DIVERSITY AND BIOACTIVE POTENTIAL OF NOVEL ACTINOBACTERIA ISOLATED FROM MARINE SEDIMENT IN PULAU TIOMAN

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ABSTRACT

Tioman Island is one of many sources for underexplored actinobacterial diversity in Malaysia. Selective isolation, molecular profiling, 16S rRNA gene sequencing and phylogenetic analyses were carried out to highlight the diversity of the marine actinobacterial community in sediment samples collected off Tioman Island. A high number of diverse actinobacteria were recovered from sediment samples pre- treated with skim milk/HEPES and UV irradiation on a mannitol-based agar medium. A total of 183 actinobacterial strains were isolated from two sediment samples. Dereplication methods produced 42 distinct groups, with members of the genera *Blastococcus*, *Salinispora* and *Streptomyces* as the major clusters. Nineteen actinobacterial strains were identified as novel species belonging to seven genera (*Agromyces*, *Blastococcus*, *Glycomyces, Mycobacterium, Nocardiopsis*, *Nonomuraea* and *Streptomyces*) and one novel genus based on 16S rRNA gene sequence analyses. Strains TPS16, TPS81 and TPS83 were identified based on polyphasic data to be novel species belonging to a novel genus within the family *Nocardiopsaceae*, as proposed in this study as *Marinitenerispora sediminis* gen. nov., sp. nov. Crude extracts from strain TPS83 was found to inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis* and *Providencia alcalifaciens*. Hierarchical clustering of the bioactivities of an active fraction revealed a unique BioMap (antibiotic mode of action) profile, which is closely related to fosfomycin. Two compounds, nocapyrone R and methylpendolmycin, were purified from the active fraction that was eluted with 80% methanol/ water solvent system. This result showed that strain TPS83 could be a novel source of nocapyrone R and methylpendolmycin. Whole genomes of novel strains TPS2, TPS3, TPS16, TPS81 and

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TPS83 were sequenced and analysed. Prediction of biosynthetic gene clusters (BGCs) from the whole genome sequences revealed involvement of BGCs in biosyntheses of various types of secondary metabolites belonging to type I- and type II- polyketide synthases (PKS) as well as non- ribosomal peptide synthases (NRPS), which were associated with antimicrobial, anti-viral, anti- tumour and anti- parasitic activities. The NRPS gene coding for methylpendolmycin/ pendolmycin was found to be present in the genomes of TPS16 and TPS81, which are clonal strains of TPS83. This study is also the first to report novel *Blastococcus* strains (TPS166, TPS357, TPS418, TPS448 and TPS459) isolated from tropical marine sediment. Phylogenetic analysis based on 16S rRNA gene sequences showed that these strains could be putatively assigned to two different novel *Blastococcus* species.

Keywords: Marine actinobacteria, Malaysia, polyphasic approach, biosynthetic gene clusters, methylpendolmycin.

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KEPELBAGAIAN DAN BIOAKTIF POTENSI AKTINOBAKTERIA BARU YANG DIPENCILKAN DARIPADA MARIN SEDIMEN DI PULAU TIOMAN

ABSTRAK

Pulau Tioman adalah salah satu sumber yang jarang diterokai untuk penyelidikan atas kepelbagaian aktinobakteria di Malaysia. Pengasingan terpilih, pemprofilan dengan teknik molekular, penjujukan gen 16S rRNA dan analisis filogenetik telah dijalankan untuk menyerlahkan kepelbagaian masyarakat aktinobakteria marin daripada sedimen yang dikumpul dari Pulau Tioman. Kebanyakan aktinobackteria adalah dipencilkan daripada sampel sedimen yang telah dirawat dengan susu skim / HEPES dan penyinaran UV pada medium berasaskan mannitol. Sejumlah 183 strain aktinobakteria telah dipencilkan daripada dua sampel sedimen, termasuk 84 aktinobakteria marin yang dikenalpasti sebagai *Salinispora* spp. yang berkait- rapat dengan *Salinispora arenicola* CNH-643^{T} berdasarkan keserupaan turutan gen 16S rRNA. Pencapjarian DNA yang diperolehi dengan pendekatan 'double digestion' mengklasifikasikan 'non-*Salinispor*a-like strains' ke dalam 41 kluster yang berbeza, dengan genera *Streptomyces* dan *Blastococcus* sebagai kluster utama. Sejumlah 17 jenis strain telah dikenalpasti sebagai spesies aktinobakteria baru dalam genus *Streptomyces* (n = 6), *Blastococcus* (n = 5), *Marinactinospora* (n = 3), *Nocardiopsis* (n = 1), *Agromyces* (n = 1), *Nonomuraea* (n = 1), *Mycobacterium* (n = 1) dan *Glycomyces* (n = 1) berdasarkan analisis gen 16S rRNA. Data polifasa daripada tiga Marinactinospora spp. menunjukkan bahawa tiga strain tersebut merupakan terbitan klon yang mewakili genus baru dalam keluarga *Nocardiopsaceae*. Strain *Blastococcus* yang merupai spesies baru berjaya dipencilkan dari sedimen marin yang dikumpulkan dari kedalaman lebih mendalam daripada sampel sedimen pantai buat kali pertama. Strain aktinobakteria TPS2 (*Nocardiopsis* sp.), TPM287 (*Glycomyces* sp.) dan TPM181 (*Mycobacterium* sp.) hanya tumbuh atas media

