A mangrove actinobacterium of Sundarbans: Taxonomical identification, exploration of metabolite production and biological activities

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**A mangrove actinobacterium of Sundarbans: Taxonomical identification, exploration of metabolite production and biological activities** "submitted by **Shri. Kaushik Biswas**, who got his name registered on 26 th June 2012 **(26/06/12)** for the award of **Ph.D. (Pharmacy)** degree of **Jadavpur University** is absolutely based upon his own work under the supervision of **Dr. Joydeep Mukherjee** and that neither his thesis nor any part of the thesis has been submitted for any degree/ diploma or any other academic award anywhere before.

(Signature of the supervisor and date with official seal)

Dedicated to…

The People of Sundarbans

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_____

Date Kaushik Biswas

List of Abbreviations

%	Percentage
°C	Degree celcius
µg	Microgram
µl	Microlitre
µM	Micromolar
Abs	Absorbance
BLAST	Basic local alignment search tool
Conc	Concentration
DNA	Deoxyribonucleic acid
RAPD	Random amplified polymorphic DNA.
MALDI-TOF/MS	Matrix-assisted laser desorption/ ionization/ time-of-flight mass spectroscopy
CICC-	Chinese Centre for Industrial Culture Collection
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylenediamine tetra acetic acid
Tris	Tris (hydroxymethyl) aminomethane
TE buffer	Tris EDTA buffer
STE buffer	Sodium chloride Tris EDTA buffer
TBE buffer	Tris borate EDTA buffer
g	Gram
GC	Gas chromatography
GCMS	Gas chromatography mass spectroscopy
TLC	Thin layer chromatography
HPLC	High performance liquid chromatography
min	Minute
hrs	Hours
km	Kilometer

kV	Kilovolt
l	Litre
m	Meter
M	Molar
MEGA	Molecular evolutionary genetics analysis
mg	Milligram
ml	Milliliter
vol.	Volume
mM	Millimolar
NCBI	National Center for Biotechnology
ng	Nanogram
nM	Nanomole
OD	Optical density
PAUP	Phylogenetic analysis using parsimony
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
Tm	Melting temperature
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
soln.	Solution
Tm	Melting temperature
G+C	Guanine plus cytosine
U	Unit
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
PCR	polymerase chain reaction

Preface

Exploration of the microbial diversity of the underexplored or extreme environment/ ecology could facilitate the discovery of new antibiotics or antimicrobials at the time when antimicrobial resistance is an emerging problem. World's largest tidal mangrove forest, Sundarbans, is enormously rich biodiverse region though its microbial diversity is not studied largely. A Gram-positive, aerobic, and non-motile actinomycete (strain MS 3/20^{T}) was isolated from sediments of the Lothian Island of the Indian *Sundarbans* mangrove forest. In the 16S rRNA gene sequence study strain MS 3/20^{T} showed 100% similarity with four microorganisms and showed more than 99% similarity with other seven microorganisms of genus *Streptomyces*. However, on the basis of low levels of DNA-DNA relatedness (27.24-53.79%) strain was differentiated from these eleven closely related species. This distinction of the strain was evidenced by other modern identification approaches like distinguished banding pattern obtained in random amplified polymorphic DNA-PCR amplification and the unique MALDI-TOF/MS profile of whole cell proteins in comparison to its phylogenetic relatives. Physiological, morphological and chemotaxonomical data supported the difference between the strain MS 3/20^{T} and its phylogenetically close relatives. Strain MS 3/20^{T} is identified as a novel species of the *Streptomyces* genus and named as *Streptomyces euryhalinus* sp. nov. The metabolite profile of the strain was explored using GCMS and it revealed the presence of several bioactive metabolites including 4-Di-tert-butylphenol, phenanthrene, 7, 9-Di-tert-butyl-1-oxaspiro (4,5) deca-6, 9-diene-2,8-dione, dibutyl phthalate, triclosan etc. The minimum inhibitory concentration (MIC)

of the extract recorded are 1 µg/ml, 2 µg/ml and 2 µg/ml against *S. aureus* MTCC 2940, *E. coli* MTCC-1195 and *C. albicans* MTCC 227. The extract showed minimum bactericidal concentration (MBC) of 4 µg/ml and 8 µg/ml against *S. aureus* MTCC 2940 and *E. coli* MTCC-1195, respectively, and minimum fungicidal concentration (MFC) was observed 6 µg/ml against *C. albicans* MTCC 227. The extract exhibited the property of cell wall damage which could be granted as the mechanism of action for the antimicrobial activity. The present study concludes the identification of a new mangrove actinobacterium, profile of the metabolite produced by the strain and the probable mechanism of action of the extract for the antimicrobial activity.

Contents

CHAPTER 1:

INTRODUCTION

1.1.Introduction

The emergence of antimicrobial resistance posed a severe threat to human health and caused a serious crisis in the world health care system including the scientific community. Antimicrobial resistance will cause the death of 10 million people with an economic loss of 100 trillion dollars in the year 2050 if it will spread at the same rate (O'Neill, 2014). It is well known that *Staphylococcus aureus*, the major reason of nosocomial and community-acquired infection in Europe, produce many enzymes like DNAse, beta-lactamase etc. caused the antibiotics resistance (Plata et al. 2009, Lowy 1998). Methicillin-resistant *Staphylococcus aureus* (MRSA) which produce beta-lactamase causes severe infections in hospitalized patients and killed 20 000 people per year in the USA only (Klein et al. 2009). Several other Gram-positive and Gram-negative organisms cause severe to serious infections to the people and cause mortality as there is no effective treatment due to the drug resistance or multidrug resistance (MDR). Now it is well known that antibiotics are going to be "endangered species" as the older antibiotics are not showing activity against these pathogens (Butler et al 2017). The invention of antibiotics revolutionized the world as it facilitates the health care system in many ways but its overuse along with other factors accelerated the antibiotic resistance. WHO celebrating "World Antibiotics Awareness Week" in every year on '12-18 November' to aware the global citizens about antimicrobial resistance and its prevention. To combat the phenomena it needs several dimensions like rational and sustainable use of antibiotics, prevention of the spread of infectious diseases and the discovery of novel antibiotics (Simpkin et al. 2017). A very important part of AMR

prevention is to discover the novel antibiotics and antibiotics with a new mechanism(s). A report of the Pew Trust showed that 39 antibiotics are in Phases I to III of clinical trial, up to March 2017 (Simpkin et al. 2017) but the new antibiotics which are in pipeline is not efficient enough to resolve the current problem of treatment of drug-resistant infectious diseases (Simpkin et al. 2017). The significant fact is none of these pipeline products have the new mechanism of actions or targeting any new chemicals as their target to show the potency against resistant organisms. Pew Charitable Trust, a non-profitable organization, are working to solve the challenges facing worldwide and also publicizing the data to inform about issues that are shaping the world. Another report of Pew Trust showed that only 31% of antibiotics under clinical trial could be active against the ESKAPE pathogen (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) and on the other hand 33% of antibiotics under clinical trial could be active against the US Centre for Disease Control urgent threat pathogen (Simpkin et al. 2017). The discovery of antibiotics is declined as the big pharmaceutical companies are unwilling to bear the high investment as there are low success rate. Fortunately, WHO and few countries like UK, USA; Australia etc. are undertaking policies and projects to combat with the antimicrobial resistance (AMR) (Butler et al. 2017). There is an immediate need of discovery of new antibiotics with novel mechanisms of action.

Discovery of antibiotics thrives the idea of exploring the diversity of microbes or the search of new microbes from the new habitats like the ocean (Thornburg et al 2010), mangrove forest (Amrita et al. 2012), desert (Bull and Asenjo, 2013) etc. In the present scenario, it is crucial to explore the underexplored habitats which could provide new species/ genus of microbes and novel metabolites. Mangroves ecosystem are unique in nature due to high salinity, high moisture content etc.(Wu and Jiang, 2012) and present in the tropical and sub-tropical region (Holguin et al. 2001). Mangrove forest is the source of the uncountable numbers of microorganisms which provides several new or novel bioactive compounds due to its specific ecological niche (Xu et al. 2014). Mangrove forest is a proven source of several new bioactive compounds like antibiotics (Xu et al. 2014; Huang et al. 2008). Sundarbans, situated in the delta of rivers the Ganges, Meghna, and Brahmaputra on the Bay of Bengal and world's largest tidal halophytic mangrove forest, is immensely richest bio-diverse region. Microbial diversity of Sundarbans is not explored well till date. Recently, a few research groups are attempting to explore the microbial diversity for the biotechnological applications. Sundarbans also showed the presence of *actinomycetes.* A couple of novel *Streptomyces* (Arumugam et al. 2011; Biswas et al. 2017) are identified from this mangrove forest. The well-documented fact is actinomycetes are the largest producer of the antibiotics and among these *Streptomyces* produced 80% of antibiotics (Pimentel-Elardo et al. 2010). In this context, we have endeavored to explore and identify the *Streptomyces* from the Sundarbans mangrove ecosystem, and exploration

of its metabolite production. Isolation and characterization of bioactive principles from the bacteria were attempted and probable mechanism of action was studied.

1.2.References

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CHAPTER 2:

REVIEW OF LITERATURE

2.1. Review of literature

2.2. Phylum *Actinomycetes* and genus *Streptomyces*

In the domain *Bacteria,* phylum *Actinobacteria* is recognized as one of the major taxonomic units among the 18 major lineages which include 6 orders, 14 suborders, and 5 subclasses (Ludwig et al. 2012). The *Actinobacteria* is mostly distributed in both the ecosystems i.e., terrestrial and aquatic (marine, mangrove), and in air also (Macagnan et al. 2006). They are Gram-positive, filamentous bacteria and their genomes have a high guanine and cytosine (G+C) content. Most of the *Actinobacteria* are aerobic physiologically and ecologically, but exceptions are present. They produce branching hyphae, mycelium and spore chain. Morphologically, physiologically and metabolically this phylum exhibit immense diversity. These saprophytic, soil-dwelling organisms (Majority of the *Actinobacteria)* spent the major portion of their life cycles (Fig. 2.1.) as semi-dormant spores, particularly in nutrient limited conditions (Mayfield et al. 1972). Genus *Micromonospora, Propionibacterium, Mycobacterium*. *Nocardia, Corynebacterium, Rhodococcus, Bifidobacterium*, *Streptomyces* etc. are placed among this phylum. Genus *Streptomyces* are drawn most attraction due to its role in the cycling of carbon from insoluble organic debris and its capacity to produce a diverse bioactive secondary metabolites, which are very important for medicine and industry (Hopwood, 2007).

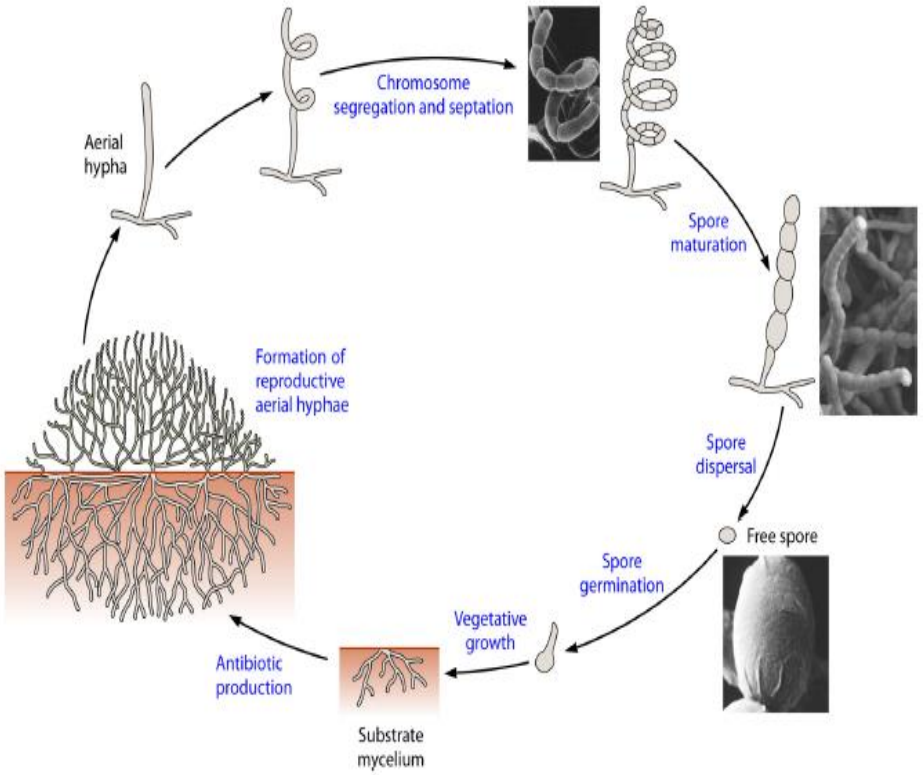

Fig.2.1. Life cycle of actinomycetes (sporulating) (Barka et al. 2015) (Republished with permission).

Two-thirds of all the known antibiotics are mainly produced by the *Actinomycetes* and among these genus *Streptomyces* are the largest producer (70-80%). This phyla and genus have very crucial microorganisms to fight against multidrug-resistant pathogens (Hopwood et al. 1995).

Previously, the taxonomy of *Actinobacteria* was based on morphology but recently with the increase of knowledge's and advancement of technology it evolved rapidly. The chemotaxonomic analysis of the organisms is an important tool for the identification. Chemotaxonomy is the cellular chemistry and used to mark the presence

of chemical entities to a group of organisms (Goodfellow and Minnikin, 1985; O'Donnell, 1988). In chemotaxonomy, most commonly identified chemical components are sugars, cell wall amino acids, menaquinones, lipids, proteins etc. (Williams et al. 1989; Goodfellow and O'Donnell, 1989;). Now, polyphasic taxonomic approaches are the basis of the classification of prokaryotes (Mayfield et al. 1972; Goodfellow and Williams, 1983; Williams and Vickers, 1988). The use of polyphasic taxonomic methods makes easier to classify the taxa belongs to the phylum *Actinobacteria* (Ventura et al. 2007). Nowadays, 16S rRNA sequencing is common tool for the identification of *Actinobacteria* or *Streptomyces*. Due to insufficient resolution capacity, intragenomic heterogeneity, intraspecific variation and frequent inconsistency with DNA-DNA relatedness study 16S rRNA sequencing analysis are not the final tool to identify the genus or species (Anderson & Wellington, 2001, Kämpfer & Labeda 2006, Kämpfer 2008, Kim et al 2012, Yamamura 2014). Currently DNA-DNA relatedness is the decisive tool to classify a prokaryotic species (Kim et al. 2012). DNA-DNA relatedness study serving as decisive tool to separate or identify the some species which are sharing a high level of similarity (more than 99%) in 16S rRNA gene sequence study among the genus *Streptomyces*. Other than these techniques, analysis of housekeeping genes, MALDITOF/MS analysis of whole cell proteins, sequences of the SsgA-like proteins (SALPs) etc. are useful methods for the classification of the genus *Streptomyces* as this is the highly congested genus.

2.3. Mangrove forest ecosystem

Mangroves, the highly productive and dynamic ecosystem, are present in the subtropical or tropical region as transitional coastal ecosystem (Chakraborty et al. 2015). World's 60-75% of the subtropical and tropical coastlines is comprised of Mangroves (Holguin et al. 2001). Here the term mangroves used to refer both communities and trees. Mangroves are not only rich in biodiversity but this productive ecosystem also supply large amount of organic matter to the coastal water and animal kingdom (Holguin et al., 2001). This ecosystem, situated at the confluence of land and sea, is dominated by the woody halophytes and subjected daily to various changing factors like tidal, temperature, salt exposure etc. (Alongi, 2002). These factors provide challenging habitat for marine and terrestrial micro biomes. This detritus based ecosystem provides a comprehensive food cycle for the organisms. The microbes and fungus degraded the detritus which are comes from fallen leaves and wood of mangroves (Alongi et al. 1989; Moran and Hodson, 1989). Healthy mangrove forests are important to good marine ecology. This ecology is very crucial due to its continuous interaction with two compartments i.e., terrestrial and marine ecosystems. Mangroves are also protecting the coastal communities from tsunami waves, storms, erosion, and provides livelihoods. Mangroves have a capacity of sequestering carbon from the atmosphere. This ecosystem has the capability of detoxification of different pollutants of different sources.

Recently mangroves are considered as the global hotspot of microbial diversity due to its different variable characteristics like tidal flooding, high rainfall, temperature, light, salinity, and other physicochemical factors (Andreote et al. 2012; Feller et al. 2010). Paradoxically mangrove ecosystems are rich in organic matter and nutrient-deficient, particularly of nitrogen and phosphorus (Alongi et al. 1993; Holguin et al. 1992; Vazquez et al. 2000) but are highly productive due to microbial activity (Alongi et al. 1993). The microbes are responsible for the transformation of nutrients and organic matter in the mangrove ecosystem (Holguin, 2001). 91% of the total biomass generated by microbes in tropical mangroves constitutes by fungi and bacteria (Alongi, 1988). Mangrove ecosystem conservation very clearly depends on the genuine relationships of microbes, nutrients and plants. Bacteria are responsible for the cycling of carbon, nitrogen, phosphorus and sulfur in the mangrove forest with other components involved in the process (Toledo et al. 1995; Vazquez et al. 2000; Rojas et al. 2001).

2.4. Mangrove ecosystem and bioactive molecules

Mangrove forests are the potential source of actinomycetes species which could be the prolific source of new bioactive metabolites. From mangrove actinomycetes 73 new and 49 known compounds were purified which includes macrolides, alkaloids and terpenoids (Xu et al. 2015) and are effective as antimicrobial, anti- tumor, anti-diabetic etc. (Xu et al. 2014; Xu et al. 2015). Ding *et al.* purified Xiamycin (Thornburg, 2010) from endophytic *Streptomyces* sp. GT 2002/1503 from the *Bruguiera gymnorrhiza* a

mangrove plant in Xiamen, China and exhibited selective anti-HIV activity. Fungichromin B (Xu et al. 2012) a new macrolide was isolated and purified from *Streptomyces albogriseolus* HA10002, which was obtained from mangrove sediment of Hainan, China. From the endophytic *Streptomyces albidoflavus* I07A-01824 antimycin A18 (Williams et al. 2005) was purified and showed superiority over blasticidin (a well know fungicide) against plant pathogenic fungi. salinosporamide A (Calcul et al. 2013) probably the most crucial secondary metabolite purified from mangrove actinomycetes. It is first time reported by Feling *et al.* with a very low IC_{50} value of 1.3 nM from the *Salinispora tropica* CNB-392 isolated from a mangrove in Chub Cay, Bahamas.

In India exploration of the bacteria from mangroves were undertaken mainly in few states like Tamil Nadu, West Bengal, Orissa, Andhra Pradesh and Kerala. Radhakrishnan et al. (2011) was isolated 49 actinomycetes from sediment samples of mangroves of Pitchavaram and Andaman & Nicobar Islands. Govindarajan et al. (2014) identified a *Streptomyces* sp. as *Streptomyces longispororuber* from Karangadu mangrove forest of Tamil Nadu using 16S rRNA study and it displayed antimicrobial activity against methicillin resistant Staphylococcus aureus. The active bioactive compound was an aromatic polyketide. Eight isolates among 54 actinomycetes isolated from the Sundarbans (Sengupta et al. 2015) were categorized as *Streptomyces*, and one isolate presented similarity of 93.57% with *Streptomyces albogriseolus* which have shown antimicrobial activity against fifteen organisms. Strain VUK-10 isolated from mangrove soil samples from Nizampatnam, Andhra Pradesh placed into the genus

Pseudonocardia (Mangamuri et al. 2012) by using polyphasic taxonomical studies and the bioactive metabolites produced by the antimicrobial activity against gram negative bacteria, gram positive and fungi. Saranya et al. (2011) isolated total 10 actinobacterial strains were from the Bhitarkanika mangrove ecosystem of Orissa which are preliminarily categorized as *Streptomyces* species and eight strains were reported for its antimicrobial activities against the human pathogens. *Streptomyces* bacteria isolated by Satheeja and Jebakumar (2011) from mangrove sediments of Manakudi estuary of Arabian Sea and only five strains showed considerable antimicrobial effects against *Salmonella typhi*, methicillin-susceptible *S. aureus* and methicillin-resistant *Staphylococcus aureus*. Alkaliphilic and halotolerant *Streptomyces* strains were isolated from Valapattanam mangrove swamp in Kerala and one strain exhibited significant antimicrobial activity against *B. cereus, S. aureus, S. citreus*, *S. marcescens* as well as fungi *Penicillium* sp., *C. albicans, C. neoformans* and *C. parapsilosis* (Das et al. 2014).

2.5. Sundarbans, microbial diversity and biotechnological applications

Sundarbans, the world's largest tidal halophytic mangrove forest, located on the delta of the Bay of Bengal formed by the rivers Ganges, Brahmaputra and Meghna. Sundarbans occupies approximately 10,200 sq. km., passed through both India and Bangladesh (Figure 2.2) (Gopal and Chauhan, 2006) and is a one of the richest biodiversity zone in the world. The name 'Sundarbans' derived from the plant 'Sundri' which actually means beautiful. In 1987, IUCN declared the Indian part of Sundarbans

as world heritage site. In 1997, the Bangladesh portion of Sundarbans was also recognized as a world heritage site, and in 1992 it was declared and enlisted as a Ramsar site. Indian Sundarbans are surrounded by many rivers like Hooghly, Matla, Harinbhanga, Raimongal, and Saptamukhi. Important characteristic of Sundarbans estuary is mixing of fresh water with saline water and segregation of sediment. Continuous alteration in geomorphological and topographical characters of the substratum plays a crucial role to maintain its diversity. Being an estuarine site, the Sundarbans has constant supply of nutrients which influence the biogeochemical cycling and causing production of biological sources in a large amount (Meire et al., 2005). Particularly, this ecosystem significantly influenced by the seasonal variations and spatial difference in biogeochemical process. The rich biodiversity of this ecosystem mainly produced and maintained by these phenomena.

Figure.2.2. Image of Indian Sundarbans

Sundarbans mangrove is recognized for its diversity of different species including, zooplankton, phytoplankton, microorganism's, invertebrates and mammals. Its eco-diversity could be depicted with the presence of famous endangered Royal Bengal tiger (*Panthera tigris tigris*) and it is said to be only mangrove tiger region in the world. The Sundarbans hosts about 300 species of birds and 350 species of vascular plants (Gopal and Chauhan, 2006). Indian Sundarbans biodiversity included 497 types of insects, 364 types of fishes, 11 types of amphibian, 47 types of mammals and 270 types of algae (Danda et al., 2017). Few other important species present are marine turtles like Olive Ridley (*Lepidochelys olivacea*), Hawksbill Turtle (*Eritmochelys imbricata*) Green Sea Turtle (*Chelonia mydas*), Estuarine Crocodile (*Crocodilus porosus*), Snubfin dolphin (*Orcella brevirostris*), Gangetic Dolphin (*Platinista gangetica*). Sundarbans mangrove is an estuarine ecosystem and said to be one of the largest detritus-based ecosystems in the world (Pillay, 1958; Ray, 2008). Constant litterfall of mangroves provide the detritus to the ecosystem and supply the nutrients to the adjacent Hooghly–Brahmaputra estuarine complex which is utilized by several commercially important shell and fin fishes. But huge human intervention begins in the of last century causes threat to the several species which become extinct or threatened or in degraded state (Gopal and Chauhan, 2006; Sodhi et al. 2007). Mangroves benefits people socially and economically and its loss will cause erosion economically and environmentally.

Microbes play the pivotal role to produce and maintain the extremely productive ecosystem of the mangrove forest. Microbes are the leaders in the

production of organic matter, nutrient transformation, nitrogen fixation, reduction of sulphates, reduction of nitrates and solubilisation of phosphates, (Ghosh et al. 2007; Santos et al. 2011). The microbes of mangroves are well known source for production of the enzymes, other bioactive substances and antibiotics. Any changes taking place in a specific ecosystem due to different influences could be traced by using Microbes. Scientists discovered 1 genus of virus, 5 genera of fungus and 34 genera of bacteria from the Sundarbans mangrove forest. According to Sridhar et al. (2004), Sundarbans mangrove fungi are considered as the second largest group among the marine fungi. Sundarbans detritus based ecosystem and climate support the growth of different genera and fungus species. Exploration of the microbial diversity and drug discovery from Sundarbans habitat could facilitate the several health, ecological or environmental threats faced by the world. Bacteria play a significant role in maintaining mangrove or any environment's biodiversity and ecosystem. Sundarbans is an enriched ecosystem based on detritus that is actually produced and maintained by bacteria. The microbial diversity of the mangrove ecosystem has not been systematically and largely studied or analyzed worldwide, and Sundarbans is not exceptional. There is little information available about the Sundarbans microbial diversity (Ghosh et al. 2010). To date, very few studies have been carried out on the characterization of culturable or non-culturable bacteria and the isolation of bioactive compounds from the Sundarbans. Sundarbans bacterial diversity from the biotechnological applications aspect was initially conducted and continued by Group of Dr. Joydeep Mukherjee in Jadavpur University (Kolkata).and Dr. D.J. Chatterjee' s group in Calcutta University (Kolkata). Few other

groups' are recently working on the microbial diversity and biotechnology applications of Sundarbans.

