Rare genera of actinobacteria like *Saccharopolyspora, Amycolatopsis* and *Prauserella* which falls under the family *Pseudonocardiaceae* were clustered together with bootstrap value of 99%. The genera of actinobacteria like *Nocardiopsis, Corynebacterium, Tsukamurella* and *Rhodococcus* from the family of *Nocardiopsaceae, Corynebacteriaceae Tsukamurellaceae* and *Nocardiaceae* respectively formed a separate clade while *Kocuria, Arthrobacter, Micrococcus* and *Zhihengliuella* which formed under the family *Micrococcaceae, and Promicromonospora, Leucobacter, Brevibacterium,* and *Brachybacterium* from the family of *Promicromonosporaceae, Microbacteriaceae, Brevibacteriaceae, Dermabacteraceae* respectively formed separate cluster with bootstrap support value of 84%.

Phylogenetic tree was also constructed for 16 different genera where a single isolate was selected representing each genus and a type strain was also recovered from NCBI Genbank database. The tree was constructed using Maximum-likelihood (ML) and Neighbour joining (NJ) method using Tamura Nei (TN93+G) parameter with lowest Bayesian Information Criterion (BIC) values (20765.71) and highest Akaike Information Criterion (AICs) value (20184.45) taking *E. coli* as an out-group. An estimated value of transition/transversion bias (R) is 1.3. The phylogenetic relationship of 16 different genera of actinobacteria were given (**Figure 3.12**).

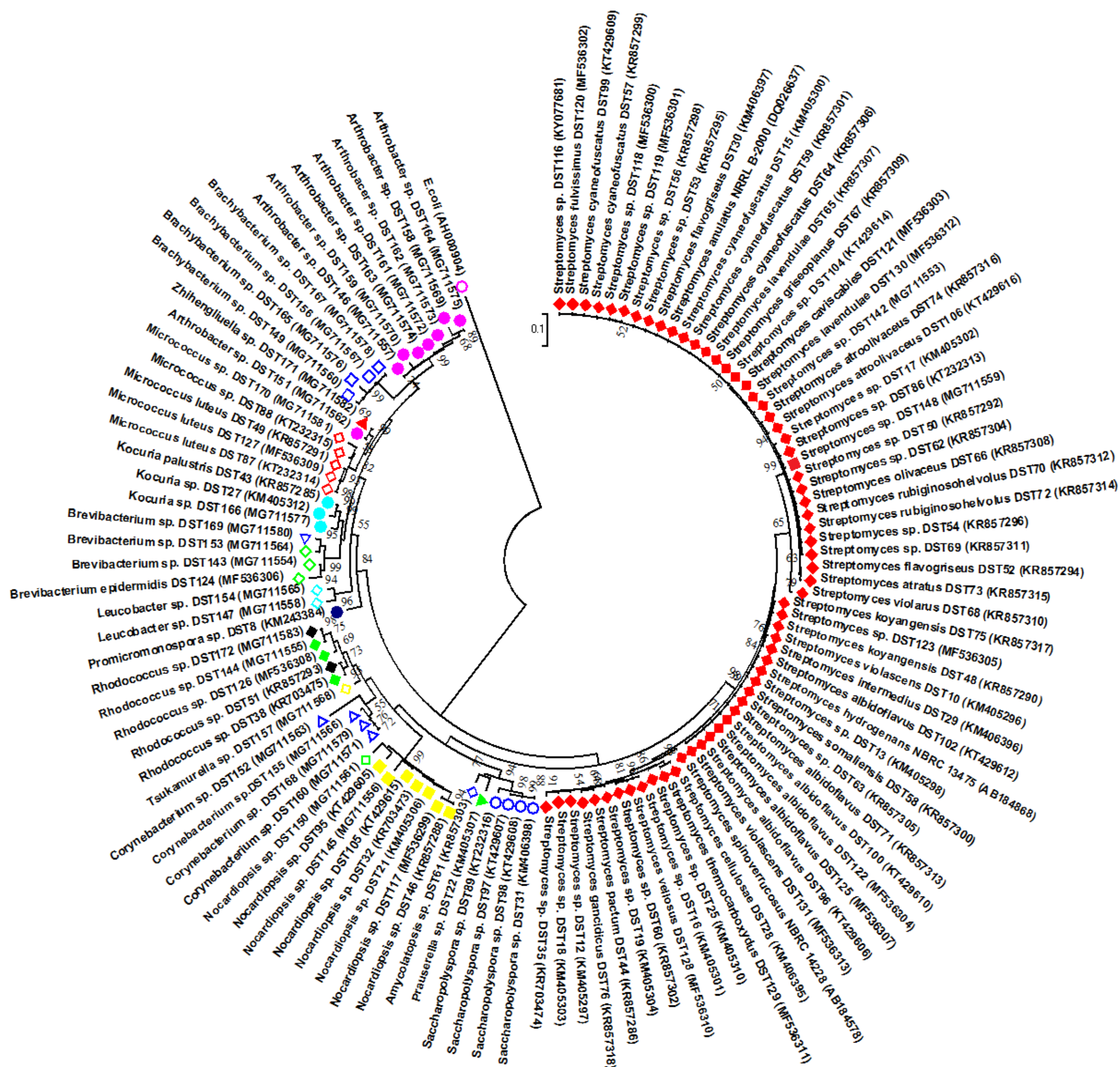

Figure 3.11: Maximum likelihood (ML) phylogenetic tree using Tamura-Nei model based on 16S rRNA gene sequences of actinobacteria showing the phylogenetic relationship between the isolates with *E. coli* as an out-group. Numbers at branches indicate bootstrap values in 1,000 replicates.

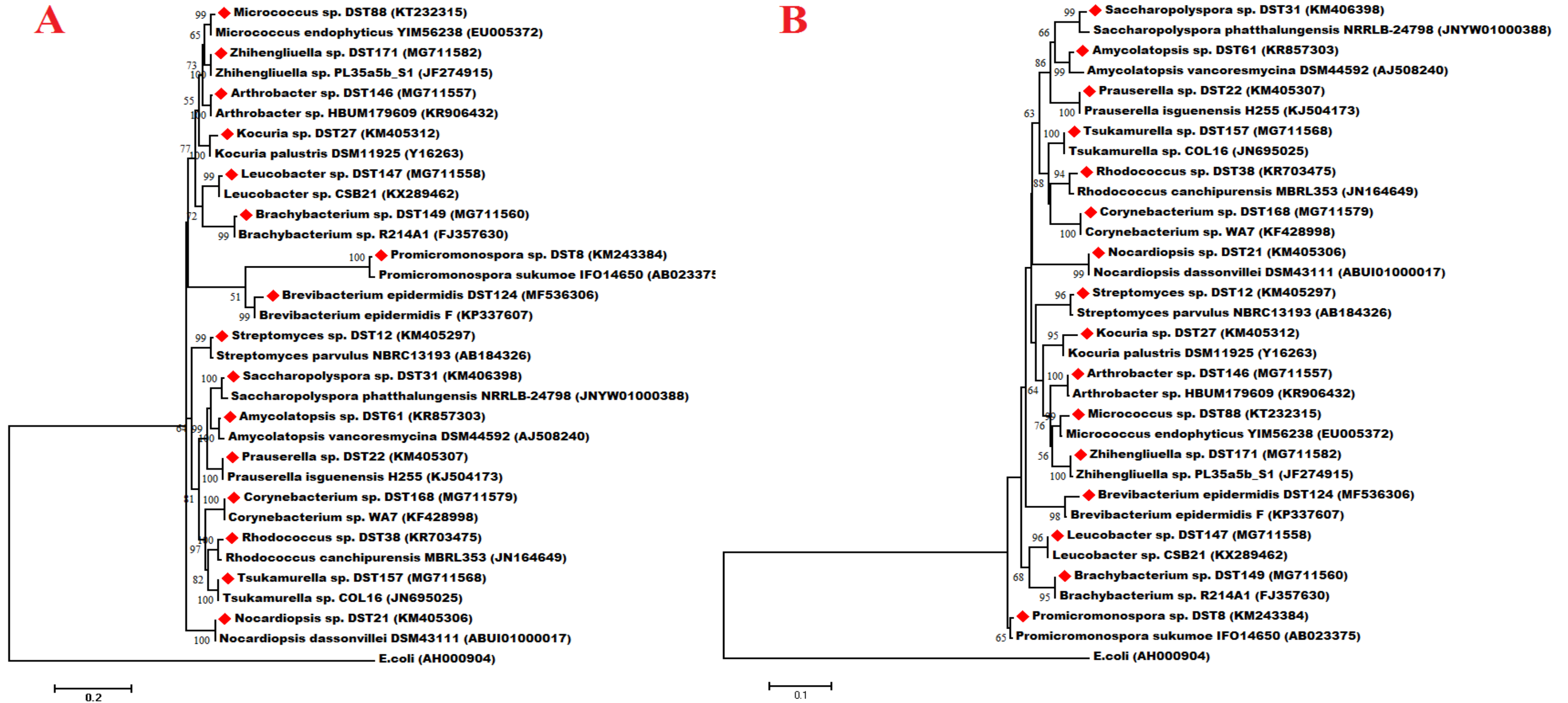

Figure 3.12: (A) Maximum likelihood (ML) phylogenetic tree using Tamura-Nei model based on 16S rRNA gene sequences of actinobacteria showing the phylogenetic relationship between different genera. (B) Neighbour joining (NJ) phylogenetic tree using Tamura-Nei model based on 16S rRNA gene sequences of actinobacteria showing the phylogenetic relationship between different genera. Numbers at branches indicate bootstrap values in 1,000 replicates and *E. coli* as an out-group.

3.4 Discussion

According to actinobacteriological research in India (Velho-Pereira and Kamat. 2013), fresh water habitats have been neglected in search of potential actinobacteria compared to marine and terrestrial ecosystem, most of which remains unexplored and is becoming a promising area for the isolation of actinobacteria associated with its bioactive compounds of pharmaceutically and biotechnologically significance. Isolation and characterization of actinobacteria from its wild habitats and its phylogenetic studies are the initial steps in the exploration research as we described in our paper (Zothanpuia et a., 2018). The diversity of actinobacteria largely depend on the isolation method, serial dilution and spread plate technique are the most commonly used method for the isolation of actinobacteria worldwide (Charousová et al., 2017) which is also used in the present investigations. Cyclohexamide and nalidixic acid are the antibiotics effectively used for the elimination of unwanted fungal and bacterial contaminants respectively supported by Ningthoujam et al. (2011) and Rizvi et al. (2012). The used of pre-treatment most commonly, heat treatment seems to affect the diversity by selectively isolating the actinobacteria. This may perhaps eliminate some rare genera of actinobacteria that could not survive under that condition, which can be isolated without performing pre-treatment, thus diminishing the diversity of isolated organisms up to certain extent.

Ribotyping using 16S rRNA gene sequencing is the most widely used and accepted method for molecular identification of actinobacteria, successfully employed and reported in various fresh water actinobacteriological research using several primers (Deshmukh and Sridhar. 2002; de Menezes et al., 2008; Sanasam et al., 2011; George et al., 2011; Iqbal1 and Sajid. 2015;), which have also been used in the diversity study of cultivable actinobacteria associated with fresh water sediments in the present investigations. The present study reported one hundred and fifteen isolates of actinobacteria from six fresh water systems of

Mizoram, Northeast India, similar studies were reported in English Lake District- Esthwaite Water and Priest Pot (de Menezes et al., 2008), River Nile in Cairo (Rifaat and El-Sayed. 2008), Tyume River in Eastern Cape Province of South Africa (Sibanda et al., 2010). To best of our knowledge, this is the first study on the comparison of fresh water actinobacteria of lakes and rivers. Maximum isolates were obtained from lakes sediment as compared to sediments of the four rivers. This could be due to the circumstance that, sediments containing actinobacteria in rivers were wiped out incessantly by running water and get deposited in different areas throughout the river. While the lake sediment are concentrated in particular areas which are not much affected by the running water. Seven different nutritional media were employed to achieve maximum diversities of actinobacteria, since the nutritional content varies among the media and nutrients uptake by the organisms also differed as described in the earlier chapter. The results showed SCA to be the best medium for the isolation of actinobacteria with maximum number of isolates, which was in accordance with previous studies on the isolation of actinobacteria from various fresh water habitats such as streams and rivers (Deshmukh and Sridhar. 2002; Rifaat. 2003; Rizvi et al., 2012).

Streptomyces represent the largest genus under the domain bacteria (Subramani and Aalbersberg, 2012) besides the Phylum actinobacteria. The present investigation also showed *Streptomyces* as the most dominant genus in fresh water sediments and was in accordance with the findings of Wohl and McArthur. (1998); Deshmukh and Sridhar. (2002); Ningthoujam et al. (2011); Sanasam et al. (2011) and Jami et al. (2015). Several genera of actinobacteria other than *Streptomyces* called rare genera, whose isolation frequency was lower compared to *Streptomyces* (Tiwari et al., 2012) were also described in the present investigation. 45% of the actinobacterial isolates belonging to fifteen different genera recovered were rare genera which include *Nocardiopsis Arthrobacter, Rhodococcus, Micrococcus, Saccharopolyspora, Brevibacterium, Brachybacterium, Corynebacterium* and

Kocuria, that were reported earlier from fresh water habitats (Jiang and Xu 1996; Sibanda et al 2010; Sanasam et al., 2011; Jami et al., 2015; Zhang et al., 2007; Liu et al., 2014). *Brevibacterium siliguriense* a novel species of actinobacteria was isolated from river water (Kumar et al., 2013). To date and best of our knowledge, *Leucobacter, Zhihengliuella, Amycolatopsis, Prauserella* and *Promicromonospora* have never been reported so far from fresh water sediments and were reported for the first time in the present study. However, a report was found in certain habitats globally like halophilic actinobacteria; *Amycolatopsis halophila* (Tang et al., 2010), *Prauserella salsuginis, Prauserella flava, Prauserella aidingensis,* and *Prauserella sediminis* (Li et al., 2009) were reported to be isolated from a salt lake Xinjiang province, north-west China. *Promicromonospora thailandica* was reported from marine sediment (Thawai and Kudo, 2012). A novel species of *Zhihengliuella aestuarii* and *Zhihengliuella flava* were also reported from tidal flat sediment (Baik et al., 2011) and sea sediment (Hamada et al., 2013) respectively. Several novel species were also reported from various habitats: *Tsukamurella soli* and *Tsukamurella spongiae* from soil and deep-water marine sponge (Olson et al., 2007), *Leucobacter zeae* and *Leucobacter margaritiformis* from the rhizosphere of maize (*Zea mays* L.) (Lai et al., 2015) and bamboo extract (Lee et al., 2012) respectively.

Phylogenetic studies using 16S rRNA gene sequencing and defining their taxonomy have been found significantly in fresh water actinobacterial research (Yokota, 1997; de Menezes et al., 2008; Sanasam et al., 2011; Iqbal1 and Sajid. 2015). Different genera of actinobacteria were clustered separately where all the *Streptomyces* formed a major separate clade which was in accordance with the the phylogenetic studies of Jami et al. (2015) and Passari et al. (2015). Rare genera of actinobacteria like *Saccharopolyspora, Amycolatopsis* and *Prauserella* which falls under the family *Pseudonocardiaceae* were clustered together supported by the findings of Passari et al. (2017). Several genera of actinobacteria which falls

under *Nocardiopsaceae, Corynebacteriaceae Tsukamurellaceae* and *Nocardiaceae* formed a separate clade while the family *Micrococcaceae, Promicromonosporaceae, Microbacteriaceae, Brevibacteriaceae, Dermabacteraceae* formed separate cluster which was in accordance with earlier studies (Sanasam et al., 2011; Iqbal1 and Sajid, 2015; Passari et al., 2015; 2017). This study provides a comprehensive investigation onto the diversity of fresh water actinobacteria and their phylogenetic affiliation which is the initial and crucial steps to study the biosynthetic potential of the organisms.

Chapter IV

Antimicrobial Potential of Actinobacteria Isolated from Fresh Water Sediments; Evaluation of Bioactive Secondary Metabolites using GC-MS/MS and UPLC ESI-MS/MS and Detection of Biosynthetic Genes

4.1 Introduction

Emerging antibiotic resistance against available drugs is one of the major reasons and challenges to look for the discovery of new and novel drugs, such as antibiotics from a natural source to fight against multidrug-resistance pathogens (Claverías et al., 2015). Natural product remains the key source for new drug compounds especially antimicrobial (Berdy, 2005). The infections caused by emerging Gram-negative multidrug-resistant pathogens globally, is an important task. Vancomycin-resistant *Enterococci* (VRE), Methicillin-resistant *Staphylococcus aureus* (MRSA), Extended-spectrum β-lactamase (ESBLs) producing Gram-negative bacteria, *Klebsiella pneumoniae* carbapenemase (KPC) producing Gram-negatives are some of the most relevant cases which are gradually becoming more and more abundant and virulent (Chambers and Deleo, 2009). Microbial secondary metabolites are the organic compounds (e.g. pigments, enzyme inhibitors, alkaloids, antibiotics, effectors of ecological competition and symbiosis, pheromones, immunomodulation agents, pesticides, antitumor agents and growth promoters of animals and plants, gibberellins, carotenoids, etc.) that are not directly involved in the normal growth and reproduction but having potential for the discovery of new drugs to fight against antibiotic resistance pathogens (David et al., 2014). They are increasingly required with the emergence and continued persistence of multiple drug resistant (MDR) disease causing microorganisms and still the bacterial infections remain the leading cause of death worldwide (Procopio et al., 2012). To overcome the present scenario, an attempt has been made by various researchers to improve the existing antibiotics

or discover the new antibiotics with improved effectiveness (Parungao et al., 2007). Not all microbes are producing biologically active secondary metabolites.

Among the microorganisms, Phylum Actinobacteria represent an outstanding and notable source for the production of new bioactive secondary metabolites including antimicrobials, antitumor agent, antiparasitic, anticancer agents and enzymes (Stach et al., 2003; Goodfellow and Fiedler, 2010; Yuan et al., 2014). Several compounds have been successfully isolated and transformed into suitable drugs, antibiotics and other chemotherapeutic agents (Zotchev, 2012). More than 22,000 secondary metabolites have been known to be derived from microbes, the phylum alone is accounting for the production of an approximately 75% of the total bioactive compounds (20% from fungi, 7% from *Bacillus spp.* and 2% from other bacteria) including antibiotics with more than 70% produced by member of the genus *Streptomyces* (Berdy, 2005; Das et al., 2010; Subramani and Aalbersberg, 2012). The genus *Streptomyces* remains prolific producers of novel compounds with a variety of biological activities including antimicrobial, anti-cancer agents and other pharmaceutically useful compounds (Berdy, 2005; Kim et al., 2008; Saurav and Kannabiran, 2012; Wang et al., 2013; Rajan and Kannabiran, 2014; Ser et al., 2015). They are widely distributed in various ecosystems like soils, fresh waters, marine environments and lakes (Goodfellow and Williams, 1983).

Lakes and rivers are important reservoir of antagonistic actinobacteria (Leiva et al., 2004). Different habitats have been largely explored in search of actinobacteria for their bioactive secondary metabolites production (Sharma et al., 2016) and still there is a continuous demand of new antibiotic compounds which may possibly be

obtained from unexplored/less explored environments (Bull and Stach, 2007). It is important to target such environments that could be highly potent source for obtaining novel and bioactive compounds. In recent years, an attempt has been made by many researchers in search of new antibiotic compounds from different unexplored habitats (Mitra et al., 2008; Goodfellow and Fiedler, 2010). Investigation of actinobacteria for their secondary metabolite productions from various ecosystems of Northeast India is reported by several researchers (Talukdar et al., 2012; Sharma et al., 2014, 2016; Passari et al., 2015). After literature review, the selected five fresh water lakes and rivers of Mizoram, North east India has been found unexplored ecosystem for actinobacterial research. A continuous screening of potential bacterial taxa for secondary metabolite production is crucial for the discovery of novel compounds (Lazzarini et al., 2000).

Gas chromatography – mass spectrometry (GC-MS) is an analytical method that used gas chromatography coupled to mass spectrometry to identify different volatile organic substances present in the sample. It is one of the most widely used analytical methods in the field of pharmaceutical and industrial research for the detection, isolation and identification of known as well as unknown volatile organic compounds present in the sample (Passari et al., 2016). It has also been successfully used for the detection and identification of the bioactive secondary metabolites present in the crude extract of various actinobacterial samples worldwide. The highly reproducibility of sensitivity of Gas chromatography – mass spectrometry with its accessibility of databases and spectral libraries for secondary metabolites identification are unique features that assist its application in metabolomics. Ultra

performance liquid chromatography (UPLC-ESI-MS/MS) is highly sensitive and excellent resolution equipped with fast separation method, successfully employed for the quantification of bioactive compounds like antibiotics, phenolic compounds, etc. (Passari et al., 2016; 2017).

Extensive arrays of enzymatic pathways that produce useful secondary metabolites in microbes are known to be encoded in biosynthetic gene clusters. Several secondary metabolite genes encoding for polyketide synthases (PKS II), aminodeoxyisochorismate synthase (*phz*E) and non-ribosomal peptide synthetases (NRPS) have been generally used for assessing the biosynthetic potential of cultivable and non-cultivable microorganism (Jami et al., 2015). Such genes are accountable for the production of most biologically active polyketide, phenazine and peptide compounds respectively.

4.2 Materials and Methods

4.2.1 Screening for antifungal activity

The actinobacterial isolates were evaluated for their antagonistic potential against four fungal phytopathogens *viz*., *Fusarium oxysporum* CABI-293942, *Fusarium udum* MTCC-2755, *Fusarium proliferatum* MTCC-286 and *Fusarium graminearum* MTCC-1893 by dual culture *in vitro* assay (Khamna et al., 2008). Fungal pathogens were inoculated at the centre of potato dextrose agar (PDA) plates and actinobacterial isolates was streaked on opposite sides, 3 cm away from the fungal pathogen. Plates without actinobacteria served as negative controls. Plates were incubated at 28^0 C for 14 days and colony growth inhibition (%) were calculated by using the formula: C - T/C $\times$ 100, where C is the colony growth of

fungal pathogen in control, and T is the colony growth of fungal pathogen in presence of actinobacteria. All isolates were tested in triplicate and mean values was calculated.

4.2.2 Antibiotic sensitivity profiling

Antibiotic sensitivity test was performed following the Kirby Bauer disk diffusion method (Williams et al., 1989) using antibiotic impregnated discs (6mm diameter). Fifteen standard antibiotics were tested against the actinobacterial isolates to determine the antibiotic sensitivity on Mueller Hinton agar medium. The isolates were inoculated in ISP medium no. 1 (tryptone yeast extract) broth and incubated at 28 ^{0}C, 150 rpm for 10-15 days. The grown cultures were spread with a sterile L-shaped spreader over the plates of Mueller Hinton agar. The antibiotic discs were placed on the plate and incubated at 37 ^{0}C for 24 hours. Antibiotic sensitivity was observed by measuring inhibition zone diameters. Based on the diameter of inhibition zone recorded to nearest mm, the organisms were categorized as resistant (R), intermediate (I) and sensitive (S) following DIFCO Manual 10th edition (1984).

4.2.3 Screening for Antimicrobial Activity

Antimicrobial screening of actinobacterial isolates were performed against five pathogenic bacterial isolates [gram positive bacteria: (*Staphylococcus aureus* MTCC-96, *Bacillus subtilis* NCIM-2097, and *Micrococcus luteus* NCIM-2170); Gram negative bacteria: (*Pseudomonas aeruginosa* MTCC-2453 and *Escherichia coli* MTCC-739) and yeast: (*Candida albicans* MTCC-3017)]. The pathogens were obtained from Microbial Type Culture Collection (MTCC), Chandigarh, and National Collection of Industrial Microorganisms (NCIM), Pune, India. Crude

extracts were prepared by inoculating a single purified colony of actinobacteria in liquid medium, Tryptone yeast extract broth (ISP medium 1) and incubated at 28º C, 150 rpm for 7-20 days. The grown cultures were subjected to centrifugation at 8,000 rpm for 3 minutes and the supernatant was used for determining antimicrobial activity by agar well diffusion method (Saadoun and Muhana, 2008). The test pathogenic bacteria were spread on nutrient agar plate and wells were prepared by using sterile cork borer of 6 mm diameter. In each of the plates, 50 µl clear supernatant of the actinobacteria were dispensed into individual wells and the plates were incubated at 28 ± 2^0 C for 24 hours. The anti-microbial activities of the isolates were observed by measuring the inhibition zone around each well. All experiments were performed in triplicate process.