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yang mengadungi sekurang-kurangnya 1% natrium klorida. Ekstrak dari strain *Marinactinospora* TPS83 dapat menghalang pertumbuhan Gram-positive (*Staphylococcus aureus* dan *Bacillus subtilis*) dan Gram-negatif pathogen (*Providencia alcalifaciens*). Pengelasan hieraki berdasarkan bioaktiviti mendedahkan profil BioMap strain TPS83 yang unik dan berkait rapat dengan fosfomisin. Dua sebatian nocapyrone R dan methylpendolmycin telah diasingkan daripada pecahan aktif yang dilarutkan dengan metanol/ air 80% semasa 'solid- phase extraction' dan kemudiannya diasingkan dengan Sephadex LH-20 dan reverse- phase HPLC. Identifikasi sebatian dipersembahkan dengan tafsiran data spektroskopik berdasarkan HRDARTMS, ^{1}H- dan ^{13}C NMR. Jujukan dan analisis genom aktinobakteria *Marinactinospora* spp. (TPS16, TPS81 dan TPS83), *Nocardiopsis* sp. (TPS2) dan *Streptomyces* sp. (TPS3) juga telah dipersembahkan dalam tesis ini. Anggaran saiz genom untuk strain TPS16, TPS2 dan TPS3 jatuh dalam julat antara 6.0 Mbp dan 10 Mbp. Ramalan kluster gen biosintetik (BGC) menunjukkan kehadiran 14- 59 BGC dalam genom strain TPS16, TPS2 dan TPS3. Analisis pada peringkat yang awal mendedahkan perkaitan BGC dengan biosintesis pelbagai jenis metabolit sekunder, termasuk type I- dan type II- polyketide synthases (PKS) dan non- ribosomal peptide synthases (NRPS). BGC yang dikenalpasti daripada tiga strain ini berkait rapat dengan metabolit sekunder yang mempunyai aktiviti antimikrob, anti-virus, anti-tumor dan anti parasit. BGC jenis NRPS yang menghasilkan methylpendolmycin/ pendolmycin juga telah dikenalpasti daripada genom TPS16 dan TPS81 yang merupakan strain saudara TPS83.

Kata kunci: aktinobakteria dari marin, Malaysia, pendekatan polifasa, kluster gen biosintetik, methylpendolmycin.

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LIST OF SYMBOLS AND ABBREVIATIONS

δ	:	Chemical shift, units in parts per million (ppm)
J	:	Coupling constant, units in Hertz (Hz)
R_f	:	Retardation factor
$H^{'}$	:	Shannon-Wiener index
V	:	Volt
$(NH_4)_2HPO_4$	:	Ammonium phosphate dibasic
AAI	:	Average amino acid identity
ANI	:	Average nucleotide identity
antiSMASH	:	antibiotics & Secondary Metabolite Analysis Shell
APG	:	Acylphosphatidylglycerol
ASW	:	Artificial sea water
ATCC	:	American Type Culture Collection
AU	:	Absorbance unit
AUC	:	Area under curve
BGC	:	Biosynthetic gene cluster
BioMap	:	Antibiotic mode of action profile
BLAST	:	Basic Local Alignment Search Tool
$CaCl_2$	:	Calcium chloride
$CaCl_2 \cdot 2H_2O$	:	Calcium chloride dehydrate
$CDCl_3$	:	Deuterated chloroform
$CoCl \cdot 6H_2O$	:	Cobalt (II) chloride hexahydrate
DAB	:	L- diaminobutyric acid
DAP	:	2,6'- diaminopimelic acid
DARTMS	:	Direct analysis in real time mass spectra
DCM	:	Dichloromethane

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LIST OF SYMBOLS AND ABBREVIATIONS

DDH	:	DNA-DNA hybridization
dDDH	:	Digital DNA-DNA hybridization
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DPG	:	Diphosphatidylglycerol
EDTA	:	Ethylenediaminetetraacetic acid
ESIMS	:	Electrospray ionization mass spectrometer
EtoAc	:	Ethyl acetate
$FeCl_3 \cdot 6H_2O$	:	Ferric chloride hexahydrate
$FeSO_4$	:	Ferrous sulfate
$FeCl_3 \cdot 6H_2O$	:	Ferrous sulphate anhydrous
$FeSO_4$	:	Ferrous sulfate
$FeSO_4 \cdot 7H_2O$	:	Ferrous sulphate anhydrous
GC	:	Gas chromatography
GC content	:	Guanine- cytosine content
GGDC	:	Genome-to-genome distance calculator
GluNU	:	Glucosamine- containing unknowns
H_2O_2	:	Hydrogen peroxide
H_2SO_4	:	Sulphuric acid
HCl	:	Hydrochloric acid
HEPES	:	4'- (2- hydroxyethyl) – 1'- piperazineethanesulfonic acid
HPLC	:	High performance liquid chromatography
HRMS	:	High resolution mass spectra
HVA	:	Humic acid vitamin agar
HVB	:	Humic acid vitamin broth