Numbers of cellulolytic, pectinolytic, amylolytic and lipolytic bacteria was isolated and reported by Biswas et al. (1986) from Sundarbans. Ten different microorganisms were isolated from coastal Sundarbans by Choudhury and Kumar (1996), three of which were enteropathogenic. In the presence of nickel, cobalt, copper, cadmium, silver, lead and zinc, enteropathogens Vibrio cholera non-01(CT+), Pseudomonas aeruginosa and *Escheichia coli* (ETEC) were able to grow. The survey conducted by Choudhury and Kumar (1996) reported that these organisms were resistant to 5- 10 antibiotics and adapted to extreme conditions such as high salinity levels and toxic metals. To find out the petroleum degrading bacteria Roy et al. (2002) was conducted a survey in the Indian Sundarbans. They identified different strains of *Klebsiella, Mycobacterium, Pseudomonas, Acinetobacter, Micrococcus* and *Nocardia* as petroleum degrading bacteria. Five strains of *Mycobacterium, Pseudomonas* and *Nocardia* were efficiently degraded 47-78% of crude oil. Highest oil degrading (75%) ability over a period of 72 hours was observed for one strain of *Pseudomonas* BBW1. Interestingly, this strain exhibited plasmid mediated resistance to various metals and antibiotics. Saha et al. (2005) isolated an actinobacterium strain MS 3/20 from Lothian Island of Sundarbans. A potent antimicrobial compound was purified by Saha et al. (2005) with molecular weight of 577.49 from strain MS 3/20 and proposed molecular formula was $C_{20}H_{35}NO_{18}$. This proposed compound demonstrated considerable

antimicrobial activity against various gram-negative, gram-positive, multidrug resistant bacteria, yeast and molds (Saha et al., 2005). From the same site Saha et al (2006) isolated one more actinomycete strain MS 1/7 and a bioactive compound of proposed molecular weight 300.2 and molecular formula $C_{20}H_{28}O_2$ (Figure. 2.3) was purified from the same strain. The strain MS 1/7 identified as *Streptomyces sundarbansensis* sp. nov. by Arumugam et al. (2011) which is the first validly published bacterium from the Sundarbans mangrove forest. Arumugam et al. (2010) purified and characterized a bioactive smoke flavoured compound 2-allyloxyphenol with molecular weight 150 ($C_9H_{10}O_2$) (Figure 2.4) first time from natural sources and the compound could be used as food preservative and for the preparation oral mouth wash. This bioactive compound displayed significant antimicrobial property against bacteria and fungi, and also demonstrated a strong antioxidant activity of IC_{50} value 22±0.12. From the Algerian coastline, *Sterptomyces sundarbansensis* identified by the Djinni et al. (2013) from a marine brown algae *Fucus* sp. and purified an anti- MRSA new polyketide compound which could be used to treat the infectious diseases. From the Sajnekhali Island of Sundarbans another strain MS 310 isolated and identified as *Streptomyces paravallus* which produces actinomycin D. Strain produced actinomycin D in a rotating disk bioreactor (RDBR) at a particular speed of one revolution per day and not displayed production of any other analogues (Sarkar et al. 2008). The concept of 'niche -mimic bioreactor' principle was used here by using of RDBR for the increase of production of antibiotics from the marine organisms. RDBR minimized the antibiotic production time at 20 hour (onset of production) for Strain MS 310 than it

produced in stirrer tank bioreactor (STBR) at 55 hour. Eleven actinomycetes (Figure 2.5) strain isolated by Mitra et al. (2011) from the nine high antagonistic potential sites of Sundarbans. All these eleven strains were exhibited antimicrobial activity against gram negative bacteria, gram positive bacteria and different fungi.

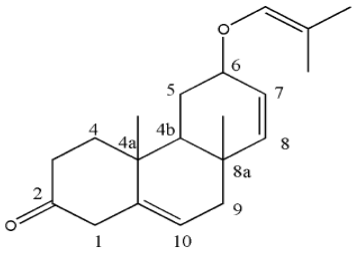

Figure 2.3. Structure of 4a, 8a-dimethyl-6- (2-methyl-propenyloxy) 3, 4, 4a, 4b, 5, 6, 8a, 9-octahydro-1Hphenanthren-2-one purified from strain MS $1/7^{T}$ isolated from Sundarbans. (Republished with permission licence no. 4511810395774).

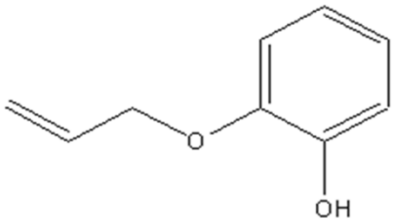

Figure 2.4. Structure of 2 allyloxyphenol purified from *Streptomyces sundarbansensis* sp. nov. (MS $1/7^{T}$) isolated from Sundarbans. (Republished with permission licence no. 4502341082392).

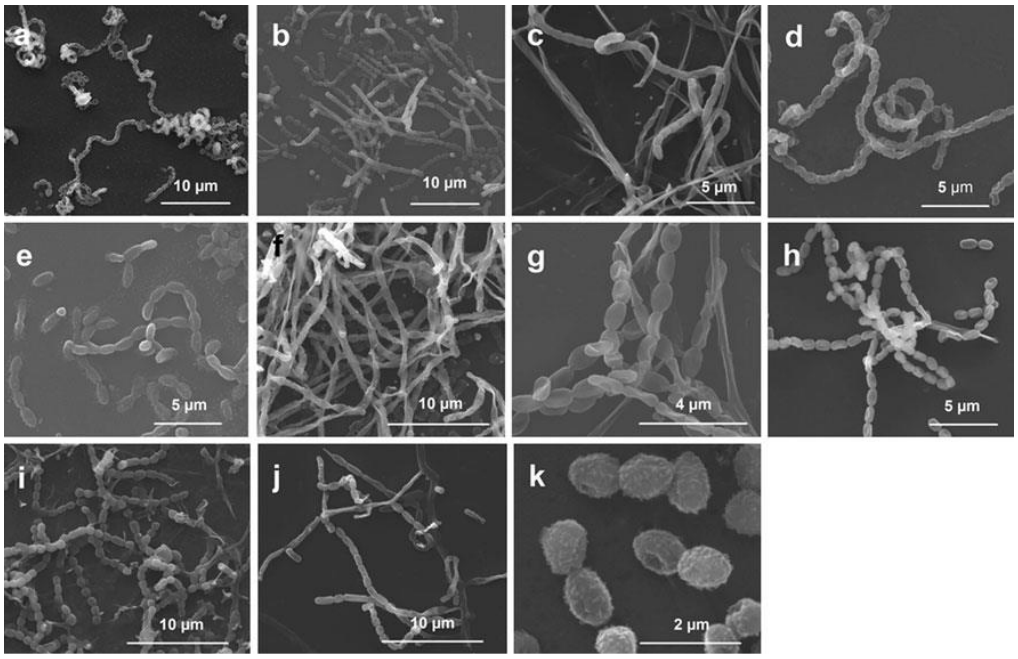

Figure 2.5. Scanning electron micrographs showing spore arrangement of eleven streptomycetes isolated from Sundarbans (a) H531 (b) AH251 (c) H524 (d) AS151 (e) ASH253 (f) ASH151 (g) H562 (h) SH521 (i) ASH853 (j) H532 and (k) ASH252 (Mitra et al., 2011) (Republished with permission license no. 4502310955663).

A *Gamma-proteobacterium* was isolated from the Lothian Island of Sundarbans by Sana et al. (2006) and purified a serine preotease enzyme from the organism which exhibited property of removing the blood and egg stains from cotton fabric in both dry and wet wash operation. This enzyme could be also useful in food processing industry for hydrolysis of soy protein and gelatin, and in leather industry for dehairing process. Sana et al. (2007) in another study purified an esterase enzyme from *Bacillus sp*. which was isolated from the Lothian Island and could be useful in industry for its property like better tolerance to solvents like DMSO, thermo stability. From the same site Sana

et al. (2008) isolated a *Bizionia* sp. (BSR01) and purified an uracil specific RNase enzyme useful as a source of DNase-free RNase in laboratory and industry.

Ramanathan et al. (2008) reported the presence of highest number microbes at Canning site (29.83×10^6 CFU.g^{-1}) than other two sites namely Jharkhali and Pakhiraloy. This study also revealed that also showed the presence of highest phosphate soluble bacteria (14.08×10^4 CFU.g^{-1}), free living nitrogen fixing and nitrate forming bacteria (13.67×10^4 CFU.g^{-1} and 50.12×10^4 CFU.g^{-1}) are present at Canning site than other two locations. But at Pakhiraloy site presence of highest number of cellulose degrading bacteria (45.15×10^4 CFU.g^{-1}) was demonstrated which refers the high litter fall and redox condition. In another study Das et al. (2012) presented the microbial diversity of Sundarbans by reporting the presence of several type of bacteria's like sulphate reducing bacteria, phosphate solubilizing bacteria, cellulose degrading bacteria, nitrifying bacteria and nitrogen fixing bacteria, fungi from three different regions of Sundarbans. A significant study on the microbial diversity was carried out by Basak et al. (2014) at three different locations (Jharkhali -Station A, Sahidnagar -Station B and Godkhali -Station C) of Sundarbans and samples were collected from the surface and sub-surface layers of sediment at two different season. At all three locations *Proteobacteria* phyla displayed the dominance with sessional variations and was demonstrated in December 2011 at about 57–72.4 % and in July 2012 49–79 %. This Dominance of *Proteobacteria* corroborates with the previous studies conducted in Sundarbans and other mangrove regions (Ghosh et al, 2010, Dos Santos et al, 2011). *Deltaproteobacteria* and *Gammaproteobacteria* are the major dominant classes among

Proteobacteria and Presence of *Deltaproteobacteria* refers the anaerobic characters of sediment. Sengupta et al. (2015) conducted a study on biodiversity of actinomycetes based on antimicrobial property in three locations of the Indian Sundarbans (Gadkhali, Bonnie camp and Kalash. Strain SMS_10, exhibited 92.5% similarity with *Corynebacterium auris* DSM 328^T and 93.57% *Streptomyces albogriseolus* NRRLB-1305^T in EzTaxon after 16 S rRNA study but showed difference in morphology. In 16 S rRNA analysis another strain SMS_7 showed 99% sequence similarity with *Streptomycete stendae* which is a nikkomycin antibiotic producer (Brillinger, 1979). All of these strains displayed considerable antimicrobial and antifungal activity. However, strain SMS_SU21 displayed highest antimicrobial (MIC 0.05 mg ml^{1}) and antioxidant activity of IC_{50} value 0.242±0.33 mg ml^{1}. Dutta et al. (2017) recently isolated a bacterium from Sundarbans paddy field and identified as *Staphylococcus sciuri* which showed potential of reduction of Cr (VI) contamination in rice plants growing in pots. This bacterium has shown capacity of 71 ± 3% of Cr (VI) and 65 ± 2% of total Cr removal from bacterium-treated soils after 8 weeks. This strain also exhibited 78 ± 4% removal of total Cr and 95 ± 5% removal of Cr (VI) from rice seeds of plants in bacterium-treated soils when compared with control rice seeds. This study of bioremediation could provide a real solution to a critical problem faced by the farmers of the Sundarbans. A bacterial strain SuMS_N03, was identified as *Staphylococcus warneri* (GenBank accession number KP771665) on the basis of 16S rRNA sequencing analysis which was isolated from the saline environment of Sundarbans (21.69N, 88.565 E) by Nag et al. (2018), and prepared a gold nano particles

(GNP) of size range 15-25 nm at room temperature using the bacterial intra cellular protein extract. This synthesized GNP exhibited a significant potential of reduction of 2-nitrophenol (2 NP), 4-nitrophenol (4 NP), 2-nitroaniline (2 NA) and 4-nitroaniline (4 NA) in presence of excess $NaBH_4$ and importantly not produced any significant by-products or side reactions. This nano-particle could be used for the degradation of industrial pollutants and for the waste water treatment. A strain $AK13^T$ was isolated by Srinivas et al. (2013) from mangrove soil of Namkhana (21˚45′ 39.35″ N 88˚ 13′ 48.05″ E) of Indian Sundarbans, and identified as *Silanimonas mangrovi* sp. nov. with the help of polyphasic taxonomic analyses which belongs to the genus *Silanimonas* proposed by Lee et al. (2005) which comes under family *Xanthomonadaceae* and the class *Gammaproteobacteria.* Chatterjee et al. (2018) conducted a very crucial study for the mosquitocidal activity against larvae of *Anopheles sundaicus, Aedes aegypti* and *Culex quinquefasciatus* and collected sample from the different locations of Sundarbans. Strain SB1 was identified as *Bacillus thuringiensis* with the help of 16S rRNA study and it demonstrated effectiveness against *A. sundaicus* larvae, *A.aegypti* and *Culex.quinquefasciatus* larvae. This study is very significant as vector borne diseases are a serious threat to Indian society.

Only 0.1-10.0% microorganism present in nature can be cultured in laboratory conditions (Zeyaullah et al. 2009) because it is difficult to maintain the specific conditions for the microbes for cultivation. Diversity of un-culturable bacteria could

be studied or understood by the metagenomics approach along with sequencing technology and bioinformatics (Tringe et al. 2005; Lauber et al.2009; Edwards et al.2006). Ghosh et al. (2010) reported about the presence of 8 main phyla of bacteria in the sediment samples of Netidhopani Island (21°55'13" N, 88°44'46" E) of the Indian Sundarbans by using metagenomics approach and major phyla present is *Proteobacteria*. In this *Proteobacteria* the most abundant is *gammaproteobacteria*. In another similar study Basak et al. (2015) reported dominance of *Proteobacteria* and *Firmicutes* from the samples of Bonnie Camp (21° 49′ 53.581″ N, 88° 36′ 44.860″ E) and Dhanchi (21° 42′ 06.41″ N, 88° 25′ 54.682″ E). Ghosh and Bhadury (2018) conducted a study based on culture independent method on samples collected from two stations of Sagar Island (21° 40′ 44.4″ N, 88° 08′ 49.5″ E and 21° 40′ 40.6″ N, 88° 09′ 19.2″ E) of the Sundarbans. confirmed the dominance of *Proteobacteria* as major phyla in the region which is echo the previous studies. In both the stations, *Bacteroidetes* and *Firmicutes* counted as the next two major phyla present in the region.

Sundarbans have a rich diversity of cyanobacteria but it is not explored well till date. Pramanik et al. (2011) first attempted to isolate the halophilic cyanobacteria of the Indian Sundarbans and screened for their antimicrobial property. This study was conducted on the various locations of Sagar Island (21˚44′7″ N, 88˚7′2″E) and Lothian Island (21˚39′ 1″N, 88˚19′ 37″E) of the Indian Sundarbans. Eight halophilic cyanobacteria (AP3, AP9F, AP17, AP20, AP24, AP25, AP3b and AP9U) (Figure 4) were selected for further study on the basis of antimicrobial property. After 16S rRNA

analysis, two strains AP17 and AP24 were showed 99% similarity with other species of *Oxynema.* But in the morphological, ultrastructural analysis and secondary structures of 16S-23S ITS regions analysis exhibited the differences with the other closest strains, and identified as a novel species under the genus *Oxynema, ie. Oxynema aestuarii* (Chakraborty et al. 2018). Veerabadhran et al. (2018) was studied about growth, biofilm formation, chlophyll a formation, EPS production and antimicrobial activity of cyanobacterial strains AP17, AP3b and AP3U isolated by Pramanik et al. (2011) on Eerlenmeyer flask (EF) and on a patented conico cylindrical flask (CCF) prepared with the polymethyl methacrylate (PMMA). Strain AP17 (*Oscillatoria* sp.) showed highest biofilm formation (0.0665 ± 0.02 g) in CCF than other two strains AP3b and AP3U and no biofilm formation was recorded in the EF. Highest antimicrobial activity was displayed by AP3b extract to against *B.subtilis* than AP3U extract in CCF compared to EF. LC-MS profile of these cyanobacteria documented various biological activities like antioxidant, anti-inflammatory, immunomodulatory, enzyme inhibitors, antimicrobial, antifouling, antiquorum sensing, antidiabetic, anticancer etc. Recently, Debnath et al. (2017) explored the diversity of cyanobacteria of the Indian Sundarbans and identified morpho type *Leptolyngbya indica* sp. nov., from the arsenic rich soil of rice field.

Fungi are playing pivotal roles in maintaining ecology and several kinds of bioprocesses (Gadd, 2007). Fungi already displayed its potential for production of novel bioactive compounds (Albersheim, 1966; Bennett, 1998; Acharya, 2007;

Chatterjee et al. 2011; Khatua et al. 2013; Bhadury et al. 2006). Kumar and Ghosh (2008) reported that increase in soil phosphorus concentration causes decrease spore density and mycorrhizal frequency. They reported that genus *Glomus* is dominant in the region. Pal and Purkaystha (1992a) demonstrated about presence of leaf inhibiting fungi of mangrove plants and *Khuskioryzae* H.J reported first time from the India. Another study by Pal and Purkayastha, (1992b) stated about isolation of two new species. From the Indian Sundarbans Bera and Parkayastha (1992) isolated three strains of *Pestalotiopsis versicolor* from *Ceriopsde candra*(Grifi) Ding Hou. From the plants of the Sundarbans De et al. (1999) isolated sixteen fungi and it was observed that growth of these fungi (*P.agallochae* ,*C.senegalensis* and *E.psidii*) was inhibited at a concentration of 0.2% tannin but growth was stimulated at concentration of 0.05 %. Macrofungal diversity study was conducted by Dutta et al. (2013) in Indian part of Sundarbans (21°32′–22°40′ latitude and 88°85′–89° longitude) and stated about presence of total of 46 genera 27 families and 62 species across. 55% of the macrofungi documented are saprophytic. *Leucocoprinus birnbaumii* (Corda) Singer isolated first time from the West Bengal (22.19° N/ 88.93° E) by Dutta et al. (2011). Rajsekhar et al. (2012) isolated *Fusarium* and *Aspergillus* species from the Indian Sundarbans and observed the antimicrobial property of the fungi maximum against *E. coli*, *Pseudomonus* sp., *Vibrio* sp. In another study Nandy et al. (2014) identified morphologically two fungi as *Aspergillus niger* and *Penicillum* sp from the Jharkhali, and when both of them were checked for the biodegradation of fish scale, *Aspergillus niger* was degraded significantly. *Aspergillus sp.* isolated from the Sundarbans was

demonstrated removal of hexavalent chromium from the aqueous solution observed by Chakroborty et al. (2018).

Sundarbans microbial diversity is less studied and only few groups attempted to explore the microbial diversity from the aspects of biotechnological applications. But it could be understand that this special ecology is promising sources for the production of new or novel compounds particularly from the microbes and may offer a best solution to the prevention of multidrug resistant phenomena.

2.6. Antimicrobial resistance (AMR) and crisis of antibiotics

Antibiotics are saving the life of people worldwide over the 70 years from the infectious diseases (Butler et al. 2017). These antibiotics or antimicrobials are comes from the microorganisms particularly from bacteria and fungi. Human kind must be thankful to these microbes for the production of life saving metabolites. But the irrational and indiscriminate use of antibiotics leads to the resistance to the pathogens and causing multidrug resistance. In addition to it many other factors like environmental factors are also involved in the antibiotic resistance phenomena. This resistance is causing death of millions of people due to the infectious diseases and affecting us economically, also. The days are coming when there will be no antibiotics to treat the infectious diseases. Different international bodies like WHO and many countries are showing their concern and initiating to fight against antibiotic crisis. Even many government agencies are

including the antibiotics resistance phenomena into natural disaster section (Dominey-Howes et al. 2014). To combat efficiently against antimicrobial resistance, it required multilayered approach. Among these very crucial one is discovery of novel antimicrobial or antibiotics with new mechanism of action (Simpkin et al. 2017). But the escape of big pharma companies from the discovery of antibiotics causes crisis or down fall in antibiotics discovery (Cooper and Shlaes, 2011). The highest antibiotic-producing genus is *Streptomyces* in the microbial world. The discovery of number of antimicrobial compounds from the species of genus *Streptomyces* per year raised exponentially for about two decades constantly but it was declined substantially in the late 1980s and 1990s.

A mathematical model (Watve et al. 2001) calculated the capability of genus *Streptomyces* for producing total number of antimicrobial compounds and that could be 100,000 among these only 3% are discovered. It could assume that lack of screening efforts for discovery of metabolites are major points with other reasons exists. Many researchers are now engaged in the search of new antibiotics or antimicrobials. But search of antibiotics from the microorganisms of traditional habitat or from the terrestrial microorganisms are counted as limited options as followed by rediscovery of old molecules and in recent times traditional habitat are failed to provide new molecules which are effective to treat the multidrug resistant infections (Xu, 2012, Debbab, A, 2010). So now, search of new antibiotics or antimicrobials are moving towards new or extreme environment or ecology like marine, desert or mangroves (Xu,

2012, Xu, 2014). Only 39 antibiotics are in Phases I to III clinical trial (Table 2.1, Figure. 2.5) as reported by the Pew Trust, up to March.

Table 2.1. Pipeline antibiotics which are in phase III clinical trial. (Republished with permission licence no. 4502370762268).

Name	Compound class	Mode of action
Solithromycin	Erythromycin	Protein synthesis inhibition
Omadacycline	Tetracycline	Protein synthesis inhibition
Sarecycline	Tetracycline	Protein synthesis inhibition
Eravacycline	Tetracycline	Protein synthesis inhibition
Surotomycin	Lipopeptide (Daptomycin)	Membrane depolarization
Plazomycin	Aminoglycoside	Protein synthesis inhibition
Cefilavancin	Cephalosporin/Vancomycin heterodimer	Cell wall biosynthesis
SQ 109	Ethambutol	Cell wall synthesis
Cadazolid	Oxazolidinone/quionolone hybrid	Protein syntheis inhibition/DNA gyrase and topoisomerase IV
Pretomanid	Nitroimidazole	DNA and cellular damage
Delafloxacin	Fluoroquinolone	DNA gyrase and topoisomerase IV
Lasqufloxacin	Quinolone	DNA gyrase and topoisomerase IV
Zabofloxacin	Fluoroquinolone	DNA gyrase and topoisomerase IV

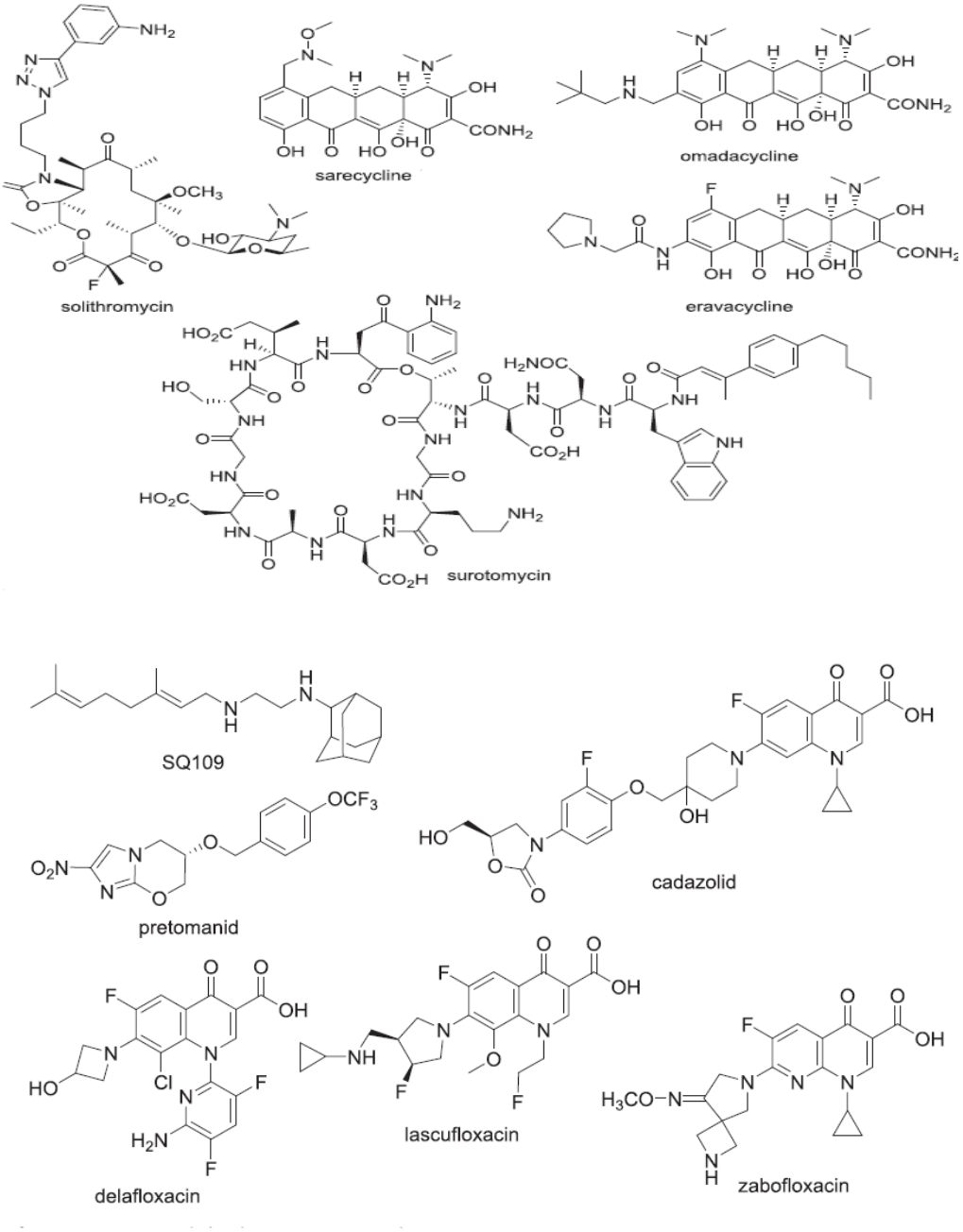

Figure 2.6. Structure of few antibiotics which are in phase III clinical trial. (Republished with permission licence no. 4502370762268).