4.2.3.1 Antimicrobial Assay using crude extract

The actinobacterial isolates selected based on antimicrobial screening were grown in ISP1 broth using 500 ml conical flask at 28º C in a shaker incubator for 30 days. The culture filtrate was used for the extraction using methanol 1:1 ratio (v/v). Rotary evaporator was used for the extraction and the extract was preserved for further investigations. Methanolic crude extract of the isolates were prepared in a concentration of 1mg, 2mg, 5mg, 20mg and 40 mg/ml with 10% di-methylsulphooxide (DMSO) and used for antimicrobial activity following agar well diffusion method and disk diffusion assay (Saadoun and Muhana, 2008; Gebreyohannes et al., 2013).

4.2.3.2 Determination of MIC

Minimum inhibitory concentration (MIC) of the selected isolates against bacterial pathogens was determined by following broth micro dilution technique using 96-well microtiter plate (Eloff, 1998). Crude extract of the isolates were dissolved with DMSO in different concentrations (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml) and were added in 96-well microtiter plate containing a bacterial culture as test. Ampicillin (1 mg/ml) amended bacterial culture was used as positive control; DMSO without actinobacterial extract was used as negative control. The plates were incubated at 37º C for 36 hours and absorbance was taken at 700 nm in spectrophotometer UV-VIS (MultiscanTM GO, Thermo Scientific, MA, USA). IC50 was expressed as the concentration (mg/ml) of crude extract at which 50% of bacterial growth was inhibited and was calculated using the calibration curve by linear regression.

4.2.4 Enzymatic screening of the isolates

4.2.4.1 Qualitative screening for cellulase-production

The selected isolates were screened for the production of cellulase and xylanases according to Teather and Wood, (1982). The isolates were streaked on carboxy methyl cellulase (CMC) agar (HiMedia, India) medium and allowed it to grow by incubating at 30 °C for 3 to 5 days (depending on culture growth). The CMC screening plates was flooded with an aqueous solution of 0.5% Congo red for 5 minutes and the plates were de-stained with running solution of 1M NaCl for 15 minutes (Kasana et al., 2008). The plates were observed for clear zone of hydrolysis around the isolates.

4.2.4.1.1 Cellulase Induction

The selected pure colonies of actinobacterial isolates were streaked on CMC induction agar plates (K_2HPO_4–0.5%, NaCl-0.5%, Yeast extract- 0.25%, Peptone-0.5%, Agar Agar -1.5% and CMC-1%) and incubated at 30°C for 3-5 days.

4.2.4.2 Qualitative screening for xylanase-production

Similarly, the actinobacterial isolates were also screened for the production of Xylanase by using Oat Spelt Agar and incubated at 28°C for 5 days (depending on culture growth) (Nanmori et al., 1990). The plates were flooded with Congo red and left for 5minutes at room temperature. After incubation, the Congo red dye was washed away using 1M NaCl and zone formation was recorded.

4.2.4.2.1 Xylanase Induction

Pure colonies of actinobacterial cultures were streaked on xylan induction agar plates (K_2HPO_4 –0.5%, NaCl-0.5%, Yeast extract- 0.25%, Peptone-0.5%, Agar Agar -1.5% ,milliQ H_2O-75% and WSOSX1 (Water Soluble Xylan from Oats for Screening Xylanase-25%) and incubated at 30°C for 3-5 days.

The grown culture actinobacterial isolates on CMCase and xylanase induction media were subjected to Congo red assay as already described.

4.2.5 Quantification of xylanase and cellulase using DNS assay

The selected isolates that showed good hydrolytic zone in screening assay were subjected to submerged fermentation in CX-induction broth incubated in a shaking incubator at 120rpm, 30°C and pH 7. The CX-induction broth was made of ISP1 broth amended with 1% beechwood xylan for xylanase and 0.5% CMC for cellulase induction.

Xylanase and cellulase activity were measured by spectrophotometric biochemical quantification and detection of reducing sugars using 3,4-Dinitrosalicylic acid (DNS) assay following the method of Ghose (1987). The grown cultures were collected and centrifuged at 9,000 rpm at 4 ºC for 10 min and the supernatant was used for enzyme assay. 0.5ml of CMC and Xylanase induction supernatants were mixed with 0.5ml of the supernatant of CMC and Xylanase induction broth respectively. The reaction mixture was then incubated at 55ºC for 1 hr. After incubation, 3 ml of DNS reagent was added and the mixture was heated at 100 ºC for 15 min. Immediately, 1ml of 40% Potassium sodium tartarate was added. On cooling the reaction mixture was further diluted with 19ml distilled water to maintain a final volume of 24ml. This was then subjected to spectrophotometric analysis against either a glucose or xylose standard. The release of reducing sugar was calculated from the OD (optical density) measured at 540 nm. One unit (U) of enzyme activity was defined as amount of enzyme capable of releasing 1 µmol of reducing sugars/min/ml.

Estimation of the concentration of enzyme was done based on the enzyme that released exactly 0.5 mg of xylose or glucose; by calculating the xylose liberated against enzyme concentration. IUPAC (International Union of Pure and Applied Chemistry) has already deemed the critical amount of glucose/xylose converted by CMCase and xylanase as 0.185 and 0.22µmol min^{-1} mL^{-1} (U mL^{-1}) respectively. Hence their activity are calculated as follows

$$\text{Xylanase Activity} = \frac{0.22}{\text{enzyme concentration to release 0.5 mg xylose}} \text{Units / mL}$$

$$\text{CMCase Activity} = \frac{0.185}{\text{enzyme concentration to release 0.5 mg glucose}}\ \text{Units / mL}$$

$$\text{Concentration} = \frac{1}{\text{dilution}}\left(= \frac{\text{volume of enzyme in dilution}}{\text{total volume of dilution}}\right)$$

4.2.5.1 Effect of incubation time on xylanase and cellulase production

To determine the effect of incubation time on xylanase and cellulase production, the selected isolates were cultured for 12 days under submerged fermentation conditions with pH 7 and 30 ^{o}C. The culture supernatant was harvested on every 1 day interval and the respective enzyme (xylanase and cellulase) assays were performed to determine the optimal day for enzyme production.

4.2.6 Amplifications of Biosynthetic Genes (PKS, *phz*E and NRPS):

The presence of biosynthetic genes [Polyketide synthase type II (PKS II) non-ribosomal peptide synthetase (NRPS) and aminodeoxyisochorismate synthase (*phz*E)] were evaluated using degenerate primers for highly conserved regions encoding enzymes associated with biosynthesis of polyketides, peptides and phenazine respectively (Ayuso-Sacido and Genilloud, 2005). The primers employed and PCR conditions for amplification of PKS-II, *phz*E and NRPS gene fragments were shown in **Table 4.1**. The conditions used in PCR were listed in **Table 4.2** and reactions mixture for the amplification of PKS II, NRPS and *phz*E genes were as follows:

PKS II (50 µL): 3 µL template, 5 µL 10× buffer (Mg2+ free), 1 µL MgCl2 (25 mM), 1 µL DMSO (10%), 5 µL dNTP (2.5 mM), 1.8 µL each primer (10 µM) and 5 U Taq DNA polymerase

NRPS (50 µL): 3 µL template, 5 µL 10× buffer (Mg2+ free), 1 µL MgCl2 (25 mM), 1 µL DMSO (10%), 5 µL dNTP (2.5 mM), 2 µL each primer (10 µM) and 5 U Taq DNA polymerase

***phz*E** (25 µL): 2 µL template, 2.5 µL 10×buffer, 2 µL BSA (1 mg/mL), 2 µL dNTP (2.5 mM), 0.8 µL each primer (10 µM) and 2 U Taq DNA polymerase

Table 4.1: Primers used for the amplification of PKS II, NRPS and *phz*E gene in the isolated actinobacterial DNA

Gene	Primer	Length (bp)	References
PKS II	KS1F 5'-TSGCSTGCTTGGAYGCSATC-3' KS1R 5'-TGGAANCCGCCGAABCCTCT-3'	~654	Wawrik et al., 2005
***phz*E**	phzEf 5'-GAAGGCGCCAACTTCGTYATCAA-3' phzEr 5'-GCCYTCGATGAAGTACTCGGTGTG-3'	~450	Schneemann et al., 2011
NRPS	A3F 5'-GCSTACSYSATSTACACSTCSGG-3' A7R 5'-SASGTCVCCSGTSGCGTAS-3'	~700	Ayuso-Sacido and Genilloud, 2005

Table 4.2: PCR conditions used for the amplification of PKS II, NRPS and *phz*E gene

Biosynthetic gene	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	
PKS II	95° - 5'	95°- 1'	58°- 1:30'	72°- 2'	72°-10'	**35 Cycle**
NRPS	95° - 5'	95°- 1'	59°- 2'	72°- 4'	72°-10'	
*phz*E	95° - 5'	94°- 1'	55°- 1'	72°- 2'	72°-18	

A negative control reaction mixture without DNA template of actinobacteria was also included with each set of PCR reactions. The amplified PCR product was

checked by using 1.5 % agarose gel electrophoresis using TAE buffer and analyzed under UV light and documented using a Bio-rad Gel Doc XR^+ system (Hercules, CA, USA) and sequenced commercially at SciGenome Pvt. Ltd. Kochin, India.

4.2.7 Detection of Antibiotics using UPLC-ESI-MS/MS

Ultra-Performance Liquid Chromatography (UPLC-ESI-MS/MS) was employed for the detection of antibiotic in the methanolic extracts of the selected isolates. Five antibiotics (nalidixic acid, trimethoprim, fluconazole, ketoconazole and rifampicin) were selected and standard solution was prepared using methanol.

4.2.7.1 Preparation of standard solution

Stock solution was prepared by mixing all the selected antibiotics with methanol and were further diluted within the ranges of 0.5 -500 $ngml^{-1}$ to prepare the working standard solutions and standard calibration curve was plotted. The standard stock solutions were maintained at -20°C till used and vortexed prior to injection as described (Passari et al., 2017).

4.2.7.2 Instrumentation and analytical conditions

Ultra performance liquid chromatography ($UPLC^{TM}$) coupled with auto-sampler and binary pump (Waters, Milford, MA) was used in this study. An analytical column Acquity BEH C18 (2.1 mm×50 mm, 1.7µm; Waters, Milford, MA) was used for the separation of compounds in the extract. The mobile phase contained two solvents: solvent A - 0.1% (v/v) formic acid in water and solvent B- acetonitrile. The following gradient program was used for the analysis:

0 - 1 min	12 - 90% B
2 - 4 min	90 - 95% B
4 - 5 min	95–12% B

Post - run (1 min)	12% B
Flow rate	0.035 ml min^{-1}
Injection volume	5 µL

Ultra performance liquid chromatography system was coupled to triple - quadrupole linear ion trap mass spectrometer [(API 4000 QTRAP™ MS/MS system) Canada] fitted with electrospray (Turbo V^{TM}) ion source was run in –ve and +ve ionization mode. The standardised parameters for +ve mode were: ion spray voltage - 5500V; turbo spray temperature - 450° C; nebulizer gas - 50psi; heater gas - 50psi; collision gas - medium; the curtain gas - 20psi. The conditions of mass spectrometer was also optimised by infusing 100 - 500 ng ml^{-1} analytes solution dissolved in methanol at 10 µl min^{-1} using Harvard '22' syringe pump (Harvard Apparatus, USA). The maximum number of precursor to product ions for every compound was selected for multiple reactions monitoring quantification. Attainment of data and instrument control was performed using analyst 1.5.1 software package (AB Sciex). The spectra covered the range from 100 to 1000 m/z for full scan Electrospray ionisation mass spectrometry analysis. Parameters for reference analytes in mass spectrometry: precursor ion (Q1), product ion (Q3), declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP) were optimized in –ve and +ve ESI mode, by flow injection analysis (FIA). MRM parameters were optimized to achieve the most abundant, specific and stable MRM transition for each compound as shown in **Table 4.3.** MS spectra and MRM extracted ion chromatogram of reference analytes was shown in **Figure 4.1** and **Fig 4.2** respectively.

Table 4.3: LC-MS/MS optimized parameters for selected antibiotics

Analytes	*rt* (min)	Q1	Q3	Ion species	DP	EP	CE	CXP
Trimethoprim	0.9	291.2	231.2	$[M+H]^+$	149	6	33	10
Fluconazole	1.07	307.1	220.1	$[M+H]^+$	59	10	27	8
Ketoconazole	1.46	532.1	82	$[M+H]^+$	106	10	68	10
Nalidixic acid	1.58	233.1	215.1	$[M+H]^+$	49	8	21	8
Rifampicin	1.83	823.5	791.4	$[M+H]^+$	53	9	24	19

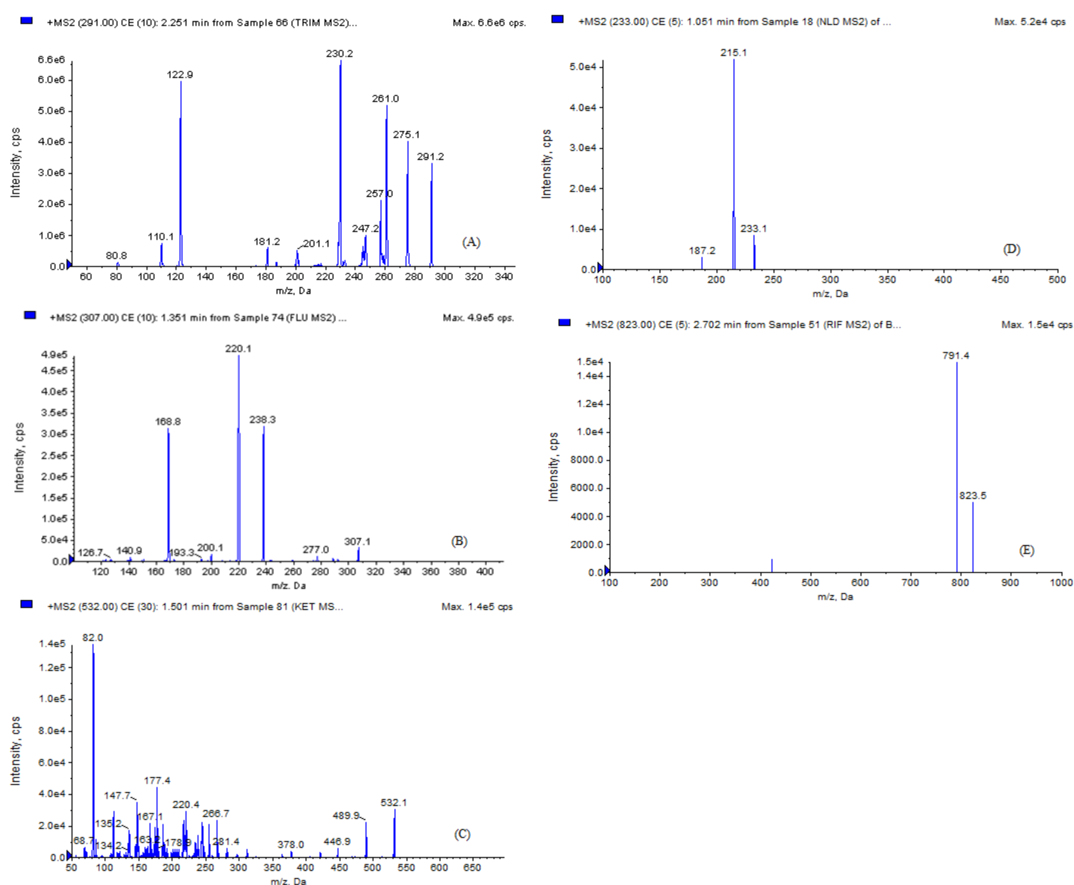

Figure 4.1: MS/MS Spectra of reference analytes; (A) trimethoprim, (B) fluconazole, (C) ketoconazole, (D) nalidixic acid, (E) rifampicin

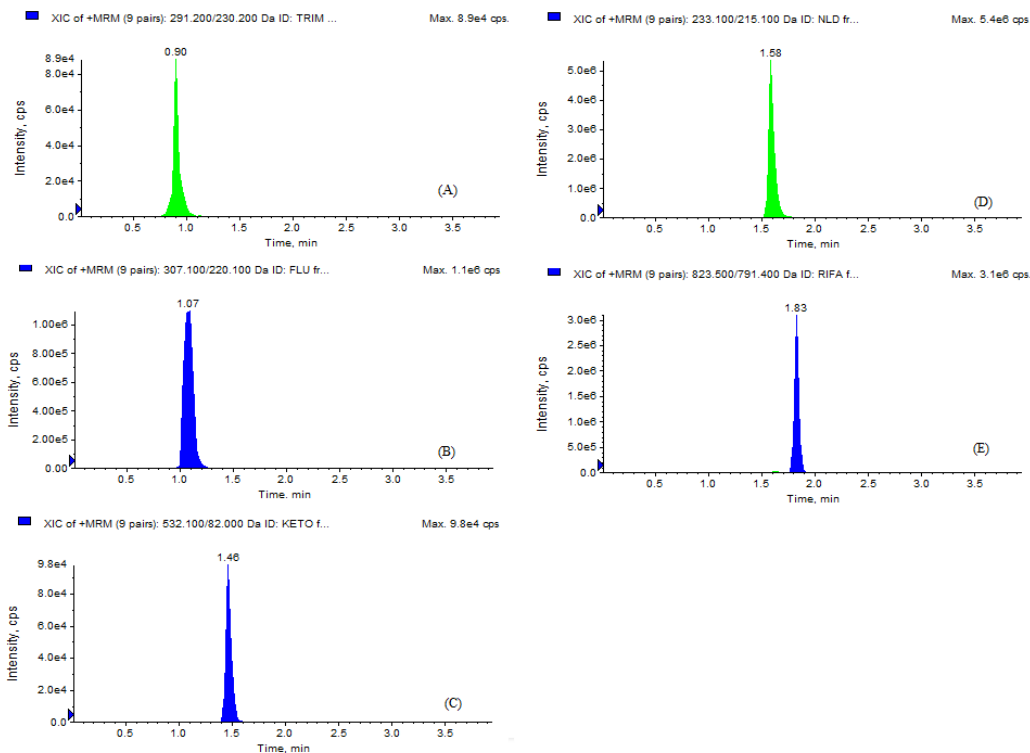

Figure 4.2: MRM extracted ion chromatogram of reference analyte: (A) trimethoprim, (B) fluconazole, (C) ketoconazole, (D) nalidixic acid, (E) rifampicin

4.2.7.3 Analytical Method Validation

The method for quantitative analysis using ultra performance liquid chromatography – Multiple reaction monitoring was validated conferring to the guidelines of international conference on harmonization (ICH, Q2R1) by linearity, limits of detection and quantification (LODs and LOQs), precision, solution stability, and recovery.

4.2.7.3.1 Linearity, LOD and LOQ

Limits of detection and quantification were determined by calibration curve method where a series of concentrations of standard solution was prepared for

creating calibration curve by plotting the peak area against the corresponding concentrations. LOD and LOQ were calculated using following equations. LOD = (3.3 × Sy. x) / S; LOQ = (10 × Sy. x) / S (Where, Sy. x = standard deviation of residuals from line; S = slope).

4.2.7.3.2 Precision, Stability and Recovery

Relative standard deviation (RSD) was employed for precision evaluation. Analytes were determined in six replicates on a single day and by duplicating the experiments over three consecutive days to evaluate intra-day and inter-day variations. The overall precision was not more than 2.01%. Sample solutions stability kept at room temperature was measured at 0, 2, 4, 8, 12, and 24 h by replicate injections. The stability RSD value of analytes is ≤2.45%. Recovery test was applied to evaluate the accuracy by spiking high, middle and lower concentration level of the analytical standards into the samples. Experiments were performed in triplicates at each level.

4.2.8 Evaluation of bioactive compounds using GC-MS

Gas Chromatography Mass Spectroscopy (GC-MS) was used to determine the volatile organic compounds (VOCs) present in the methanolic crude extracts of the selected actinobacterial isolates. For GC-MS, the Clarus 680 GC was used in the analysis employed with fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250μm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1μL of extract sample injected into the instrument and the oven temperature was as follows:

60° C (2 min); followed by 300° C at the rate of 10° C min^{-1}; and 300° C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240° C; ion source temperature 240° C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 600 Da were analysed. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

4.2.9 Statistical analysis

All experiments were performed in triplicate process and repeated thrice. Readings were taken as the mean ± standard deviation of mean of three replicates calculated using Microsoft Excel XP 2010.

4.3 Results

4.3.1 Antifungal activity of actinobacterial isolates

The antifungal activity of actinobacterial isolates was tested against four fungal phytopathogens: *F. udum, F. oxysporum, F. graminearum* and *F. proliferatum.* From a total of 115 actinobacterial isolates tested, 49.7% (n=57) of the isolates showed inhibition activity against at least one of the tested pathogens **(Table 4.4).** 14 isolates *viz. Streptomyces cyaneofuscatus* DST 15, *Streptomyces* sp. DST 16, *Streptomyces* sp. DST25, *Saccharopolyspora* sp. DST31, *Streptomyces griseoplanus* DST53, *Streptomyces* sp. DST54, *Streptomyces cyaneofuscatus* DST64, *Streptomyces albidoflavus* DST71, *Streptomyces* sp. DST86, *Streptomyces albidoflavus* DST102, *Streptomyces* sp. DST104, *Nocardiopsis* sp. DST105, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST119 showed activity against all

the tested four *Fusarium* pathogens. Maximum inhibitory activity of the actinobacterial isolates was found against *F. graminearum* (33.9%, n=39) [**Figure 4.3 (I)**], followed by *F. proliferatum* (30.4%, n=35) [**Figure 4.3 (II)**], *F. oxysporum* (29.5%, n=34) [(**Figure 4.3 (III)]** and *Fusarium udum* (26.9%, n=31) [(**Figure 4.3 (IV)**] with percentage of inhibition ranging from 20-87.2. Among all the isolates, *Streptomyces* sp. DST25 showed maximum percentage of inhibition against *Fusarium Udum* (87.20%)

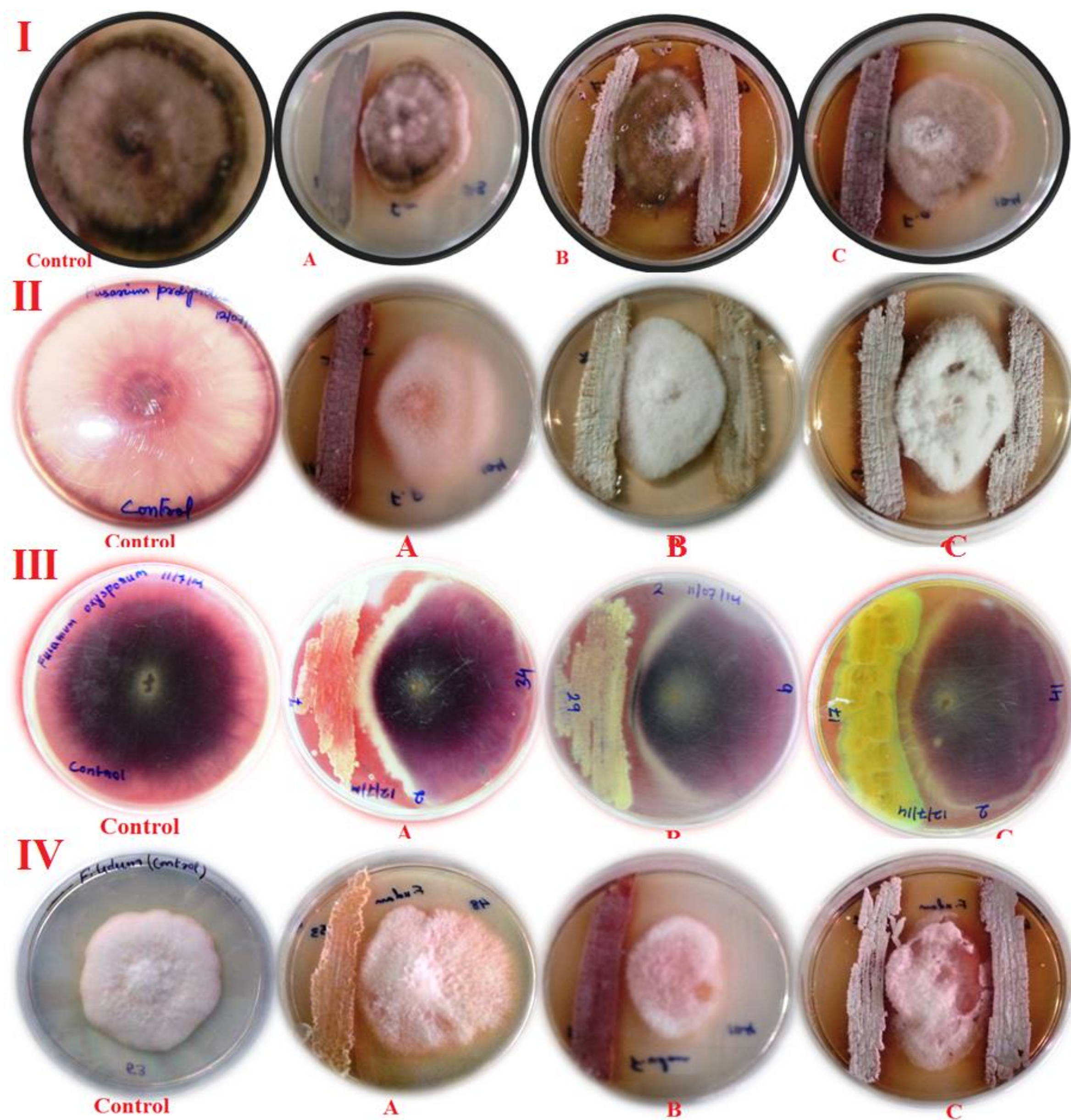

Figure 4.3: (I) Antifungal activity of actinobacterial isolates against *F. graminearum* **(II)** *F. proliferatum* **(III)** *F. Oxysporum* and **(IV)** *F. udum*

Table 4.4: *In vitro* antagonistic activity of actinobacterial isolates against four fungal phytopathogens.