2017 (Simpkin et al. 2017). But these antibiotics are not efficient enough to meet the current clinical demand (Simpkin et al 2017). It is noticed that these antibiotics do not possess new mechanisms of action. In recent times, few initiatives followed internationally to incentivising the antibiotic R & D like the Joint Programming Initiative on Antimicrobial, New Drugs for Bad Bugs (ND4BB) programme, Resistance (JPIAMR), Research and Development Authority's (BARDA) Broad Spectrum, The Innovative Medicines Initiative's (IMI's), Biomedical Advanced, Antimicrobials Program and Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator (CARB-X) (Renwick et al. 2016). It is the high time to focus on the search of new antimicrobials or antibiotics from the microorganisms of the new habitats to provide a remedy to the antimicrobial resistance (AMR) or multidrug resistance (MDR) phenomena.

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CHAPTER 3:

AIMS AND OBJECTIVES

3.1. Aims and objectives

Discovery of new or novel antimicrobial or antibiotics has been declining for several years but the hard fact is that antibiotic resistance is growing rapidly which causing a severe threat to the humankind. This fact leads to the urgent need for new antibiotics or secondary metabolites to prevent the death of people caused by the antimicrobial resistance (AMR). Discovery of a new antibiotic could be accelerated by exploration of the new bacteria or microorganisms. Actinomycetes are economically as well as biotechnologically crucial and valuable prokaryotes. They are the producer of the maximum discovered bioactive secondary metabolite like antibiotic and anti-tumor agents. It is becoming important to explore the microbial diversity of unique ecology or unexplored region for the exploration of new microorganisms. Mangrove forests are relatively unexplored or under-explored for its bacterial diversity and for the discovery of new metabolites. It is already reported that various compounds of potential medicinal or other use were already isolated from mangrove actinobacteria, fungus etc. in recent years with diverse pharmacological activity. World's largest tidal mangrove forest, Sundarbans, lies in the delta of the Ganges, Meghna and Brahmaputra rivers and is an enormously rich biodiverse region. Till date the microbial diversity of the Sundarbans has not been studied in details, only a few groups endeavored to explore the culturable and un-culturable microorganisms. Hence the interests in screening and searching of such microorganisms for novel antibiotics or bioactive metabolites are needed. However, it is difficult to discover clinically and economically significant

antibiotics or bioactive secondary metabolites from known actinomycetes as this method causes rediscovery of known antibiotics and bioactive compounds which is justifying the need of isolation, characterization, and screening of unexplored actinomycete species. The most important task in this regard is the identification of the microorganisms. Identification of the microorganisms can be determined by employing different methods like morphological, physiological, biochemical, chemotaxonomical and molecular approach. Identification of micro-organism will enhance the production of bioactive compounds. In this context the objectives of this work were:

1. **Complete identification of an actinobacterium by polyphasic taxonomic approach isolated from the Sundarbans mangrove forest**

   Phenotypic studies (Morphological, physiological and biochemical), chemotaxonomic studies were performed along with the molecular phylogenetic analysis (16 S rRNA studies), DNA-DNA relatedness study, RAPD banding pattern, MALDI TOF / MS analysis of whole-cell proteins. The polyphasic taxonomic approach was employed for the identification of the strain MS 3/20.

2. **Exploration of metabolite production of the bacterial strain MS 3/20^{T} using analytical tools**

Production of the metabolite was observed using specific production media and extracted with the organic solvents. The metabolites produced by the strain were analyzed using gas chromatography mass spectrometry (GCMS).

3. **Investigation of antimicrobial property by minimum inhibitory concentration and minimum bactericidal concentration, and mechanism of action of the extract**

   The metabolite produced by the strain MS 3/20 ^{T} was checked for the antimicrobial property considering by minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration. The probable mechanisms of action were evaluated for the antimicrobial property of the extract by checking the cell membrane integrity or permeability studies. Other biological properties like antioxidant, anti-inflammatory, and hemolysis were determined.

CHAPTER 4:

IDENTIFICATION OF A MANGROVE ACTINOBACTERIUM STRAIN MS 3/20

4.1. Introduction

Screening and isolation of microorganisms from the new or extreme habitat is crucial for the discovery of new antibiotics when antimicrobial resistance is a growing and life threating phenomena. Exploration of microbial diversity or isolation and identification of the microorganisms from new or extreme environment like Sundarbans is an important task to be fulfilled. Genus *Streptomyces* are the largest producers of the antibiotics or antimicrobials. So the isolation and identification of actinomycetes from the underexplored ecology, Sundarbans, could provide new microbes and metabolites. Identification of *Actinobacteria* is difficult task as this phylum is heavily congested. In this chapter, identification of an actinobacterium is described by using polyphasic taxonomic approach.

4.2. Materials and methods:

4.2.1. Sample Collection, isolation, purification and maintenance of the bacterium

Strain MS 3/20 was originally collected from sediment sample of the Lothian Island of Indian *Sundarbans* mangrove forest (Lat. 20°50′N, Long. 88°19′E) (Figure 4.1) by using corer. Samples were subjected to serial dilution by dissolving it into natural sea water and plating the samples on different agar medium followed by incubation for 4 days at 28 °C on the enrichment medium mentioned earlier (Saha et al 2005). The strain was preserved on storage medium (SM) finally as it has shown best growth (Fig.

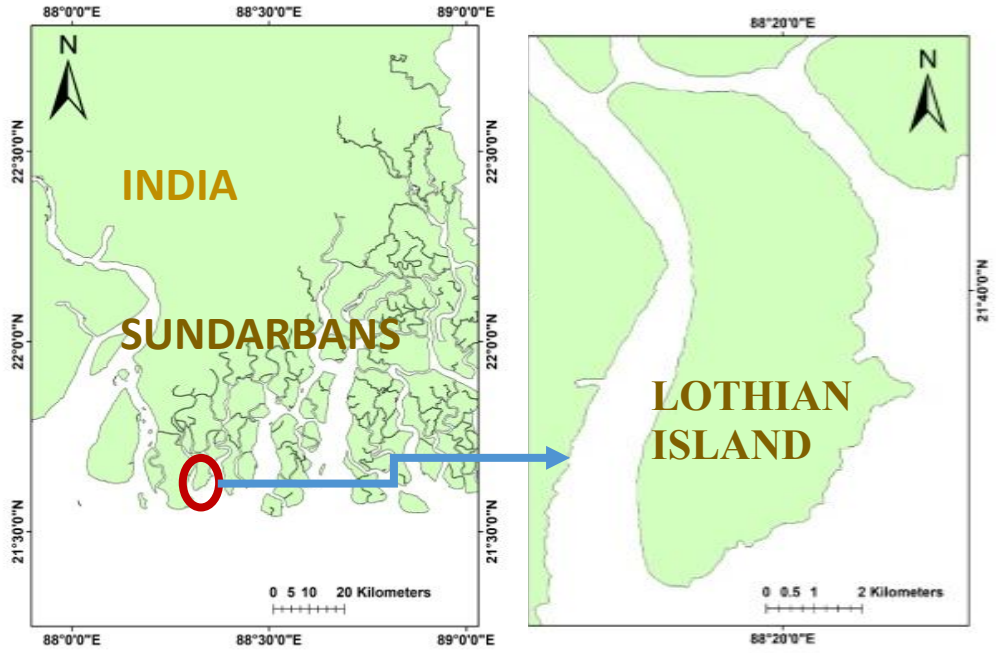
4.2). The strain was preserved in -20 °C freezers as a glycerol stock (10-15%) and also in -80 °C freezers, since its isolation and the strain was also preserved as a lyophilized powder. The strain MS 3/20 were also maintained at 0-4°C for the regular experimental uses. Strain MS 3/20 was submitted to Chinese Centre for Industrial Culture Collection (CICC 11032^{T}) and Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures (DSM 103378^{T}).

Composition of storage media for strains (g/L)

Dipotassium phosphate-	0.5g
Casein (protein rich) -	3.0 g
Starch (potato) -	10.0 g
Peptone-	1.0g
Yeast extract-	1.0g
Malt extract-	10.0g
Distilled water-	500ml
Artificial sea water-	500 ml
Agar powder -	2%

Composition of artificial sea water (g/L)

Sodium chloride	23.92 g
Sodium sulphate anhydrous	4.008 g
Potassium chloride	0.677 g
Sodium hydrogen carbonate	0.196g
Potassium bromide	0.098 g
Boric acid	0.026 g
Sodium fluoride	0.003 g
Calcium chloride, dihydrate	1.519 g
Magnesium chloride, hexahydrate	10.83 g
Strontium chloride, hexahydrate	0.24 g
Distilled water	1000 ml



4.1. Map of Indian Sundarbans and sample collection site (Lothian Island at Sundarbans).

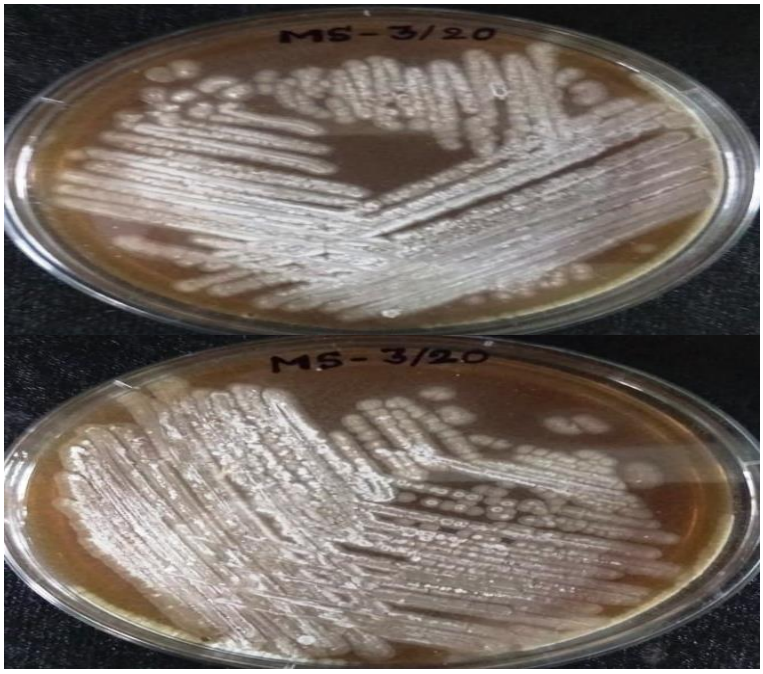


Figure 4.2. Image of the strain MS 3/20 after growth at storage medium.

4.3. Genotypic characterization of strain MS 3/20

4.3.1. Genomic DNA isolation

Strain MS 3/20 cultures were grown in modified production medium described by Saha et al. (2005) at 28 °C for 48-96 hrs. In a 2-ml microfuge tube 1 ml of cultured broth was taken and was centrifuged at 10,000 rpm for 10 minutes to get adequate cell mass. For inadequate amount of cell mass, same process was repeated 2 to 3 times to obtain sufficient amount of cell mass. The cell mass was dissolved in double distilled water by vortexing and washed twice by centrifugation. Supernatant was discarded and 1.5 ml of STE buffer added to it and solution kept at -20°C for overnight. The solution was centrifuged at 10,000 rpm and 0.3 ml 50:10 TE buffer [10 mM Tris: 1 mM EDTA; pH 8.0] was added. To this mixture added 50 µl of lysozyme (25 mg/ml) and incubated for 2 hrs. at 37 °C. After incubation 75 µl of 10% SDS and 3µl of Proteinase K (20 mg/ml) was added to it and incubated for 1.5 hrs. -2 hrs. at 60°C. After centrifugation of this mixture at 10,000 rpm for 10 min, the supernatant was transferred into a new tube. 3 µl of 10 mg/ml pre-heated RNase dissolved in 0.5 M NaCl was added and incubated at 37 °C for 1hr. Standard phenol-chloroform extraction method was performed for Purification of DNA according to Moore and Dowhan (2003). An equal amount of phenol: chloroform isoamyl alcohol (25:24:1) (prepared with Tris-saturated buffered phenol) was added to the mixture after incubation. Before centrifugation at 10,000 rpm for 10 min the mixture was mixed well by vortexing. The DNA containing upper aqueous layer was carefully transferred to a fresh tube. Equal volume of

Chloroform was added to it and was centrifuged for 10 min at 10,000 rpm. Pipette out upper aqueous layer and 1/10th volume of 3M of sodium acetate (pH 5.2) was added to the solution. Ice-cold 100% ethanol of 1 ml was added to it and mixed by vortexing and was kept at -20 °C overnight. Then it was centrifuged at 10,000 rpm for 10 min and supernatant was discarded. It was washed twice with 70% ethanol. Washed pellets were air dried for 30 min. The final product (DNA) was re-suspended in 50 µl of sterile double distilled water and stored at -4 °C.

4.3.2. Gel electrophoresis of MS 3/20 genomic DNA

The quantity and quality of total genomic DNA was checked in horizontal gel electrophoresis. Genomic DNA (10 µl) were mixed with 2 µl of 6× loading buffer and sample(s) loaded into the slots to a 5 cm-long well on an agarose gel (1%, w/v) dissolved in 1×TBE buffer. The bands of DNA were visualized with the help of UV trans illuminator.

4.3.3. Amplification of 16S rRNA gene and sequencing

16S rRNA gene of strain MS 3/20 was amplified and sequenced by the DSMZ Identification Service (Braunschweig, Germany). The amplification of 16S rRNA gene sequence was carried out by polymerase chain reaction (PCR). The reaction mixture was composed of 200 µM of each dNTP, 2 U of Taq polymerase, 50 - 100 ng of genomic DNA, 0.5 µg each of universal primers 27f(5´GAGTTTGATCCTGGCTCAG3´) and 1525r (5´AGAAAGGAGGTGATCCAGCC3´). The reaction cycles were as followed: initial denaturation at 98 °C for 3 min, denaturation (28 cycles) for 1 min at 94 °C, primer

annealing (28 cycles) for 1 min at 52 °C and extension (28 cycles) at 72 °C for 2 min. The final extension step was carried out for 5 min at 72 °C (Rainey et al. 1996). PCR amplificants was checked by agarose gel electrophoresis. By using direct sequencing method 16SrRNA gene of strain MS 3/20 was sequenced. Then sequencing of purified PCR products are conducted by using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) as per protocol of manufacturer. Electrophoresis of sequenced reactions was carried out using the Applied Biosystems 3500xl Genetic Analyzer.

4.3.4. Phylogenetic analysis of sequenced gene

16S rRNA gene sequence (1503 bp) was submitted and checked using EzTaxon-e-server (http://eztaxon-e.ezbiocloud.net/) (Kim et al. 2012) for the identification of the close relatives of strain MS 3/20. Reference strains for phenotypic, genomic and phylogenetic analyses were selected from the top hits of this analysis and obtained from ARS Culture Collection (NRRL), United States Department of Agriculture, Peoria (USA). The 16S rRNA gene sequence (1503-bp) of strain MS 3/20 and closely-related *Streptomyces* type strains was aligned using the CLUSTAL W program (Thompson et al. 1994) in Molecular Evolutionary Genetics Analysis (MEGA) 6 software (Tamura et al. 2013). The neighbor-joining (NJ) algorithm (Saitou, N. and Nei, M. 1987) and MEGA 6 (Tamura et al. 2013) was used to construct the phylogenetic tree. Kimura's two parameter model (Kimura. 1980) was employed to determine the evolutionary

distance matrix. Bootstrap analysis was carried out using 1000 re-samplings (Felsenstein. 1985).

4.3.5. DNA-DNA hybridization study

DNA-DNA relatedness was performed thrice for delineation of species by dot blot method (Choudhury et al. 2015) using DIG High Prime DNA detection kit (Roche Applied Science, Benzberg, Germany) with digoxigenin-labeled DNA following manufacturer's instructions. DNA-DNA hybridization experiments were carried with eleven type strains which have shown highest 16S rRNA gene sequence similarities. The genomic DNA of strain MS 3/20 was used to prepare the genomic DNA probe. Digoxigenin–11-dUTP (DIG) was used to label the template DNA after overnight incubation. Saline sodium citrate (SSC) buffer were used to dissolve the total DNA from strain MS 3/20 and reference strains to make a final concentration of 6×SSC and then for denaturation it was boiled for 10 min. Spots of equal amounts of DNA of all the strains were placed on an uncharged nylon membrane and then subjected to treatment with denaturation solution (1.5 M NaCl/0.5 M NaOH) (10 min) and neutralization solution (1M NaCl/0.5 M Tris HCl, pH 7.0) (5 min), consecutively. The membrane was baked at 80 °C for 30 min after drying. The formulas applied for determination of optimum hybridization temperature are: Tm= 49.82+0.41 (% G+C)-(600/l) (l= length of hybrid in base pairs) and Topt=Tm -20 to 25°C. This calculated temperature was used to hybridize the nylon membrane overnight with DIG-labeled DNA probe. After hybridization, the membrane was passed through low stringency wash (2× SSC/0.1% SDS for 2×5 min under continuous agitation and at room

temperature) and high stringency wash (0.5×SSC/0.1% SDS for 2×15 min at 65°C with continuous agitation). Following blocking treatment, Anti-Digoxigenin-AP (antibody raised against Digoxigenin, attached with alkaline phosphatase enzyme) was applied to the membrane. Finally, with the help of NBT/BCIP, the colorimetric detection was performed. ImageJ software (http://rsb.info.nih.gov/ij/index.html) was used for the determination of color intensities by measuring relative intensity values of all the organisms in equal sized circles of DNA. For comparison self-hybridization value was counted as 100%.

4.3.6. Random amplified polymorphic DNA (RAPD-PCR) amplification

Primer selection for finger printing technique random amplified polymorphic DNA (RAPD-PCR) and amplification was carried out as explained by Lee et al, (2005). Three primers, AM 50, AM62, 70-34 were selected for this study. The PCR mixture contained 2.5 µl of 10 X buffer Taq buffer, 1.0 µl 50 mM magnesium chloride, 1.0 µl of 10 mM dNTP ,1.o µl of 100 pmol/ µl primer, 1.0 µl Taq polymerase, 1.5 µl DMSO, 16.0 µl double distilled water and 1.0 µl DNA. Amplification was carried out for 4 min at 95 °C, 40 cycles of 40 s at 94 °C, 45 s at 38 °C and 90 s at 72 °C and a final extension was carried out for at 72 °C for 5 min. The amplified PCR fragments were subjected to agarose gel electrophoresis and visualized by ethidium bromide solution. The images were captured with the help of E-Gel imager (Thermo Fisher Scientific, Waltham, MA, USA).

4.3.7. Matrix assisted laser desorption/ ionization time-of-flight mass spectroscopy (MALDI-TOF/MS) analysis

MALDI-TOF/MS study was carried out by Microbial Culture Collection (MCC), Pune, India. The bacterial strains were harvested on ISP 2 medium at 28 °C for 2-3 days. To obtain the whole cell protein the cells were subjected to extraction using ethanol, formic acid and actonitrile as solvents. Then the whole cell protein extracts were analyzed with an Autoflex TOF/TOF mass spectrometer and mass spectra obtained was visualized using MALDI Biotyper software 3.0 (BrukerDaltonik GmbH).

4.3.8. Determination of DNA G+C content

The thermal denaturation method of Marmur and Doty (1962) was applied to determine the DNA G+C content of strain MS 3/20. UV/visible spectroscopy (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA) was used to calculate the melting temperature (Tm). DNA was dissolved in a buffer composed of 0·15 M NaCl and 0·015 M sodium citrate (pH 7·0±0·3). Temperature of the chamber was increased to about 5°C below the calculated melting point for the estimation of the Tm of the DNA. Again the temperature was increased at a time about 1 °C. Samples optical density was measured at 260 nm. The absorbance increased sharply at a point when the DNA denatures and no increase in the optical density after further increasing of the temperature corroborates as the completion of denaturation. The ratio of optical density at each temperature and the optical density at 25 °C were plotted against the temperature of the sample. G+C content was determined by applying the formula: Tm

= 69·3+0·41 (G+C), where Tm denotes the melting temperature in degree Celsius and G+C content expressed as the mole percentage of guanine plus cytosine bases.

4.4. Morphological, physiological and biochemical characteristics of strain MS 3/20

4.4.1. Morphological Characteristics

Morphological characteristics like spore chain morphology, spore surface ornamentation and color of aerial and substrate mycelium were determined after growth in different International S*treptomyces* project medium (Shirling and Gottlieb, 1966). Compositions of different ISP medium are as follows:

Composition of Yeast extract –malt extract agar (ISP2) medium

Yeast extracts	4.0 g
Malt	10.0 g
Dextrose	4.0 g
Distilled Water	1000 ml
Agar	20.0 g
pH	7.2 ±0.2

Composition of Oatmeal agar (ISP3) medium

Oat meal	20.0 g
Distilled Water	1000 ml
Trace Solution	1 ml
Agar	20.0 g

The 20 gm. oatmeal cooked in 1000 ml of distilled water for 20 minutes and then filtered it with the help of cheese cloth. Finally 1 ml of trace solution added to the medium pH of the medium should be 7.2.

Composition Inorganic salts –starch agar (ISP4) medium

Soluble starch	10.0 g
Dipotassium Phosphate	1.0 g
Magnesium sulphate	1.0 g
Sodium chloride	1.0 g
Ammonium sulphate	2.0 g
Calcium carbonate	2.0 g
Distilled water	1000 ml
Trace solution	1.0 g

Agar	20.0 g
pH	7.0-7.4 ±0.2

Composition Glycerol asparagine agar (ISP 5)

L-asparagine	1.0 g
Glycerol	10.0 g
K2HPO4	1.0 g
Trace solution	1.0 g
Distilled water	1000 ml
Agar	20.0 g
pH	7.0-7.4 ±0.2

Composition of Peptone yeast extract iron agar (ISP 6)

Peptic digest	5.00 g
Protease peptone	5.0 g
Yeast	1.0 g
Ferric ammonium citrate	0.5 g
Dipotassium phosphate	1.0 g
Sodium triphosphate	0.080 g

Distilled water	1000 ml
Agar	20.0
pH	7.0-7.2±0.2

Composition of Tyrosine agar (ISP 7)

Glycerol	15.0 g
L-tyrosine	0.5 g
L-asparagine	1.0 g
K2HPO4	0.5 g
Magnesium sulphate, 7 H2O	0.5 g
Sodium chloride	0.5 g
Ferrous sulphate	0.001 g
Distilled water	1000 ml
Trace salt solution	1.0 ml
Agar	20.0 g
pH	7.2-7.4±0.2

Composition of Trace salt solution

Ferrous sulphate, 7 H2O	0.1 g
Manganese chloride, 4 H2O	0.1 g
Zinc sulphate, 7H20	0.1 g
Distilled water	1000 ml

4.4.2 Scanning electron microscopy

Scanning electron microscopy was performed to determine the spore chain morphology and spore surface morphology. Scanning electron microscopy (Jeol JSM-6700F, Jeol, Tokyo, Japan) was performed after growth of sample on ISP 2 medium at 28 °C for 14 days (Shirling and Gottlieb, 1966). At the time of inoculation thin (1cm x1 cm) glass slide was inserted into the plate. After 14 days of growth glass slide was taken out and washed with phosphate buffer saline (PBS) thrice. Glass slide then dipped into 4% glutarldehyde prepared in sodium phosphate buffer (pH 7.2) and incubated at room temperature for overnight. Then it is washed with sodium phosphate buffer for thrice. Sample was subjected to dehydrolysis by different concentration of alcohol and at 100 % alcohol it was incubated for 1 hour. Finally after 1 hour alcohol discarded and dried at room temperature. This glass slide was coated with gold palladium and used for scanning electron microscope.

Composition of phosphate buffer saline (PBS)

Disodium hydrogen phosphate	10.9 g
Sodium dihydrogen phosphate	3.2 g
Sodium chloride	90 .0 g
Double distilled water	1000
pH	7.2±0.2

Composition of sodium phosphate buffer

100 ml of 0.1M Disodium hydrogen phosphate and 100 ml of 0.1 M Sodium dihydrogen phosphate was prepared. Then 68.4 ml of 0.1M Disodium hydrogen phosphate was mixed with 31.6 ml of 0.1 M Sodium dihydrogen phosphate. pH was maintained at 7.2 ±0.2.

4.4.3. Light microscopy

Light microscopy was used to determine the spore chain arrangement, color of aerial and substrate mycelium of the strain and eleven closest reference strains. In case of aerial and substrate mycelium color determination light microscopy (Leica DM750, Leica Microsystems, Buffalo Grove, IL, USA) was performed after growth on ISP 2, ISP 3, ISP 4, ISP 5 and ISP 6 at 28 °C for 2-3 weeks according to Shirling and Gottlieb, 1966. Spore chain morphology was observed after growth on ISP 2 medium at 28 °C for 14 days.

4.5. Physiological and biochemical characteristics

For the physiological and biochemical comparison study, eleven closest strains have been selected as reference strains based on 16 S r RNA gene sequence similarity.

4.5.1 Collection and maintenance of reference strains:

The reference strains *Streptomyces variabilis* NRRL B-3984^T, *Streptomyces erythrogriseus* NRRL B-3808^T, *Streptomyces griseoincarnatus* NRRL B-5313^T, *Streptomyces labedae* NRRL B-5616^T, *Streptomyces griseorubens* NRRL B-3982^T, *Streptomyces althioticus* NRRL B-3981^T,*Streptomyces griseoflavus* NRRL B-5312^T, *Streptomyces matensis* NRRL B-2576^T, *Streptomyces viridochromogenes* NRRL B-1511^T, *Streptomyces albogriseolus* NRRL B-1305^T and *Streptomyces paradoxus* NRRL B-3457^T were received from the ARS Culture Collection (NRRL), United States Department of Agriculture, Peoria, IL (USA) for comparative studies and were cultured on International *Streptomyces* Project (ISP) 2 medium at 28 °C. These strains were preserved as glycerol stock in -80 °C as well as -20 °C.

4.5.2. Physiological characteristics

Growth of strain MS 3/20 on temperature ranging from 15 °C to 45 °C was recorded on ISP 2 medium using petriplates for 14 days. The effect of temperature from 15 °C to 19 °C and 41°C to 45 °C was measured at an interval of 2 °C. In case of 20°C to 40 °C effect of temperature was measured at an interval of 1 °C. Growth at different temperature was observed visually. Tolerance to pH ranging from 1 to 12 at an interval of 0.5 unit was measured spectreophotometrically (OD 600 nm) using 1SP 2 medium after 14 days and culture media was incubated in BOD shaker incubator at 28 °C on a

speed of 100 rpm. The pH was adjusted by using 1 N HC1 and NaOH. Effect of NaCl on growth also recorded spectreophotometrically (OD600 nm) on ISP 2 medium for 14 days ranging from 0-25% w/v at an interval of 1% increment.

4.5.3. Biochemical characteristics

Nitrate reduction

Culture tubes containing Nutrient broth supplemented with 0.2/w/v potassium nitrate and agar inoculated and incubated for 14 days. Nitrate production was determined after14 days, by adding 0.2 ml of each Griss-Ilosavy I and II reagents (Williams et al. 1983). Development of a pin-red color is the indicator of positive result. For negative results a trace of zinc powder was added and change color into red indicates the no nitrate reduction.

Composition of nutrient broth for nitrate reduction

Beef extracts	1.0 g
Yeast extracts	2.0 g
Peptone	5.0 g
Potassium nitrate	2.0 g
Distilled water	1000 ml
Agar	8 .0 g

Hydrogen sulphide production

Peptone yeast extract iron agar (ISP 6) slant culture tube were inoculated and incubated for 7 days. After incubation period lead acetate paper inserted into the each tube and

if paper color turns into black or brown it indicates the formation of hydrogen sulphide production (Williams et al. 1983).

Composition of Peptone yeast extract iron agar (ISP 6)

Peptic digest	15.0
Protease peptone	5.0 g
Yeast extracts	1.0 g
Ferric ammonium citrate	0.5 g
Dipotassium phosphate	1.0 g
Sodium triphosphate	0.080 g
Distilled water	1000 ml
Agar	12.0 g
pH	7.0-7.2

Catalase production test

Catalase production test was observed after inoculation of strain into the modified Bennett's agar medium. After 7 days few drops of hydrogen peroxide was added to the plate and bubble formation was considered as positive result (Williams et al. 1983).

Oxidase activity test

Modified Bennett's agar medium was used for the oxidase activity and strains were incubated in the medium at 28 ˚c for 7 days. A loop full colony of well grown bacterial strains were taken and rubbed into the oxidase stripes (Sigma Aldrich, USA). Color changed into dark black or purple are considered as positive to oxidase activity.

Urea decomposition

Sterilized urea solution (15%) was added to the urease broth and slants tubes were prepared for urea decomposition activity (Gordon et al. 1974). Bacterial strains were inoculated and observed for two to four weeks for alkaline activity which was indicated by appearance of pink color. A negative control medium without urea was prepared and observed.

Composition of urease agar medium

Disodium hydrogen phosphate	9.5g
Mon potassium phosphate	10.0 g
Yeast extracts	1.0g
Phenol red	20.0 ml
Distilled water	1000 ml
pH	6.7±0.5

Esculin degradation

Esculin broth was inoculated and incubated at 28 °C. After two and four weeks the blackening of medium was monitored and considered as positive result (Gordon et al. 1974). A negative control medium without esculin was prepared and observed.

Composition of esculin broth

Esculin	1.0 g
Ferric citrate	0.5 g
Peptone	10.0 g
NaCl	5.0 g
Artificial seawater	500 ml
Distilled water	500 ml
pH	7.4 ± 0.2

L-tyrosine degradation

0.4 gm. of l-tyrosine was added to the nutrient agar medium and used for the l tyrosine degradation test. Bacterial strains were inoculated and incubated at 28 °C and observed for two and four weeks for disappearance of zone which was considered as positive result (Gordon et al. 1974).

Composition of nutrient agar medium with L-tyrosine

L-tyrosine	0.4 g

Beef extract	1.0 g
Yeast extracts	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Water	1000 ml
Agar	18.0 g
pH	7.0 ±0.5

Gelatin degradation

Culture tubes containing Modified Bennett's agar medium supplemented with 0.4% w/v gelatin inoculated and incubated for 7 days. After growth of organisms the tubes were kept in refrigerator for 2 hours and observed for the liquefaction of the tubes. A control tube was used without inoculation for the study.

Composition of Modified Bennett's agar medium with gelatin

Gelatin	0.4 g
Beef extract	1.0 g
Glycerol	10.0 g
Enzymatic digest of casein	2.0 g

Yeast extracts	1.0 g
Distilled water	1000.0 ml
Agar	15.0 g
pH	7.3± 0.5

Starch hydrolysis

Modified Bennett's agar medium supplemented with 1.0 % w/v of starch were inoculated and incubated for 7 days. After 7 days the plates were flooded with acidified magnesium chloride and iodine solutions respectively. Clear zone around the growth indicate the hydrolysis of starch.

Composition of Modified Bennett's agar medium with starch

Starch	1.0 g
Beef extract	1.0 g
Glycerol	10.0 g
Enzymatic digest of casein	2.0 g
Yeast extracts	1.0 g
Distilled water	1000.0 ml
Agar	15.0 g

pH 7.3± 0.5

Melanin production

Culture tubes containing ISP 6 medium were inoculated and incubated for melanin production test (Shirling and Gottlieb, 1966). Melanoid pigments production were observed after 2 and 4 days and compared with an un-inoculated control tube. Formations of brown or brownish black or greenish brown color were considered as appositive result.

Carbon source utilization

Ability of the microorganisms to utilize the different compounds as carbon source were tested by supplementing 1% carbon sources into basal medium ISP 9 (Shirling and Gottlieb 1966). The plates were inoculated and incubated for 10-16 days. A control plates which is prepared using only basal medium without supplementing carbon sources was used for the comparison of growth. Growth of bacterial strains on agar plates determined visually and considered as positive result.

Nitrogen source utilization

To assess the ability of the strains to use the different amino acids as sole source of nitrogen were tested on basal medium supplemented with 0.1% different amino acids (Williams et al. 1983). Growth was recorded after 15 days visually and compared with both negative and positive control.

Composition of basal medium

D-glucose	10.0 g
Magnesium sulphate, 7H20	0.5 g
Sodium chloride	0.5 g
Ferrous sulphate, 7H20	0.01 g
Dipotassium hydrogen phosphate	1.0 g
Agar	18.0 g
pH	7.4±0.5

4.6. Antibiotic susceptibility test

Bacterial strains were tested for susceptibility or resistance to different antibiotics by the diffusion plate technique. Nutrient agar was used as basal medium. Sterile disk of antibiotics were placed into the plates and incubated at 28˚ C. The plates were then observed for 3, 5 and 7 days for the zone of inhibition around the growth. A control was tested without antibiotic disk and compared. Antibiotics used for this study are followings ampicillin (10 µg), carbenicillin (100µg), chloramphenicol (30µg), gentamicin (50 µg), kanamycin (30 µg), lincomycin (15µg), neomycin (30 µg), oleandomycin (15 µg), polymyxin B (300 units), streptomycin (10 µg), tetracycline (30 µg),carbenicillin (100µg) and vancomycin (30 µg).

4.7. Chemotaxonomic analysis

4.7.1. Whole cell sugar determination

Whole cell sugar analysis was carried as described by Staneck and Roberts (1974). About 25 mg of bacterial cells were taken into ampoule filled with 1.5 ml of 1 N sulphuric acid. It was heated for 2 hour and then saturated barium hydroxide was added after cooling until pH was reached 5.2 to 5.5. After centrifugation supernatant was collected and subjected to evaporation for dryness. The residue was dissolved into water and subjected to ascending thin layer chromatography (TLC) by using n-butanol-distilled water-pyridine –toluene (10:6:6:1 v/v) as solvent system. The thin layer chromatography (TLC) spots were visualized by spraying acid aniline phthalate and heating at 100 °C for 4 minutes. The 1% solutions of standard sugars are spotted into the TLC sheet for the identification of the whole cell sugar.

4.7.2. Di-amino pamelic acid analysis

Di-amino pamelic acid analysis also carried out according to the methods of Staneck and Roberts (1974). Bacterial cell was transferred to an ampoule filled with 1 ml of 6 N hydrochloric acid. Sample was heated at 100 °C and cooled to filter through Whatman no 1filter paper. Then supernatant was subjected to dryness and after dissolving into water it was spotted into the thin layer chromatography (TLC) sheet using methanol-distilledwater-6 N HCl-pyridine (80:26:4: 10 v/v) as solvent system. Finally spots were detected by spraying 0.2 % ninhydrin into acetone.

4.7.3. Fatty acid methyl ester analysis

Fatty acid analyses were performed by the Royal Life Sciences (affiliated to MIDI Sherlock), Secundrabad, India. Strains were cultivated on the specified media. About40 mg cells were harvested from petridishes and processed for saponification, methylation and extraction using small modifications to the methods of Miller (1982) and Kuykendall et al (1988) to obtain the fatty acid methyl ester. The fatty acid methyl ester mixtures were separated using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, New York, DE 19711 USA) which consisted of an Agilent model 6890N gas chromatographic system. Names and percentage of fatty acids present were determined using MIS Standard Software (Microbial ID). In this analysis strain MS 3/20 were compared with phylogenetically very closest four strains for the identification.

4.7.4. Polar lipid analysis

Polar lipids were extracted using methanol/chloroform/saline (2:1:0.8, by vol.) as described by Bligh and Dyer (1959) and with minor modifications of Card. G. L. (1973). Two-dimensional thin layer chromatography (TLC) was used for separation of lipids where chloroform-methanol-water (65:25:4 by vol.) used as solvent system in the one direction and chloroform-acetic acid-methanol- water (80:15:12:4 by vol.) used in the another direction (Tindall 1990). The TLC plates were dried and subjected to spraying of 5% ethanolic molybdophosphoric acid, ninhydrin, molybdenum blue, dragendorff, and α-naphthol for the identification of polar lipids present in the strain.

4.7.5. Quinone estimation

Microbial quinone compounds were identified by the China Center of Industrial Culture Collection (Beijing, China), usingFMIC-QO01-008 analytical method (Ruan and Huang 2011, Xu et al. 2007). Respiratory quinones were extracted using methanol –chloroform as solvents and checked in thin layer chromatography. Finally, it is isolated by preparative thin layer chromatography and subjected to high pressure liquid chromatography (HPLC) for purification and analysis. It is compared with its database in high pressure liquid chromatography (HPLC) or standard quinones and identified. Often data obtained from HPLC was rechecked and confirmed using mass spectroscopy.

4.8. Results and Discussions

4.8.1. Growth and maintenance of strain MS 3/20

The organism grows well in described storage medium along with ISP2, ISP3, ISP4, ISP5, and ISP6. It grows as spore forming bacteria which is the characteristics of classical *Streptomyces*.

4.9. Molecular phylogenetic analyses

4.9.1. Phylogenetic analysis of the 16S rRNA gene

The preliminary identification of the strain MS 3/20 was determined by using of 16S rRNA gene sequence study. Nearly complete 16S rRNA gene sequence (1503 base pair) was analyzed in this study. The comparison of the gene sequence of the strain was carried out by using EzTaxon-e server. The comparison indicated that the strain

belongs to the genus *Streptomyces* and has shown 100 % gene sequence similarity with four organisms namely, *Streptomyces variabilis* NBRC 12825^T, *S. erythrogriseus* LMG 19406^T, *S. griseoincarnatus* LMG 19316^{T}and *S. labedae* NBRC 15864^T. The strain also shared more than 99% similarity with other organisms of the genus and these were *S. griseorubens* NBRC12780^T (99.73%), *S. althioticus* NRRL B-3981^T (99.52%), *S. griseoflavus* LMG 19344^T (99.45%), *S. matensis* NBRC 12889^T (99.45%), *S. viridochromogenes* NBRC 3113^T (99.11%), *S. albogriseolus* NRRL B-1305^T (99.05%), *S. heliomycini* NBRC 15899^T (99.04%), *S. paradoxus* NBRC 14887^T (99.04%) and *S. viridodiastaticus* NBRC 13106^T (99.04%).

The NJ-phylogenetic tree (Figure 4.3) again affiliated that the strain MS 3/20 belongs to the genus *Streptomyces*. The phylogenetic tree refers that the strain MS 3/20 clustered with *S. griseoincarnatus* LMG 19316^T, *S. erythrogriseus* LMG 19406^T, *S. variabilis* NBRC 12825^T and *S. labedae* NBRC 15864^T. This 16S rRNA gene sequence study creates ambiguity to identify the strain. It is well know that the genus *Streptomyces* contains largest number of organisms and it is highly congested. In case of delineation of species or genus 16S rRNA gene sequence study are not currently considered as a gold standard techniques (Stackebrandt & Ebers 2006). The 16S rRNA gene sequence technique has few limitations for species delineation particularly within the genus *Streptomyces* due to insufficient resolution capacity, intragenomic heterogeneity, intraspecific variation and frequent inconsistency with DNA-DNA relatedness study(Anderson and Wellington, 2001, Kämpfer and Labeda, 2006, Kämpfer 2008, Kim et al 2012, Yamamura et al. 2014). Labeda *et al.* (2012)

recommended that it is difficult to propose any definite16S rRNA gene sequence similarity value for the species delineation. In the view of the insufficient resolution capacity of the 16S rRNA molecule, Kämpfer *et al*. (2008) stated about simple treeing method for the genus *Streptomyces* and recommended that "tree is only a visual aid to place a novel species in its approximate neighborhood". Kämpfer*et al*. (2008) reiterated that description of new *Streptomyces* species should be based on the combination of phenotypic and genotypic data, and the "sufficient" evidence must be provided.

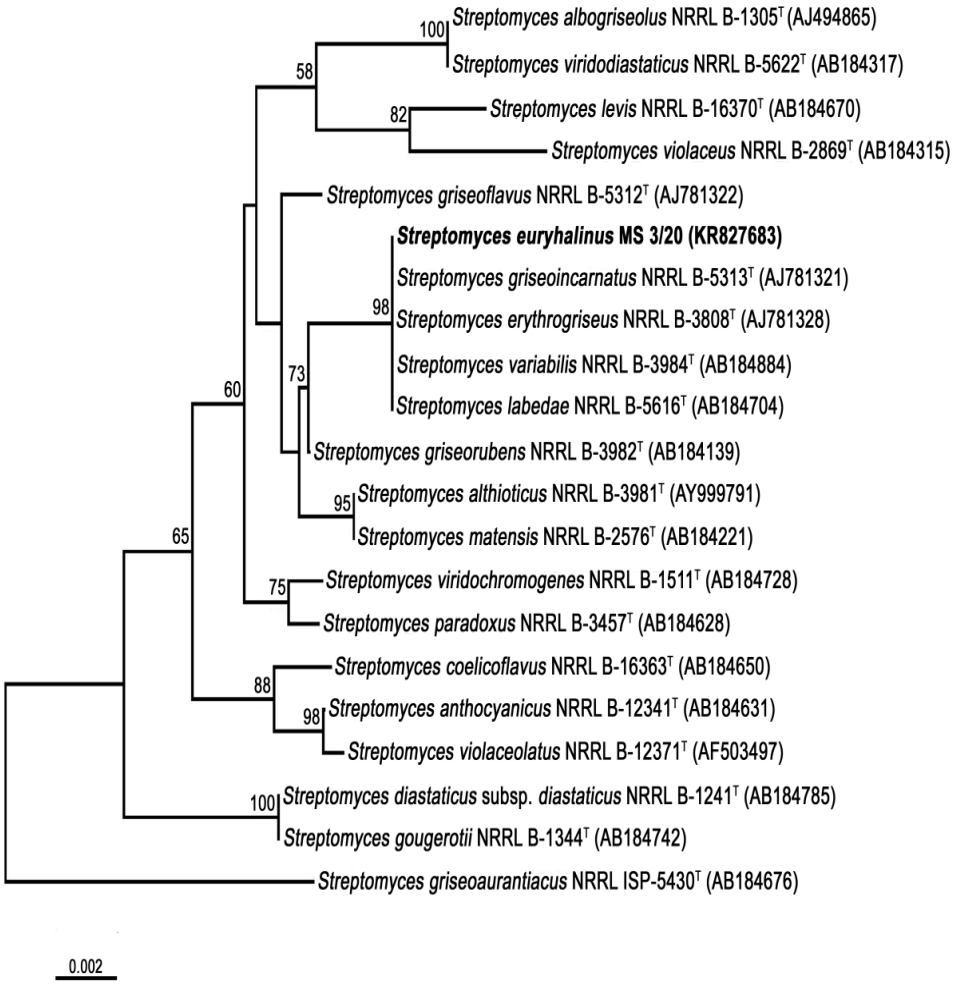


Figure 4.3.Neighbor-joining phylogenetic tree indicating the position of *Streptomyces euryhalinus* MS 3/20 within the *Streptomyces* genus based on 16S rRNA gene sequences. Levels of bootstrap support (percentages of 1000 replicates) indicated by numbers at nodes; greater than 50% values are shown. GenBank accession numbers are mentioned.

4.9.2. DNA-DNA hybridization study

It is currently mandatory to conduct the DNA-DNA relatedness study for species delineation as Labeda et al.(2012) advocated for no specific values of 16S rRNA gene sequences similarity for species delineation in case of *Streptomyces* genus. DNA-DNA relatedness study of the strain MS 3/20 was showed maximum relatedness (53.79±3.46%) with *S. griseoflavus* LMG 19344^T, which is significantly lower than the general 70% cut off value for species differentiation (Wayne et al. 1987) and presently 80% cut off value recommended for identification of a novel species within the genus *Streptomyces* (Labeda et al, 2012). The DNA-DNA re-association values of strain MS 3/20^T with the other phylogenetically close type strains differed between 27.24% to 35.48% (Table 4.1.). This result undoubtedly testified that strain MS 3/20 is distinct from the reference type strains and could be a new species.

The proposed cut-off value (97% 16S rRNA gene sequence similarity) for DNA-DNA hybridization is disputable as reported by Ray et al (2013) while proposing *Streptomyces chilikensis* as a new species and citing Stackebrandt and Ebers (2006) in their favor. In addition, Stackebrandt and Ebers suggested a higher (98.7-99%) 16S rRNA gene sequence similarity value in comparison to the previously accepted 97% for DNA-DNA hybridization in situations where high-quality almost full-length 16S rRNA gene sequences are in question. However, Labeda *et al*. (2012) contested the proposal stating that for the genus *Streptomyces*, the previously proposed 16S rRNA gene sequence similarity level (Stackebrandt and Ebers, 2006) appears to be invalid. DNA-DNA relatedness is currently applied as the decisive criterion to define a

prokaryotic species (Kim et al. 2012). In many situations, some species share a high level of 16S rRNA gene sequence similarity (99%), even though they are distinctly separated by DNA-DNA hybridization (Kim et al. 2014). Within the genus *Streptomyces*, the 16S rRNA gene sequence similarity between *Streptomyces setonii* and *Streptomyces griseus* was 99.8%, whereas the DNA-DNA relatedness between the two strains was 43% (Kim et al. 2012). To cite few other examples *Streptomyces hundungensis* sp. nov. (Nimaichand et al. 2013), *Streptomyces hyaluromycini* sp. nov.(Harunari et al 2015) and *Streptomyces lactacystinicus* sp. nov.(Také et al. 2015) and *Streptomyces chilikensis* sp. nov.(Ray et al.2013) showed 99.6%, 99.72%, 99.7% and 99.53% 16S rRNA gene sequence similarities respectively with their closest neighbors but were established as novel species by low levels of DNA-DNA relatedness. This study is the first case where a novel *Streptomyces* species was established in spite of 100% 16S rRNA sequence similarity with four phylogenetic neighbors.

Table 4.1. DNA-DNA relatedness values between strain MS 3/20 and its phylogenetically closest strains.

Relative strains	**NRRL Accession numbers**	**Pairwise similarity of 16S rRNA (%)**	**DNA-DNA hybridization (%)**			**Average value(±SD)**
			I	**II**	**III**	
S. variabilis	NRRL B-3984^T	100	28.4	36.8	29.6	31.6 (±4.6)
S.erythrogriseus	NRRL B-3808^T	100	24.2	39.3	27.0	30.2 (±8.0)
S.griseoincarnatus	NRRL B-5313^T	100	23.4	37.3	26.4	29.0 (±7.3)
S. labedae	NRRL B-5616^T	100	28.6	36.3	28.1	31.0 (±4.6)
S. griseorubens	NRRL B-3982^T	99.73	25.5	36.8	27.7	30.0 (±6.0)
S. althioticus	NRRL B-3981^T	99.52	35.1	38.9	32.4	35.5 (±3.3)
S. griseoflavus	NRRL B-5312^T	99.45	57.2	50.3	53.9	53.8 (±3.5)

S. matensis	NRRL B-2576^{T}	99.45	23.2	31.0	27.5	27.2 (±3.9)
S.viridochromogenes	NRRL B-1511^{T}	99.11	25.6	33.7	27.1	28.8 (±4.3)
S.albogriseolus	NRRL B-1305^{T}	99.05	25.0	31.3	26.9	27.7 (±3.2)
S.paradoxus	NRRL B-3457^{T}	99.04	27.8	35.0	28.2	30.3 (±4.0)

4.9.3. Random amplified polymorphic DNA (RAPD-PCR) amplification

Fingerprinting techniques like RAPD is a notable technique for tracking and differentiation of species within a genus (Roberts and Crawford, 2000). RAPD techniques subsequently used with other important differentiating tools to determine the species within genus *Streptomyces* like in case of delineation of *Streptomyces koyangensis* sp. nov. (Lee et al, 2005). Here, RAPD was employed to construct the difference between strain MS 3/20 and other phylogenetically close relatives which have shown distinction in DNA-DNA relatedness study. Banding pattern of the randomly amplified DNA of strain MS 3/20 showed recognizable differences from the pattern of its relatives (Figure 4.4. a, b, c). Bands at 700 bp and 1600 bp generated from DNA of strain MS 3/20 by amplification with primer AM 62 were not detected in other reference strains. When primer AM 50 was applied, bands at 400 bp and 1300 bp were observed in the amplified DNA of strain MS 3/20, which were not observed in the other strains. Similarly, when primer 70-34 was applied, amplified DNA of strain MS 3/20 displayed bands at 300 bp, 600 bp, 950 bp and1100 bp while other strains did not produce bands in the same regions. RAPD banding pattern profoundly indicates the significant difference between the strain MS 3/20 with other phylogenetic close relatives which could be leads to establish the strain as novel species.