Sl no.	Isolate name	Isolate no.	Antifungal properties C-T/CX100			
			F. udum	***F. oxysporum***	***F. graminearum***	***F. proliferatum***
1	*Streptomyces* sp.	DST13	-	48.50±0.29	20.40±0.65	46.00±0.30
2	***Streptomyces cyaneofuscatus***	**DST 15**	**74.40±0.16**	**54.60±0.42**	**42.00±0.25**	**55.00±0.63**
3	***Streptomyces* sp.**	**DST 16**	**61.00±0.69**	**45.50±0.44**	**52.00±0.69**	**62.20±0.33**
4	*Streptomyces* sp.	DST 17	59.00±0.03	-	61.29±0.98	-
5	*Streptomyces* sp.	DST 18	-	-	-	46.50±0.93
6	*Streptomyces* sp.	DST19	61.54±0.49	39.40±0.13	-	47.00±0.38
7	*Prauserella* sp.	DST22	48.71±0.14	-	35.50±0.41	37.84±0.29
8	*Streptomyces* sp.	DST23	-	73.68±0.01	-	57.89±0.36
9	*Streptomyces parvus*	DST24	-	50.68±0.00	-	67.12±0.18
10	***Streptomyces* sp.**	DST**25**	**87.20±0.37**	**69.69±0.41**	**54.84±0.84**	**72.97±0.075**
11	*Kocuria* sp.	DST27	73.68±0.01	45.20±0.09	-	48.65±0.53
12	*Streptomyces cellulosae*	DST28	43.59±0.46	-	32.25±0.69	-
13	*Streptomyces intermedius*	DST29	57.89±0.36	35.48±0.28	-	52.63±0.00
14	*Streptomyces flavogriseus*	DST30	-	48.49±0.24	-	-
15	***Saccharopolyspora* sp.**	**DST31**	**58.97±0.33**	**51.52±0.68**	**48.39±0.24**	**56.76±0.35**
16	*Streptomyces* sp.	DST35	-	-	23.60±0.29	-
17	*Rhodococus* sp.	DST38	-	54.55±0.5	-	45.95±0.25
18	*Streptomyces pactum*	DST44	-	-	22.58±0.64	-
19	*Streptomyces koyangenesis*	DST48	-	-	-	45.94±0.21
20	*Streptomyces* sp.	DST50	-	-	35.48±0.41	-
21	*Rhodococcus* sp.	DST51	54.00±0.85	45.45±0.49	41.94±0.38	-
22	*Streptomyces flavogriseus*	DST52	48.72±0.49	-	19.35±0.79	32.43±0.59
23	***Streptomyces griseoplanus***	**DST53**	**58.97±0.63**	**58.00±0.33**	**48.50±0.39**	**56.76±0.44**

24	***Streptomyces* sp.**	**DST54**	**58.97±0.98**	**57.58±0.62**	**48.40±1.39**	**56.76±0.44**
25	*Streptomyces* sp.	DST56	-	-	41.94±0.71	-
26	*Streptomyces somaliensis*	DST58	43.59±0.14	-	-	-
27	*Streptomyces cyaneofuscatus*	DST59	-	39.39±0.36	22.58±0.39	45.95±0.38
28	*Streptomyces* sp.	DST60	-	39.39±0.61	29.03±0.5	40.54±0.96
29	*Streptomyces* sp.	DST63	59.00±0.12	-	48.39±0.56	59.46±0.99
30	***Streptomyces cyaneofuscatus***	**DST64**	**58.97±0.02**	**39.39±0.35**	**35.48±0.52**	**59.46±0.76**
31	*Streptomyces lavendulae*	DST65	-	-	54.84±0.28	-
32	*Streptomyces olivaceus*	DST66	-	-	35.48±0.16	-
33	*Streptomyces griseoplanus*	DST67	-	-	41.94±0.73	-
34	*Streptomyces* sp.	DST69	-	-	35.50±0.34	43.24±0.73
35	***Streptomyces albidoflavus***	**DST71**	**53.8±0.76**	**45.45±0.44**	**35.48±1.29**	**51.35±0.57**
36	*Streptomyces rubiginosohelvolus*	DST72	38.46±0.44	-	-	-
37	*Streptomyces atratus*	DST73	-	40.35±0.81	9.68±0.45	43.24±0.73
38	*Streptomyces atroolivaceus*	DST74	53.85±0.16	39.40±1.39	41.94±0.06	-
39	*Streptomyces koyangenesis*	DST75	61.53±0.53	42.50±0.43	51.61±0.56	-
40	***Streptomyces* sp.**	**DST86**	**66.67±0.37**	**39.39±0.61**	**41.93±0.38**	**37.84±0.48**
41	*Saccharopolyspora* sp.	DST89	53.85±0.09	45.45±0.55	35.48±0.62	-
42	*Nocardiopsis*	DST95	-	-	-	35.14±0.64
43	*Streptomyces albidoflavus*	DST96	-	37.88±0.25	-	-
44	*Saccharopolyspora* sp.	DST97	-	-	22.60±0.27	-
45	*Streptomyces cyaneofuscatus*	DST99	-	51.52±0.32	35.48±0.71	-
46	*Streptomyces albidoflavus*	DST100	-	-	41.94±0.37	-
47	***Streptomyces albidoflavus***	**DST102**	**49.52±0.39**	**51.52±0.25**	**48.45±0.17**	**40.54±0.71**
48	*Streptomyces cyaneofuscatus*	DST103	48.72±0.19	54.55±0.25	-	45.95±0.95
49	***Streptomyces* sp.**	**DST104**	**66.67±0.84**	**51.52±0.38**	**48.40±0.54**	**64.86±0.44**
50	***Nocardiopsis***	**DST105**	**60.26±0.26**	**51.52±0.29**	**49.50±0.77**	**45.95±0.42**

51	*Streptomyces atroolivaceus*	DST106	-	-	23.00±0.52	45.95±0.56
52	***Streptomyces* sp.**	**DST116**	**48.71±0.48**	**51.52±0.21**	**29.03±1.06**	**45.95±0.63**
53	*Streptomyces griseus*	DST118	-	39.39±0.69	-	43.24±0.42
54	***Streptomyces* sp.**	**DST119**	**49.00±0.39**	**45.45±0.52**	**51.61±0.39**	**56.76±0.55**
55	*Streptomyces fulvissimus*	DST120	50.50±0.48	39.39±0.43	-	51.35±0.57
56	*Streptomyces* sp.	DST142	42.50±0.19	-	-	-
57	*Nocardiopsis* sp.	DST145	28.00±0.08	-	-	-

4.3.2 Antibiotic sensitivity of actinobacteria isolates

Fifteen different standard antibiotic discs procured from Himedia (India) were used against actinobacteria isolates to check the antibiotic sensitivity pattern by using Mueller Hinton agar media. Antibiotics used include: *viz.* Ampicillin (Amp^{10}), Streptomycin (S^{10}), Erythromycin (E^{15}), Norfloxacin (NX^{10}), Tetracycline (TE^{30}), Gentamycin (Gen^{10}), Penicillin G (P^{2}), Neomycin (N^{10}), Chloramphenicol (C^{10}), Polymixin B (PB^{50}), Ciprofloxacin (CIP^{10}), Vancomycin (VA^{10}), Trimethoprim (TR^{10}), Clindamycin (CD^{10}), Levofloxacin (LE^{5}). Among the 69 isolates tested for their antibiotic sensitivity, the isolates were highly resistant to trimethoprim (R=62, S=7) followed by Penicillin (R=52, S=3, I=14) and Ampicillin (R=42, S=14, I=13) whereas a high sensitivity was found against Streptomycin, Erythromycin, Norfloxacin, Tetracycline, Gentamycin, Neomycin, Chloramphenicol, Polymixin B, Ciprofloxacin, Vancomycin, Clindamycin and Levofloxacin. Majority of the isolates were sensitive to Levofloxacin (R=1, I=4, S=64). *Streptomyces* sp. DST13, *Streptomyces* sp. DST19, *Streptomyces intermidus* DST29, *Saccharopolyspora* sp. DST89, *Nocardiopsis* sp. DST95 and *Nocardiopsis* sp. DST105 showed resistance to ten antibiotics (**Table 4.5; Figure 4.4**).

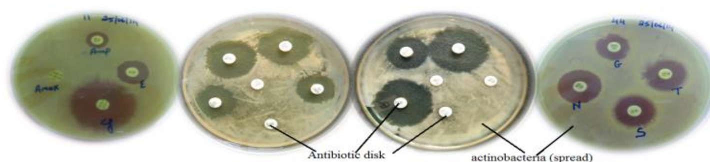

Figure 4.4: Antibiotic sensitivity profile of some actinobacteria against 15 standard antibiotics

Table 4.5: Antibiotic sensitivity profile of actinobacterial isolates against fifteen selected standard antibiotics

Isolates	**Amp^{10}**	**S^{10}**	**E^{15}**	**NX^{10}**	**Te^{30}**	**Gen^{10}**	**P^{2}**	**N^{10}**	**C^{1O}**	**PB^{50}**	**Cip^{10}**	**VA^{10}**	**Tr^{10}**	**CD^{10}**	**LE^{5}**
DST8	S	S	R	I	S	S	S	S	S	R	S	S	S	S	S
DST10	R	I	R	I	I	I	R	R	I	R	S	I	R	I	I
DST12	R	S	I	R	I	S	R	I	I	R	S	I	R	S	S
DST13	I	R	R	R	R	R	R	I	I	R	R	I	R	S	S
DST15	I	I	I	R	S	I	I	S	S	I	S	S	R	S	S
DST16	I	I	I	R	I	I	S	I	S	R	I	I	S	S	S
DST17	I	I	I	I	I	I	R	S	S	I	I	I	R	S	S
DST18	R	R	R	R	R	S	I	R	I	I	S	I	R	S	I
DST19	R	R	R	R	R	R	R	I	S	R	R	I	R	S	S
DST21	R	S	R	I	S	S	R	I	I	R	I	I	R	S	S
DST22	R	S	I	S	S	S	R	I	I	R	S	I	R	S	S
DST23	R	S	I	S	S	S	R	I	I	R	S	I	R	S	S
DST24	R	S	S	I	I	R	R	I	R	I	I	I	R	S	R
DST25	I	S	S	R	S	I	R	I	S	I	S	I	R	S	S
DST27	R	R	I	I	I	S	R	I	S	R	S	I	R	S	S
DST28	R	S	I	I	I	I	R	S	S	R	I	S	S	S	S
DST29	R	R	R	R	R	R	R	R	S	R	S	I	R	S	S
DST30	R	S	I	S	R	R	I	S	S	I	S	I	R	S	S
DST31	S	I	S	R	I	I	I	I	I	I	S	R	R	R	S
DST32	I	S	S	S	I	I	R	I	S	I	S	S	R	S	S
DST35	R	S	I	R	R	S	R	I	S	I	S	R	R	R	S
DST38	R	I	I	I	S	S	S	I	I	R	S	I	R	S	S
DST43	R	S	I	I	S	S	R	I	I	R	S	I	R	S	S
DST44	R	S	S	R	I	I	R	I	I	I	I	R	R	R	S
DST46	R	I	S	S	I	I	R	I	R	I	S	S	R	I	S

DST48	R	S	R	I	S	S	R	S	S	R	S	I	R	S	S
DST49	R	S	S	I	I	I	I	I	R	R	I	S	R	S	S
DST51	I	S	S	S	S	S	I	S	S	R	S	S	R	S	S
DST52	S	S	S	I	I	S	R	S	S	R	S	S	R	S	S
DST53	R	I	S	S	I	S	R	I	S	I	S	I	R	S	S
DST54	I	I	S	I	S	S	I	S	I	I	R	S	R	S	S
DST55	S	R	S	R	R	S	R	R	I	R	S	R	R	R	S
DST56	S	R	I	R	I	I	R	R	I	R	I	R	R	R	I
DST57	S	S	R	S	S	S	S	I	S	R	S	S	S	S	S
DST58	R	S	S	S	S	S	R	S	S	I	S	I	R	S	S
DST59	I	S	S	S	S	S	I	S	S	R	S	S	R	S	S
DST60	I	S	S	R	I	S	R	S	I	I	S	S	R	S	S
DST61	R	R	S	S	R	I	R	R	I	R	S	R	R	I	S
DST62	R	R	I	S	R	S	R	I	I	R	S	I	R	S	I
DST63	R	I	S	I	I	I	R	R	S	R	S	I	R	I	S
DST64	R	S	I	S	S	S	R	I	S	I	S	I	R	R	S
DST66	R	S	S	R	S	S	R	R	S	R	S	S	R	S	S
DST67	R	I	S	S	S	S	R	I	I	R	S	I	R	S	S
DST68	R	I	I	I	I	I	R	S	S	R	S	S	R	S	S
DST69	R	I	I	I	I	I	R	I	S	R	S	R	S	S	S
DST70	S	I	S	I	I	S	I	I	S	I	S	R	R	R	S
DST71	R	I	I	I	R	R	R	R	S	R	I	I	R	S	S
DST72	I	S	S	S	S	S	I	I	R	R	S	R	R	R	S
DST74	R	R	I	R	I	R	R	R	S	R	S	I	R	S	S
DST75	R	S	I	I	S	I	R	I	I	R	S	S	R	I	S
DST76	R	S	I	R	I	S	R	S	S	I	I	S	R	S	S
DST86	S	R	I	I	I	I	R	I	I	R	I	I	R	S	S
DST87	S	I	R	R	I	I	R	S	I	I	I	S	R	R	S

DST89	R	R	R	R	R	R	R	I	I	R	R	I	R	S	S
DST95	R	R	R	R	R	R	R	I	I	R	R	I	R	S	S
DST96	I	S	R	S	S	S	I	S	S	I	S	S	R	S	S
DST98	R	I	I	I	I	I	R	I	R	R	I	R	R	S	S
DST99	I	S	S	S	I	S	I	S	S	R	S	S	R	S	S
DST100	R	S	S	S	S	S	R	S	S	R	S	S	R	S	S
DST102	S	S	S	S	S	R	R	S	I	R	S	I	R	S	S
DST103	R	S	S	S	S	S	R	R	I	R	S	R	R	S	S
DST104	S	S	R	S	S	I	I	S	I	R	S	S	R	S	S
DST105	R	R	I	I	R	R	R	R	R	R	S	R	R	I	S
DST106	R	I	S	I	I	R	R	R	I	R	S	I	R	S	S
DST116	S	I	R	R	I	R	R	I	I	I	I	I	R	I	S
DST117	S	S	S	S	S	S	S	I	S	R	S	S	S	S	S
DST118	R	I	I	I	I	S	R	S	I	R	S	S	S	I	S
DST119	S	S	S	R	S	S	I	S	S	I	S	R	R	R	S
DST120	R	S	S	S	I	S	R	I	I	R	S	I	R	S	S

Degree of susceptibility according to CLSI guidelines. Amp^{10} – Ampicillin, S^{10} - Streptomycin, E^{15} – Erythromycin, NX^{10} – Norfloxacin, TE^{30} – Tetracycline, Gen^{10} – Gentamycin, P^{2} – Penicillin G, N^{10} – Neomycin, C^{10} – Chloramphenicol, PB^{50} – Polymixin B, CIP^{10} – Ciprofloxacin, VA^{10} – Vancomycin, TR^{10} – Trimethoprim, CD^{10} – Clindamycin, LE^{5} - Levofloxacin.

4.3.4 Antimicrobial activity of actinobacterial isolates

All the actinobacterial isolates (n=115) were screened for their antimicrobial activity against five bacterial pathogens and one yeast pathogen (*S. aureus*, *B. subtilis, M. luteus*, *P. aeruginosa, E. coli* and *C. albicans*). Among the tested isolates, 66.1% (n=76) of the actinobacterial isolates were found to inhibit the growth of at least one tested pathogens (**Figure 4.5**). Among the pathogens used in the study, *E. coli* was found to be the most susceptible pathogen against the isolated actinobacteria, 76 isolates inhibited the growth of *E. coli* within the inhibition range of 7.4 mm to 15.5 mm diameter. The susceptibility of the pathogens against the isolated actinobacterial isolates was followed by *P. aeruginosa* where (60.1%, n=70) of the isolates showed activity, followed by *C. albicans* (50.43%, n=58), *B. subtilis* (48.7%, n=56), and *S. aureus* (43.5%, n=50). Only 22.6% (n=26) of the isolates showed activity against *M. luteus* and found to be the most resistant pathogens. The maximum inhibition diameter was recorded by *Streptomyces flavogriseus* DST30 (18.8 mm) followed by *Streptomyces cyaneofuscatus* DST57 (15.95 mm) and *Streptomyces albidoflavus* DST71 (15.9 mm) against *M. luteus, C. albicans* and *B. subtilis*, respectively. Nine actinobacterial isolates namely *Streptomyces* sp. DST25, *Streptomyces cellulosae* DST28*, Streptomyces intermidus* DST29, *Streptomyces flavogriseus* DST52, *Streptomyces albidoflavus* DST71, *Streptomyces cyaneofuscatus* DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST119 were selected based on their broad-spectrum antimicrobial activities and these isolates were selected as potential candidates for further investigation. The antimicrobial activity of 76 actinobacterial isolates were given in **Table 4.6**

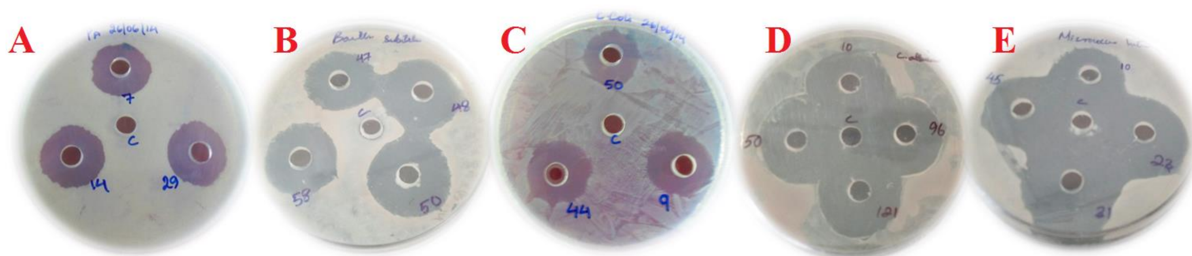

Figure 4.5: Antimicrobial activity of actinobacterial isolates against **A**. *P. aeruginosa*, **B**. *B. subtlis*, **C.** *E. coli*, **D**. *C. albicans*, and **E**. *M. luteus*

Table 4.6: Antimicrobial activity of actinobacterial isolates against 5 bacterial pathogens and one yeast pathogen; detection of biosynthetic PKS type II, NRPS and *phz*E gene

Isolate no.	**Antibacterial properties Zone of inhibition [mean (mm) ± sd]**					**Yeast**	**Biosynthetic genes**		
	E. coli	***P. aeruginosa***	***S. aureus***	***M. luteus***	***B subtilis***	***C. albicans***	**PKS-II**	**NRPS**	***pheZ***
DST8	14.5±0.05	09.0±0.05	05.6±0.20	-	-	12.2±0.10		-	-
DST10	10.4±0.05	11.0±0.01	8.34±0.03	-	13.2±0.20	11.0±0.10	-	-	-
DST12	08.0±0.30	04.0±0.01	08.0±0.26	-	-	-	-	-	-
DST13	09.5±0.01	10.5±0.05	-	-	-	9.00±0.50	+	+	-
DST15	9.25±0.05	12.1±0.01	9.43±0.03	05.2±0.10	-	10.5±0.50	+	+	-
DST16	10.1±0.06	08.0±0.01	10.1±0.01	13.4±0.10	10.4±0.03	-	+	+	-
DST17	10.4±0.05	-	9.00±0.05	05.4±0.20	13.5±0.10	12.1±0.10	-	-	-
DST18	08.0±0.00	09.0±0.03	-	11.2±0.10	14.5±0.30	13.8±0.30	-	-	-
DST19	13.5±0.05	09.0±0.01	12.3±0.10	-	12.3±0.10	13.8±0.20	-	-	-
DST21	10.6±0.02	10.0±0.01	8.62±0.02	-	-	-	-	-	-
DST22	10.5±0.05	09.0±0.50	-	-	9.00±0.50	-	-	-	-
DST23	10.4±0.05	11.0±0.01	8.34±0.03	-	-	-	+	+	
DST24	10.7±0.02	10.0±0.01	8.62±0.02	-	-	-	+	+	
DST25	**09.5±0.03**	**12.0±0.02**	**9.00±0.06**	**13.2±0.10**	**12.5±0.20**	**12.5±0.20**	+	+	-
DST27	10.2±0.05	10.4±0.05	9.00±0.10	-	-	10.0±0.10	+	+	
DST28	**09.5±0.01**	**10.0±0.01**	**10.0±0.10**	**12.6±0.10**	**12.5±0.10**	**11.5±0.50**	-	-	-
DST29	**14.5±0.04**	**08.5±0.01**	**10.2±0.10**	**-**	**0.80±0.04**	**9.25±0.05**	+	+	
DST30	10.4±0.03	06.4±0.02	10.5±0.01		14.8±0.30	12.8±0.10	-	+	-
DST31	12.0±0.09	12.5±0.03	9.20±0.05	-	12.5±0.20	12.2±0.10	-	-	-
DST32	14.0±0.02	10.0±0.05	07.5±0.20	-	13.0±0.20	12.0±0.05	-	-	-
DST35	10.0±0.03	09.0±0.05	-	12.5±0.10	10.4±0.05	12.2±0.10	-	-	-