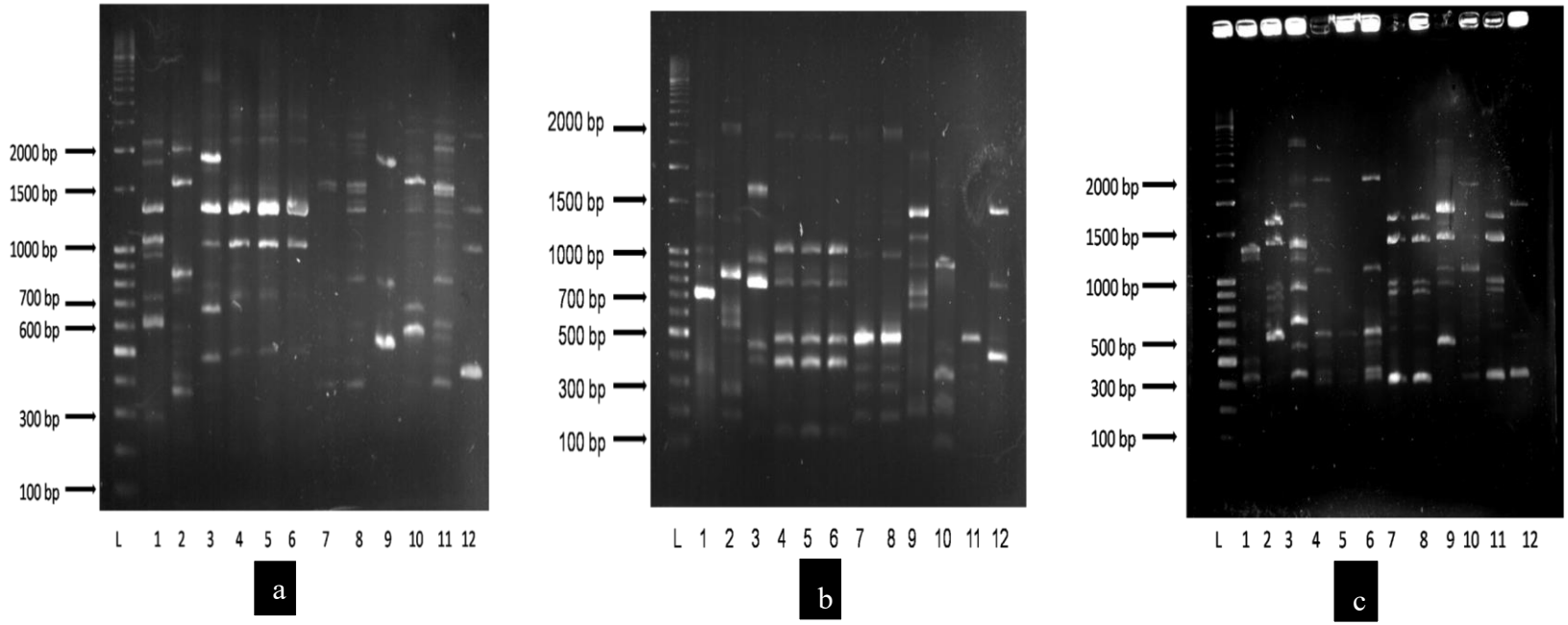
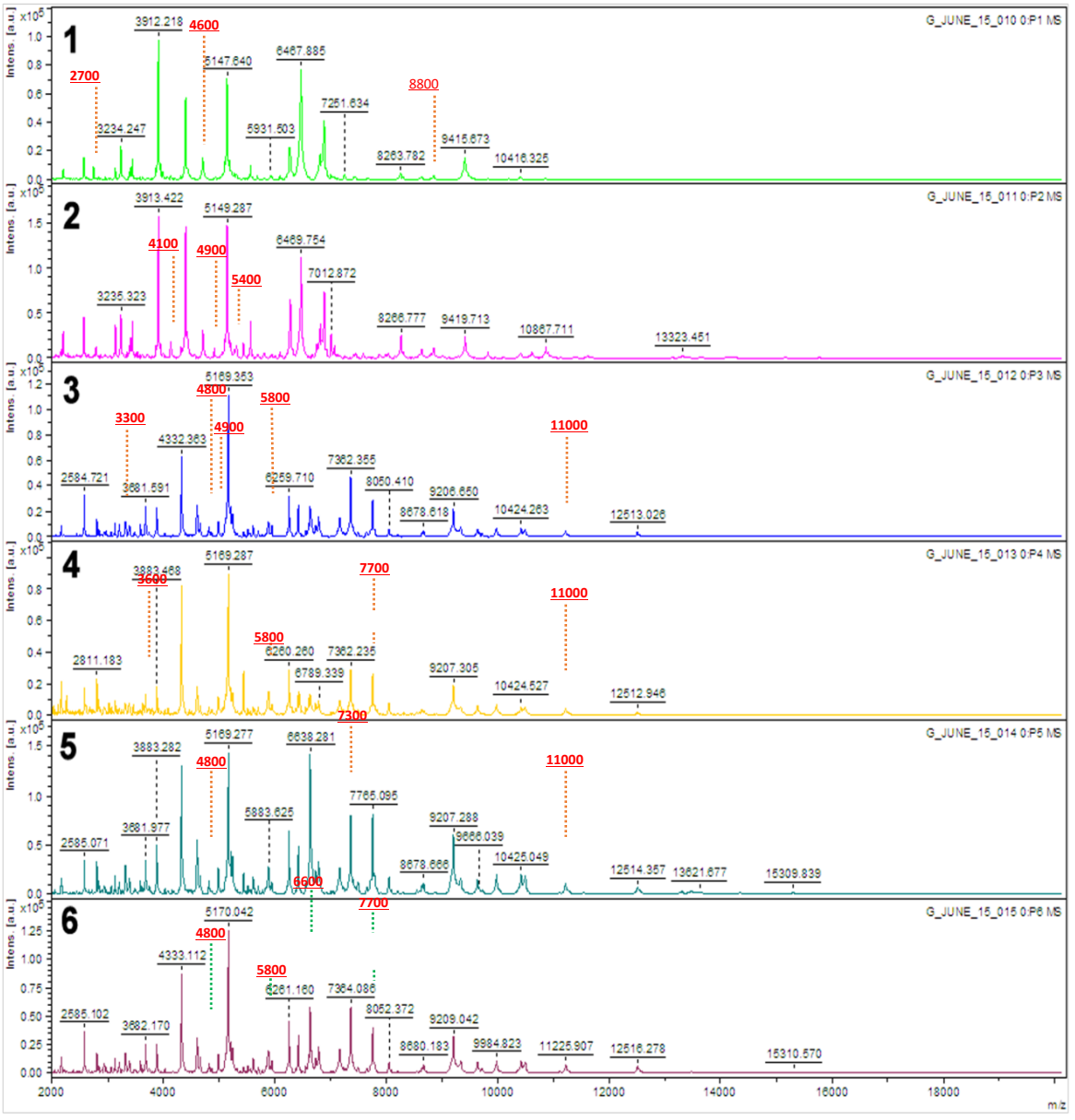


Figure 4.4. Agarose gel electrophoretic image of RAPD fingerprint profile of PCR-amplified DNA of strain MS 3/20 and its phylogenetically closest strains using primer a) AM 62,b) AM 50, c) 70-34, captured on an E-Gel imager (Thermo Fisher Scientific). Lanes represent: L Marker, **1** MS 3/20; **2** *Streptomyces variabilis* NRRL B-3984^{T}; **3** *Streptomyces erythrogriseus* NRRL-B-3808^{T}; **4** *Streptomyces griseoincarnatus* NRRL-B-5313^{T}; **5** *Streptomyces labedae* NRRL-B-5616^{T}; *Streptomyces griseorubens* NRRL-B-3982^{T}; **7** *Streptomyces althioticus* NRRL B-3981^{T}; **8** *Streptomyces griseoflavus* NRRL-B-5312^{T}; **9** *Streptomyces matensis* NRRL-B-2576^{T}; **10** *Streptomyces viridochromogenes* NRRL-B-1511^{T}; **11** *Streptomyces albogriseolus* NRRL B-1305^{T}; **12** *Streptomyces paradoxus* NRRL-B-3457^{T}.

4.9.4. MALDI-TOF/MS analysis

MALDI-TOF/MS is an excellent and effective technique employed for bacterial identification and for differentiation of closely related species (Murray, 2010). MALDI-TOF/MS spectra of strain MS 3/20 was exhibited distinction in comparison with corresponding spectra of other phylogenetically close relatives. The peaks for whole cell protein of Strain MS 3/20 was observed at the regions (m/z values) of 2700, 3900, 4600, 5000, 5300, 7200, 8200, 8800 and 9400 (Figure 4.5, Table 4.2). It was noticed that few of these peaks (m/z values) are present in other reference strains but never occurred simultaneously. A few peaks at regions (m/z values) of 3300, 3600, 4100, 4800, 4900, 5400, 5800, 6600, 7300, 7400, 7700, 11000, 12000, 13000, 15000 and 18000 were present in other type strains but absent in strain MS 3/20^{T}(Figure 4.4., Tabl2 4.2). MALDI-TOF/MS data was played a decisive role during the re-classification of *Streptomyces spheroides* and *Streptomyces laceyi* in the genus *Streptomyces*. Thus, the results obtained from DDH, RAPD-PCR banding pattern, and MALDI-TOF/MS profile of strain MS 3/20 and other reference strains significantly indicates the substantial differences, and the strain MS 3/20 was proclaimed as a novel species in the genus *Streptomyces*.


1
G_JUNE_15_010 0:P1 MS
3912.218
4600
5147.640
6467.885
2700
3234.247
7251.634
8800
5931.503
8263.782
9415.673
10416.325
2
G_JUNE_15_011 0:P2 MS
3913.422
5149.287
4100
4900
6469.754
5400
7012.872
3235.323
8266.777
9419.713
10867.711
13323.451
3
G_JUNE_15_012 0:P3 MS
5169.353
4800
5800
3300
4900
11000
4332.363
7362.355
2584.721
3681.591
6259.710
8050.410
9206.650
8678.618
10424.263
12513.026
4
G_JUNE_15_013 0:P4 MS
5169.287
3883.468
3600
7700
11000
5800
6260.260
7362.235
2811.183
6789.339
9207.305
10424.527
7300
12512.946
5
G_JUNE_15_014 0:P5 MS
5169.277
6638.281
3883.282
4800
11000
7765.095
5883.625
9207.288
9666.039
3681.977
2585.071
8678.666
10425.049
6600
12514.357
13621.677
15309.839
6
G_JUNE_15_015 0:P6 MS
5170.042
7700
4800
4333.112
5800
6261.160
7364.086
2585.102
8052.372
3682.170
9209.042
8680.183
9984.823
11225.907
12516.278
15310.570
Intens. [a.u.]
m/z

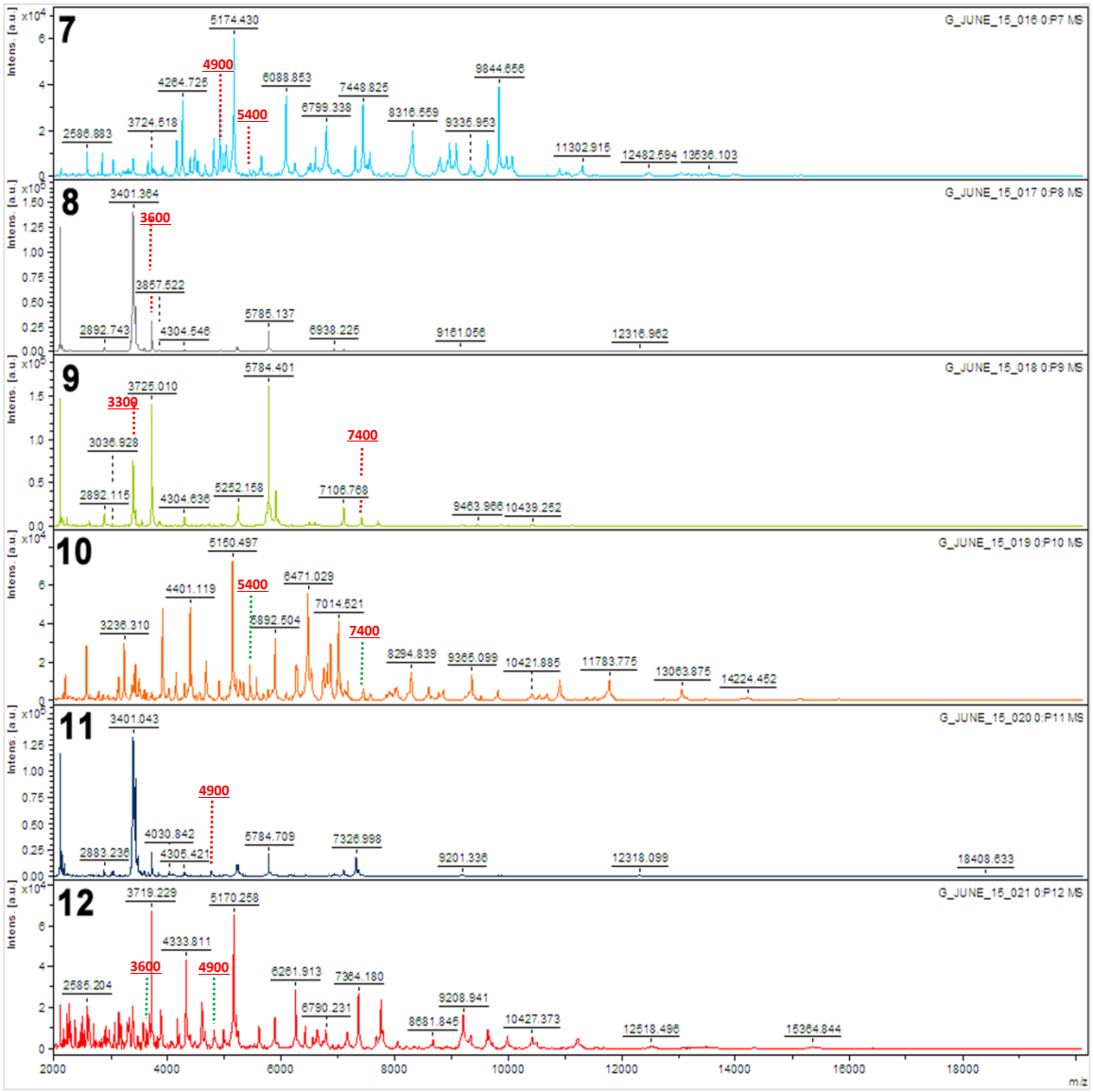


Figure 4.5. MALDI-TOF/MS spectra of whole-cell proteins of strain MS 3/20 and its phylogenetically closest strains.**1** MS 3/20; **2** *Streptomyces griseoincarnatus* NRRL B-5313^{T}; **3** *Streptomyces variabilis* NRRL B-3984^{T}; **4***Streptomyces erythrogriseus* NRRL B-3808^{T}; **5** *Streptomyces labedae* NRRL B-5616^{T}; **6** *Streptomyces griseorubens* NRRL B-3982^{T}; **7** *Streptomyces matensis* NRRL B-2576^{T}; **8** *Streptomyces griseoflavus* NRRL B-5312^{T};**9** *Streptomyces althioticus* NRRL B-3981^{T}; **10** *Streptomyces viridochromogenes* NRRL B-1511^{T};**11** *Streptomyces albogriseolus* NRRL B-1305^{T}; **12** *Streptomyces paradoxus* NRRL B-3457^{T}. Whole-cell proteins of strain MS 3/20 demonstrated peaks (*m/z* values) at the regions of 2700, 3900, 4600, 5000, 5300, 7200, 8200, 8800 and 9400 (**shown in red**). A few of these peaks shown presence in other

type strains but never takes place simultaneously. Peaks (*m/z* values) at regions of 3300, 3600, 4100, 4800, 4900, 5400, 5800, 6600, 7300, 7400, 7700, 11000, 12000, 13000, 15000 and 18000 were not present in strain MS 3/20 but shown presence in other phylogenetically close relatives (**shown in red**).

Table4.2. Comparison of spectral data obtained from MALDI-TOF analyses of whole-cell proteins of strain MS 3/20 and its phylogenetically closest strains. **1** MS 3/20; **2** *Streptomyces griseoincarnatus* NRRL B-5313^{T}; **3** *Streptomyces variabilis* NRRL B-3984^{T}; **4***Streptomyces erythrogriseus* NRRL B-3808^{T}; **5** *Streptomyces labedae* NRRL B-5616^{T}; **6** *Streptomyces griseorubens* NRRL B-3982^{T}; **7** *Streptomyces matensis* NRRL B-2576^{T}; **8** *Streptomyces griseoflavus* NRRL B-5312^{T};**9** *Streptomyces althioticus* NRRL B-3981^{T}; **10** *Streptomyces viridochromogenes* NRRL B-1511^{T};**11** *Streptomyces albogriseolus* NRRL B-1305^{T}; **12** *Streptomyces paradoxus* NRRL B-3457^{T}. Green represents *m/z* values of whole-cell proteins of strain MS 3/20 which were present in other type strains but never appeared simultaneously. Red indicates m/z values which were not present in strain MS 3/20 but present in other phylogenetically close relatives.

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2199.116	2166.554	2165.829	2166.430	2166.000	2166.475	2131.808	2089.695	2089.981	2200.179	2089.891	2107.737
2564.411	2199.481	2584.721	2261.625	2585.071	2585.102	2586.883	2106.726	2106.596	2575.292	2106.789	2114.360
2573.521	2565.395	2811.227	2267.666	2811.324	2811.835	2853.575	2137.722	2115.916	2784.654	2144.869	2166.004

2751.081	2574.599	2839.137	2584.981	2839.204	2942.661	3044.642	2144.903	2128.543	2804.270	2183.641	2224.627
3136.269	2783.844	3130.540	2811.183	3130.517	3131.119	3303.889	2182.349	2144.887	3122.305	2253.654	2268.579
3142.045	3143.164	3211.972	2839.200	3211.996	3212.574	3399.640	2250.575	2158.778	3137.609	2258.800	2295.691
3234.247	3235.323	3320.205	3021.622	3319.922	3320.383	3656.844	2294.941	2229.178	3144.144	2883.236	2373.589
3240.365	3241.374	3325.928	3131.339	3326.593	3395.905	3724.518	2626.703	2625.923	3236.310	3007.366	2500.534
3253.162	3252.983	3395.676	3681.681	3395.546	3581.910	3913.669	2883.205	2892.115	3242.862	3045.964	2535.613

3406.825	3407.860	3581.633	3883.468	3581.489	3682.170	4158.836	2892.743	3036.928	3378.183	3358.104	2585.204
3443.003	3444.432	3681.591	4332.233	3681.977	3883.764	4264.725	3045.870	3400.244	3409.067	3385.670	2612.306
3870.892	3872.272	3883.212	4369.198	3883.282	4333.112	4402.542	3299.213	3416.496	3438.289	3401.043	2626.473
3898.476	3898.918	4332.363	4604.770	3897.074	4369.459	4465.662	3357.379	3439.627	3507.474	3416.393	2705.583
3912.218	3913.422	4604.268	4992.653	4331.906	4605.193	4485.203	3401.364	3477.566	3529.320	3439.897	2905.399
3949.490	3930.353	4620.582	5116.764	4370.530	4669.384	4542.940	3416.913	3553.741	3586.023	3455.980	2975.355

3992.791	3951.415	4668.453	5126.663	4604.638	4820.171	4668.823	3439.754	3725.010	3914.424	3477.480	3075.250
4398.883	4133.841	4819.025	5169.287	4622.061	4993.029	4818.107	3471.047	3741.338	3928.025	3497.227	3131.367
4418.203	4319.934	4992.022	5190.568	4668.669	5128.035	4842.829	3477.781	3761.504	4022.552	3515.666	3144.293
4434.619	4400.067	5128.795	5210.934	4819.865	5170.042	4922.457	3489.716	3775.599	4148.547	3554.368	3182.163
4451.338	4426.944	5169.353	5230.142	4992.567	5188.157	4940.674	3499.862	3846.923	4302.846	3584.239	3295.846
4698.552	4454.200	5190.583	5246.707	5126.206	5192.907	4986.989	3515.871	3856.019	4355.571	3663.560	3308.906

4709.410	4710.536	5211.542	5439.433	5169.277	5213.107	5037.069	3540.424	3870.492	4401.119	3725.562	3320.223
4726.163	4916.778	5232.042	5869.193	5192.024	5247.229	5047.131	3547.258	4304.636	4428.380	3741.674	3328.623
5074.604	5106.790	5245.995	5883.178	5212.353	5613.615	5132.371	3554.178	4732.578	4683.680	3762.850	3394.264
5105.886	5149.287	5517.463	5953.993	5246.267	5869.782	5152.321	3572.912	4940.588	4911.073	3845.143	3567.453
5147.640	5170.656	5612.492	6231.597	5439.279	5884.456	5174.430	3589.491	5223.520	5108.072	4030.842	3633.599
5184.436	5186.221	5868.506	6260.260	5612.502	5954.766	5196.866	3725.660	5252.158	5150.497	4097.392	3682.151

5210.266	5210.561	5882.928	6423.112	5869.684	6261.160	5212.218	3741.394	5270.610	5186.960	4305.421	3719.229
5227.687	5280.532	5953.668	6448.685	5883.625	6424.244	5454.505	3762.430	5278.305	5212.203	4780.474	3738.826
5279.341	5306.971	6259.710	6608.197	5953.771	6555.898	5624.340	3857.522	5568.441	5279.526	4999.838	3883.966
5306.576	5317.779	6422.383	6639.177	6259.863	6609.495	5651.269	3872.276	5696.005	5344.961	5211.437	3899.678
5567.050	5434.605	6553.920	6738.183	6423.058	6639.766	6046.269	4030.542	5740.766	5456.280	5226.655	4177.552
5688.348	5454.504	6608.088	6789.339	6554.608	6654.829	6088.853	4304.546	5784.401	5569.579	5252.544	4214.238

5931.503	5568.238	6638.930	7161.996	6608.519	6739.897	6115.540	4581.680	5820.248	5765.971	5275.652	4333.811
6270.214	6244.094	6653.544	7177.440	6638.281	6790.334	6124.684	4781.378	5855.938	5849.957	5289.522	4398.750
6283.484	6273.021	6666.212	7329.350	6653.767	6808.323	6240.223	4940.819	5870.493	5892.504	5297.847	4605.775
6308.704	6286.038	6738.217	7362.235	6716.661	7120.998	6471.823	5227.290	5899.581	6273.592	5327.242	4669.747
6322.081	6311.408	6789.129	7397.542	6738.707	7162.872	6490.445	5252.436	5915.058	6287.479	5740.061	4821.066
6352.665	6469.754	7161.547	7726.021	6788.844	7177.831	6521.660	5371.229	5952.926	6374.238	5784.709	4992.943

6367.636	6483.669	7175.567	7764.988	6806.864	7238.845	6594.192	5741.446	5970.892	6471.029	5802.027	5170.258
6467.885	6504.476	7362.355	7793.459	6824.848	7323.842	6607.710	5755.264	6503.930	6485.121	5821.148	5192.562
6480.791	6754.256	7394.671	8050.766	7161.505	7364.086	6650.129	5785.137	6564.102	6532.335	6159.897	5213.519
6501.112	6789.961	7420.234	9207.305	7177.012	7394.545	6704.906	5802.093	6595.548	6740.969	6937.632	5247.134
6755.105	6815.096	7654.192	9245.836	7362.298	7422.487	6782.766	5821.635	6675.511	6755.341	6977.738	5613.305
6799.444	6844.360	7723.968	9279.909	7396.260	7494.115	6799.338	5848.947	7063.513	6774.073	7106.821	5885.023

6812.914	6854.995	7749.075	9289.094	7422.118	7656.192	6841.272	5900.461	7106.768	6816.226	7142.515	6261.913
6848.670	6888.100	7764.546	9306.374	7492.277	7725.510	6874.563	5915.774	7141.644	6841.178	7326.998	6424.447
6857.319	6913.578	7793.662	9335.395	7724.515	7766.227	7312.280	5925.036	7422.868	6875.438	7364.963	6555.411
6885.709	6925.451	8050.410	9638.698	7765.095	7793.490	7448.825	6505.427	7693.991	6993.375	7391.075	6610.328
6921.557	7012.872	8642.993	9982.606	7793.381	8052.372	7484.923	6920.618	7709.739	7014.521	7401.358	6641.053
6946.414	7054.523	8678.618	10424.527	8051.221	8645.134	7509.337	6938.225	7737.998	7051.143	7424.216	6655.461

7009.624	7077.876	9134.519	10490.349	8643.125	8680.183	7528.974	7107.892	9202.875	7051.161	9160.786	6790.231
7251.634	8266.777	9206.650	11223.275	8678.666	9209.042	7567.932	7325.390	9463.966	7113.467	9201.336	7163.090
8263.782	8290.419	9243.864	12512.946	9207.288	9247.584	8297.979	7423.419	9880.344	7133.174	9214.662	7178.122
8634.222	8303.930	9256.258		9244.459	9296.965	8316.559	7712.212	9998.324	7171.450	9822.938	7364.180
8850.307	8638.853	9282.090		9284.525	9338.011	8351.885	9161.056	10426.590	7453.347	12249.547	7395.123
9415.673	8853.848	9296.588		9304.962	9640.389	8776.469	9865.227	10439.252	7909.348	12296.393	7683.961

9454.817	9419.713	9335.296		9319.568	9668.398	8808.709	9880.724	11138.869	7930.839	12318.099	7728.664
10416.325	9456.584	9638.037		9336.166	9727.435	8893.505	12316.962		7951.450	12347.842	7766.830
10864.883	9832.253	9666.346		9638.240	9984.823	8929.705			8016.604	12371.009	7794.574
	10421.760	9724.716		9666.039	10425.920	8968.856			8028.282	18368.450	8052.894
	10612.412	9982.001		9983.229	10492.093	9004.501			8044.214	18384.379	8681.845
	10639.133	10384.825		10019.355	11225.907	9022.325			8230.850	18408.633	9183.730

	10867.711	10424.263		10425.049	11256.866	9086.321			8249.167	18440.427	9208.941
	10908.758	10489.655		10491.663	12516.278	9335.953			8280.077		9228.538
	13323.451	11222.799		10523.953	12533.441	9635.904			8294.839		9338.839
	13367.145	11252.472		11223.450	13477.279	9673.373			8604.275		9640.705
		12513.026		11253.690	15310.570	9844.656			8774.126		9668.309
		12533.507		11265.677		9883.033			8855.340		9727.888

				12514.357		9911.172			9306.709		9969.482
				13314.135		9973.660			9323.096		9984.728
				13478.621		9995.340			9365.099		10427.373
				13621.677		10030.652			9820.336		10493.667
				15309.839		10074.089			10421.885		11226.566
						10909.230			10554.973		11279.584

						11017.664			10687.043		12518.496
						11260.781			10911.034		13481.211
						11283.672			10951.611		15364.844
						11302.915			11783.775		
						12482.594			11839.336		
						13044.780			13063.875		

						13536.103			14224.452		
						13970.079			14259.711		
									15140.259		

4.10. Morphological, physiological and biochemical characteristics of strain MS 3/20

4.10.1. Light microscopy and scanning electron microscopy

Scanning electron microscopy and light microscopy of strain MS 3/20 was carried out after growth on ISP 2 medium for 14 days. Scanning electron microscopy at low and high magnification (4.6. a, b, c), and light microscopy image (4.7. a-b) showed spores are retinaculum-apertum in arrangement with long chain spiny surface.