DST38	10.5±0.02	07.8±0.20	10.0±0.10	-	13.0±0.30	12.5±0.15	-	-	-
DST43	11.0±0.05	08.2±0.05	09.6±0.02	-	-	-	+	+	-
DST44	8.50±0.15	08.0±0.02	07.2±0.10	06.4±0.30	13.4±0.10	-	+	+	-
DST46	12.0±0.20	-	-	09.8±0.10	12.2±0.30	11.0±0.10	-	+	-
DST48	09.2±0.10	09.5±0.01	-	11.2±0.20	13.4±0.10	13.2±0.20	-	+	-
DST49	12.5±0.05	10.0±0.03	07.2±0.10	12.8±0.10		14.5±0.20	-	+	-
DST50	14.0±0.05	-	12.1±0.30	07.2±0.30	12.4±0.10	14.2±0.20	-	+	+
DST51	10.4±0.10	09.5±0.04	07.5±0.05	14.3±0.20	13.5±0.05	14.2±0.05	-	+	-
DST52	**15.0±0.01**	**08.0±0.10**	**08±0.050**	**10.8±0.10**	**08.4±0.01**	**15.5±0.05**	+	+	-
DST53	09.5±0.01	10.8±0.30	05.8±0.30	-	07.6±0.04	12.2±0.20	+	+	-
DST54	14.5±0.02	-	04.5±0.05	05.1±0.01	06.8±0.10	10.2±0.20	+	+	+
DST56	11.9±0.05	9.00±0.50	-	-	12.4±0.40	10.4±0.10	+	+	+
DST57	15.5±0.05	09.0±0.35	06.0±0.10	-	15.6±0.30	15.2±0.10	+	+	+
DST58	10.2±0.10	-	-	12.2±0.30	10.8±0.10	12.4±0.20	+	+	+
DST59	13.0±0.04	06.0±0.30	-	-	10.4±0.10	11.8±0.10	+	+	-
DST60	13.5±0.15	08.0±0.25	-	-	12.0±0.10	14.3±0.05	-	+	+
DST61	15.0±0.05	10.0±0.35	-	-	-	13.4±0.20	+	+	-
DST62	08.2±0.00	12.2±0.02	-	10.8±0.10	06.8±0.20	13.0±0.30	-	+	-
DST63	11.5±0.10	9.00±0.35	-	12.4±0.10	11.2±0.50	11.5±0.20	-	+	-
DST64	09.8±0.20	09.8±0.20	12.2±0.20	-	9.80±0.10	12.1±0.40	-	+	+
DST65	08.5±0.05	09.0±0.05	-	-	9.00±0.50	-	+	+	-
DST66	10.5±0.15	05.0±0.10	07.4±0.10	-	12.5±0.01	10.4±0.10	-	+	+
DST67	07.5±0.20	10.5±0.20	-	-	10.8±0.20	10.4±0.20	-	+	+
DST68	07.4±0.05	10.1±0.10	-	-	09.4±0.40	12.0±0.20	-	-	-
DST69	14.0±0.15	08.0±0.10	-	-	11.9±0.10	12.2±0.15	+	+	-
DST70	09.4±0.10	10.4±0.30	-	-	07.4±0.30	10.1±0.40	-	+	-
DST71	**13.0±0.03**	**08.0±0.05**	**06.6±0.04**	**06.2±0.20**	**15.9±0.20**	**13.8±0.10**	-	+	+
DST72	10.2±0.04	0.8±0.04	09.8±0.10	07.8±0.10	-	12.2±0.30	-	+	-

DST73	07.8±0.05	10.5±0.05	-	-	-	-	-	+	-
DST74	12.5±0.15	09.0±0.05	05.2±0.30	-	09.4±0.04	13.2±0.05	+	+	+
DST75	09.1±0.16	09.2±0.03	12.5±0.10	-	15.0±0.20	-	-	+	-
DST76	10.0±0.10	10.6±0.03	8.37±0.02	-	15.2±0.30	13.4±0.20	+	+	+
DST86	10.5± 0.05	8.30±0.05	9.10±0.01	-	-	-	-	-	-
DST87	08.0±0.05	7.05±0.05	04.0±0.10	-	15.0±0.30	-	-	-	-
DST88	14.0±0.25	08.5±0.05	-	-	-	-	-	-	-
DST89	10.0±0.10	07.8±0.15	10.1±0.05	-	-	-	-	-	-
DST95	8.95±0.05	9.00±0.32	7.95±0.05	-	10.5±0.30	12.4±0.10	+	+	-
DST96	14.5±0.10	9.00±0.10	-	09.2±0.10	14.5±0.20	11.8±0.10	-	+	-
DST97	10.0±0.05	9.18±0.02	10.0±0.10	-	-	-	-	+	-
DST98	9.00±0.05	10.1±0.03	10.1±0.20	-	-	11.5±0.05	-	-	-
DST99	15.5±0.01	07.5±0.03	10.4±0.03	-	12.2±0.10	10.6±0.20	+	+	+
DST100	13.0±0.04	9.8±0.05	10.6±0.30	-	07.3±0.01	12.5±0.10	-	+	-
DST102	12.0±0.10	05.0±0.30	15.0±0.20	-	10.2±0.10	12.4±0.30	-	+	+
DST103	**15.0±0.03**	**08.0±0.25**	**07.0±0.10**	-	**11.2±0.10**	**12.6±0.05**	+	+	+
DST104	13.5±0.10	-	5.60±0.01	05.3±0.50	15.0± 0.50	11.5±0.20	-	+	-
DST105	13.0±0.05	05.0±0.40	-	-	08.4±0.20	11.5±0.20	+	+	-
DST106	14.0±0.05	08.5±0.10	05.4±0.10		12.6±0.10	12.3±0.05	+	-	+
DST116	**10.5±0.03**	**05.8±0.20**	**09.2±0.25**	**18.8±0.10**	**14.4±0.20**	**12.9±0.05**	+	+	+
DST117	12.5±0.03	12.2±0.10	-	-	11.6±0.10	11.5±0.20	-	+	-
DST118	08.0±0.02	7.90±0.10	03.0±0.30	10.8±0.10	14.2±0.10	12.2±0.20	+	+	+
DST119	**08.1±0.01**	**07.0±0.10**	**8.00±0.10**	**14.3±0.10**	**14.2±0.10**	**13.1±0.10**	+	+	+
DST120	14.5±0.05	10.0±0.05	-	-	12.4±0.20	14.8±0.20	+	+	+
DST142	**14.0±0.05**	**7.50±0.02**	**8.5±0.01**	**09.0±0.05**	**07.0±0.04**	**09.0±0.05**	+	+	+
DST143	12.0±0.05	08.0±0.03	-	-	-	-	-	-	-
DST145	13.0±0.03	8.50±0.02	-	-	-	-	-	-	-

4.3.4.1 Antimicrobial assay using methanol crude extract

The methanolic crude extract of the selected isolates that were tested for their antimicrobial activity showed adequate inhibition zones at 20 mg ml^{-1} and 40 mg ml^{-1} (**Figure 4.6**) for all the samples, while all the isolates showed no activity in 1 mg ml^{-1} and 2 mg ml^{-1}. The agar well diffusion assay showed better results compared to the filter paper disk diffusion assay.

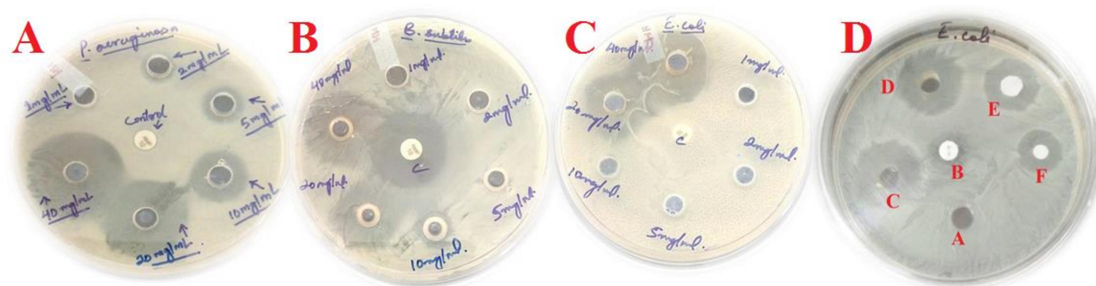

Figure 4.6: Antimicrobial activity of mathanolic crude extract of actinobacterial isolates at different concentrations against **A.** *P. aeruginosa,* **B.** *B. subtilis,* **C**. *E.coli* and **D.** comparison of disk diffusion assay and filter paper disk diffusion assay

4.3.4.2 MIC of selected isolates

The methanolic crude extracts of the nine selected isolates were subjected to antimicrobial activity quantification by determining the minimum inhibitory concentration (MIC) of each isolates against six pathogens. Among the selected isolates, *Streptomyces cyaneofuscatus* showed highest activity against *E. coli* (EC50=0.002 $mgml^{-1}$) followed by *Streptomyces flavogriseus* DST52 ((EC50=0.052 $mgml^{-1}$) and *Streptomyces* sp. DST142 (EC50=0.062 $mgml^{-1}$). The EC50 of *Streptomyces cyaneofuscatus* was found to be lowest against *P. aeruginosa* (EC50=0.009 $mgml^{-1}$) followed by *Streptomyces* sp. DST119 (EC50=0.015 $mgml^{-1}$)

and *Streptomyces* sp. DST142 (EC50=0.024 $mgml^{-1}$). *Streptomyces* sp. DST119 showed highest activity against *S aureus* (EC50=0.015 $mgml^{-1}$) among the isolates while *Streptomyces flavogriseus* DST52 and *Streptomyces cyaneofuscatus* DST103 showed highest activity against *M. luteus* (EC50=0.003 $mgml^{-1}$) and *C. albicans* (EC50=0.025 $mgml^{-1}$) respectively (**Table 4.7**).

Table 4.7: EC50 reading of nine *Streptomyces* isolates against five bacterial pathogens and one yeast pathogen

	EC50 mg/ml					
Isolates	***E. coli***	***P. aeuginosa***	***S. aureus***	***B. subtilis***	***M. luteus***	***C. albicans***
Streptomyces sp. DST25	0.086	0.144	0.070	**0.009**	0.286	0.070
Streptomyces cellulosae DST28	1.673	2.353	1.085	0.804	0.331	0.900
Streptomyces intermidus DST29	1.046	1.224	0.862	0.028	0.043	0.860
Streptomyces cyaneofuscatus DST103	**0.002**	**0.009**	0.043	0.032	0.012	**0.025**
Streptomyces flavogriseus DST52	0.056	0.267	0.040	0.170	**0.003**	1.600
Streptomyces albidoflavus DST71	0.102	0.050	0.138	0.650	0.190	0.075
Streptomyces sp. DST116	0.235	0.231	0.110	0.227	0.051	0.069
Streptomyces sp. DST119	0.260	0.015	**0.015**	0.278	0.950	1.195
Streptomyces sp. DST142	0.062	0.024	0.094	0.324	0.044	0.054

4.3.5 Detection and amplification of biosynthetic genes

Out of the 115 isolates screened for three biosynthetic genes (PKS II, NRPS and *phz*E), PKS type II was detected in 31 isolates (27%) (**Figure 4.7**), NRPS was detected in 47% (n=54) of the isolates (**Figure 4.8**) and *phz*E was detected in 17.4% (n=20) of the isolates (**Figure 4.9**). A total of 11 isolates (DST45, DST47, DST54, DST56, DST57, DST58, DST74, DST76, DST77, DST99, DST101 and DST142) were found to have all three genes that were amplified using PCR (**Table 4.6**). The biosynthetic genes for selected *Streptomyces* isolates were sequenced and deposited in NCBI database and Genbank accession number were given as KX595190, MG200184 - MG200188 for NRPS; KX595189, MG200189 - MG200192 for PKSII; KX894555, MG200193- MG200194 for *phz*E.

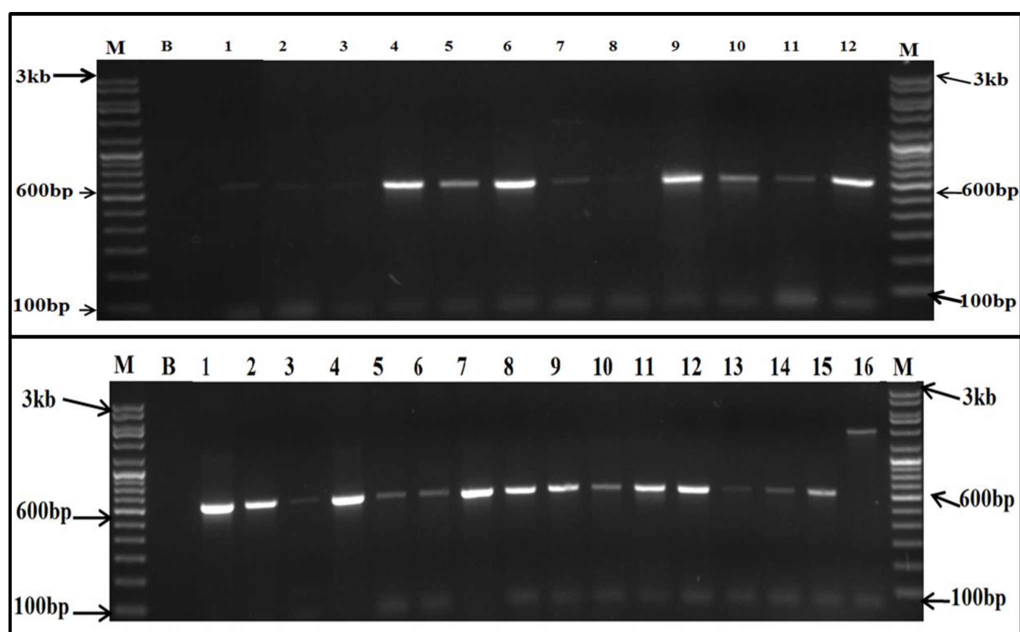

Figure 4.7: PCR amplification of **NRPS gene** of potential isolates**. M:** Low range DNA**;** Numbers indicates different isolates

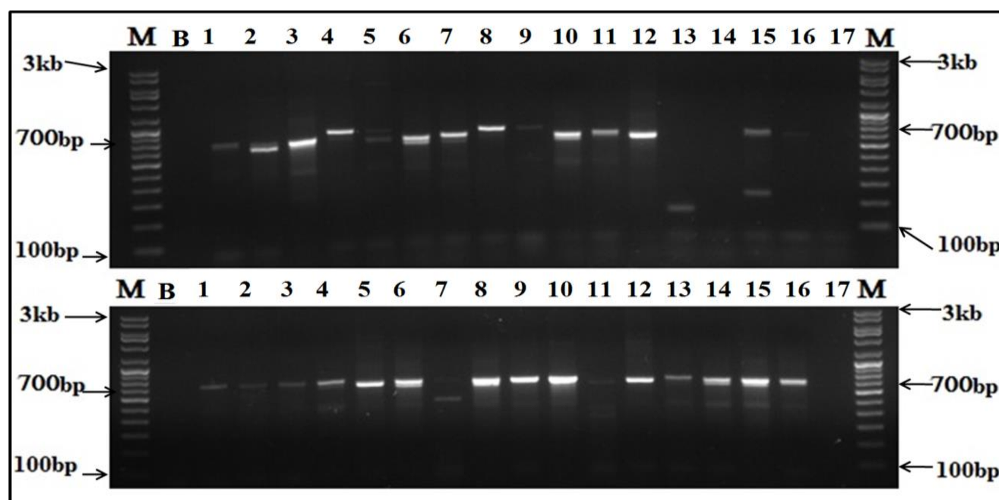

Figure 4.8: PCR amplification of **PKSII gene. M:** Low range DNA; Numbers indicates different isolates

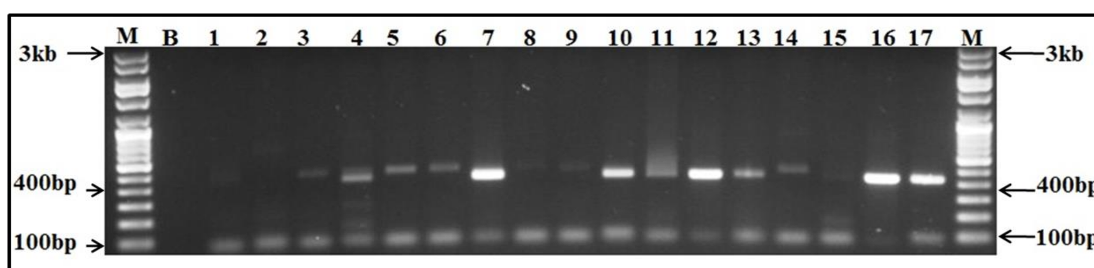

Figure 4.9: PCR amplification of ***phz*E gene** of potential isolates**. M:** Low range DNA; Numbers indicates different isolates.

4.3.5.1 Phylogenetic analysis of biosynthetic genes

The nucleotide sequences of three biosynthetic genes (PKS II, NRPS and *phz*E) showed 82-92% similarity with the type strain from NCBI-BLASTn database. The transition and transversion bias ratio of PKSII, NRPS and *phz*E gene was 0.55, 0.33 and 0.17 respectively whereas the maximum log likelihood for the substitution computation was -2765.453, -501.484 and -801.607 respectively. The phylogenetic tree constructed using PKS II sequences revealed that *Streptomyces* sp. DST29

formed separate clade with *Streptomyces* sp. MM48 *Streptomyces gobitricini* with bootstrap values of 99% while *Streptomyces* sp. DST116, *Streptomyces* sp. DST52 and *Streptomyces* sp. DST119 each formed a separate clade with a bootstrap support value of 99-100% (**Figure 4.10A**). Similarly the NRPS gene sequences of *Streptomyces* sp. DST116 *Streptomyces* sp. DST25, *Streptomyces* sp. DST71 and *Streptomyces* sp. DST119 formed separate clade with *Streptomyces* sp. CAH29-18, *Streptomyces albidus* NBRC14052, *Streptomyces cyaneofuscatus* DST103, *Streptomyces bamensis* NBRC14727 and *Streptomyces* sp. BSH50-42 respectively with a bootstrap value of 84-89% (**Figure 4.10B**). Similarly *Streptomyces* sp. DST119 and *Streptomyces* sp. DST71 were clustered separately in *phz*E gene sequences forming same clade with *Streptomyces* sp. HB291 and *Streptomyces* sp. 13-33-9 respectively with a bootstrap value of 100% (**Figure 4.10C).**

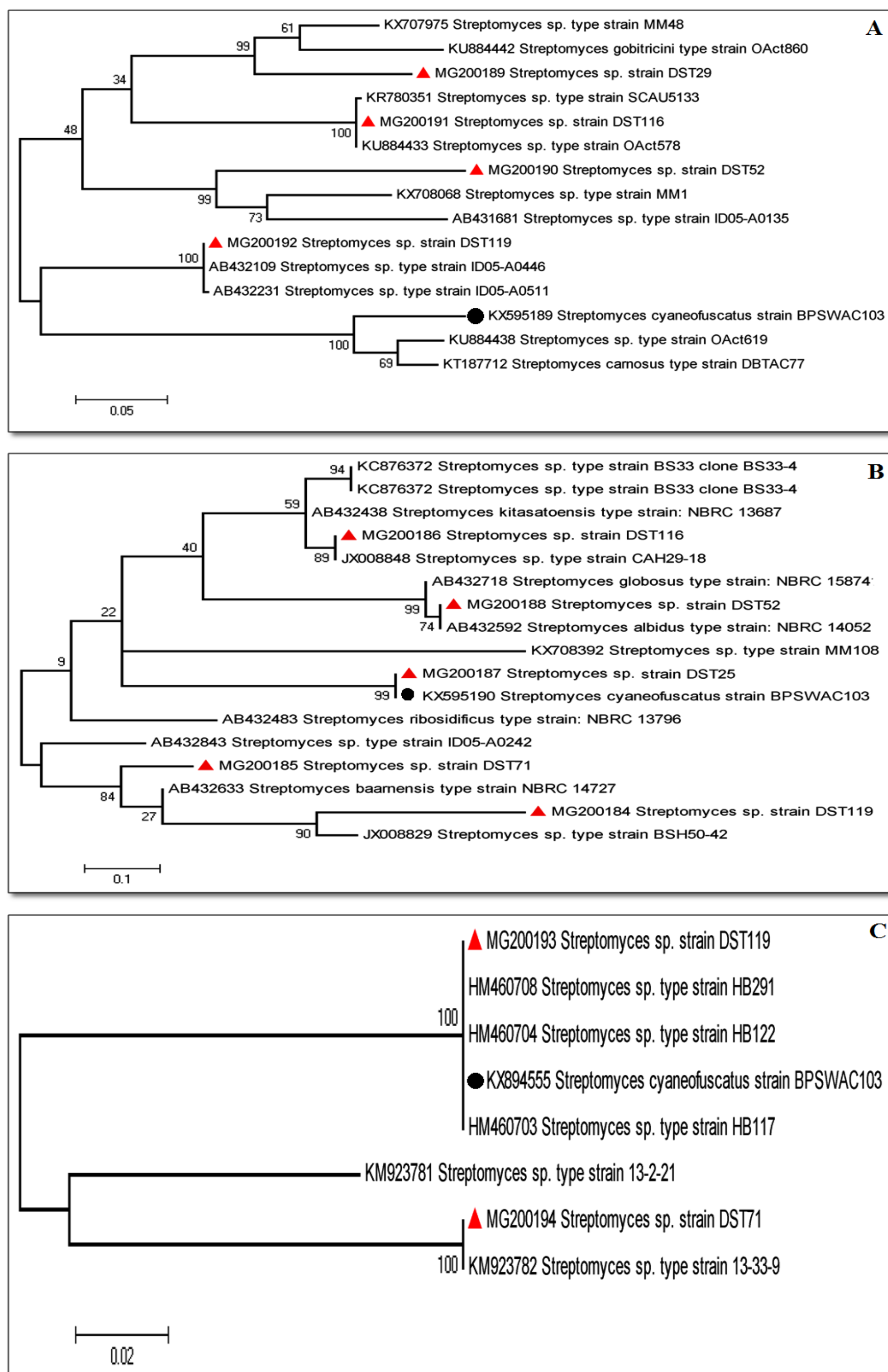

Figure 4.10: Maximum likelihood (ML) phylogenetic tree constructed using amino acid sequences for **(A)** PKS type II gene; **(B)** NRPS gene and **(C)** *phz*E gene. The scale bar represents the amino acid changes.

4.3.6 Enzymatic activity of actinobacterial isolates

The actinobacterial isolates were screened for their ability to hydrolyze xylan and cellulose (**Table 4.8**). Nineteen isolates which showed good activity in preliminary screening were induced by growing them in induction media (**Figure 4.11**). From nineteen isolates, seven isolates (*Streptomyces* sp. DST25, *Streptomyces cellulosae* DST28, *Streptomyces flavogriseus* DST30, *Streptomyces flavogriseus* DST52, *Streptomyces cyaneofuscatus* DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST142) showed both cellulose and xylanase activity. The hydrolytic zones formed by the actinobacterial isolates for cellulose and xylan ranges from 0.04cm-4cm and 0.08cm-6.6cm respectively. Maximum cellulolytic zone was formed by *Streptomyces* sp. DST25 (4cm) followed by *Streptomyces flavogriseus* DST52 (1cm) whereas maximum xylanolytic zone was formed by *Streptomyces* sp. DST142 (6.6cm) followed by *Streptomyces cyaneofuscatus* DST103 (3.8cm).