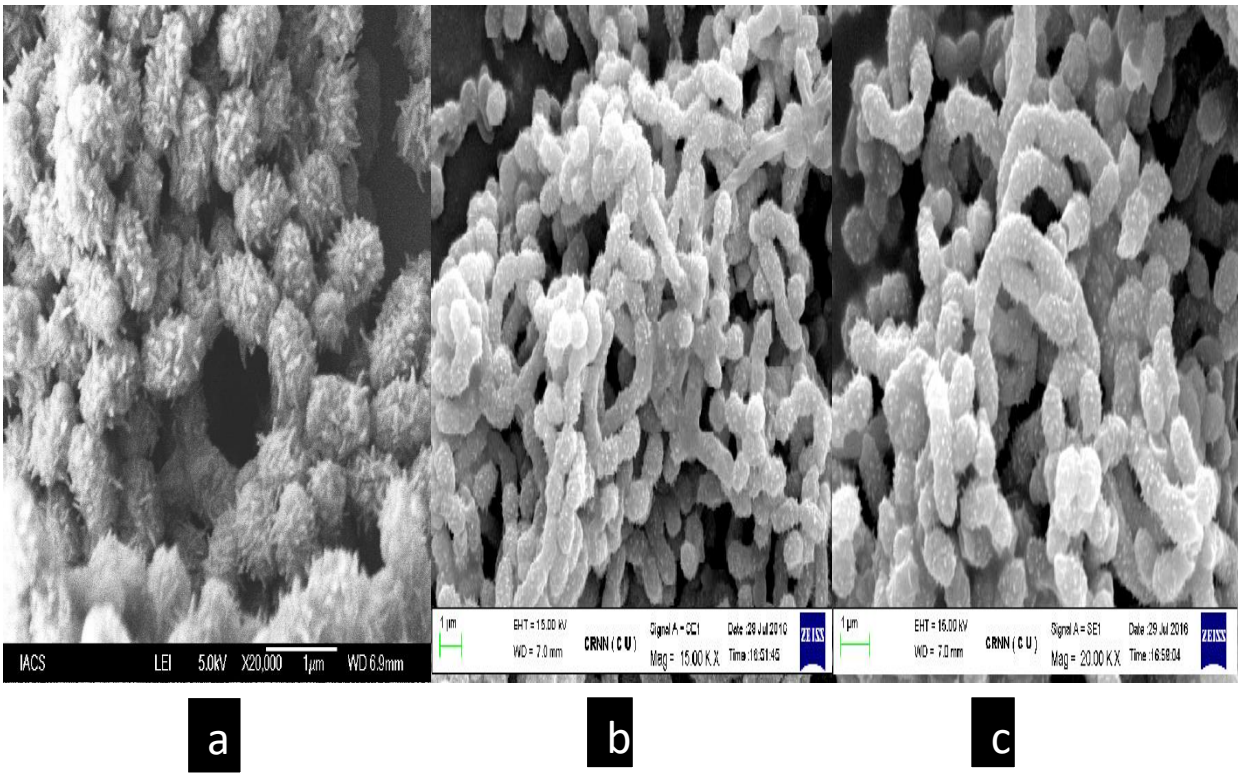


Figure 4.6. Scanning electron microscopic image[a) at high magnification, b-c) at low magnification] of strain MS 3/20 after growth on International *Streptomyces* Project 2 medium at 28 °C for 14 days

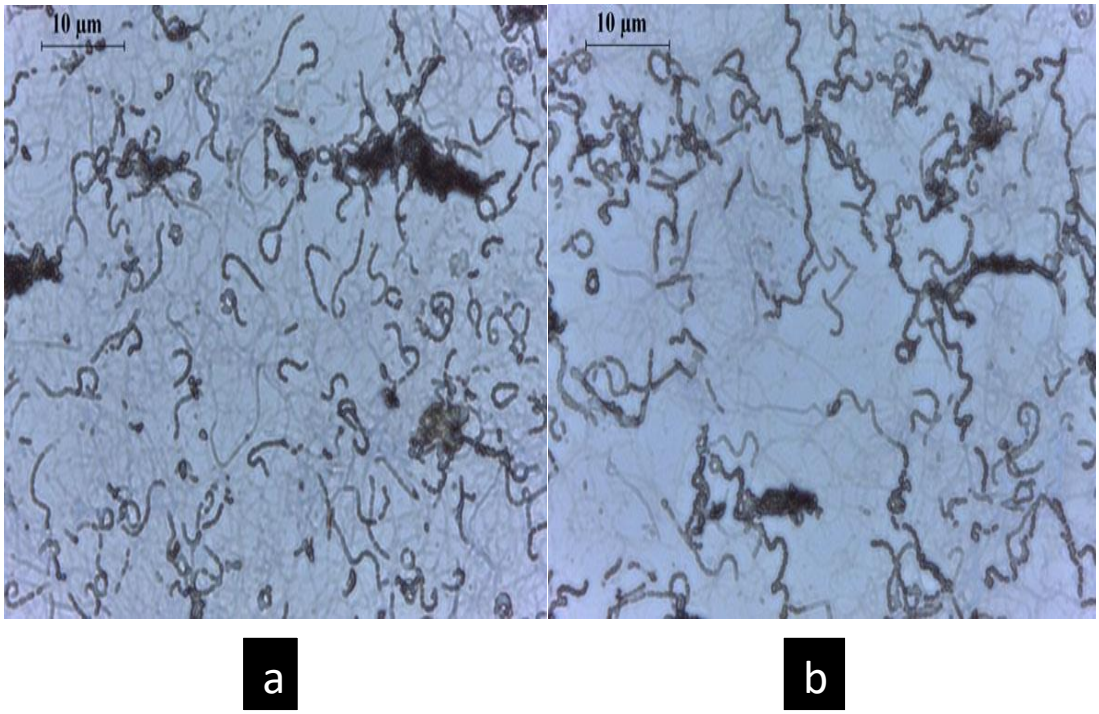


Figure 4.7. Image of strain MS 3/20 captured on light microscope (under oil immersion) after growth on International *Streptomyces* Project 2 medium at 28°C for 14 days.

4.10.2. Growth of strain MS 3/20 and its phylogenetically close relatives in different International *Streptomyces* Project (ISP) media.

The spore chain of MS 3/20 was a retinaculum-apertum, which was distinct from the spore chains of related species (spiral to flexuous) showing 100% 16S rRNA gene sequence similarity. The spore surface of MS 3/20 was spiny, which was common to *S. variabilis* and *S. griseoincarnatus* but different from *S. erythrogriseus* and *S. labedae.* Aerial mass colors of MS 3/20 grown on ISP 2, 3, 4 and 5 media were distinct from related species which are showing 100% 16S rRNA gene sequence similarities (Table 4.3.). The aerial mass color of MS 3/20 grown on ISP 6 medium was similar to *S. griseoincarnatus* but different from the other three closely related species. The substrate mycelium colors of MS 3/20 grown on ISP 2 and 3 media were similar to *S. erythrogriseus* but different from the other three species with 100% 16S rRNA gene sequence similarities. Substrate mycelium colors of MS 3/20 grown on ISP 4, 5 and 6 media were distinct from the four closely related species (Table 4.3.).

Table 4.3. Comparison of growth pattern of strain MS 3/20 and its phylogenetically closest strains in different International Streptomyces Project (ISP) media. **1** MS 3/20; **2** *Streptomyces variabilis* NBRC 12825^T; **3** *Streptomyces erythrogriseus* LMG 19406^T; **4** *Streptomyces griseoincarnatus* LMG 19316^T; **5** *Streptomyces labedae* NBRC 15864^T; **6** *Streptomyces griseorubens* NBRC 12780^T; **7** *Streptomyces althioticus* NRRL B-3981^T; **8** *Streptomyces griseoflavus* LMG 19344^T; **9** *Streptomyces matensis* NBRC 12889^T; **10** *Streptomyces viridochromogenes* NBRC 3113^T; **11** *Streptomyces albogriseolus* NRRL B-1305^T; **12** *Streptomyces paradoxus* NBRC 14887^T

ISP Medium	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
ISP 2												
Aerial mass color	Yellowish brown to red	Gray to yellow	Gray to yellow	Red	Gray to yellow	Gray to yellow	Yellow	Yellowish brown to gray	Gray	Gray to yellow	Yellow to brown	Brown to red
Substrate myceliu m color	Yellow to brown	Brown	Yellow to brown	Brown to red	Brown to blue	Gray	Yellow to brown	Yellow to blue	Brown	Brown to blue	Brown	Brown
ISP 3												
Aerial mass color	Gray to brown	Gray	Gray to yellow	Brown to red	Gray	Gray to yellow	Gray	Gray	Gray to yellow	Yellow	Gray	Gray

Substrate myceliu m color	Yellow to brown	Yellow to gray	Yellow to brown	Gray to red	Brown to red	Gray	Gray to yellow	Yellow to pink	Gray to brown	Gray to yellow	Gray to yellow	Gray
ISP 4												
Aerial mass color	Yellow to brown	Yellow to gray	Yellow	Brown	Yellow to gray	Gray to yellow	Gray to yellow	Gray	Yellow to gray	Yellow	Gray to yellow	Yellow to brown
Substrate myceliu m color	Gray to brown	Brown	Brown to pink	Gray to yellow	Yellow to brown	Gray	Gray to yellow	Yellow to brown	Brown	Brown	Gray to yellow	Yellow to brown
ISP 5												
Aerial mass color	Yellow to brown	Gray to yellow	Gray to brown	Brown to red	Gray to brown	Yellow to brown	Yellow to blue	Gray to brown	Brown to blue	Yellow to blue	Gray	Brown
Substrate myceliu m color	Yellowish brown to blue	Brown	Gray to brown	Red	Gray to brown	Gray	Gray	Brown	Gray	Yellow	Gray to yellow	Yellow
ISP 6												
Aerial mass color	Yellow to brown	Gray	Brown	Yellow to brown	Brown to gray	Gray to yellow	Gray	Yellow to brown	Yellow to brown	Yellow	Yellow to brown	Brown to blue

Substrate myceliu m color	Brown to red	Gray to blue	Gray to brown	Red	Brown to blue	Gray	Yellow	Yellow to brown	Yellow	Brown	Brown	Brown

4.10.3. Physiological and biochemical test

The strain showed positive activity for oxidase, catalase, urea decomposition, starch and esculin degradation. The strain showed negative results for melanin production, gelatin liquefaction, nitrate reduction, hydrogen sulfide production and L-tyrosine degradation. Resistance to penicillin and susceptibility to ampicillin, neomycin and polymixin B were observed for the strain MS 3/20. The strain demonstrated to utilize arabinose, sucrose, lactose and sodium citrate as sole carbon sources whereas it failed to utilize sorbitol and sodium acetate. The strain as sole amino acid sources utilized tyrosine, valine, tryptophan, leucine, isoleucine and glutamine. It showed growth at temperatures between 24 to 35 °C and between pH 5.0 to 9.0. The strain can grow in presence of 20% sodium chloride concentration.

The strain MS 3/20^{T} displayed 29 differentiating characteristics morphologically, physiologically and biochemically when compared with eleven phylogenetically closest relatives (Table 4.4.). It exhibited a minimum of 11 distinguishing characteristics with respect to *S. griseoflavus* NRRL B-5312^{T}and a maximum of 18 differentiating characteristics in respect to *S. erythrogriseus* NRRL B-3808^{T} in total out of the 29 characteristics. Strain MS 3/20^{T} exhibited a very crucial disparity that it can grow in media containing 0 to 20% of NaCl whereas other type strains failed to show the property.

Table 4.4. Comparison of different phenotypic characteristics of strain MS 3/20 and its phylogenetically closest strains of genus *Streptomyces.*Strains: **1** MS 3/20; **2** *Streptomyces variabilis* NBRC 12825^{T}; **3** *Streptomyces erythrogriseus* LMG 19406^{T}; **4** *Streptomyces griseoincarnatus* LMG 19316^{T}; **5** *Streptomyces labedae* NBRC 15864^{T}; **6** *Streptomyces griseorubens* NBRC 12780^{T}; **7** *Streptomyces althioticus* NRRL B-3981^{T}; **8** *Streptomyces griseoflavus* LMG 19344^{T}; **9** *Streptomyces matensis* NBRC 12889^{T}; **10** *Streptomyces viridochromogenes* NBRC 3113^{T}; **11** *Streptomyces albogriseolus* NRRL B-1305^{T}; **12** *Streptomyces paradoxus* NBRC 14887^{T}. **F**: flexuous; **S**: spiral; **R**: rectus; **RA**: retinaculum-apertum. In case of antibiotic: **R**: resistant; **S**: susceptible.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Spore chain	RA	F-S*	S*	F-S*	S*	S*	S*	S*	RA-S*	S-RA*	F*	F-RA*
Surface spore	Spiny	Spiny *	Warty *	Spiny *	Warty *	Spiny *	Spiny*	Spiny*	Spiny*	Smooth *	Warty *	Smooth *
Aerial mass color on ISP 2 medium	Yellowish brown to red	Gray to yellow	Gray to yellow	Red	Gray to yellow	Gray to yello w	Yello w	Yellowish brown to gray	Gray	Gray to yellow	Yello w to brown	Brown to red
Substrate mycelium color on ISP 2 medium	Yellow to brown	Brown	Yello w to brown	Brow n to red	Brown to blue	Gray	Yello w to brown	Yellow to blue	Brown	Brown to blue	Brown	Brown
pH range for growth	5-9	5-10	5-10	5-10	5-9	5-10	6-10	5-10	5-10	5-10	6-10	5-10
Growth temperature range (°C)	24-35	24-37	25-40	22-37	25-37	25-40	25-37	24-37	24-37	25-37	24-40	24-37

Growth in NaCl concentration range (%)	0-20	0-12	0-9	0-9	0-11	0-10	0-9	0-10	0-10	0-10	0-12	0-8
Growth on sole carbon source												
Arabinose	+	-	-	-	-	-	+	+	-	-	-	+
Sucrose	+	+	-	+	+	-	+	+	+	+	+	-
Sodium acetate	-	+	+	-	+	-	+	-	+	+	-	-
Sodium citrate	+	+	-	-	-	+	+	+	-	-	+	-
Lactose	+	+	+	-	+	-	-	-	-	+	+	-
Sorbitol	-	+	-	+	+	-	-	-	-	-	-	+
Growth on sole amino acid as nitrogen source												
Tyrosine	+	+	-	-	+	+	-	+	+	+	-	+
Isoleucine	+	+	-	-	+	+	+	+	+	+	-	+

Glutamine	+	+	-	-	+	+	-	+	+	+	-	+
Tryptophan	+	+	+	+	-	+	-	+	+	+	-	+
Valine	+	+	-	-	+	+	-	+	+	+	+	+
Leucine	+	-	+	+	+	+	-	+	+	+	-	-
Antibiotic resistance/susceptibility												
Penicillin (10 units)	R	R	R	S	R	R	R	R	R	R	R	R
Ampicillin (10 µg)	S	R	R	R	R	R	R	R	R	R	R	R
Polymixin B (300 units)	S	S	S	S	R	S	S	R	R	S	S	R
Neomycin (30 µg)	S	R	S	S	S	S	S	S	R	S	S	S

Catalase activity	+	-	-	+	+	-	+	+	+	+	+	+
Oxidase activity	+	-	-	-	-	-	-	-	-	+	-	-
Nitrate reduction	-	-	+	-	+	+	+	+	+	+	+	-
Urea decomposition	+	-	-	+	-	-	+	+	-	-	-	-
Gelatin liquefaction	-	-	-	+	-	-	-	-	-	-	-	-
Melanin production	-	-	-	-	-	-	-	-	+	-	-	-

*Data from Kämpfer et al. (2012).

4.11. Chemotaxonomic analysis

LL-diaminopimelic acid (LL-DAP) was present in the cell wall of the strain. Whole cell hydrolysate showed presence of galactose. Polar lipids present in the strain was glycolipid (GL), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), unidentified phospholipid (UPL) and unidentified amino lipid (UAL) (Figure 4.8.). Kim et al (2003) stated that major menaquinones in genus Streptomyces are MK-9 (H_8) and MK-9 (H_6). The strain was showed presence MK-9(H_8): 52.3% and MK-9(H_6): 35.6% as predominant menaquinones along with MK-9(H_4): 8.5% and MK-9(H_2): 3.6%. The predominant cellular fatty acids present in the strain were iso-C16:0 (23.89%), anteiso-C15:0 (17.53%) and anteiso-C17:0 (10.29%) (Table 4.5). Furthermore, the cellular fatty acid profile of MS 3/20 was distinct from that of its phylogenetically close relatives. These three fatty acids were not present concomitantly in any of the four close relatives as principal fatty acids. Anteiso-$C_{17:1}$ was found in MS 3/20 (3.28%) but not in any of the close relatives showing 100% 16S rRNA gene sequence similarity (Table 4.5). The G+C content of strain was determined as 77.3 mol%. These Chemotaxonomic data indicates strain MS 3/20 as a member of the genus *Streptomyces*.

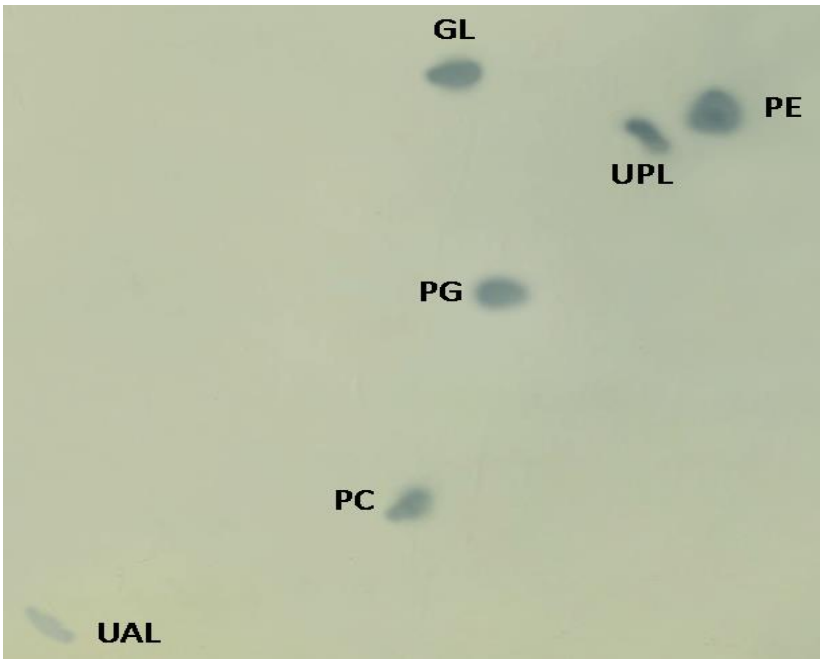


Figure 4.8. Image of polar lipids present in the strain MS 3/20. GL:glycolipid, PG:phosphatidylglycerol, PE:phosphatidylethanolamine, PC:phosphatidylcholine, UPL:unidentified phospholipid, UAL:unidentified amino lipid.

Table 4.5. Fatty acid profile. Numbers indicate content in percent (%). **1** MS 3/20; **2** *Streptomyces variabilis* NRRL B-3984^T; **3** *Streptomyces erythrogriseus* NRRL B-3808^T; **4** *Streptomyces griseoincarnatus* NRRL B-5313^T; **5** *Streptomyces labedae* NRRL B-5616^T. Three major fatty acids of each organism are shown in bold.

Fatty acids	**1**	**2**	**3**	**4**	**5**
$C_{9:0}$	0.73	-	-	-	-
$C_{9:0}$ 3OH	-	7.56	-	-	-
$C_{10:0}$ 3OH	-	**9.76**	-	0.90	-
$C_{10:0}$	-	-	-	1.14	-
iso $C_{10:0}$	-	-	-	0.82	-
anteiso $C_{11:0}$	0.70	-	-	1.25	-
$C_{11:0}$	-	8.05	1.43	-	-
iso $C_{12:0}$	0.84	-	2.38	-	-
iso $C_{13:0}$	0.76	-	3.60	0.96	4.27
anteiso $C_{13:0}$	0.64	-	3.17	1.92	-
iso $C_{13:0}$ 3OH	-	-	1.24	-	-
$C_{12:0}$ 2OH	0.42	2.31	-	-	-

$C_{12:1}$ 3OH	0.25	-	-	-	-
iso $C_{14:0}$	3.79	4.14	**10.46**	2.66	7.87
$C_{14:0}$	0.70	5.35	3.49	0.57	0.44
anteiso $C_{14:0}$	-	-	1.54	-	-
iso $C_{15:0}$	6.23	3.18	**14.39**	**8.69**	**24.62**
iso $C_{15:1}$ G	-	-	-	0.73	-
iso $C_{15:1}$ F	0.52	-	-	-	1.57
anteiso $C_{15:0}$	**17.53**	**8.91**	6.28	**13.82**	**19.01**
$C_{15:1}$ B	0.50	-	-	0.34	-
$C_{15:0}$	0.81	-	1.08	1.60	-
$C_{15:1}$cis 10	-	-	1.91	-	-
iso $C_{16:1}$ H	5.46	-	-		0.88
iso $C_{16:0}$	**23.89**	6.03	**11.67**	**18.84**	**19.74**
iso $C_{16:0}$ H	-	-	-	6.39	-
iso $C_{16:1}$ E	-	-	0.77	-	-
anteiso $C_{16:0}$	0.36	-	1.27	-	-

$C_{16:1}$cis 9	5.46	6.09	-	5.97	1.77
$C_{16:0}$	6.22	**17.10**	5.19	6.06	6.40
iso $C_{15:0}$ 3 0H	0.55	-	-	-	-
$C_{16:0}$ 9 methyl	2.77	-	-	5.02	-
$C_{16:0}$ methyl	-	-	-	-	-
anteiso $C_{17:1}$	3.28	-	-	-	-
iso $C_{17:1}$ E	-	-	0.99	-	-
iso $C_{17:1}$ H	-	-	1.99	-	-
anteiso $C_{17:0}$	**10.29**	3.88	1.67	7.87	4.87
anteiso $C_{17:1}$ A	-	-	0.86	-	-
anteiso $C_{17:1}$ C	3.28	-	-	4.54	-
iso $C_{17:0}$	2.27	2.21	2.82	3.50	3.69
$C_{17:1}$cis 9	0.66	-	1.09	1.82	1.10
$C_{17:0}$cyclo	1.59	2.91	0.81	1.31	0.97
$C_{17:0}$	0.53	-	0.93	1.12	1.13
iso $C_{18:0}$	-	-	0.61	0.61	-

$C_{17:0}$ 10 methyl	0.35	-	-	-	-
$C_{18:0}$	-	-	0.88	-	-
$C_{18:1}$cis 9	0.21	-	1.92	0.39	0.50

4.12. Conclusion

16S rRNA gene sequence analysis of strain MS 3/20 was showed 100% sequence similarity with four *Streptomyces* strain and more than 99% similarity with other seven strains. DNA-DNA relatedness study, RAPD banding patter and MALDI TOF/MS study of strain MS 3/20 were showed clear difference with phylogenetically closely related strains. In addition to it several morphological, physiological and chemotaxonomic properties of strain revealed significant disparities with others phylogenetically close relatives. The major disparities strain showed is tolerance to sodium chloride (0-20%) whereas other related strains can't tolerate up to 20% of sodium chloride concentration. These results demonstrated that strain MS $3/20^{T}$ is a novel species and it is named as *Streptomyces euryhalinus* sp.nov.

Description of *Streptomyces euryhalinus* sp. nov.

Streptomyces euryhalinus (eu.ry.ha'linus Gr. adj. *eurus*, broad; Gr. n. *halos*, salt; L. suff.-*inus*suffix implying sense of belonging to; N.L. masc. adj. *euryhalinus*, living in a broad range of salinity).

Strain MS 3/20 is a Gram-positive, aerobic, non-motile actinomycetes. It forms long chain spiny spores and present in a retinaculum-apertum arrangement. It grows well on ISP 2, ISP 3, ISP 4, ISP 5 and ISP 6 media. On ISP 2 medium it produces yellowish brown to red color aerial mycelium and on IPS 3 medium gray to brown. In case of ISP 4, ISP 5 and ISP 6 media aerial mycelium color is yellow to brown. It produces yellow to brown color Substrate mycelium on ISP 2 and ISP 3 media. Strain produces

gray to brown substrate mycelium on ISP 4 medium, on ISP 5 yellow-brown to blue medium and on ISP 6 medium brown to red. It is not produces any melanin and diffusible pigment in any ISP medium. Cell wall of the strain contains LL-DAP and galactose as whole cell hydrolysate. Polar lipids identifiedin strain are phosphatidylglycerol (PG), glycolipid (GL), phosphatidylcholine (PC), phosphatidylethanolamine (PE), unidentified phospholipid (UPL) and unidentified amino lipid (UAL). Predominant fatty acids present are anteiso-C15:0, iso-C16:0 and anteiso-C17:0.Strain MS 3/20^{T} is positive for oxidase, catalase, urea decomposition, starch and esculin degradation. It shows negative results for gelatin liquefaction nitrate reduction, hydrogen sulfide production and L-tyrosine degradation. Strain is resistant to penicillin (10 units) and susceptible to ampicillin (10 µg), gentamicin (30 µg), streptomycin (10 µg), tetracycline (30 µg), vancomycin (30 µg), polymixin B (300 units), chloramphenicol (30 µg), kanamycin (30 µg), carbenicillin (100 µg), neomycin (30 µg), lincomycin (15 µg), andoleandomycin (15 µg). It utilizes sucrose, lactose, starch, mannose, arabinose, galactose, maltose, xylose, inositol, cellobiose, trehalose, mannitol and sodium citrate as sole carbon source, but fails to utilize sorbitol, sodium succinate and sodium acetate. Strain also utilizes alanine, leucine, isoleucine, lysine, valine, tryptophan, arginine, asparagine, histidine, tyrosine, glutamine,methionine, proline, threonine and glutamine as sole amino acid source but not utilizescysteine. It can tolerate0 to 20% NaCl (optimum 5%). It grows at pH range between 5.0 to 9.0 (optimum 7.2 to 7.5) and at temperature range between 24 to 35 ºC (optimum 28 ºC)

The type strain MS 3/20^{T} (=CICC 11032^{T} =DSM 103378^{T}) was originally isolated from sediments of Lothian Island of *Sundarbans* mangrove forest, India and the DNA G+C content of the type strain determined is 77.3 mol%.