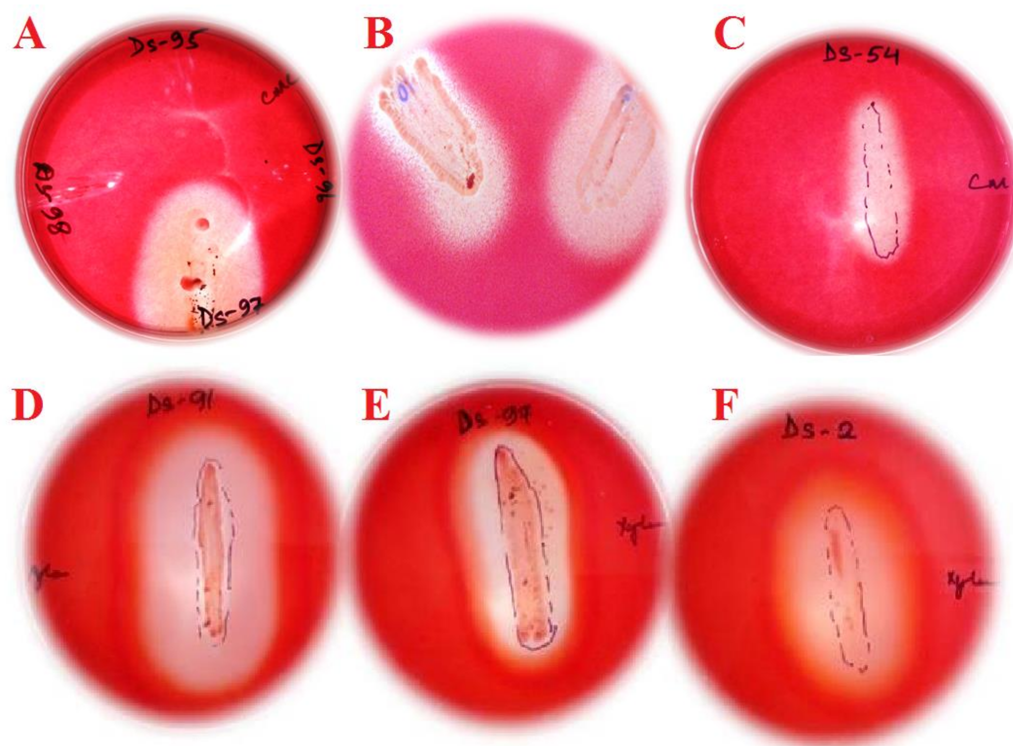

Figure: 4.11 A, B & C – Cellulolytic activity of actinobacterial isolates, **D, E & F** – Xylanolytic activity of actinobacterial isolates

Table 4.8: Cellulase and xylanase activity of selected actinobacterial isolates

Isolates	**[A]** **Diameter of the zone (cm)**	**[B]** **Diameter of the colony (cm)**	**[C]** **Diameter of the clear zone (cm)**	**[C] / [B]** **Hydrolysis capacity**
Cellulase				
DST8	2.2	2	0.2	0.1
DST17	2.3	1.6	0.8	**0.5**
DST21	2.1	2	0.1	0.05
DST25	**5.0**	**1.0**	**4.0**	**4.0**
DST28	**3.3**	**2.8**	**0.5**	**0.178**
DST30	**2.8**	**2.1**	**0.7**	**0.333**
DST50	2.6	2.5	0.1	0.04
DST52	**1.0**	**0.5**	**0.5**	**1.0**

DST103	**2.4**	**1.9**	**0.5**	**0.263**
DST116	**3**	**2.3**	**0.7**	**0.304**
DST142	**2.5**	**2.3**	**0.2**	**0.086**
Xylanase				
DST25	**1.5**	**1.0**	**0.5**	**0.5**
DST28	**4.0**	**1.2**	**2.8**	**2.3**
DST30	**2**	**1.3**	**0.7**	**0.54**
DST47	4.4	1.0	3.4	3.4
DST52	**3.8**	**1.0**	**2.8**	**2.8**
DST56	2.2	0.6	1.6	2.6
DST71	2.5	1.0	1.5	1.5
DST98	2.7	2.5	0.2	0.08
DST103	**4.8**	**1.0**	**3.8**	**3.8**
DST105	5.0	1.0	4.0	4.0
DS-106	2.5	1.0	1.5	1.5
DST116	**3.2**	**2.2**	**1**	**0.45**
DST117	4.0	1.4	2.6	1.85
DST142	**3.8**	**0.5**	**3.3**	**6.6**
DST145	3.0	1.0	2.0	2.0

4.3.7 Quantification of cellulase

The amount of enzymes (CMCase) present in the sample was quantified and the optimum incubation time was shown in **Table 4.9**. On day 8 *Streptomyces* sp. DST17 produced maximum amount of cellulase i.e. 0.61 U/ml, similarly on day 8 *Streptomyces cyaneofuscatus* DST103 produced 0.19 U/ml, whereas in *Streptomyces flavogriseus* DST30 and *Streptomyces* sp. DST116 produced maximum amount on 4th days and 6th days respectively **(Figure 4.12)**

Table 4.9: Cellulase (CMCase) activity of isolates at 30 ^{o}C and pH 7

Isolates	No. of days incubated	CMCase activity (U/ml)
Streptomyces sp. DST17	2	0.37
	4	0.46

	6	0.54
	8	**0.61**
Streptomyces cyaneofuscatus DST103	2	0.08
	4	0.16
	6	0.16
	8	**0.19**
Streptomyces sp. DST116	2	0.26
	4	0.31
	6	**0.44**
	8	0.35
Streptomyces flavogriseus DST30	2	0.38
	4	**0.61**
	6	0.43
	8	0.37

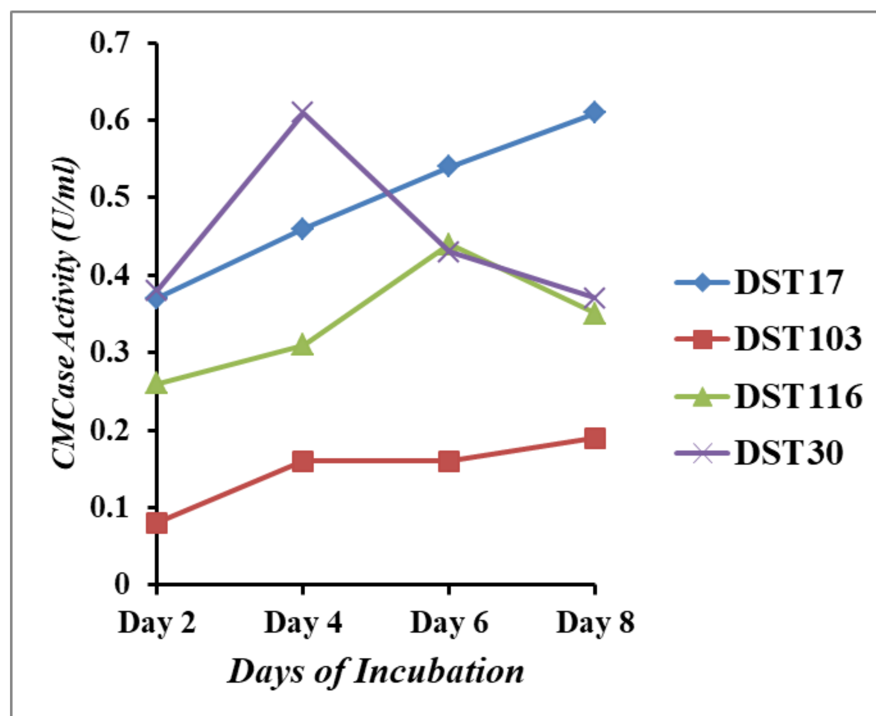

Figure 4.12: Optimization study for cellulase production from the selected isolates

4.3.8 Quantification of Xylanase

The amount of xylanase from selected actinobacterial isolates were determined **(Table 5.0)** where maximum amount was quantified on 8th day in *Streptomyces cyaneofuscatus* DST 103 (16.6 U/ml) whereas in *Streptomyces flavogriseus* DST30 and *Saccharopolyspora* sp. DST98 maximun amount of enzyme was quantified on the 6th day (32.7 U/ml and 21.6 U/ml respectively). In *Streptomyces* sp. DST116, on 4th day 25.6 U/ml of enzyme was quantified which is maximum **(Figure 4.13)**.

Table 5.0: Xylanase activity of Isolate 30 °C and pH 7

Isolates	No. of days incubated	CMCase activity (U/ml)
Streptomyces cyaneofuscatus DST 103	2	8.08
	4	8.52
	6	10.8
	8	**16.5**
Streptomyces flavogriseus DST30	2	9.30
	4	14.4
	6	**32.7**
	8	8.62
Streptomyces sp. DST116	2	3.16
	4	**25.6**
	6	4.91
	8	2.25
Saccharopolyspora sp. DST98	2	6.01
	4	12.4
	6	**21.6**
	8	7.70

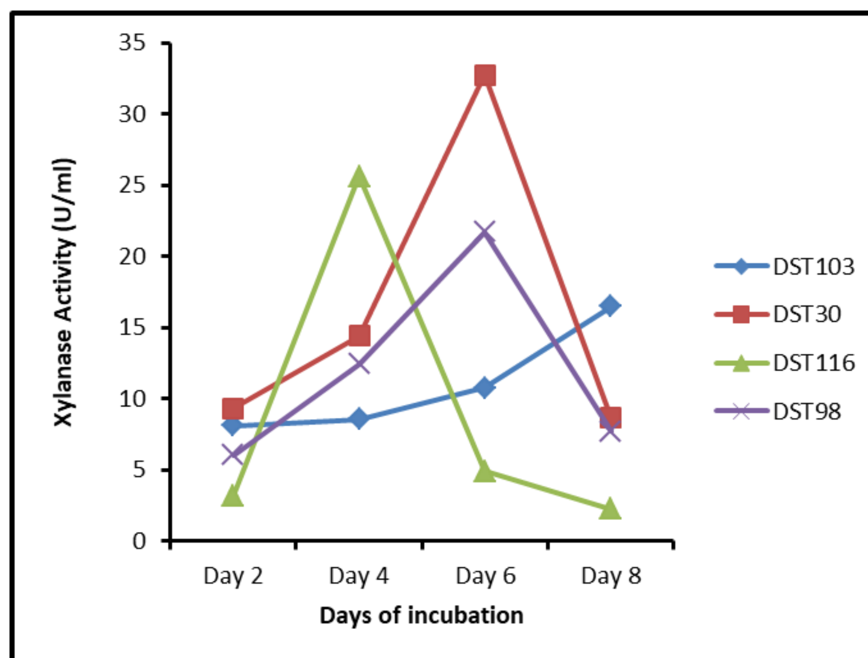

Figure 4.13: Optimization study for Xylanase production from the selected isolates

4.3.9 GC-MS analysis

The methanolic crude extracts of nine selected actinobacterial isolates were investigated to determine their volatile organic compounds (VOCs) using GC-MS, which revealed sixty-three VOCs**.** Fourteen compounds were detected from the extract of *Streptomyces albidoflavus* DST71 within the retention time of 15 to 29 minutes (**Figure 4.14**). Among the compounds, hexanal constituted the maximum amount, which accounted for 23.2% of the total volume. Six VOCs, valine, glutaraldehyde, D-leucine, 3,3-dimethyl-4-methylamino-butan-2-one, pentadecylamine, cyclopropane and 1-butyl-2-(2-methylpropyl)-, were detected from the extract of *Streptomyces* sp. DST25, and glutaraldehyde was the most abundant followed by an amino acid, valine (**Figure 4.15**). Only one compound (di-n-octyl

phthalate) was detected in extracts of *Streptomyces cellulosae* DST28 (**Figure 4.16**). Two major compounds were detected in the extract of *Streptomyces* sp. DST29 in which Di-n-octyl phthalate consists of more than 70%. Seven compounds were determined from the extract of *Streptomyces flavogriseus* DST52, of which carbonic acid, 2, 2, 2-trichloroethyl undec-10-enyl ester alone constituted 49.78% (**Figure 4.17**). Four compounds were detected from *Streptomyces cyaneofuscatus* DST103 where 2-decene, 3-methyl-, (z) - was found in higher concentration compared to other three compounds. Only 2-methoxy-4,5-diphenyl-6-(2'-phenylethyl)pyrimidine was detected in the extract of *Streptomyces* sp. DST116 (**Figure 4.18**), while six compounds were detected in the extract of *Streptomyces* sp. DST119 in which 2-benzylthio-8-methyl-7-phenylpyrano [2, 3-f] benzoxazol-6(h)-one constituted the maximum amount (42.66%) (**Figure 4.19**). The methanolic crude extract and ethyl acetate crude extract was compared in *Streptomyces* sp. DST142 for their secondary metabolites content where 17 compounds were determined from the methanolic extract of DST142 while only 5 compounds were retrieved from ethyl acetate extract of DST142 **(Table 5.1).**

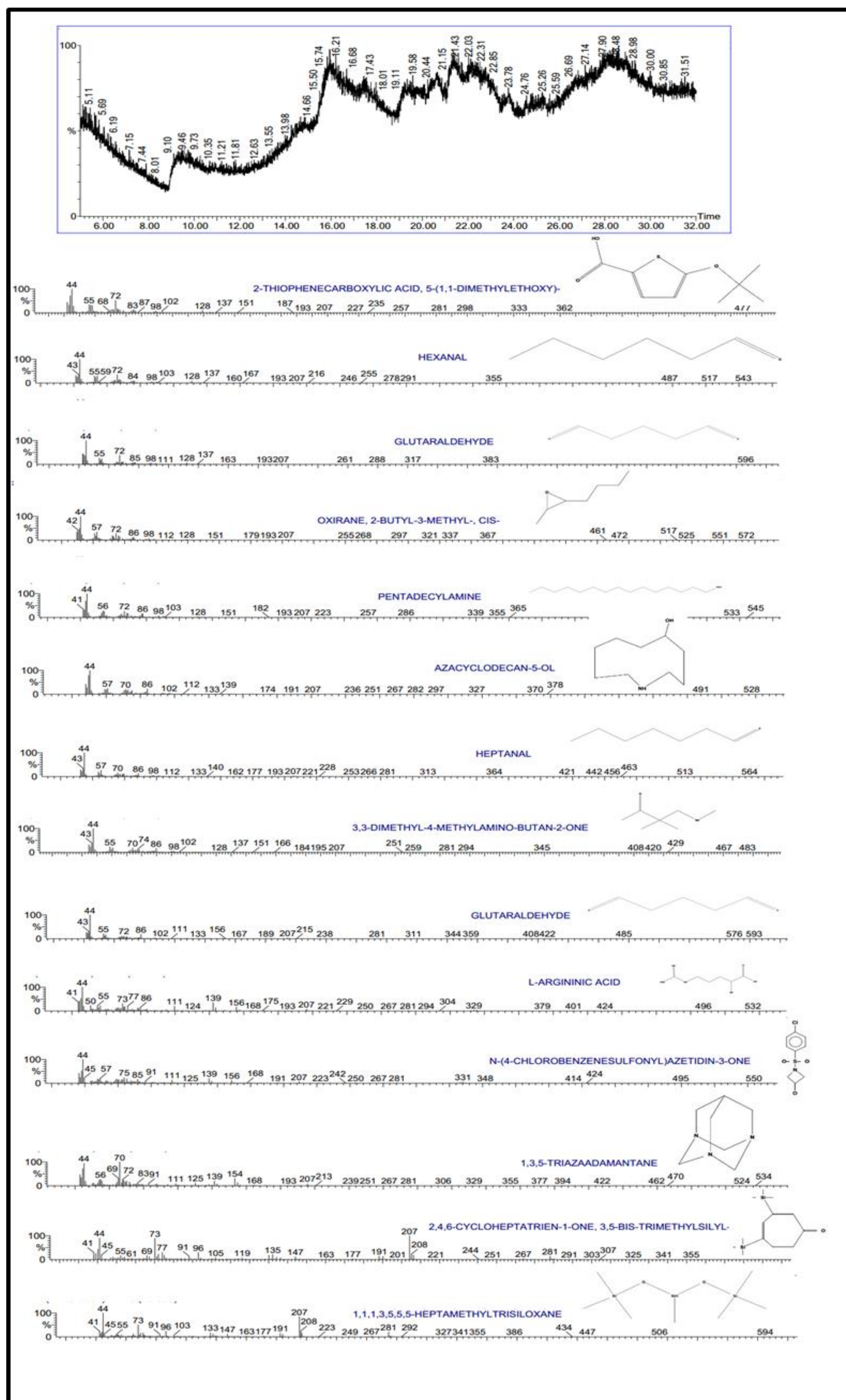

Figure 4.14: VOCs determined using GC/MS in extract of *Streptomyces albidoflavus* DST71

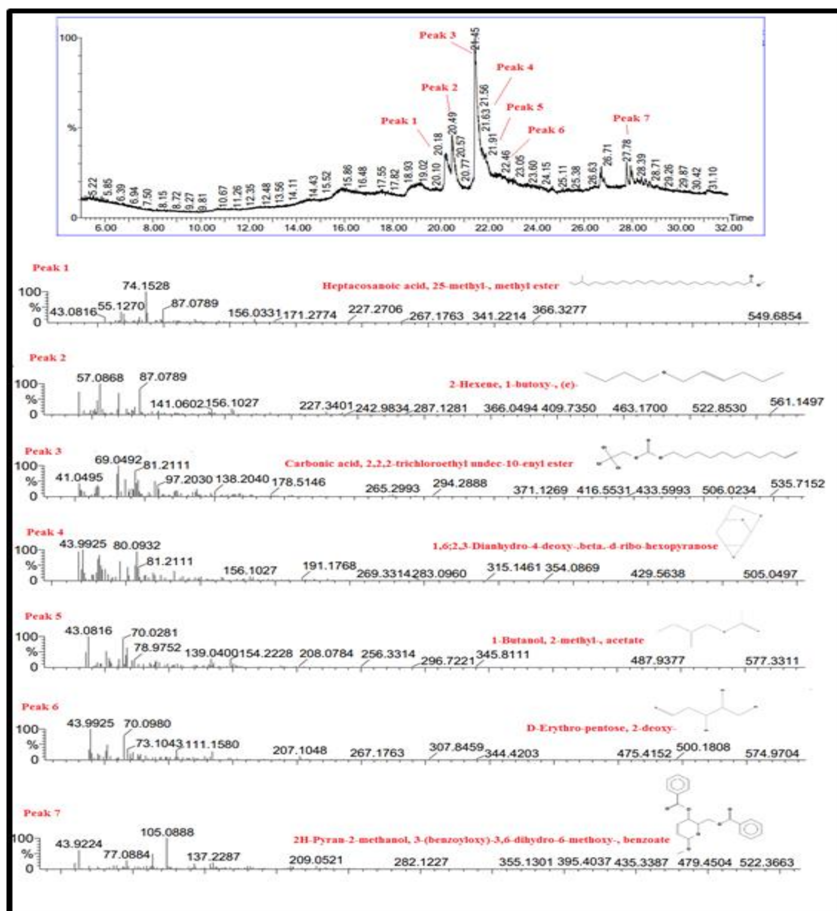

Figure 4.15: VOCs determined using GC/MS in extract of *Streptomyces* sp. DST25

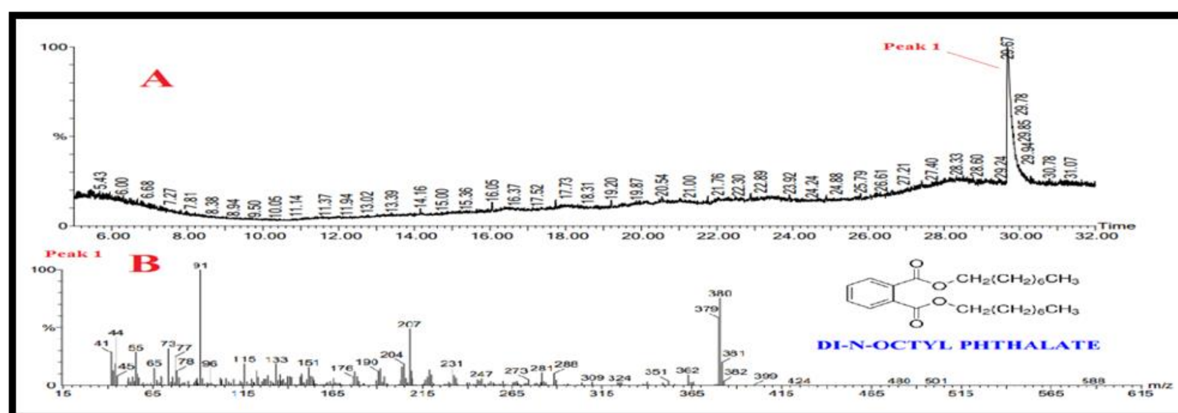

Figure 4.17: VOCs determined using GC/MS in extract of *Streptomyces cellulosae* DST28

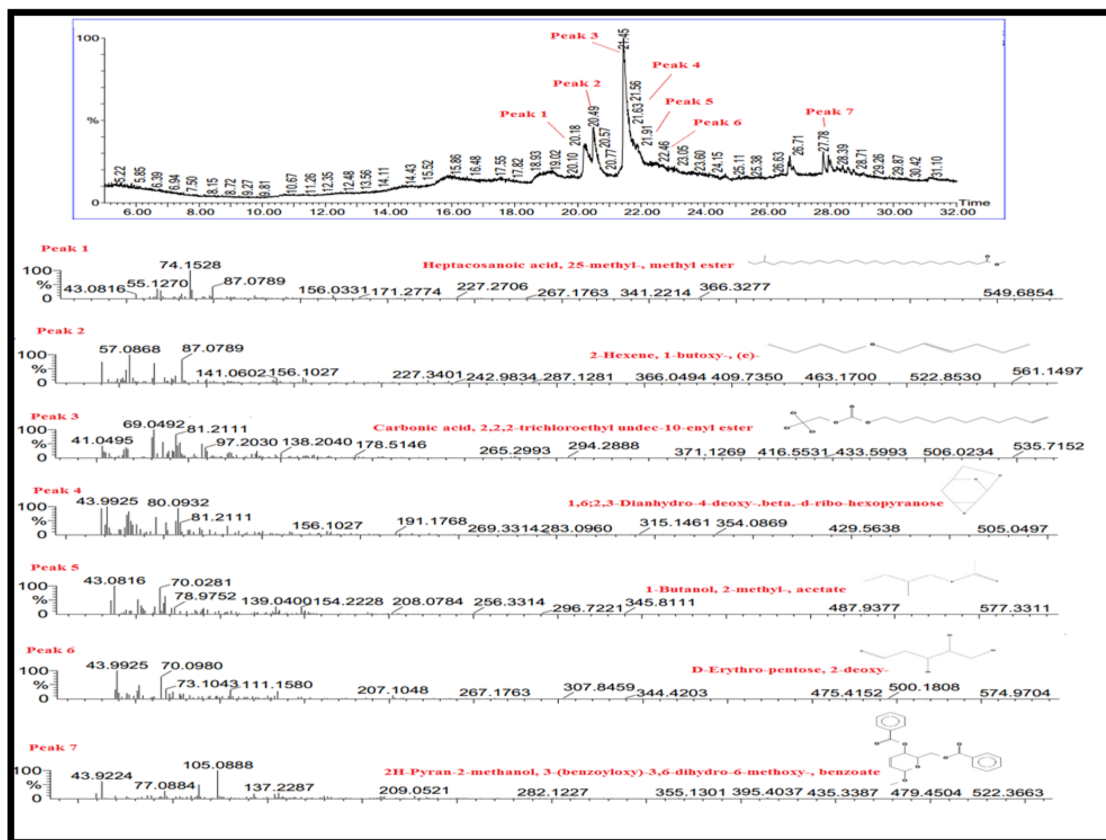

Figure 4.17: VOCs determined using GC/MS in extract of *Streptomyces flavogriseus* DST52