4.13. References

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CHAPTER 5:

EXPLORATION OF METABOLITE PRODUCTION OF STRAIN MS 3/ 20^{T} AND BIOLOGICAL ACTIVITIES

5.1. Introduction

Mangrove ecology is the producer of several new antimicrobials and antibiotics. New actinobacteria isolated and identified from the ecology of underexplored Sundarbans mangrove forest could be the producer of new metabolites. Exploration of new ecology or environment is important not only for the exploration of microorganisms but also for the exploration of the potential metabolites present in that ecology. So the present research demands for the transcendence of the microbial diversity into the metabolite diversity which will be helpful for the discovery of new secondary metabolites. In this context, the present chapter explored the metabolite production and profile of the new actinobacterium strain MS 3/20^{T} and biological activities like antimicrobial property is investigated extensively.

5.2. Materials and methods

5.2.1. Fermentation and extraction

Cultivation of the strain MS 3/20^{T} for the production of the antimicrobial compounds was carried out into the modified production medium (MPM) (all units g /L-starch 2.0, glucose 2.0, soybean meal 2.0, yeast extract 0.5, NaCl0.25, CaCO3 0.32, CuSO4 0.005, MnCl2 0.005, ZnSO4 0.005, pH7.2, distilled water 1,000 ml.) as described by Saha et al (2005) earlier using conical flask. The cultivation was conducted after incubation at 30 °C for 72-96 hours at 120 revolutions per minute. The cultivated broth was filtrated and extraction of filtrated broth was done thrice using an equal volume of ethyl acetate

as a solvent. The extracted portion were concentrated under rotary vacuum evaporator at 45 °C to produce a crude extract

5.3. GC-MS analysis of the extract

Gas chromatography (GC) analysis was carried out using Perkin Elmer Clarus SQ8C gas chromatography equipped with DB 5 MS capillary standard nonpolar column (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The injector temperature was set at 250 °C and the oven temperature was initially set at70 °C. Helium was used as a carrier gas with a flow rate of1mL/min. One microliter of the sample was injected in the split mode in the ratio of 1:12.The mass spectrometer was operated in the electron impact mode at positive (+VE) Ion source. The mass spectra were obtained by centroid scan of the mass range from 40 to 650 amu. The peaks obtained were identified by NIST library data.

5.4. Microorganisms and culture conditions

Staphylococcus aureus MTCC 2940, *Escherichia coli* MTCC-1195 and *Candida. albicans* MTCC 227 were obtained from the Institute of Microbial Technology, Chandigarh, Punjab, India. The bacterial strains were maintained on agar slant after culturing at nutrient agar and the fungal strain was maintained on agar slant of sarbound dextrose broth. These slants were placed at 4^{o}C freezer and periodically subcultured.

5.4.1. Antimicrobial activity by agar well diffusion method

Agar well diffusion method (Hriduyatulla et al. 2018) was used to evaluate the antimicrobial activity of the extract. The bacterial strains were inoculated on nutrient broth for overnight growth. The fungi were inoculated into sarbound dextrose broth and subjected to grow overnight. The exponential phase cultures of *S. aureus* MTCC 2940, *E. coli* MTCC-1195 and *C. albicans* MTCC 227 were used for the study and 20 µl of microbial culture spread over the nutrient agar or sarbound dextrose agar plate. Holes of 6 mm diameter were prepared on the plates and 30 µl test sample with different concentration (for bacteria 25 µg/ml, 50 µg/ml and for fungi 10 µg/ml, 25 µg/ ml, 50 µg/ ml) was introduced into the well. The plates were placed at 4 ^{o}C for 30 minutes for the diffusion of the test material. The plates were then kept overnight in an incubator at 37 ^{o}C and zone of clearance (in mm) was measured for the antimicrobial activity.

5.4.2. Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

Antimicrobial properties of extract of the strain MS 3/20^{T} were tested by micro broth dilution method using 96 well microtiter plates (Sarker et al. 2007, Andrews, 2001). The MIC was measured against *S. aureus* MTCC 2940, *E.coli* MTCC-1195 and *C. albicans* MTCC 227. The fresh cultures of microbes were grown on their respective culture media described earlier and density of the culture media was adjusted to 0.5 McFarland standards as recommended by the Clinical & Laboratory Standards Institute (CLSI) guideline. The bacterial strains were cultivated in Muller–Hinton Broth (MHB)

and the fungal strain were cultivated in sarbound dextrose broth (SDB), and then incubated overnight at 37°C. The microbial cultures were adjusted to the turbidity of 10^6 CFU per ml. 50 µl of MHB or SDB were placed into each well followed by 50 µl of extracts and 10 µl of microbial cultures were introduced to make a concentration of 5 X 10^5 CFU per ml. The plates then subjected to incubation at 37°C for overnight and resazurin (10 µl) was added. The color change was monitored visually and no color change was recorded as a MIC value. Then Minimum bactericidal concentration was recorded after seeding the cultures from the well to the nutrient agar plate by incubating the plates at 37°C for 24 hours. Minimum fungicidal concentration was recorded by spreading cultures from the well into the SDA plates and plates were incubated at 37°C for 24-48 hours. No colony growth in the plates was counted as the MBC or MFC of the samples (Li et al. 2014; Sundararaman et al. 2013).

5.5. Membrane permeability

5.5.1. Integrity of cell membrane

Cell membrane integrity of bacterial strains *S. aureus* MTCC 2940 and *E. coli* MTCC-1195 was observed by the release of internal material absorbing at 260 nm (Li et al. 2014). Bacterial cultures were centrifuged for 10 minutes at 8000 rpm, washed twice and dissolved with 0.5% NaCl. To the cell suspension 1/2 MIC, MIC and 2 MIC of extracts were added and internal material release absorbing at 260 nm was recorded using UV-Visible spectrophotometer (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA) over the time period. Cell suspensions without test samples were counted as a control.

5.5.2. Crystal violet assay

Crystal violet assay (Li et al. 2014) investigated to evaluate the membrane permeability alternation in *S. aureus* MTCC 2940 and *E. coli* MTCC-1195. The bacterial strains are allowed to grow in nutrient broth and cell mass was collected by centrifugation at 9,300g for 5 minutes. The cell mass was washed and resuspended in 0.5% NaCl solution. Test samples were added to the cell suspension at a concentration of 1/2 MIC, MIC, 2 MIC and incubated at 37 ˚ C for 6 hours and cells were collected again by centrifugation at 9300g for 5 minutes. The cells are again resuspended in 0.5 % NaCl solution containing crystal violet (10 µg/ ml) and incubated for 10 minutes at 37 ˚ C. The cells were centrifuged at 13400 g for 15 minutes and optical density was determined at OD_{590} using UV-Visible spectrophotometer (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA). Crystal violet uptake percentage of samples was calculated using the formula:

(OD value of the sample)/ (OD value of crystal violet solution) X 100.

5.5.3. Inner membrane permeability assay

Inner membrane permeabilization was evaluated by determining the release of β-galactosidase activity of *S. aureus* MTCC 2940 and *E. coli* MTCC-1195 into the culture medium supplemented with ONPG as substrate (Ibrahim et al. 2000). Logarithmic phase culture grown in LB supplemented with 2% lactose was centrifuged at 11000 g for 10 minutes were collected, washed and resuspended in the 0.5% NaCl solution. 200 µl of cell suspension (adjusted to 1.2 absorbance at 420 nm) was taken into 96 well

plate followed by 10 µl of ONPG and samples of different concentration (1/2 MIC, MIC, 2 MIC). After incubation at 37 ^{o}C, inner membrane permeability was examined by measuring the release of o-nitrophenol by means of increasing absorbance at 415 nm using a spectrophotometer (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA). Bacterial culture with ONPG was counted as a control.

5.6. Inhibition of biofilm formation assay

The Antibiofilm formation assay of extract at different concentration was carried out in 96 well microtiter plate. The overnight grown cultures of *S. aureus* MTCC 2940 and *E. coli* MTCC-1195 was introduced in the 96 well microtiter plate along with Luria broth and different concentrations (1/2 MIC, MIC, 2 MIC) of extracts, and incubated statically at 37 ^{o}C for 24 h. Then the planktonic cells and the excess broth was discarded, washed with PBS (7.2 pH) and dried at 60 ^{o}C for 30 minutes. The biofilm formed in the wells were stained with 1% crystal violet for 15 minutes, washed with distilled water and 200 µl of ethanol was added to re-solubilize the crystal violet bounded to the biofilm formed and the absorbance was recorded at 595 nm using microplate reader (SpectraMax M5, Molecular Device, USA) (Wu et al. 2015, Wu et al. 2016).

5.7. Hemolysis assay

Hemolytic toxicity or hemolysis of the extract was examined by using murine erythrocyte. The murine blood of about 10 ml was centrifuged at 2,500 rpm for 5 minutes and pellets were washed thrice with Phosphate buffered saline (PBS). A 3% suspension of erythrocyte was prepared with PBS buffer. A fresh culture of *E.coli* MTCC-1195 was centrifuged and redissolved in saline solution. Different concentrations (1/2 MIC, MIC, and 2 MIC) of extracts were added to the mixture of erythrocyte suspension and cell culture. The absorbance of the supernatant, obtained after centrifugation of mixture at 2,500 rpm for 5 minutes, was recorded spectrophotometrically (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA) at 540 nm for determination of release of hemoglobin after incubation at 37°C for 45 minutes (Lee et al, 2017). As positive and negative controls, distilled water and normal saline were counted respectively.

5.8. Secreted aspartyl proteinase (Sap) inhibition

Inhibition of Candidal Sap activity of extract was determined by the protocol of Sundararaman et al. (2013). 100 µl of *C. albicans* culture was added to the 20 ml of Sap induction medium (23.4 g yeast carbon base, 2.0 g yeast extract, and 4.0 g BSA made up to 1 l in distilled H2O, pH 5.0) containing different concentrations of extract (1/2 MIC, MIC, 2 MIC) and incubated for 48 h at 37 ^{o}C with shaking.. The proteinase inhibition was evaluated by adding 0.1 ml culture supernatant to 0.9 ml of 0.1 M citrate buffer (pH 3.2) containing 0.2% (w/v) BSA, and incubated at 37 ^{o}C for 1 h. The

reaction was terminated by introducing 1 ml of 5% (w/v) ice-cold TCA. After centrifugation, the absorbance of the supernatant was recorded at 280 nm on a UV Visible Spectrophotometer (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA). Distilled water was considered as a control.

5.9.1. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

The antioxidant activity potential of extract was evaluated based on the scavenging activity of the stable DPPH free radical (Barca et al, 2001). To the 3.0 mL of methanolic (0.004% methanolic solution) solution of DPPH, 50µL of extract of different concentrations (25-100 µg/ml) were added. After 30 minutes of incubation absorbance at 517 nm was determined spectrophotometrically (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA), and the percentage inhibition activity was estimated. IC50 value (50% scavenge of DPPH free radicals) was calculated by plotting the percentage of free radicals scavenged versus the antioxidant concentration.

5.9.2. Measurements of reductive ability

Extract of different concentration (25-100 µg/ ml) were mixed with 3.5 ml phosphate buffer (0.2M; pH 6.6) and $K_3Fe(CN)_6$ (1.0%, w/v) solution, and incubated for 20 minutes at 50^{o}C. To the mixture, 2.5 ml of trichloroacetic acid (TCA) was added and centrifuged for 10 minutes at 3000 rpm. Then 0.5 ml of $FeCl_3$ solution (0.1%, w/v) was added to the supernatant and absorbance was taken at 700nm (Oyaizu et al 1986).

5.10. Anti-inflammatory activity by membrane stabilization method

Human red blood cell (HRBC) was taken into Alsever solution from a healthy volunteer and centrifuged at 2,500 rpm for 10 minutes. The packed cell was washed with sterile saline solution (0.9% NaCl solution) and a 10% v/v suspension was prepared with a saline solution. Test extract samples were added to the 1 ml of phosphate buffer, 2 ml of hyposaline solution and 0.5 ml of HRBC suspension. This mixture incubated at 37^{o}C for 30 minutes and centrifuged for 20 minutes at 3000 rpm. Finally, the absorbance of supernatant measured at 560 nm spectrophotometrically (Lambda 25 UV/visible spectrophotometer; PerkinElmer, USA), and the percentage of inhibition of hemolysis was calculated (Parvin et al. 2015). Diclofenac sodium used as the standard drug and solution without test sample counted as a control.

5.11. Statistical analysis:

The experiments are conducted thrice and results were showed as mean ±SD. Microsoft office excel (version 2007) was used for the data analysis.

5.12. Results and Discussion

5.12.1. GCMS profile

The GCMS profile of ethyl acetate extract recorded to explore the presence of biologically potential and important compounds. The profile (Table.5.1) showed the presence of several components but based on percentage (%) area few of them are reported here. Among these 2,4-Di-tert-butylphenol, phenanthrene, 7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione, benzenepropanoic acid 3,5-bis (1,1-

dimethylethyl)-4-hydroxy- methyl ester , heptadecanoic acid, 16-methyl-, methyl ester , 17-pentatriacontene , dibutyl phthalate , triclosan etc. are known compounds with antimicrobial, antibacterial, antifungal, antioxidant and Antibiofilm property (Padmavathia et al. 2015,Varsha et al. 2015, Kovács et al. 2008). Molecular weight, molecular formula, retention time are documented along with name of the compounds present in the extract.

Table 5.1.Bioactive compounds identified in the ethyl acetate extract using GCMS analysis.

Retention Time	**Name of the Compounds**	**Theoretical Formula**	**Molecular Weight**(g/mol)
11.987	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.329
15.949	2-Propenoic acid, tridecyl ester	$C_{16}H_{30}O_2$	254.414
17.644	Dibenzo[b,E][1,4]dioxin, 2,7-dichloro-	$C_{12}H_6Cl_2O_2$	253.078

17.804	Phenanthrene	$C_{14}H_{10}$	178.234
19.375	Phthalic acid, decyl isobutyl ester	$C_{22}H_{34}O_4$	362.51
19.695	8-Pentadecanone	C15H30O	226.404
20.240	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2, 8-dione	$C_{17}H_{24}O_3$	276.376
20.766	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	$C_{18}H_{28}O_3$	292.419 g/mol
21.271	Dibutyl phthalate	C16H22O4	278.348
21.736	Triclosan	$C_{12}H_7Cl_3O_2$	289.536

22.696	à-D-Galactopyranoside, methyl 2,6-bis-O-(trimethylsilyl)-, cyclic butylboronate	$C_{17}H_{37}BO_6Si_2$	404.457
23.817	Hexadecen-1-ol, trans-9-	$C_{16}H_{32}O$	240.431
24.607	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	298.511
26.138	17-Pentatriacontene	$C_{35}H_{70}$	490.945
28.659	[1,1'-Biphenyl]-2,3'-diol, 3,4',5,6'-tetrakis(1,1-dimethylethyl)-	$C_{28}H_{42}O_2$	410.632
29.259	Hexanedioic acid, dioctyl ester	$C_{22}H_{42}O_4$	370.574
31.325	Phthalic acid, di(2-propylpentyl) ester	$C_{24}H_{38}O_4$	390.564

5.12.2.1. MIC, MBC and MFC of the extract

The extract exhibited the significant zone of inhibition of 25 mm, 20 mm and 14 mm. (Table 5.2), respectively against *S. aureus* MTCC 2940, *E. coli* MTCC-1195 and *C. albicans* MTCC 227 in agar well diffusion method. The minimum inhibitory concentration of extract determined against *S. aureus* MTCC 2940, *E. coli* MTCC-1195 and *C. albicans* MTCC 227 are1 µg/ ml, 2 µg/ ml and 2 µg/ ml (Table 5.2). The minimum bactericidal concentration determined is 4 µg/ml and 8 µg/ml against *S. aureus* MTCC 2940 and *E. coli* MTCC-1195, respectively. The minimum fungicidal concentration is 6 µg/ml against *C. albicans* MTCC 227.

Table 5.2. MIC, MBC and MFC value of extract and standard drug determined against bacteria and fungi.

Name of the organisms	**Zone of inhibition showed by extract (mm)**	**MIC (µg/ml) of Extract**	**MBC (µg/ml)/ MFC (µg/ml) of Extract**
E. coli MTCC-1195	20	2	8
S. aureus MTCC 2940	25	1	4
C. albicans MTCC 227	14	2	6

5.12.2.2. Cell membrane integrity

The bacterial membrane damage could be measured by the release of UV absorbing materials like intracellular components. Upon treatment with extract on bacterial suspension showed the release of intracellular components which increased with the increment of concentration and for a certain period of time. The extract showed the highest activity of membrane damage at a concentration of 2 MIC against both the bacteria. In case of *E. coli*, at 2 MIC concentration, the OD value increased up to 0.412 from 0.081 whereas in MIC concentration it was increased up to 0.379. At a dose of 1/2 MIC, the OD increased up to 0.256 which is the lowest compared to the other two concentrations. Upon treatment with extract at a 2 MIC concentration on *S. aureus* the OD value increased from 0.194 to 0.496 whereas upon treatment with MIC the OD value increased up to 0.417. In case of gram-negative bacteria, the extract showed an increase of absorbance slowly and remains unchanged over a long period of time after 8 hours whereas in case of gram-positive bacteria extract exhibited the abrupt increase in absorbance (Figure 5.1, 5.2). This result refers to the interaction of the extract with the cytoplasmic membrane and alteration or damage of the membrane (Denyer, 1990). Due to the damage in the membrane the low molecular weight components like K^+, PO_4^- comes out first from the membrane followed by genetic materials like DNA, RNA etc.(Li et al. 2014). This rapid increase of OD value in case of Gram-positive bacteria is due to the difference in membrane composition and structure compared to the Gram-negative bacteria. It is well known that membrane of Gram-positive organisms is made

up of peptidoglycan layer with several pores through which the antimicrobial enters inside the cell and causes damage (Tao et al. 2011).

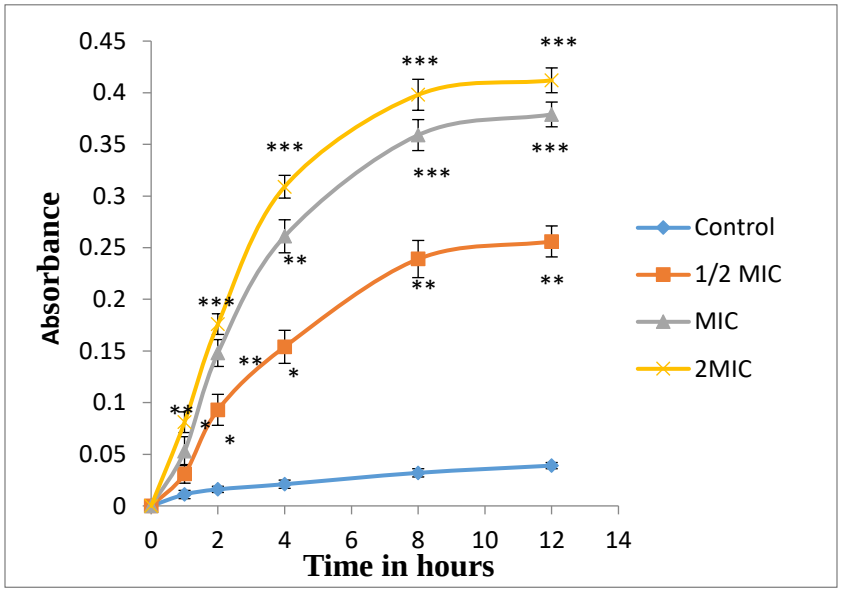

Figure 5.1.Effect of extract on cell membrane permeability of *E.coli* MTCC-1195. * p value <0 .05, ** p value < 0.005, *** p value < 0.001

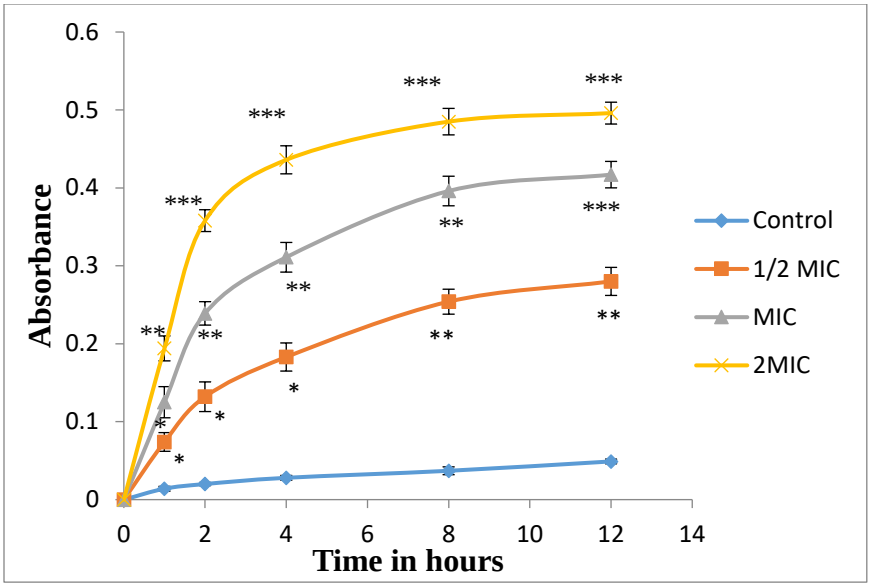

Figure 5.2.Effect of extract on cell membrane permeability of *S. aureus* MTCC 2940. * p value<0 05, ** p value< 0.005, *** p value < 0.001.

5.12.2.3. Crystal violet assay

Crystal violet assay facilitates to judge the membrane alternation in bacteria caused by the antimicrobial agents. Crystal violet penetrates the membrane of the bacteria poorly in normal condition but if the membrane is altered or damaged it penetrates easily. The extract showed an increase of crystal violet uptake from 18.59 % (in control) to 55.51 % and 74.49 % upon MIC and 2 MIC concentrations, respectively in the case of *E.coli* (Figure 5.3). The uptake of crystal violet in the case of *S.aureus* increased from 19.09 % (in control) to 63.19% and79.63 % after treatment with extract (Figure 5.3). This result indicates that the extract significantly damaged or altered the membrane of bacteria and increased the membrane permeability.

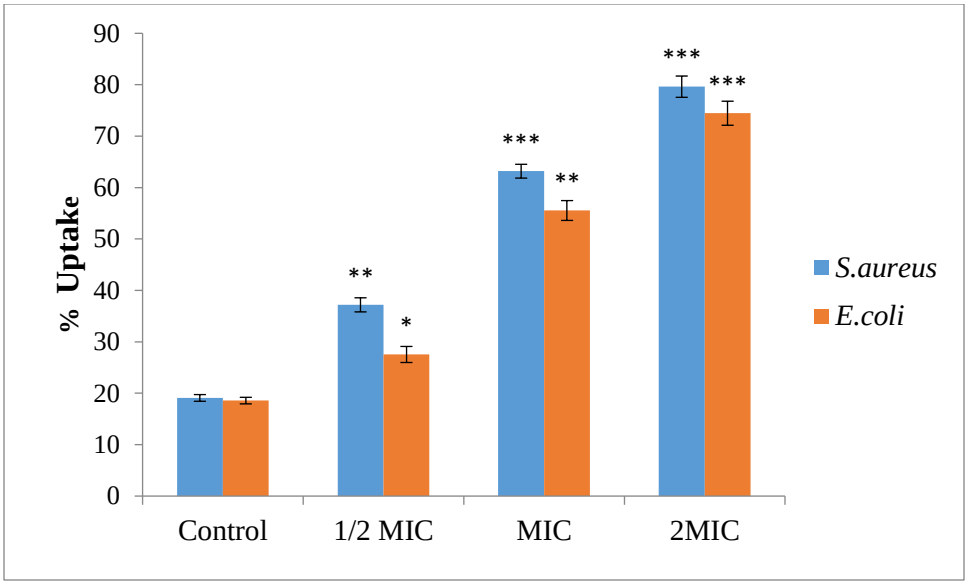

Figure 5.3. Crystal violet uptake of *S. aureus* MTCC 2940 *and E.coli* MTCC-1195 upon treatment of extract. * p value<0 .05, ** p value< 0.005, *** p value< 0.001.

5.12.2.4. Inner membrane permeability

The inner membrane permeability of the extract against gram-positive and gram-negative bacteria is evaluated in respect to release of cytoplasmic β-galactosidase, after growth of bacteria in lactose-containing medium. The release of cytoplasmic β-galactosidase was dose and time-dependent as showed in Figure 5.4 and 5.5. Upon treatment of cells at a dose of MIC and 2 MIC, the release of β-galactosidase was observed up to 80 minutes progressively to reach a steady state. 2MIC concentration showed the greater absorbance than MIC concentration which corresponding the greater inner membrane permeability effect of the extract. The inner membrane of the gram-negative bacteria comprised of phosphatidylglycerol and cardiolipin (Je and Kim, 2006). The release of cytoplasmic β- galactosidase is the result of a change in the integrity of the inner membrane as well as the outer membrane.

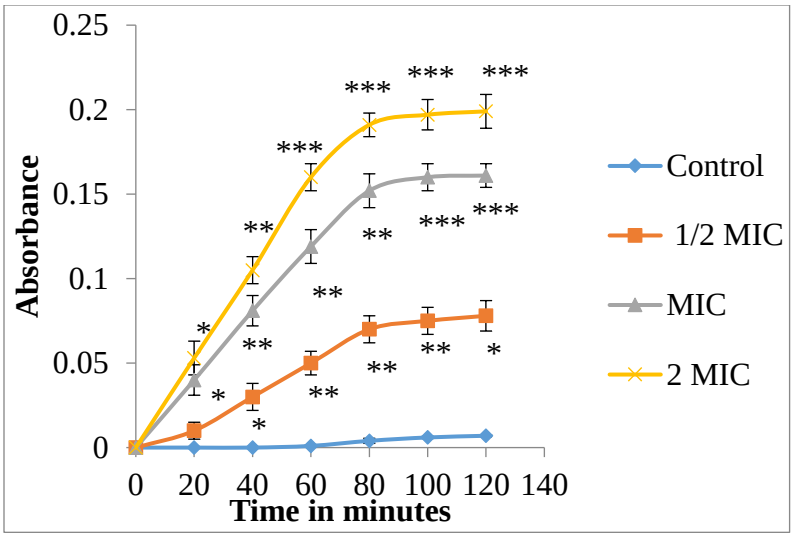

Figure 5.4. Inner-membrane permeability study determined through release of cytoplasmic β- galactosidase from *E. coli* MTCC-1195 after treatment with the extract. * p value<0 .05, ** p value< 0.005,*** p value< 0.001.

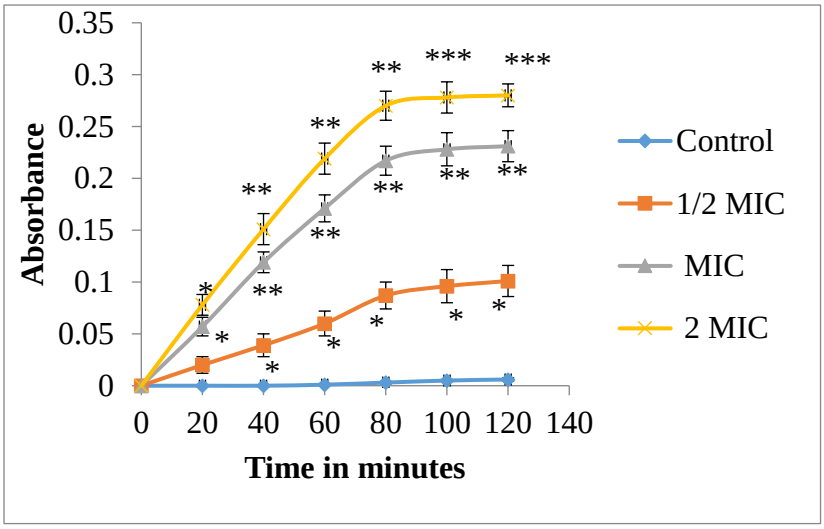

Figure 5.5. Inner-membrane permeability study determined through release of cytoplasmic β- galactosidase from *S. aureus* MTCC 2940 after treatment with the extract. * p value<0 .05, ** p value< 0.005,*** p value< 0.001.

5.12.3. Biofilm formation inhibition assay

Biofilm formed by the microbial communities are responsible for the antibiotic resistance (Michalska and Wolf, 2015; Simones et al. 2009). A significant fact is that approximately 80% of the human infections are related with biofilms (Ricucci and Siqueira, 2010). The extract of strain MS 3/20^{T} shown significant biofilm inhibition property in a dose-dependent manner. The extract at 2 MIC dose exhibited 78. 49 % (±0.037) inhibition against *S.aureus* while MIC concentration showed inhibition of 62.99% (±0.049) (Figure 5.7). In case of *E. coli*, extract showed inhibition of biofilm formation of 53.64% (±0.60) and 67.90% (± 0.027) at MIC and 2 MIC dose (Figure 5.6). This result indicates a considerable biofilm inhibition property of the extract against both Gram-positive and Gram-negative bacteria. Antibiofilm compound is very urgently needed to combat against pathogenicity of microbes and to prevent the antibiotic resistance.

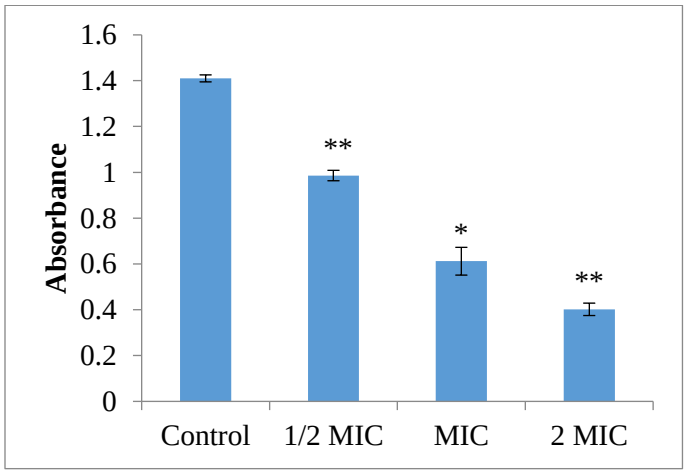

Figure 5.6.Effect of the extract on the biofilm formation by *E. coli* MTCC-1195 measured at 595 nm. *p<0 .005, **p<0.001.

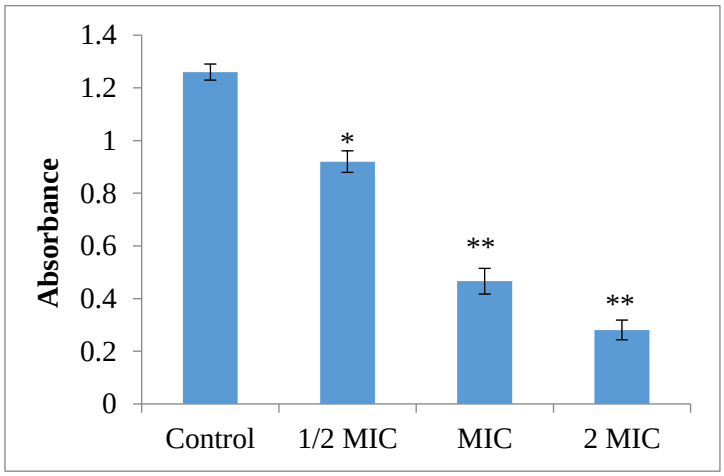

Figure 5.7. Effect of the extract on the biofilm formation by *S. aureus* MTCC 2940 measured at 595 nm. *p<0 .005, **p<0.001.

5.12.4. Hemolysis reduction activity of the extract

The extract exhibited a concentration dependent reduction of hemolysis against *S.aureus and E. coli.* At 2 MIC concentration in case of *S.aureus*, extract reduced hemolysis from 61.29% (±0.0009) to 18.28% (±0.0002) (Figure 5.8). In case of E. coli the reduction of hemolysis is from 68.88% (±0.002) to 19.6% (±0.001) at 2 MIC concentration (Figure 5.8). Hemolysis of erythrocyte caused due to the production of the hemolysin by the bacteria which is considered as a virulence factor and facilitating the infection (Kupferwasser et al. 2003). So the reduction of hemolysis by the extract could be attributed to its role in inhibition of the virulence factor.

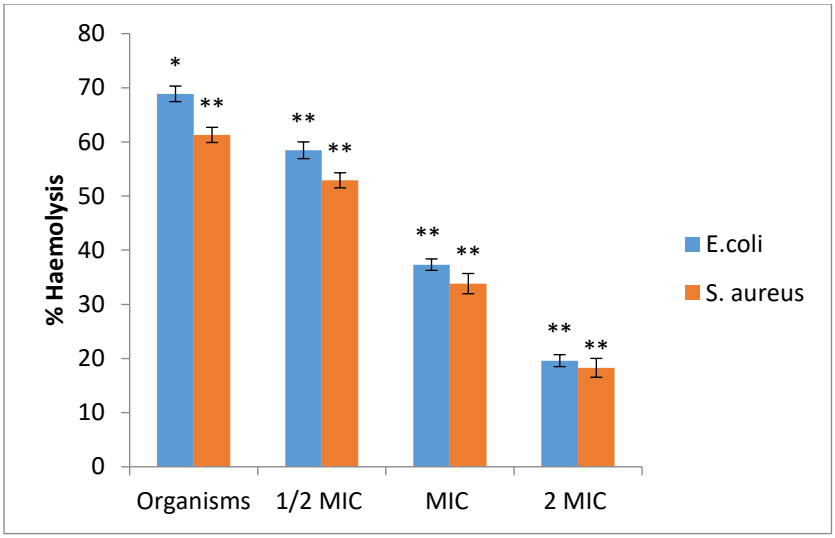

Figure 5.8.Effect of the extract on the hemolysis of erythrocyte (virulence factor) by *S. aureus* MTCC 2940 and *E. coli* MTCC-1195. * p value< 0.005, ** p value< 0.005.

5.12.5. Secreted aspartyl proteinase (Sap) inhibition

The inhibition effect of the extract on virulence factor of candida was evaluated against concentrations of 1/2 MIC, MIC, and 2 MIC (Figure 5.9). The extract showed the gradual decrease of Sap activity with increase of concentrations. The 2 MIC dose was showed the highest inhibition property in comparison with the other two concentrations but much less than standard drug. Proteinase activity of *C. albicans* is working as the virulence factor (MacDonald and Odds, 1983; Kwon-Chung et al. 1985). This activity attributed to the tissue invasion and damage capacity of the organism (Borg and Rüchel, 1988; Schaller et al. 1999).

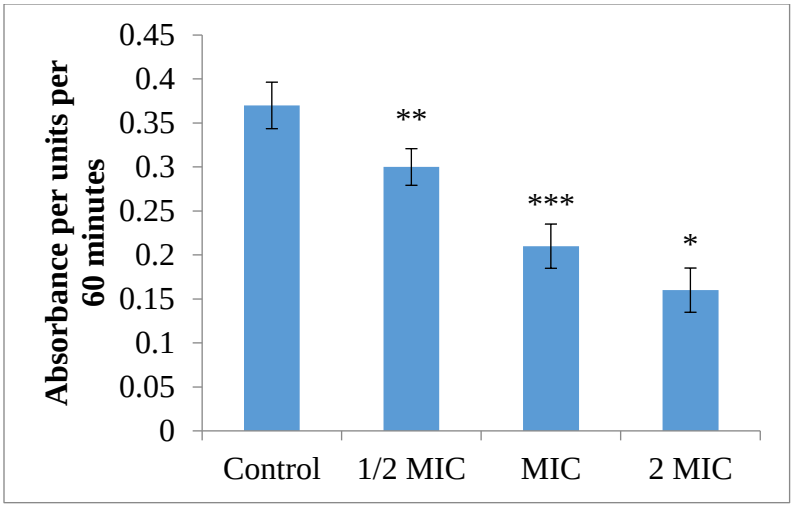

Figure 5.9. Effect of the extract on the secreted aspartyl proteinase (virulence factor) by *C. albicans* MTCC 227. * p value< 0 .05,** p value< 0.005, *** p value< 0.001.

5.12.6. Antioxidant activity

The extract was shown a significant antioxidant activity in a dose-dependent manner. The extract exhibited 82.22% (±0.68) inhibition in 100 µg/ml concentration whereas standard ascorbic acid exhibited 91.85% (±0.30) inhibition (Table 5.2). The IC_{50} value of the extract is 31.58 µg/ml which is higher than the standard ascorbic acid 7.08 µg/ml. The extract also demonstrated a significant reducibility potential in concentration-dependent manner. The 100 µg/ml concentration for both standard and extract exhibited increase of the absorbance which could be counted as reductive property the extract. The result is presented on the table 5.2.

5.12.7. Anti-inflammatory activity

The results of anti-inflammatory activity recorded by the HRBC membrane stabilization property was shown on table 5.3. The extract exhibited inhibition property in a concentration dependent manner. At concentration 200 µg/ml extract exhibited

73.19% (±2.08) inhibition which is less than 88.73% (±0.89) inhibition observed in standard Diclofenac sodium. This inhibition is significant as compared to the standard drug. The extract is effective in inhibition of hypotonicity induced hemolysis may be due to the inhibition of the release of lysosomal content of the neutrophil.

Table 5.2. Antioxidant property of the extract determined by DPPH assay and reductive assay *p< 0.01, **p <0.005, ***p <0.001

Concentration(µg/ml) of the extract	Reductive assay		DPPH assay			
	Optical density of standard	Optical density of extract	Average % inhibition of Standard	IC_{50} (µg/ml)	Average % inhibition of the extract	IC_{50} (µg/ml)
10	0.236±0.005	0.218±0.008**	50.96±0.68		37.71±0.86***	
25	0.293±0.004	0.247±0.01***	56.55±0.75		46.17±0.75*	
50	0.331±0.007	0.283±0.006**	73.24±0.80		62.93±0.74*	
100	0.353±0.005	0.331±0.006*	91.85±0.30	7.08	82.22±0.68***	31.58

Table 5.3. Anti-inflammatory activity of the extract. * p value <0 .01, ** p value <0 .001.

Concentration(µg/ml)	Percentage inhibition of Standard (Diclofenac sodium)	Percentage inhibition of Extract
50	57.65 ±0.71	47.09±1.44*
100	72.12±1.09	61.09±0.89**
200	88.73±0.89	73.19±2.08*

5.13. Conclusion

The metabolite profile of the extract was explored by using GCMS study and exhibited presence of numerous antibacterial, antifungal, antioxidants etc. compounds. There are possibilities that many other chemicals present in the extract may not be detected by the GCMS analysis. The extract exhibited a significant MIC value, MBC and MFC value. The mechanism of the extract for its antimicrobial and antifungal activity was proposed in this study. The extract showed its potential to damage the outer membrane as well as inner membrane of the both Gram-positive and Gram-negative bacteria. The biofilm inhibition property of the extract indicates its possibilities to use against the resistant pathogens. The capacity of the extract for inhibition of the virulence factor secreted by the bacteria and fungi was an important property and indicates the mechanism of action.

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CHAPTER 6:

ATTEMPT FOR PURIFICATION OF BIOACTIVE COMPOUNDS

6.1. Materials and methods

6.2. Extraction

The filtrated broth obtained after fermentation was extracted using ethyl acetate as solvent and concentrated using rotary vacuum evaporator under reduced pressure. The obtained crude extract was used for the purification of the bioactive compounds.

6.3. Thin layer chromatography (TLC) of the crude extract and Purification of bioactive compound

The crude extract obtained was subjected to the thin layer chromatography in TLC silica gel 60 F 254 aluminium sheet (Merck, Germany) using hexane: ethyl acetate: methanol: acetic acid (1:1.5:0.5:0.5)as solvents for the study. The Rf value was calculated for the bioactive compound. The bioactive spot was scrapped from the TLC plates and filtered with methanol, and dried for the further analysis.

6.4. Characterization of the bioactive compound

The dried compound was then subjected to the EI mass spectroscopy (ESI positive mode), ^{1}H NMR (300 MHz), ^{13}C NMR (300 MHZ) study for the characterization of the compound.

6.5. Results

6.6. Extraction and TLC

The yield of the crude obtained after extraction was very low. The Rf value of the pink colored spot E was determined 0.75. This spot was scrapped from the TLC plates and dissolved in methanol.

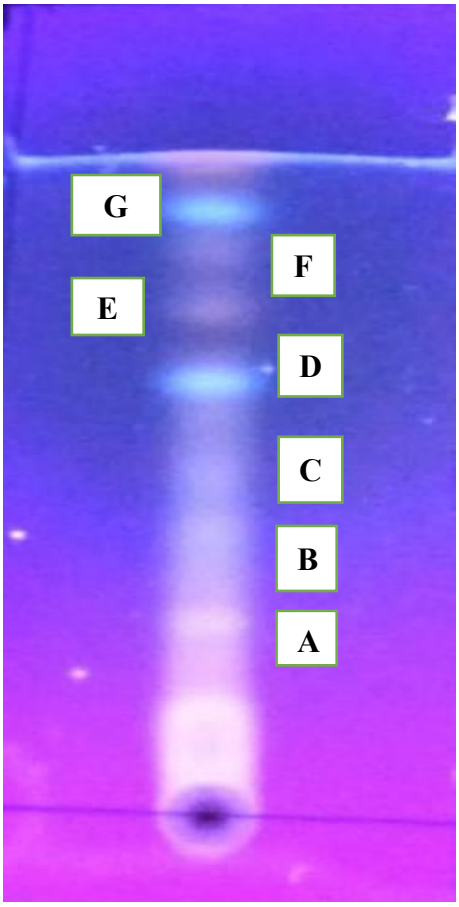

Figure 6.1.TLC profile of the crude extract.

6.7. Characterization of the bioactive compound

The EI mass spectrometry (positive mode) (Figure 6.2)**,** ^{1}H NMR (Figure 6.3), ^{13}C NMR (Figure 6.4) data revealed that the bioactive compound contains impurities. Due to this reason the interpretation of the analytical data for structure elucidation was not possible. The characterization remains incomplete.

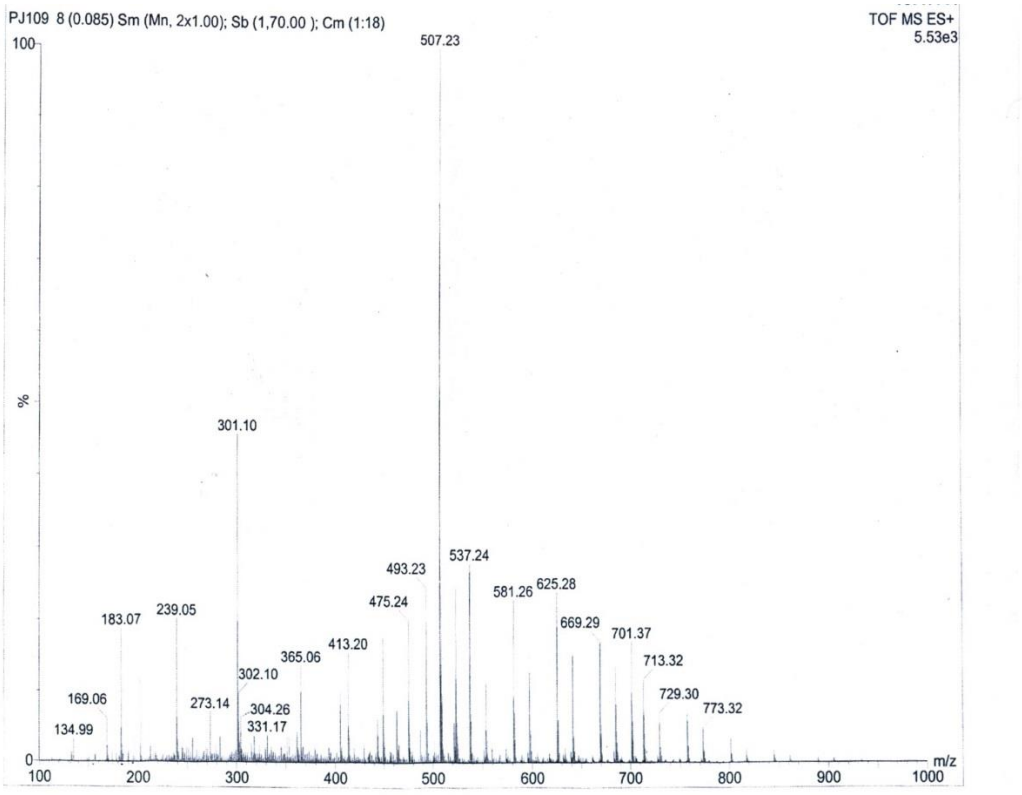

Figure 6.2. EI mass spectra (Positive mode) of the bioactive spot.

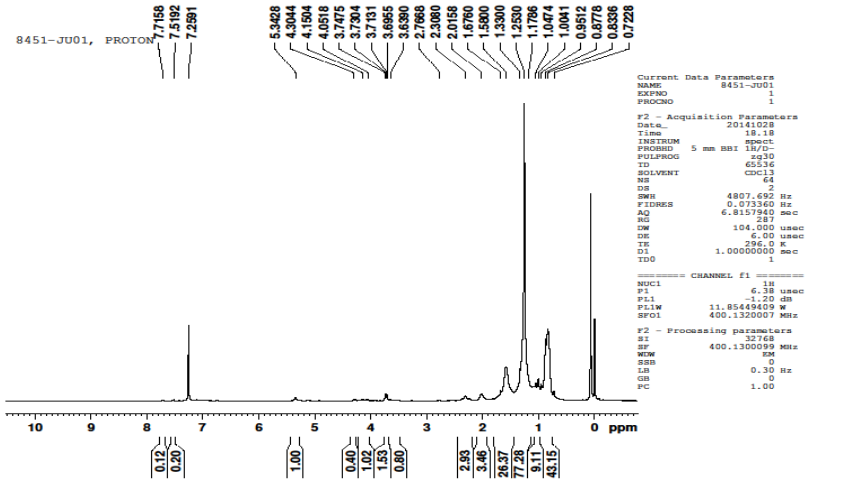

Figure 6.3. Proton NMR of bioactive compound.

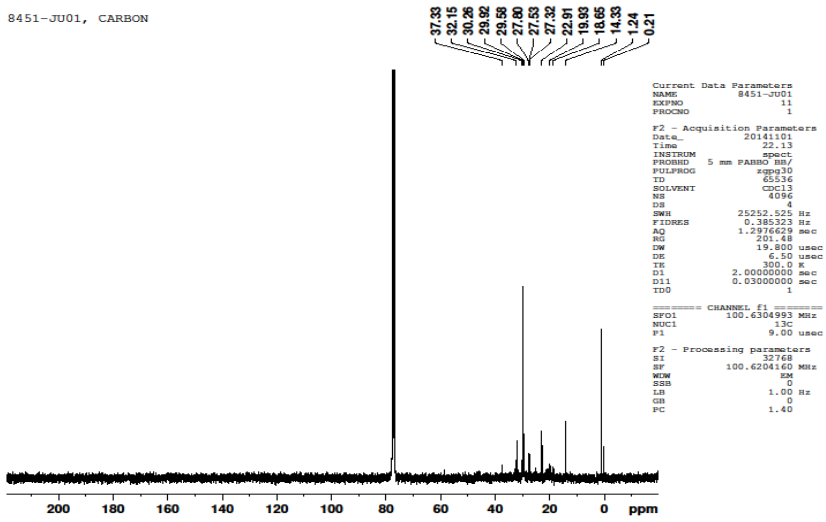

Figure 6.4. Carbon NMR of bioactive compound.

6.8. Conclusion

The isolation and purification of the compound is essential to contribute in the search of new antimicrobials. Purification of the compound was attempted but due to the presence of the impurities, it remains incomplete. The purification process is required to be standardized and more analytical methods have to undertake to elucidate the complete structure.

CHAPTER 7:

CONCLUSIONS AND FUTURE SCOPE

7.1. Conclusions and future scope

The present study undertakes many aspects and objectives. The new ecology or underexplored ecology is an important environment for the discovery of new microorganisms and new bioactive metabolites. This context was evidenced by the identification of the new actinomycetes *Streptomyces euryhalinus* sp nov. from the Sundarbans mangrove forest. Sundarbans microbial diversity was not studied largely. But the mangrove forests are the supplier of the many novel microbes and new bioactive principles. The identification of the actinobacteria is critical as the genus *Streptomyces* is the largest and congested. The preliminary identification of the strain MS 3/20^{T} was determined by using of 16S rRNA gene sequence study. Due to various limitations, 16S rRNA gene sequence study is not the ultimate tool for the identification. Currently, DNA-DNA relatedness study is the decisive tool for the identification of the actinobacteria. In this study, we have employed the DNA-DNA relatedness study, RAPD analysis, MALDI/TOF MS analysis along with chemotaxonomic and phenotypic study to identify the actinobacterium. Polyphasic taxonomy is carried out for the identification.

Metabolite profile of the bacteria was explored with the help of GCMS analysis. Metabolite profile revealed the presence of many biologically significant compounds. The extract was evaluated for the antimicrobial, antifungal property and the probable mechanism of action was explored. The biofilm inhibition property of the extract is significant and it may relate to its application in the treatment of infections caused by

resistant pathogens. The extract showed outer and inner membrane damage capability and inhibited the virulence factors secreted by the microorganisms. Purification of the bioactive compound remains incomplete due to the presence of impurities and low yield of the extract. The attempt of purification provides various ideas to characterize the compound further.

The future scope of the study lies in many directions. Though the identification of the strain MS 3/20^{T} is complete, whole genome sequence study may be carried out in future to identify the biochemical pathway for the production of the antibiotics or the secondary metabolites. Identification of the antibiotic-producing genes and pathway (s) are important for the search of antibiotics. This could be helpful in the enhancement of the production of the antibiotics. The purification and characterization of the antibiotics is an important task for the future study. The establishment of the mechanisms of action of the compound has to study in details. The study of the compound against pathogenic organisms particularly from the aspects of the virulence factors causes infection and damage are needed to undertake for future study.