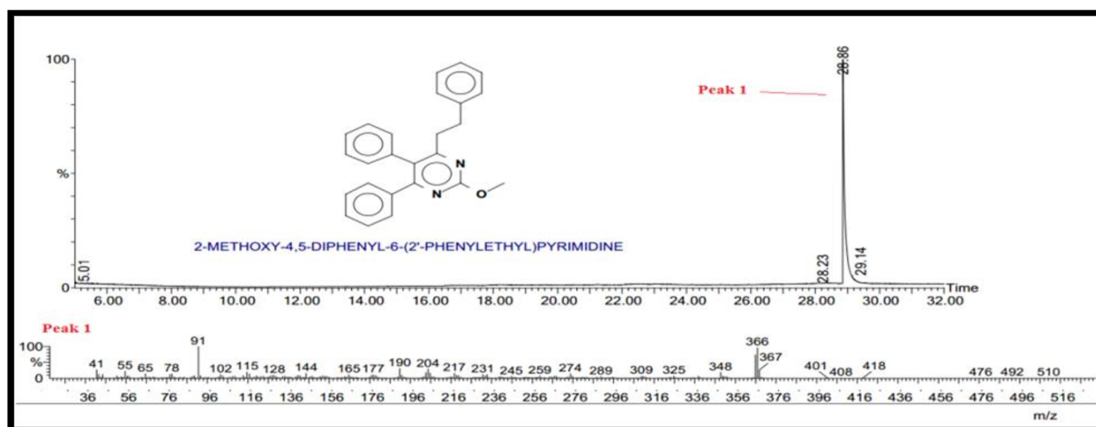

Figure 4.18: VOCs determined using GC/MS in extract of *Streptomyces* sp. DST116

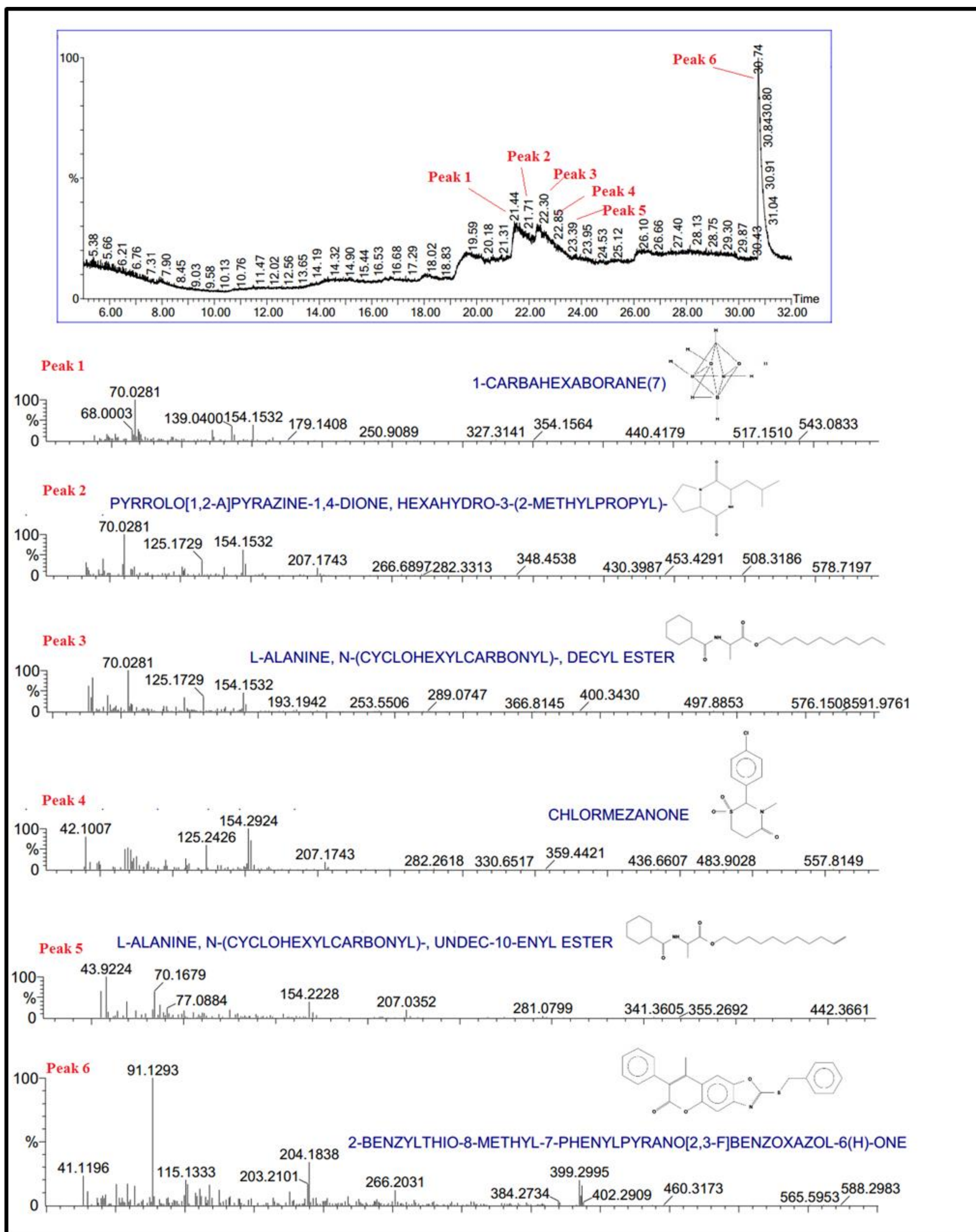

Figure 4.19: VOCs determined using GC/MS in extract of *Streptomyces* sp. DST119

Table 5.1: Volatile secondary metabolites detected from the crude extract of selected actinobacterial isolates

Sl no.	Compound name	Retention time (min)	Area (%)	Molecular Formula	Molecular weight (MW)	Quality (%)	Activity	Reference
***Streptomyces* sp. DST25**								
1	Valine	15.383	23.413	$C_5H_{11}O_2N$	117	75	antimicrobial and cytotoxic activity	Lee et al., 2014
2	Glutaraldehyde	15.668	50.075	$C_5H_8O_2$	100	80	disinfectant, antimicrobial	Lerones et al., 2004; Hill et al., 1991
3	D-Leucine	16.958	11.942	$C_6H_{13}O_2N$	131	80	antibacteria	Fox et al., 1944; Ahmad et al., 2014
4	3,3-Dimethyl-4-methylamino-butan-2-one	17.458	3.585	$C_7H_{15}ON$	129	86	antimicrobial	Dineshkumar et al., 2017
5	Heptanal	17.799	5.813	$C_7H_{14}O$	114	84	antimicrobial	Al-Wathnani et al., 2012
6	Cyclopropane, 1-butyl-2-(2-methylpropyl)-	22.390	5.172	$C_{11}H_{22}$	154	80	-	-
***Streptomyces cellulosae* DST28**								
7	Di-N-Octyl phthalate	29.673	100	$C_{24}H_{38}0_4$	390	85	Antimocrobial	Philip et al., 2011; Shafaghat et al., 2012)
***Streptomyces* sp. DST29**								
8	Di-n-octyl phthalate	22.33	71.31	$C_{24}H_{38}0_4$	390	85%	Antimocrobial	Ushadevi, 2008;

								Philip et al., 2011; Senthilkumar et al., 2011; Shafaghat et al., 2012)
9	1-Bromo-3,7-Dimethyloctane	25.28	28.69	$C_{10}H_{21}Br$	220	80%	-	-
***Streptomyces flavogriseus* DST52**								
10	Heptacosanoic acid, 25-methyl-, methyl ester	20.235	13.177	$C_{29}H_{58}O_2$	438	81	Larvicidal	Balasubramani et al., 2015
11	2-Hexene, 1-butoxy-, (e)-	20.495	13.827	$C_{10}H_{20}O$	156	69	-	-
12	Carbonic acid, 2,2,2-trichloroethyl undec-10-enyl ester	21.445	49.782	$C_{14}H_{23}O_3Cl_3$	344	70	antibacteria	Musini et al., 2013
13	1,6;2,3-Dianhydro-4-deoxy-.beta.-d-ribo-hexopyranose	21.880	12.522	$C_6H_8O_3$	128	71	Anticancer (actino)	Zhou et al., 2017
14	1-Butanol, 2-methyl-, acetate	22.290	2.313	$C_7H_{14}O_2$	130	61	antimicrobial	Ezra and Strobel. 2003 Ezra et al., 2004
15	D-Erythro-pentose, 2-deoxy-	22.350	6.155	$C_5H_{10}O_4$	134	60	-	-
16	2H-Pyran-2-methanol, 3-(benzoyloxy)-3,6-dihydro-6-methoxy-, benzoate	27.778	2.224	$C_{21}H_{2}0O_{6}$	368	76	-	-
***Streptomyces albidoflavus* DST71**								

17	2-Thiophenecarboxylic acid, 5-(1,1-dimethylethoxy)-	15.838	8.764	$C_9H_{12}O_3S$	200	85	antimicrobial	Al-Wathnani et al., 2012; Perveen and Alwathnani. 2013
18	Hexanal	15.943	23.187	$C_6H_{12}O$	100	82	antimicrobial	Rattanakom and Yasurin 2014; Sitarek et al., 2017
19	Glutaraldehyde	16.208	4.578	$C_5H_8O_2$	100	82	disinfectant, antimicrobial	Lerones et al., 2004; Hill et al., 1991
20	Oxirane, 2-butyl-3-methyl-, cis	16.808	5.268	$C_7H_{14}O$	114	79	-	-
21	Pentadecylamine	17.013	5.176	$C_{15}H_{33}N$	227	81	antimicrobial	Kabara et al., 1972
22	Azacyclodecan-5-ol	17.408	5.820	$C_9H_{19}ON$	157	84	-	-
23	Heptanal	17.433	7.692	$C_7H_{14}O$	114	88	antimicrobial	Al-Wathnani et al., 2012
24	3,3-Dimethyl-4-methylamino-butan-2-one	17.548	5.962	$C_7H_{15}ON$	129	84	antimicrobial	Dineshkumar et al., 2017
25	Glutaraldehyde	17.748	5.582	$C_5H_8O_2$	100	82	antimicrobial	Lerones et al., 2004; Hill et al., 1991
26	L-Argininic acid	20.384	4.491	$C_6H_{13}O_3N_3$	175	73	antimicrobial	Sepahi et al., 2017
27	N-(4-Chlorobenzenesulfonyl)azetidin-3-one	20.645	6.772	$C_9H_8O_3N$	245	70	-	-
28	1,3,5-Triazaadamantane	21.305	7.769	$C_7H_{13}N_3$	139	72	-	-
29	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	28.128	3.529	$C_{13}H_{22}OSi_2$	250	73	antioxidant, antimicrobial	Ramakrishnan and Venkataraman . 2011;

								Foo et al., 2017
30	1,1,1,3,5,5,5-Heptamethyltrisiloxane	28.892	5.411	$C_7H_{22}O_2Si_3$	222	81	antimicrobial	Dehpour et al., 2011
***Streptomyces cyaneofuscatus* DST103**								
31	2-propen-1-amine, n-2-propenyl-	17.545	8	$C_6H_{11}N$	97	71	Antibacterial	Senbagam et al., 2016
32	2-propenal, 3-(1-aziridinyl)-3-(dimethylamino)-	17.820	8.96	$C_7H_{12}ON_2$	140	76	Antimicrobial	Sheoran et al., 2015
33	2-decene, 3-methyl-, (z)-	18.995	30.00	$C_{11}H_{22}$	154	78	Antimicrobial	Kalaiselvan et al., 2012; Idramsa et al., 2016
34	5-pyrrolidino-2-pyrrolidone	19.991	28.03	$C_8H_{14}ON_2$	154.209	74	-	-
***Streptomyces* sp. DST116**								
35	2-Methoxy-4,5-diphenyl-6-(2'-phenylethyl)pyrimidine	28.858	100	$C_{25}H_{22}ON_2$	366	65	-	-
***Streptomyces* sp. DST119**								
36	1-Carbahexaborane(7)	21.435	3.218	CH_7B_5	74	70	-	-
37	Pyrrolo[1,2-a]Pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	21.535	21.466	$C_{11}H_{18}O_2N_2$	210	59	Antimicrobial, antioxidant	Sheoran et al., 2015; Melo et al., 2014; Durai et al., 2013, Jinfeng et al., 2017
38	L-Alanine, n-(Cyclohexylcarbonyl)-, Decyl ester	22.105	3.030	$C_{20}H_{37}O_3N$	399	63	-	-

39	Chlormezanone	22.301	25.682	$C_{11}H_{12}O_3N$	273	49	anxiolytic, muscle relaxant	Asif. 2015
40	L-Alanine, n-(cyclohexylcarbonyl)-, undec-10-enyl ester	23.132	3.935	$C_{21}H_{37}O_3N$	351	67	-	-
41	2-Benzylthio-8-methyl-7-phenylpyrano[2,3-f]benzoxazol-6(h)-one	30.739	42.669	$C_{24}H_{17}O_3NS$	399	77	-	-
***Streptomyces* sp. DST142 (Ethyl acetate extract)**								
42	2-Butanol, 3-Methyl-	20.34	70.405	$C_5H_{12}O$	88	75%	Antimicrobial	Alwathnani and Perveen, 2017
43	Cathinone	20.81	4.337	$C_9H_{11}ON$	149	57%	Antibacteria	Siddiqui et al., 2012
44	Butanamine, 2,2-dinitro-n-methyl-	21.97	5.391	$C_5H_{11}O_4N_3$	177	76	-	-
45	Heptanedioic acid, dimethyl ester	22.01	16.187	$C9H16O_4$	188	63	Antimicrobial	Foo et al., 2017
46	1H-cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan-2-yl)-, (3aR,1-trans,6a-cis)-	28.03	3.68	$C_{10}H_{12}O_4$	196	63	Antibiofilm	Kiran et al., 2010
***Streptomyces* sp. DST142 (methanol extract)**								
47	3-ethoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasilox	13.942	3.460	$C_{17}H_{50}O_7Si_7$	562	77	anticancer	Mohansrinivasan et al., 2015
48	Cyclooctasiloxane, hexadecamethyl-	15.868	8.873	$C_{16}H_{48}O_8Si_8$	592	72	Antimicrobial	Jinfeng et al., 2017

49	1,1,1,3,5,7,9,11,11,11-decamethyl-5-(trimethylsiloxy)hexasiloxane	17.523	8.248	$C_{18}H_{52}O_7Si_7$	576	77	-	-
50	Heptasiloxane, hexadecamethyl-	18.984	8.021	$C_{16}H_{48}O_6Si_7$	532	61	Antibacterial	El-Din and El-Ahwany, 2016
51	1,1,1,3,5,7,7,7-octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	20.315	6.538	$C_{14}H_{42}O_5Si_6$	458	75	-	-
52	Cyclononasiloxane, octadecamethyl-	21.525	6.149	$C_{18}H_{54}O_9Si_9$	666	79	Biocontrol	Ahsan et al., 2017
53	Mercaptoacetic acid, bis(trimethylsilyl)-	22.660	7.639	$C_8H_{20}O_2SSi_2$	236	81	-	-
54	Hexasiloxane, tetradecamethyl-	23.736	8.632	$C_{14}H_{42}O_5Si_6$	458	75	-	-
55	Tetracosamethyl-cyclododecasiloxane	24.761	8.784	$C_{24}H_{72}O_{12}Si_{12}$	888	68	Antifungal	Omoruyi et al., 2014
56	Acetic acid, [o-(trimethylsiloxy)phenyl]-, trimethylsilyl ester	25.697	8.141	$C_{14}H_{24}O_3Si_2$	296	68	-	-
57	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)oxy]-	26.547	5.746	$C_{12}H_{36}O_4Si_5$	384	74	Antioxidant	Khan et al., 2016
58	Cyclononasiloxane, octadecamethyl-	27.347	4.745	$C_{18}H_{54}O_9Si_9$	666	54	Antibacteria	Dehbashi et al., 2015
59	3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetras	28.098	4.821	$C_{18}H_{52}O_7Si_7$	576	77	-	-

60	Cyclotrisiloxane, hexamethyl-	28.723	2.331	$C_6H_{18}O_3Si_3$	222	74	Antibacteria	Priyanka et al., 2015
61	1,2,4-benzenetricarboxylic acid, 1,2-dimethyl ester	28.798	2.902	$C_{11}H_{10}O_6$	238	70	Biocontrol	Ahsan et al., 2017
62	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	28.888	3.168	$C_{12}H_{38}O_5Si_6$	430	66	-	Jayashree et al., 2015A
63	3,5-dimethoxyphenylacetic acid, trimethylsilyl ester	28.948	1.804	$C_{21}H_{26}O_2NF_5Si_2$	475	65	-	-

4.3.10 Detection and Quantification of Antibiotics using the UPLC-MRM Method

The analytical method used in UPLC-ESI-MS/MS was validated by linearity, LOD, LOQ, precision, solution stability, and recovery; the observed validations were shown in **Table 2.** All the calibration curves showed good linearity with correlation coefficients (r^2) from 0.9989 to 0.9999 within the test ranges. The LOD and LOQ for each reference analyte was less than 0.52 ngml^{-1} and 0.78 ngml^{-1}, respectively. The overall recovery of analytical method developed is in the range of 97.98% – 102.12% (RSD ≤ 1.45%) for all analytes which signifies a good accuracy (**Table 5.12**).

Table 5.12: Method Validation Parameters for selected antibiotics

Analytes	**Regression Equation**	r^2	**Linear range ng/ml**	**LOD ng/ml**	**LOQ ng/ml**	**Precision RSD (%)**		**Stability**	**Recovery RSD (%)**
						Intra-day (n=6)	Inter-day (n=6)	RSD (n = 5)	
Trimethoprim	y = 1116.5x - 2399.2	0.9989	1-100	0.52	0.78	1.23	0.99	1.55	1.44
Fluconazole	y = 16698x - 980.72	0.9999	0.5-50	0.11	0.42	1.11	0.58	2.45	1.27
Ketoconazole	y = 641.95x - 104.06	0.9996	0.5-50	0.13	0.38	1.08	1.03	1.29	1.45
Nalidixic acid	y = 55514x + 9135.4	0.9996	0.5-100	0.18	0.42	0.78	2.01	1.35	1.09
Rifampicin	y = 7410.9x - 5216.3	0.9995	0.5-50	0.10	0.36	0.81	1.67	0.79	0.96

The antibiotics content in the methanolic crude extracts of the selected actinobacterial isolates were indicated in **Table 5.13.** which consists of two antifungal (fluconazole and ketoconazole) and three antibacterial (nalidixic acid, rifampicin and trimethoprim). Nalidixic acid was detected and quantified in highest concentration in most of the methanolic extract of the selected isolates except in the extract of *Streptomyces* sp. DST25 and *Streptomyces* sp. DST116 where ketoconazole and rifampicin was detected and quantified in higher amount compared

to nalidixic acid in the extract of the two isolates respectively. Among the selected isolates, trimethoprim was detected and quantified more amount in the methanolic crude extract of *Streptomyces flavogriseus* DST52 (39 µg/g). Extract of *Streptomyces cellulosae* DST28 contains maximum amount of Ketoconazole (17 µg/g) and Fluconazole (µg/g) among the selected isolates while extract of *Streptomyces* sp. DST25 and *Streptomyces* sp. DST116 contains more amount of Nalidixic acid (7920 µg/g) and Rifampicin (86 µg/g) respectively.

Table 5.13: Antibiotic content in the methanol extract of selected actinobacterial isolates (µg/g)

Isolates	Trimethoprim	Fluconazole	Ketoconazole	Nalidixic acid	Rifampicin
DST116	26.0	10.0	49.0	30.0	**86.0**
DST28	21.0	**17.0**	**50.0**	4870.0	74.0
DST25	17.0	8.0	29.0	**7920.0**	51.0
DST47	28.0	6.0	32.0	9.0	64.0
DST52	**39.0**	5.0	28.0	7090.0	78.0
fDST71	27.0	16.0	35.0	393.0	68.0
DST103	18.0	6.0	18.0	135.0	56.0

4.4 Discussion

The potential contributions of actinobacteria in various pharmaceutical and agricultural industries have been highlighted by several researchers globally (Yuan et al., 2014; Passari et al., 2015; Sharma et al., 2016) and a number of habitats have been explored in search of new and novel microbial compounds (Ningthoujam et al., 2011). The demand for new bioactive compounds especially antibiotics still increases since the disease causing pathogens were resistance to most of the commonly used antibiotics (Jami et al., 2015). However, bacterial infections were reported to be one of the leading causes of death all over the world (Sanasam et al., 2011). With all the

challenges, researchers explored various habitats like soil, medicinal plants, marine water, etc. in search of novel drug compounds. Furthermore, nature remains the best supplier of biologically active compounds and fresh water bio-resources were less explored compared to other habitats especially in bioprospection studies of actionobacteria. The present study described the antimicrobial screening of 115 isolates of actinobacteria from fresh water sediments of Mizoram, Northeast part of India, which have never been reported earlier for its actinobacteriological research.

Initially, the antifungal activity was checked to understand the inhibition ability of the isolated actinobacteria against various fungal pathogens by dual culture *in vitro* assay, which is a method widely used for preliminary screening. 49.7% of the isolated organisms showed activity against at least one of the tested pathogens which clearly showed the antifungal potential of actinobacteria from fresh water sediments supported by the findings of Rifaat, (2003) that demonstrated the anti-mycotic activity of 114 fresh water actinobacteria from Nile River. We have reported the antifungal potential of two actinobacterial isolates *Streptomyces* sp. DST23 and *Streptomyces parvus* DST24 against *F. oxysporum, F. proliferatum* and *F. oxy. ciceri* from fresh water Tuichang river (Zothanpuia et al., 2015) which was in accordance with the isolation of *Streptomyces* from Krishna river showing antibacterial and antifungal activity (Ellaiah et al., 2002). Similar reports were also found from Lake Baikal, the largest fresh water lake worldwide that described more than 70% of the isolates having antifungal activity (Protasov et al., 2017). Literature regarding the study of antifungal activity of fresh water actinobacteria was so rare and researchers should emphasise more in this area.

The antibiotic sensitivity of the isolated actionobacteria was studied against 15 commonly used selected antibacterial antibiotics. Similar studies were reported on twenty six actinobacteria, screened for their sensitivity against 12 antibiotics (Gousterova et al., 2014). Antibiotics resistance to penicillin and ampicillin have been largely reported by several researchers worldwide. The mechanisms of resistance to beta-lactam antibiotics like penicillin and cephalosporin was explained by the fact that the beta lactamase enzymes produced by bacteria hydrolysed the antibiotics making them inactive (Dever and Dermody, 1981). The present study also showed high resistance to ampicillin and penicillin antibiotics supported by the findings of Falcao et al. (2004), Scoaris et al. (2008), Reboucas et al. (2011) and Passari et al. (2015). The highly resistant of endophytic actinobacteria to antibiotics (penicillin and ampicillin) and highly sensitive to nalidixic acid, tetracycline and erythromycin was recently reported by Passari et al. (2017) which supported the present investigations.

Actinobacteria are known to produce two-third of the antibiotics used in clinical and agricultural field (Gebreyohannes et al., 2013) and are one of the potential candidates to fight multidrug resistance pathogens (Claverias et al., 2015). Several antimicrobial compounds from actinobacteria have been reported worldwide from different habitats (Sharma et al., 2016). Investigations on the potential aspects of fresh water actinobacteria especially antimicrobial (antibacterial and antifungal) study are becoming an important challenge to meet the current scenario of antibiotic resistance hitches. Broad spectrum antimicrobial activity against five bacterial pathogens and one yeast pathogens were reported from fresh water lakes and rivers

of Mizoram in the present investigations. 76 out of 115 actinobacterial isolates showed *in vitro* antimicrobial activity against at least one of the tested pathogens. Similar studies and reports from fresh water habitats were also found from several researchers worldwide (Johnston and Cross, 1976; Rifaat and El-Sayed; 2008; de Menezes et al., 2008; Sibanda et al., 2010; Radhika et al., 2011; Sanasam et al., 2011; Rizvi et al., 2012; Gebreyohannes et al., 2013; Jami et al., 2015; Iqbal and Sajid. 2015; Mullowney et al., 2015; Shaikh et al., 2015). The antimicrobial activity of 69 actinobacterial isolates against *B. subtilis* and *C. albicans* were also reported from the lakes and rivers of Southern Chile (Leiva et al., 2004). Rare genera of actinobacteria such as *Kocuria*, *Nocardiopsis*, *Amycolatopsis*, *Saccharopolyspora*, *Rhodococcus*, *Prauserella*, *Promicromonospora* and *Micrococcus* were also reported for its antimicrobial potential in the current study. Among the rare genera of actinobacteria screened for the antimicrobial potential, *Saccharopolyspora* sp. DST31, *Nocardiopsis* sp. DST32, *Rhodococus* sp. DST38 and *Nocardiopsis* sp. DST95 showed activity against five of the tested six pathogens which was in consistent with the reports of *Sachharopolyspora* and *Actinosynemma* from Tyume River, South Africa having antibacterial activity against both Gram positive and Gram negative bacteria (Sibanda et al., 2010). A number of other rare genera of actinobacteria except *Amycolatopsis, Prauserella* and *Promicromonospora* were already reported for their antimicrobial activity from fresh water habitat (Chou et al., 2008; Ningthoujam et al., 2011; Jami et al., 2015). *Streptomyces* which is the dominant genus among the phylum actinobacteria remains the dominant producers which accounts for more than 70% of the antibiotics produced by actinobacteria

(Sharma et al., 2016). The present investigations also selected the best nine isolates from a total of 115 actinobacterial isolates based on their antimicrobial screening and the selected isolates were all *Streptomyce*s species which clearly supports and signifies the potential of this genus as described earlier. The broad spectrum antimicrobial potential of nine *Streptomyces* isolates was further investigated using their methanolic crude extract which showed better activity using agar well diffusion method compared to filter paper disk diffusion assay supported by the findings of Gebreyohannes et al. (2013). The antimicrobial potential of *Streptomyces cyaneofuscatus* from fresh water sediments of Tamdil Lake was in accordance with the isolation of *Streptomyces cyaneofuscatus* M-27 having antitumor and anti-inflammatory compounds from the Central Cantabrian Sea (Afredo et al., 2014). *Streptomyces* sp. AZ-NIOFD1 with broad-spectrum antimicrobial activity was also reported from river Nile (Atta et al., 2009). Several potential isolates of *Streptomyces* were also described from various fresh water habitats (Nwodo et al., 2012; Singh et al., 2014; Zhao et al., 2015). Methanolic crude extract of *Streptomyces flavogriseus* DST52 showed antimicrobial activity with MIC value of 0.003 mg ml^{-1} which was lower than the actinobacterial isolate SMS_SU21, that showed antimicrobial activity with MIC value of 0.05 mg ml^{-1} reported from mangrove ecosystem (Sengupta et al., 2015). The MIC of *Streptomyces* sp. DST119 was 0.015 mg ml^{-1} against *S aureus* while that of *Streptomyces flavogriseus* DST52 was 0.056 mg ml^{-1} which was far lower than that of the MIC of actinobacterial crude extract 1.65 mg ml^{-1} and 1.84 mg ml^{-1} against *S. aureus* and *E. coli* respectively (Gebreyohannes et al., 2013). Based on the MIC of five bacterial pathogens and one yeast pathogen, the crude extract of

Streptomyces cyaneofuscatus DST103 was the best isolate which showed lowest MIC against three pathogens compared to other selected isolates.

The correlation of genes (biosynthetic genes) and its bioactive secondary metabolites have been discussed in a variety of microbes such as actinobacteria by various researchers (Yuan et al., 2014; Passari et al., 2015) especially PKS type II, *phz*E and NRPS genes which have been widely described as involved in the synthesis of bioactive polyketides, phenazine and peptides compounds respectively (Schwarzer et al., 2003; Ayuso-Sacido and Genilloud, 2005; Yuan et al., 2014). The correlation of biosynthetic genes and its antimicrobial properties of fresh water actinobacteria have been described in the present study. Among the selected actinobacterial isolates which showed antimicrobial activity against all the tested pathogens, biosynthetic genes such as PKS type II, *phz*E and NRPS were all detected and amplified with the expected size in *Streptomyces* sp. DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST119 whereas none of the genes were detected in *Streptomyces cellulosae* DST28 which showed antimicrobial activity against all the tested pathogens. This clearly explained that the isolates showing antimicrobial properties should not necessarily contain a PKS-II, *phz*E or NRPS gene which was in agreement with the previous studies (Qin et al., 2009; Passari et al., 2015).

Quantitation and detection of non-volatile organic compounds using high performance liquid chromatography (HPLC) was common compared to ultra-performance liquid chromatography. Antibiotics such as rifamycin and trimethoprim were detected and quantified from the crude methanol extract of selected *Streptomyces* isolates which was supported by the findings of Passari et al. (2016).

Nalidixic acid, Fluconazole and ketoconazole were also quantified in all the selected extract of *Streptomyces* isolates which was reported from *Streptomyces cyaneofuscatus* from Tamdil Lake. The synthetic and semi-synthetic compounds such as trimethoprim, fluconazole, ketoconazole, nalidixic acid and rifampicin detected in the study were very minute and might not be significant since no studies were found in support of the present investigations. Further investigating on more antibiotic non-volatile compounds using HPLC or UPLC will certainly acknowledge the potential aspects of the actinobacterial extracts which can be subjected for downstream processing. However, targeting interesting compounds, identification, quantitation and purification of the compounds will be taken care in future studies.

GC-MS is efficiently used for bioactive secondary metabolite profiling and is becoming one of the bases in the field of biological sciences that has been successfully employed for the determination of VOCs from various microbial and plant samples (Tan et al., 2015; Sharma et al., 2016). The phylum actinobacteria has been reported as prolific producers of diverse bioactive secondary metabolites. The present investigations determined 63 VOCs from nine methanolic crude extracts of *Streptomyces* isolates using GC-MS, out of which maximum compounds were retrieved from the methanolic extract of *Streptomyces* sp. DST142 which consist of 17 compounds, more than 50% of the compounds detected were not found in the earlier literatures while 41% of the compounds detected in the methanolic extract DST142 were reported earlier for its antimicrobial and antioxidant activites (Omoruyi et al., 2014; Mohansrinivasan et al., 2015; Jinfeng et al., 2017; El-Din and El-Ahwany, 2016; Jayashree et al., 2015; Priyanka et al., 2015; Dehbashi et al.,

2015; Ahsan et al., 2017). Among the 14 compounds detected from the extract of *Streptomyces albidoflavus* DST71, except Oxirane, 2-butyl-3-methyl-, cis, Azacyclodecan-5-ol, N-(4-Chlorobenzenesulfonyl)azetidin-3-one and 1,3,5-Triazaadamantane, the rest of the detected compounds have the antimicrobial activity as reported by earlier workers (Al-Wathnani et al., 2012; Sitarek et al., 2017; Lerones et al., 2004; Kabara et al., 1972; Dineshkumar et al., 2017; Sepahi et al., 2017; Ramakrishnan and Venkataraman . 2011; Foo et al., 2017; Dehpour et al., 2011). In the present study the amount of hexanal in methanol extract of *Streptomyces albidoflavus* DST71 was found to be maximum (23.2%) and this compound was reported as one of the constituent in crude extract of *Leonurus sibiricus's* root for its antibacterial, anti-inflammatory, antioxidant, and antiproliferative properties (Sitarek et al., 2017). Antimicrobial activity of 2-Thiophenecarboxylic acid, 5-(1,1-dimethylethoxy)- and heptanal were also observed in the extract of *Phormidium autumnale* and *Chlorella vulgaris* respectively (Al-Wathnani et al., 2012). The antimicrobial activity of glutaraldehyde was also discussed earlier (Hill et al., 1991; Lerones et al., 2004) and was also determined in the extract of *Streptomyces* sp. DST25. All the compounds extracted from the crude extract of *Streptomyces* sp. DST25 except Cyclopropane, 1-butyl-2-(2-methylpropyl)- were earlier reported in the antimicrobial studies (Lee et al., 2014; Ahmad et al., 2014; Dineshkumar et al., 2017). Amino acid valine was also determined as one of the major compound next to glutaraldehyde in the present study and this compound increases the production of glycopeptide antibiotic as reported by Beltrametti et al. (2004) in actinobacteria isolate *Nonomuraea* sp. Only Pyrrolo[1,2-a] Pyrazine-1,4-dione, hexahydro-3-(2-

methylpropyl) out of six compounds detected from extract of *Streptomyces* sp. DST119 was reported earlier for its antimicrobial activity (Sheoran et al., 2015; Jinfeng et al., 2017). Two of the seven compounds; Carbonic acid, 2,2,2-trichloroethyl undec-10-enyl ester and 1-Butanol, 2-methyl- acetate from the extract of *Streptomyces flavogriseus* DST52 were reported earlier for its antimicrobial activity (Ezra and Strobel. 2003 Ezra et al., 2004; Musini et al., 2013). Only one compound was determined in the extracts of *Streptomyces cellulosae* DST28 and *Streptomyces* sp. DST116 with a single peak. Di-N-Octyl phthalate obtained from *Streptomyces cellulosae* DST28 was reported earlier by various researchers for its antimicrobial activity (Philip et al., 2011; Shafaghat et al., 2012) while no activity was reported for 2-Methoxy-4,5-diphenyl-6-(2'-phenylethyl)-pyrimidine obtained from extract of *Streptomyces* sp. DST116 for its antimicrobial activity. Majority of the compounds detected in the extract of *Streptomyces cyaneofuscatus* DST103 were earlier reported in the antimicrobial studies of several microbial and plants extracts (Senbagam et al., 2016; Sheoran et al., 2015; Kalaiselvan et al., 2012; Idramsa et al., 2016)

From all the secondary metabolites detected (n=63) using GC-MS in the present investigation, 52.4% of the compounds were earlier reported for its antibacterial, antifungal, antioxidant and cytotoxic activity and to our knowledge, no activity reported in 46% of the detected compounds. Majority of the compounds reported earlier were found to be related with the study of medicinal plants, seaweeds, cyanobacteria, green algae and several varieties of plant species. Among the compound determined, to best of our knowledge, only two compounds were

found to be reported earlier from actinobacteria. Compound 1,6;2,3-Dianhydro-4-deoxy-.beta.-d-ribo-hexopyranose was detected in the methanolic crude extract of ***Streptomyces flavogriseus* DST52** which was reported for its anticancer activity in the extract of *Streptomyces carpaticus* BTSS-501 isolated from human gut microbiota (Zhou et al., 2017). 1H-cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan- 2-yl)-, (3aR,1-trans,6a-cis)- which was detected in the ethyl aceteate extract of *Streptomyces* sp. DST142 was also earlier reported in the study of antibiofilm activity of a glycolipid biosurfactant isolated from the marine Actinobacterium *Brevibacterium casei* MSA19 (Kiran et al., 2010). Interestingly, none of them were earlier reported from fresh water actinobacteria which clearly explained the novelty of the study and needs to explore more in future for its potency in drug industries. Passari et al. (2015) explained that bioactive compounds having antimicrobial activities were extracellular which could further be isolated and quantified that may help in the discovery of new drug.

Thus, from this study we conclude that further investigation on the purification of these potent compounds detected and transformation into drugs will certainly explicate their efficacy in the pharmaceutical industry. The usage of fresh water bio-resource hence can be an ideal source for the isolation of actionbacterial cultures with rare and unique properties that could certainly add to the ever growing pharmaceutical needs and other biotechnological applications.

Summary and Conclusion

Actinobacteria are well known antibiotic producers providing two third of the world's commercially available antibiotics in clinical use, and different habitats have been largely explored in search of potential actinobacteria for its bioactive compounds especially antibiotics. However, with the inappropriate use of antibiotics and other several means, the antibiotic resistant microbes are becoming more prevalent in the environment. The present scenario of antibiotic resistance among the pathogens can only be undertaken with the discovery of new and novel drugs having different mechanisms of actions, which urges in the bio-prospection of unexplored habitats for new and more effective antimicrobial antibiotic compounds. Actinobacteria have long been reported from fresh water since the early 1969 but then remains less explored compared to other habitats, which is becoming promising source for the isolation of novel bioactive secondary metabolites. Hence, after literature review, the present study entitled "**Molecular Characterization and Evaluation of Antimicrobial Potential of Actinobacteria Isolated from Lakes and Rivers of Mizoram**" was designed which aims to isolate actinobacteria from fresh water sediments, screen them for their antimicrobial activity and their ability to produce bioactive secondary metabolites. However, the discovery of naturally derived antibiotics begins with the isolation of organisms from its wild habitats, screening for antimicrobial biologically active potential and subjected to downstream process.

The present investigation reported the abundance of fresh water actinobacteria from four rivers *viz.* Tlawng River, Tuirial River, Tuichang River,

Murlen stream and two lakes *viz.* Tamdil Lake and Rihdil Lake of Mizoram, Northeast India. A total of 115 isolates of actinobacteria were isolated from water sediment samples, identified using 16S rRNA gene sequencing and differentiated the isolates into 16 different genera. *Streptomyces* constitutes the dominant genus comprising of 55% (n=63) of the total isolates followed by *Nocardiopsis* (n=8), *Arthrobacter* (n=8), *Rhodococcus* (n=5), *Micrococcus* (n=5), *Saccharopolyspora* (n=4), *Brevibacterium* (n=4), *Brachybacterium* (n=4), *Corynebacterium* (n=4), *Kocuria* (n=3), *Leucobacter* (n=2), *Promicromonospora* (n=1), *Prauserella* (n=1), *Amycolatopsis* (n=1), *Tsukamurella* (n=1) and *Zhihengliuella* (n=1). Maximum diversities were observed at Rihdil Lake where 31 actinobacteria belonging to thirteen different genera were obtained followed by Tamdil lake [31 isolates (eight different genera)], Tuirial river [19 isolates (three genera)], Tlawng river [19 isolates (two genera)], Tuichang river [9 isolates (2 genera)] and Murlen stream [6 isolates (2 genera)]. The genus *Streptomyces* is the most versatile among the isolates, observed in the sediment samples of the entire selected study site. Among the seven nutritional media employed for isolation, SCA was proposed as best medium for the isolation of fresh water actinobacteria, which yields maximum number of isolates accounting for 43% of the total isolates, followed by AIA (29%), ISP7 (10%), ISP5 (7%), SA (6%), TWYE (3%) and ISP2 (2%).

Initially, the antifungal activity of actinobacterial isolates was tested against four fungal phytopathogens: *F. udum, F. oxysporum, F. graminearum* and *F. proliferatum.* 49.7% of the isolates showed inhibition activity against at least one of the tested pathogens. Among them, 14 isolates *viz.* *Streptomyces cyaneofuscatus* DST 15, *Streptomyces* sp. DST 16, *Streptomyces* sp. DST25, *Saccharopolyspora* sp.

DST31, *Streptomyces griseoplanus* DST53, *Streptomyces* sp. DST54, *Streptomyces cyaneofuscatus* DST64, *Streptomyces albidoflavus* DST71, *Streptomyces* sp. DST86, *Streptomyces albidoflavus* DST102, *Streptomyces* sp. DST104, *Nocardiopsis* sp. DST105, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST119 showed activity against all the tested fungal plant pathogens. Maximum inhibitory activity of the actinobacterial isolates was found against *F. graminearum* (33.9%, n=39), followed by *F. proliferatum* (30.4%, n=35), *F. oxysporum* (29.5%, n=34) and *Fusarium udum* (26.9%, n=31) with percentage of inhibition ranging from 20-87.2%. Maximum percentage of inhibition was observed in *Streptomyces* sp. DST25 against *Fusarium udum* (87.20%). In view of the above observations and further exploration besides in depth analysis of the potential actinobacteria from fresh water sediments will certainly help in the discovery of effective anti-mycotic agent.

Fifteen different standard antibiotic discs procured from Himedia (India) were used to understand the antibiotic sensitivity of actinobacterial isolates, which includes Ampicillin (Amp^{10}), Streptomycin (S^{10}), Erythromycin (E^{15}), Norfloxacin (NX^{10}), Tetracycline (TE^{30}), Gentamycin (Gen^{10}), Penicillin G (P^{2}), Neomycin (N^{10}), Chloramphenicol (C^{10}), Polymixin B (PB^{50}), Ciprofloxacin (CIP^{10}), Vancomycin (VA^{10}), Trimethoprim (TR^{10}), Clindamycin (CD^{10}), Levofloxacin (LE^{5}). The isolates were highly resistant to trimethoprim (R=62, S=7) followed by Penicillin (R=52, S=3, I=14) and Ampicillin (R=42, S=14, I=13) whereas a high sensitivity was found against Streptomycin, Erythromycin, Norfloxacin, Tetracycline, Gentamycin, Neomycin, Chloramphenicol, Polymixin B, Ciprofloxacin, Vancomycin, Clindamycin and Levofloxacin. Majority of the isolates were sensitive to Levofloxacin (R=1, I=4, S=64). The selected antibiotics tested against the

actinobacterial isolates were all antibacterial, which provides information on the antibiotic susceptibility of the isolated organisms from fresh water habitats.

The antimicrobial activity of actinobacterial isolates were screened against five bacterial pathogens and one yeast pathogen (*S. aureus*, *B. subtilis, M. luteus*, *P. aeruginosa, E. coli* and *C. albicans*). 66.1% of the actinobacterial isolates were found to inhibit the growth of at least one tested pathogens and *E. coli* was found to be the most susceptible pathogen where 76 isolates inhibited the growth of *E. coli* within the inhibition range of 7.4 mm to 15.5 mm diameter followed by *P. aeruginosa*, *C. albicans*, *B. subtilis* and *S. aureus*. Only 22.6% of the isolates showed activity against *M. luteus* and found to be the most resistant pathogens. Overall, the actinobacterial isolates showed good antimicrobial activity against tested pathogens. Nine actinobacterial isolates namely *Streptomyces* sp. DST25, *Streptomyces cellulosae* DST28*, Streptomyces intermidus* DST29, *Streptomyces flavogriseus* DST52, *Streptomyces albidoflavus* DST71, *Streptomyces cyaneofuscatus* DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST119 were selected based on their broad-spectrum antimicrobial activities and these isolates were selected as potential candidates for further investigation. Additionally, the methanolic crude extract of the selected isolates showed adequate inhibition zones against tested pathogens at 20 mg ml^{-1} and 40 mg ml^{-1} for all the samples. Minimum inhibitory concentration of the extracts of selected isolates against the pathogens were described where *Streptomyces cyaneofuscatus* showed highest activity against *E. coli* (EC50=0.002 $mgml^{-1}$) followed by *Streptomyces flavogriseus* DST52 (EC50=0.052 $mgml^{-1}$) and *Streptomyces* sp. DST142 (EC50=0.062 $mgml^{-1}$). The EC50 of *Streptomyces cyaneofuscatus* was found to be lowest against *P. aeruginosa* (EC50=0.009 $mgml^{-1}$)

followed by *Streptomyces* sp. DST119 (EC50=0.015 $mgml^{-1}$) and *Streptomyces* sp. DST142 (EC50=0.024 $mgml^{-1}$). *Streptomyces* sp. DST119 showed highest activity against *S aureus* (EC50=0.015 $mgml^{-1}$) among the isolates while *Streptomyces flavogriseus* DST52 and *Streptomyces cyaneofuscatus* DST103 showed highest activity against *M. luteus* (EC50=0.003 $mgml^{-1}$) and *C. albicans* (EC50=0.025 $mgml^{-1}$) respectively.

PKS type II, *phz*E and NRPS genes which have been widely described as involved in the synthesis of bioactive polyketides, phenazine and peptides compounds respectively were detected and amplified in the present investigation. Out of the 115 isolates, PKS type II was detected in 31 isolates (27%), NRPS was detected in 47% (n=54) of the isolates and *phz*E was detected in 17.4% (n=20) of the isolates. A total of 11 isolates of actinobacteria (DST45, DST47, DST54, DST56, DST57, DST58, DST74, DST76, DST77, DST99, DST101 and DST142) were found to constitute all the three genes. Among the actinobacterial isolates which showed antimicrobial activity against all the tested pathogens, biosynthetic genes such as PKS type II, *phz*E and NRPS were all detected and amplified with the expected size in *Streptomyces* sp. DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST119 whereas none of the genes were detected in *Streptomyces cellulosae* DST28 which showed antimicrobial activity against all the tested pathogens. This clearly explains that the isolates showing antimicrobial properties may not necessarily contain PKS-II, *phz*E or NRPS gene.

The actinobacterial isolates were further screened for their ability to hydrolyze xylan and cellulose. Nineteen isolates which showed good activity in preliminary screening were subjected to cellulase and xylanase induction by allowing

them to grow in induction media. Seven isolates (*Streptomyces* sp. DST25, *Streptomyces cellulosae* DST28*, Streptomyces flavogriseus* DST30, *Streptomyces flavogriseus* DST52, *Streptomyces cyaneofuscatus* DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST142) showed both cellulase and xylanase activity. The hydrolytic zones formed by the actinobacterial isolates for cellulose and xylan ranges from 0.04cm-4cm and 0.08cm-6.6cm respectively. Maximum cellulolytic zone was formed by *Streptomyces* sp. DST25followed by *Streptomyces flavogriseus* DST52 whereas maximum xylanolytic zone was formed by *Streptomyces* sp. DST142 followed by *Streptomyces cyaneofuscatus* DST103. Further purification of the extracellular enzymes produced by the potential isolates will have positive impact in the breakdown and degradation of organic matter such as cellulosic and hemicelulosic biomasses. Researchers should emphasise more on the enzymatic potential of fresh water actinobacteria, an untapped bioresources.

The methanolic crude extracts of nine selected actinobacterial isolates were investigated to determine their volatile organic compounds (VOCs) using GC-MS, which revealed sixty-three VOCs**.** Maximum compounds were detected in the methanolic extract of *Streptomyces* sp. DST142 which comprise of 17 compounds while only 5 compounds were retrieved from ethyl acetate extract of DST142. 14 compounds were detected from the extract of *Streptomyces albidoflavus* DST71. Six VOCs, from the extract of *Streptomyces* sp. DST25, Only one compound (di-n-octyl phthalate) was detected in extracts of *Streptomyces cellulosae* DST28. Two compounds were detected in the extract of *Streptomyces* sp. DST29. Seven compounds were determined from the extract of *Streptomyces flavogriseus* DST52, of which carbonic acid, 2, 2, 2-trichloroethyl undec-10-enyl ester alone constituted

49.78%. Four compounds were detected from *Streptomyces cyaneofuscatus* DST103, only 2-methoxy-4,5-diphenyl-6-(2'-phenylethyl)pyrimidine was detected in the extract of *Streptomyces* sp. DST116, while six compounds were detected in the extract of *Streptomyces* sp. DST119. From all the secondary metabolites detected using GC-MS in the present investigation, 54% of the compounds were earlier reported for its antibacterial, antifungal, antioxidant and cytotoxic activity and no activities were earlier reported in 46% of the detected compounds. Among them, to best of our knowledge, only two compounds [1,6;2,3-Dianhydro-4-deoxy-.beta.-d-ribo-hexopyranose and 1H-cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan- 2-yl)-, (3aR,1-trans,6a-cis)-] were found to be reported earlier from actinobacteria described for its anti-cancer and anti-biofilm activity from human gut actinobacteria and marine actinobacteria respectively. Furthermore, to best of our knowledge, the compounds detected have never been reported so far from fresh water actinobacteria.

The antibiotics content in the methanolic crude extracts of the selected actinobacterial isolates was studied using two antifungal (fluconazole and ketoconazole) and three antibacterial (nalidixic acid, rifamycin and trimethoprim) antibiotics. Nalidixic acid was detected and quantified in highest concentration in most of the methanolic extract of the selected isolates except in the extract of *Streptomyces* sp. DST25 and *Streptomyces* sp. DST116 where ketoconazole and rifampicin was detected and quantified in higher amount compared to nalidixic acid in the extract of the two isolates respectively. Among the selected isolates, trimethoprim was detected and quantified more amount in the methanolic crude extract of *Streptomyces flavogriseus* DST52 (39 µg/g). Extract of *Streptomyces*

cellulosae DST28 contains maximum amount of Ketoconazole (17 µg/g) and Fluconazole (µg/g) among the selected isolates while extract of *Streptomyces* sp. DST25 and *Streptomyces* sp. DST116 contains more amount of Nalidixic acid (7920 µg/g) and Rifampicin (86 µg/g) respectively. We believed that further employing more antibiotics standard in the study will certainly help in the efficacy of the potential isolates which could be done in further studies.

To date and to the best of our knowledge, rare genera of actinobacteria such as *Leucobacter, Zhihengliuella, Amycolatopsis, Prauserella* and *Promicromonospora* have never been reported so far from fresh water sediments and were reported for the first time in the present investigation. Bioactive secondary metabolite compounds determination and study of biosynthetic genes in fresh water actinobacteria were very rare according to literature review; this study provides a baseline data for fresh water actinobacteriological research. Thus, from the present investigation we conclude that further exploration of fresh water actinobacteria for its potential besides investigation on the purification of potent compounds detected and transformation into drugs will certainly explicate their efficacy in pharmaceutical industry. The usage of fresh water bio-resource hence can be an ideal source for the isolation of actinobacterial cultures with rare and unique properties that could certainly add to the ever growing pharmaceutical needs and other biotechnological applications.

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Appendices

Appendix I: Paper published in peer reviewed journal

1. **Zothanpuia**, Passari AK, Chandra P, Leo VV, Mishra VK, Kumar B & Singh BP. (2018). Bioprospection of actinobacteria derived from freshwater sediments for their potential to produce antimicrobial compounds. ***Microbial Cell Factories***, 17. 68. https://doi.org/10.1186/s12934-018-0912-0.
2. **Zothanpuia,** Passari AK, Gupta VK & Singh BP. (2016). Detection of antibiotic-resistant bacteria endowed with antimicrobial activity from a freshwater lake and their phylogenetic affiliation. ***Peer J,*** 4:e2103.
3. **Zothanpuia**, Passari AK & Singh BP. (2015). Molecular characterization of actinomycetes isolated from Tuichang river and their biosynthetic potential. ***Science Vision,*** 15:136.
4. **Zothanpuia**. Passari AK & Singh BP. (2018) *In vitro* Evaluation of Antimicrobial Activities and Antibiotic Susceptibility Profiling of Culturable Actinobacteria from Fresh Water Streams. ***Indian Journal of Experimental Biology,*** *56,* 665-673.
5. Passari AK, Chandra P, **Zothanpuia,** Mishra VK, Leo VV, Gupta VK, Kumar B & Singh BP. (2016). Detection of biosynthetic gene and phytohormone production by endophytic actinobacteria associated with *Solanum lycopersicum* and their plant-growth promoting effect. ***Research in Microbiology,*** 167:692-705.
6. Passari AK, Lalsiamthari PC, **Zothanpuia,** Leo VV, Mishra VK, Yadav MK, Gupta VK & Singh BP. (2018). Biocontrol of *Fusarium* wilt of *Capsicum annuum* by rhizospheric bacteria isolated from turmeric endowed with plant

growth promotion and disease suppression potential. ***European Journal of Plant Pathology,*** 150 (4) 831-846. DOI 10.1007/s10658-017-1325-3.

7. Ghatak S, Lallawmzuali D, Lalmawia, Sapkota R, **Zothanpuia**, Pautu JL, Muthukumaran RB & Kumar NS. **(2014).** Mitochondrial D-loop and Cytochrome Oxidase C subunit I polymorphisms among the breast cancer patients of Mizoram, Northeast India. ***Current Genetics,*** 60 (3) 202-212.
8. Joanne Zote, Passari A.K, **Zothanpuia**, Chandra Nayaka Siddaiah et al. (2018). Phylogenetic affiliation and determination of bioactive compounds of bacterial population associated with organs of mud crab, Scylla olivacea. ***Saudi Journal of Biological Sciences***, https://doi.org/10.1016/j.sjbs.2018.08.025

Book chapter

9. **Zothanpuia**, Passari AK, Leo VV & Singh BP (2018) Freshwater Actinobacteria: Potential Source for Natural Product Search and Discovery. *New and Future Developments in Microbial Biotechnology and Bioengineering Actinobacteria: Diversity and Biotechnological Applications*. ***Elsevier*****.** 67-77 doi.org/10.1016/B978-0-444-63994-3.00004-7
10. Passari AK, Mishra VK, **Zothanpuia** & Singh BP (2018) Molecular Markers Used for Identification and Genomic Profiling of Plant Associated Endophytic Actinobacteria. *New and Future Developments in Microbial Biotechnology and Bioengineering Actinobacteria: Diversity and Biotechnological Applications*. ***Elsevier*****.** 42-65 DOI: https://doi.org/10.1016/B978-0-444-63994-3.00003-5
11. Leo VV, Asem D, **Zothanpuia** and Singh B.P (2018) Actinobacteria: A Highly Potent Source for Holocellulose Degrading Enzymes. *New and Future Developments in Microbial Biotechnology and Bioengineering Actinobacteria:*

Diversity and Biotechnological Applications. ***Elsevier*.** 190-202 DOI: https://doi.org/10.1016/B978-0-444-63994-3.00013-8

12. Passari AK, Mishra VK, **Zothanpuia,** Leo VV & Singh BP (2017) Plant endophytic bacterial diversity for production of useful metabolites and their effect on environmental parameters. *Phytoremediation of Environmental Pollutants* Boca Raton: CRC Press Taylor and Francis group.

Appendix II: List of presentation in conference/symposium/seminar

1. **Presented paper** on Antimicrobial activity of Fresh water sediment derived Actinobacteria *Streptomyces sp.* DST25 and screening for their biosynthetic potential at ***International conference on Contemporary antimicrobial research 2016***, Assam University, Silchar, India (November 14-17, 2016).
2. **Presented paper** on *In Vitro* Evaluation of Antimicrobial Potential of Fresh Water Derived *Streptomyces sp.* strain DST107: Detection of Biosynthetic Genes and their Secondary Metabolites production in ***57th Annual conference of Association of Microbiologist of India & International symposium on "Microbes what's new and what's next"*** at Guwahati University, Assam, India (24-27, November, 2016).
3. **Presented paper on** Exploration of Actinobacteria from Fresh Water Sediments and their Applications in Pharmaceutical Industry ***58th Annual conference of Association of Microbiologist of India & International symposium on Microbes for Sustainable Development: Scope & Applications*** (16-19 November, 2017).
4. **Presented paper** on Antimicrobial and biosynthetic potential of fresh water actinobacteria isolated from Teirei river and their phylogenetic affiliation in ***Mizoram Science Congress*** at Mizoram University (13-14 October, 2016)
5. **Presented paper** on Bioprospecting of fresh water actinobacteria an excellent source for natural product search and discovery at ***National conference on Biodiversity, Conservation and Utilization of Natural Resources with reference to North East India (BCUNRNEI)*** (30-31 March, 2017).

6. **Presented paper** on Fresh water sediments: an alternative source for the isolation of actinobacteria having secondary metabolites production ability at ***National conference on "Recent Advances in Biotechnology"*** (Nov 09-10, 2017).
7. **Presented paper** on *In-vitro* Evaluation of Actinobacteria for its Potential in Bio-control of Fungal Plant Pathogens ***4th National Conference On Plant Growth-Promoting Rhizobacteria (PGPR) for Sustainability of Agriculture and Environment*** at Mizoram University, Mizoram, India.

Awards

- Best poster award in ***International symposium on Microbes for Sustainable Development: Scope & Applications*** at Lucknow, India (16-19 Nov 2017)
- Best poster award in ***4th National Conference On Plant Growth-Promoting Rhizobacteria (PGPR) for Sustainability of Agriculture and Environment*** at Mizoram University, Mizoram, India.

Apppendix III: List of seminar/symposium/conference/workshops attended

1. Participated India-UK Scientific Seminar on **“Structural Elucidation of Microbial Natural Products: Opportunities and Challenges”** in Department of Biotechnology, Mizoram University, India jointly funded by DST, New Delhi and The Royal Society, London, UK during 22nd -24th January, 2014
2. Participated International workshop on **“Molecular Entomology, Molecular Phylogeny and Next-Generation Sequencing”** jointly organised by Global Initiative for Academic Networks (GIAN), MHRD Govt. of India and Mizoram University during 19-28 June, 2017.
3. Attended National workshop on **HPLC, HPTLC and GC-MS/MS** held at Sophisticated and Instrumentation Centre for Applied Research and Testing (SICART) Vallabh Vidyanagar, Gujarat (28-30 July 2016)
4. Participated in one week course on **Research methodology for Research scholar** held from 20-26 June 2016 organized by University Grants Commission (HRDC), MZU.
5. Attended National workshop on **“Statistical and computing method for life science data analysis”** organized by The Biological Anthropology Unit, Indian Statistical Institute, Kolkata (09-16 February, 2015) at Pachhunga University College, Mizoram University.
6. Participated in a workshop on **Statistical and Computing Methods for Life Science Data Analysis** jointly organized by Biological Anthropology Unit, Indian Statistical Institute, Kolkata and Department of Botany, Mizoram University, Aizawl (5th-10th March, 2018).

7. Participated in National workshop on **"Application of NGS in Microbial Ecology"** held during 30th October to 2nd November, 2017 organizedby BIF, Dept. of Biotech, MZU sponsored by DBT New Delhi.
8. Attended a National workshop on "**Advances in Cancer Genomics"** held from 30-31 May, 2014 Organized jointly by Mizoram State Cancer Institute, Aizawl and Department of Biotechnology, Mizoram University Coordinated by Indian Institute of Technology, Guwahati under the scheme of Program support for North East Institutions.
9. Attended a National workshop on **"Capacity Building in Effective Management of Intellectual Property Rights"** organized by BCIL, Govt. of India, Department of Biotechnology, Mizoram University. 27-28 Aug, 2014.
10. Attended **North-East Life Science Entrepreneurship Workshop** held from October 12th to 14th 2012 Gangtok, Sikkim organized by Department of Biotechnology, Ministry of Science and Technology, Govt. of India and Association Of Biotechnology Led Enterprises (ABLE).

ABSTRACT

MOLECULAR CHARACTERIZATION AND EVALUATION OF ANTIMICROBIAL POTENTIAL OF ACTINOBACTERIA ISOLATED FROM LAKES AND RIVERS OF MIZORAM

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2018

ABSTRACT

Molecular Characterization and Evaluation of Antimicrobial potential of Actinobacteria Isolated from Lakes and Rivers of Mizoram

Thesis submitted in partial fulfilment of the requirement for the degree of
Doctor of Philosophy in Biotechnology

By

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Ph.D. Registration No: MZU/Ph. D. /711 of 22.05.2015

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Abstract

Actinobacteria are Gram positive microbes, ubiquitous in nature that constitute one of the largest taxonomic units under the domain bacteria and are major producers of antibiotics, industrially important enzymes and many pharmaceutically important bioactive compounds. With the emergence of antibiotic resistant pathogens, there is need to search novel antimicrobial compounds and has been emphasized that new group of microorganism from unexplored habitats can serve as sources of novel antibiotics and other therapeutic agents. Isolation of antimicrobial compounds from freshwater environment is in advance interest in recent times to isolate novel bioactive compounds. Prior to the present investigation, no comprehensive work has been carried on studies of fresh water actionbacteria in Mizoram. Accordingly, the present study entitled “**Molecular Characterization and Evaluation of Antimicrobial Potential of Actinobacteria Isolated from Lakes and Rivers of Mizoram”** was designed with the following objectives:

- Identification and phylogenetic analysis of actinobacteria by using 16S rRNA gene sequencing.
- Antimicrobial activities of actinobacteria isolated from selected lakes and rivers of Mizoram.
- Screening for the production of extracellular enzymes and detection of biosynthetic genes; nonribosomal peptide synthetase (NRPS) and polyketide synthase (PKSII) from the potential isolates.

The study was initiated by sampling sediments samples from four rivers [Tlawng river (24º 52´N; 92º 36´E), Tuirial river (24º 21’N 92º 53′), Tuichang river (23°33’ N, 93°06’ E), Murlen stream (23° 37′ N 93° 18′ E)] and two lakes [Tamdil Lake (23º 44´N; 92º 57´ E), Rihdil (23^{0} 20’ N, 93^{0} 23’ E)]. The samples were pre-treated, subjected to serial dilution and spread plate technique using seven different nutritional media. 115 isolates of actinobacteria were obtained identified using 16S rRNA gene sequencing which differentiate the isolates into 16 different genera. *Streptomyces* constitutes the dominant genus which comprises 55% of the total

isolates followed by *Nocardiopsis, Arthrobacter, Rhodococcus, Micrococcus, Saccharopolyspora, Brevibacterium, Brachybacterium, Corynebacterium, Kocuria, Leucobacter, Promicromonospora, Prauserella, Amycolatopsis, Tsukamurella,* and *Zhihengliuella.* Among the media used for isolation, Starch casein agar (SCA) yields maximum number of isolates which accounts for 43% of the total isolates, Most of the isolates were observed with yellow, brownish white to blackish white; some isolates formed brown and purple coloured pigments on the media and majority of the aerial mycelia of actonobacterial isolates produced long and spiral spore chains when observed using field emission gun-scanning electron microscopy (FEG-SEM).

Maximum diversities were observed at Rihdil Lake where 31 isolates belonging to thirteen different genera were obtained followed by Tamdil Lake, Tuirial River, Tlawng River, Tuichang River and Murlen Stream. The genus *Streptomyces* represent the most versatile organisms among the isolated actinobacteria that were observed in the sediment samples of the entire selected study site. Phylogenetic studies using 16S rRNA gene sequences have been found significantly in the study, the tree constructed based on Maximum-likelihood (ML) and Neighbour joining (NJ) method using Tamura Nei (TN93+G) parameter separated different genera forming separate clusters, the genus *Streptomyces* formed a major clade with a bootstrap support value of 98%.

The actinobacterial isolates were evaluated for their antagonistic potential against four fungal phytopathogens *viz*., *Fusarium oxysporum* CABI-293942, *Fusarium udum* MTCC-2755, *Fusarium proliferatum* MTCC-286 and *Fusarium graminearum* MTCC-1893 by dual culture *in vitro* assay. 49.7% of the isolated actinobacteria showed inhibitory activity against at least one of the tested pathogens with percentage of inhibition ranging from 20-87.2. Among all the isolates *Streptomyces cyaneofuscatus* DST 15, *Streptomyces* sp. DST 16, *Streptomyces* sp. DST25, *Saccharopolyspora* sp. DST31, *Streptomyces griseoplanus* DST53, *Streptomyces* sp. DST54, *Streptomyces cyaneofuscatus* DST64, *Streptomyces albidoflavus* DST71, *Streptomyces* sp. DST86, *Streptomyces albidoflavus* DST102, *Streptomyces* sp. DST104, *Nocardiopsis* sp. DST105, *Streptomyces* sp. DST116 and

Streptomyces sp. DST119 showed activity against all the tested four fungal pathogens.

Fifteen standard antibiotics were tested against the actinobacterial isolates to determine the antibiotic sensitivity, the isolates were highly resistant to trimethoprim (R=62, S=7) followed by Penicillin (R=52, S=3, I=14) and Ampicillin (R=42, S=14, I=13) whereas a high sensitivity was found against Streptomycin, Erythromycin, Norfloxacin, Tetracycline, Gentamycin, Neomycin, Chloramphenicol, Polymixin B, Ciprofloxacin, Vancomycin, Clindamycin and Levofloxacin. Majority of the isolates were sensitive to Levofloxacin (R=1, I=4, S=64). *Streptomyces* sp. DST13, *Streptomyces* sp. DST19, *Streptomyces intermidus* DST29, *Saccharopolyspora* sp. DST89, *Nocardiopsis* sp. DST95 and *Nocardiopsis* sp. DST105 showed resistance to ten antibiotics

Antimicrobial screening of actinobacterial isolates were performed against five pathogenic bacterial isolates [gram positive bacteria: (*Staphylococcus aureus* MTCC-96, *Bacillus subtilis* NCIM-2097, and *Micrococcus luteus* NCIM-2170); Gram negative bacteria: (*Pseudomonas aeruginosa* MTCC-2453 and *Escherichia coli* MTCC-739) and yeast: (*Candida albicans* MTCC-3017)]. 66.1% (n=76) of the actinobacterial isolates were found to inhibit the growth of at least one tested pathogens. *E. coli* was found to be the most susceptible pathogen against the isolated actinobacteria followed by *P. aeruginosa, C. albicans, B. subtilis* and *S. aureus.* The maximum inhibition diameter was recorded by *Streptomyces flavogriseus* DST30 (18.8 mm) followed by *Streptomyces cyaneofuscatus* DST57 (15.95 mm) and *Streptomyces albidoflavus* DST71 (15.9 mm) against *M. luteus, C. albicans* and *B. subtilis* respectively.

Nine actinobacterial isolates namely *Streptomyces* sp. DST25, *Streptomyces cellulosae* DST28*, Streptomyces intermidus* DST29, *Streptomyces flavogriseus* DST52, *Streptomyces albidoflavus* DST71, *Streptomyces cyaneofuscatus* DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST119 were selected based on their broad-spectrum antimicrobial activities and these isolates were selected as potential candidates for further investigation. Minimum inhibitory concentration (MIC) of the selected isolates against bacterial pathogens was determined following broth micro

dilution technique using 96-well microtiter plate against six pathogens. *Streptomyces cyaneofuscatus* showed highest activity against *E. coli* (EC50=0.002 $mgml^{-1}$) followed by *Streptomyces flavogriseus* DST52 (EC50=0.052 $mgml^{-1}$) and *Streptomyces* sp. DST142 (EC50=0.062 $mgml^{-1}$). The EC50 of *Streptomyces cyaneofuscatus* was found to be lowest against *P. aeruginosa* (EC50=0.009 $mgml^{-1}$) followed by *Streptomyces* sp. DST119 (EC50=0.015 $mgml^{-1}$) and *Streptomyces* sp. DST142 (EC50=0.024 $mgml^{-1}$). *Streptomyces* sp. DST119 showed highest activity against *S. aureus* (EC50=0.015 $mgml^{-1}$) among the isolates while *Streptomyces flavogriseus* DST52 and *Streptomyces cyaneofuscatus* DST103 showed highest activity against *M. luteus* (EC50=0.003 $mgml^{-1}$) and *C. albicans* (EC50=0.025 $mgml^{-1}$) respectively.

The actinobacterial isolates were screened for their ability to hydrolyze xylan and cellulose. Seven isolates (*Streptomyces* sp. DST25, *Streptomyces cellulosae* DST28*, Streptomyces flavogriseus* DST30, *Streptomyces flavogriseus* DST52, *Streptomyces cyaneofuscatus* DST103, *Streptomyces* sp. DST116 and *Streptomyces* sp. DST142) showed both cellulase and xylanase activity. The hydrolytic zones formed by the actinobacterial isolates for cellulose and xylan ranges from 0.04cm-4cm and 0.08cm-6.6cm respectively. Maximum cellulolytic zone was formed by *Streptomyces* sp. DST25 (4cm) followed by *Streptomyces flavogriseus* DST52 (1cm) whereas maximum xylanolytic zone was formed by *Streptomyces* sp. DST142 (6.6cm) followed by *Streptomyces cyaneofuscatus* DST103 (3.8cm). Quantitative studies is also carried out with selected isolates and the incubation temperature of different isolates were optimized.

Biosynthetic genes [Polyketide synthase type II (PKS II) non-ribosomal peptide synthetase (NRPS) and aminodeoxyisochorismate synthase (*phz*E)] were evaluated using degenerate primers for highly conserved regions encoding enzymes associated with biosynthesis of polyketides, peptides and phenazine respectively. PKS type II was detected in 31 isolates (27%), NRPS was detected in 47% (n=54) of the isolates and *phz*E was detected in 17.4% (n=20) of the isolates. A total of 11 isolates (DST45, DST47, DST54, DST56, DST57, DST58, DST74, DST76, DST77,

DST99, DST101 and DST142) were found to have all three genes that were amplified using PCR.

Ultra-Performance Liquid Chromatography (UPLC-ESI-MS/MS) was employed for the detection of antibiotic in the methanolic extracts of the selected isolates. The antibiotics content in the methanolic crude extracts of the selected actinobacterial isolates consists of two antifungal (fluconazole and ketoconazole) and three antibacterial (nalidixic acid, rifamycin and trimethoprim). Nalidixic acid was detected and quantified in highest concentration in most of the methanolic extract of the selected isolates except in the extract of *Streptomyces* sp. DST25 and *Streptomyces* sp. DST116 where ketoconazole and rifamycin was detected and quantified in higher amount compared to nalidixic acid in the extract of the two isolates respectively. Trimethoprim was detected and quantified more amount in the methanolic crude extract of *Streptomyces flavogriseus* DST52 (39 µg/g). Extract of *Streptomyces cellulosae* DST28 contains maximum amount of Ketoconazole (17 µg/g) and Fluconazole (µg/g) while extract of *Streptomyces* sp. DST25 and *Streptomyces* sp. DST116 contains more amount of Nalidixic acid (7920 µg/g) and Rifamycin (86 µg/g) respectively.

Methanolic crude extracts of nine selected actinobacterial isolates were investigated to determine their volatile organic compounds (VOCs) using Gas Chromatography Mass Spectroscopy (GC-MS), which revealed sixty-three VOCs**.** Fourteen compounds were detected from the extract of *Streptomyces albidoflavus* DST71 within the retention time of 15 to 29 minutes. Among the compounds, hexanal constituted maximum amount accounted for 23.2% of the total volume. Six VOCs, valine, glutaraldehyde, D-leucine, 3,3-dimethyl-4-methylamino-butan-2-one, pentadecylamine, cyclopropane and 1-butyl-2-(2-methylpropyl)-, were detected from the extract of *Streptomyces* sp. DST25, and glutaraldehyde was the most abundant followed by an amino acid, valine. Only one compound (di-n-octyl phthalate) was detected in extracts of *Streptomyces cellulosae* DST28. Two major compounds were detected in the extract of *Streptomyces* sp. DST29 in which Di-n-octyl phthalate consists of more than 70%. Seven compounds were determined from the extract of *Streptomyces flavogriseus* DST52, of which carbonic acid, 2, 2, 2-trichloroethyl

undec-10-enyl ester alone constituted 49.78%. Four compounds were detected from *Streptomyces cyaneofuscatus* DST103 where 2-decene, 3-methyl-, (z) - was found in higher concentration compared to other three compounds. Only 2-methoxy-4,5-diphenyl-6-(2'-phenylethyl)pyrimidine was detected in the extract of *Streptomyces* sp. DST116, while six compounds were detected in the extract of *Streptomyces* sp. DST119 in which 2-benzylthio-8-methyl-7-phenylpyrano [2, 3-f] benzoxazol-6(h)-one constituted the maximum amount (42.66%). The methanolic crude extract and ethyl acetate crude extract was compared in *Streptomyces* sp. DST142 for their secondary metabolites content where 17 compounds were determined from the methanolic extract of DST142 while only 5 compounds were retrieved from ethyl acetate extract of DST142**.**

This work highlighted the abundance and distribution of various genera of actinobacteria from unexplored fresh water habitats which provides a baseline data for fresh water actinobacteriological research in Mizoram, Northeast India. The antifungal, antimicrobial and enzymatic potential besides the volatile organic compounds and non-volatile organic compounds from the crude methanolic extract of actinobacteria were described which clearly showed the potential of fresh water actinobacteria. Hence, the usage of freshwater bio-resources can be an ideal source for the isolation of actinobacterial cultures with rare and unique properties that could certainly add to the ever-growing pharmaceutical needs and other biotechnological applications.