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LIST OF SYMBOLS AND ABBREVIATIONS

IDBA-UD	:	Iterative de Bruijn Graph *de novo* assembler for short reads sequencing data with highly uneven sequencing depth
i.e.	:	id est (that is)
ISP	:	International *Streptomyces* Project
ISP 1	:	Tryptone-yeast extract agar
ISP 2	:	Yeast extract malt extract agar
ISP 3	:	Oatmeal agar
ISP 4	:	Inorganic salts-starch agar
ISP 5	:	Glycerol-asparagine agar
ISP 6	:	Peptone-yeast extract iron agar
ISP 7	:	Tyrosine agar
ISP 9	:	Carbon utilisation medium
ITS	:	Internal transcribed spacer
K_2HPO_4	:	Potassium phosphate dibasic
KCl	:	Potassium chloride
KH_2PO_4	:	Potassium dihydrogen phosphate
KNO_3	:	Potassium nitrate
KS	:	Ketosynthases
LB	:	Luria broth
LM	:	Luedemann medium
M3	:	Modified peptone asparagine agar
M2	:	Mannitol arginine agar
MBA	:	Mannitol supplemented modified Bennett's agar
$MgSO_4$	:	Magnesium sulfate
MHA	:	Mueller-Hinton agar

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LIST OF SYMBOLS AND ABBREVIATIONS

MHB	:	Mueller-Hinton broth
MHz	:	Megahertz
MIC	:	Minimum inhibitory concentration
$MnCl_2 \cdot 4H_2O$	:	Manganese chloride anhydrous
$MnSO_4H\ _2O$	:	Manganese (II) sulfate monohydrate
MRSA	:	Multiple-drug resistant *Staphylococcus aureus*
mult.	:	Multiplicity or splitting pattern of the peak
MW	:	Molecular weight
NA	:	Nutrient agar
Na_2CO_3	:	Sodium carbonate
Na_2HPO_4	:	Disodium hydrogen phosphate
$Na_2MoO_4 \cdot 2H_2O$	:	Sodium molybdate dihydrate
$NaHCO_3$	:	Sodium bicarbonate
NaOH	:	Sodium hydroxide
nl	:	Nanolitre
nm	:	Nanometre
NMR	:	Nuclear magnetic resonance
NRPS	:	Non-ribosomal peptide synthetase
nt	:	Nucleotide
OAT	:	Orthologous Average Nucleotide Identity Tool
PC	:	Phosphatidylcholine
PE	:	Phosphatidylethanolamine
PG	:	Phosphatidylglycerol
PI	:	Phosphatidylinositol
PIMs	:	Phosphatidylinositolmannosides

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LIST OF SYMBOLS AND ABBREVIATIONS

PME	:	Phosphatidylmethylethanolamine
ppt	:	Parts-per-thousand
Q-TOF	:	Quadrupole time of flight mass spectrophotometer
RAST	:	Rapid Annotation using Subsystem Technology
RFLP	:	Restriction fragment length polymorphisms
rpm	:	Revolutions per minute
rRNA	:	Ribosomal RNA
SEM	:	Scanning electron microscope
SPE	:	Solid phase extraction
SPR	:	Subtree-pruning-regrafting
subsp.	:	Subspecies
TAE	:	Tris-acetate-EDTA
TLC	:	Thin layer chromatography
TMS	:	Tetramethylsilane
TSB	:	Tryptic soy broth
trans- AT	:	*trans*- type acyltransferase
TSA	:	Tryptic soy agar
T1 PKS	:	Type-I polyketide synthases
T2 PKS	:	Type-II polyketide synthases
UPGMA	:	Unweighted pair group method with arithmetic mean
UV	:	Ultraviolet
vol/vol	:	Volume percent
YCS- Glc	:	Yeast extract- casein- starch- glucose medium
YG- Glyc	:	Yeast extract- glucose- glycerol medium
$ZnSO_4 \cdot 7H_2O$	:	Zinc sulphate anhydrous

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CHAPTER 1: INTRODUCTION

The class *Actinobacteria* comprises members that are Gram- stain positive, most of which contains high GC content within the genome and are characterised by a homologous insertion of 100 nucleotides between helices 54 and 55 of the 23S ribosomal RNA (rRNA) gene (Ventura *et al.*, 2007). The terrestrial originated actinobacteria are extraordinarily diverse and this extends the interest of researchers to study those from the marine environment. The ocean covers three-quarters of the earth's surface and therefore is believed to have the greatest diversity. Existence of indigenous marine actinobacteria was supported by the discoveries and descriptions of marine species, *Dietzia maris*, *Rhodococcus marinonascens*, *Salinibacterium amurskyense*, *Williamsia maris* as well as isolation of the first obligate marine genus *Salinispora* spp. (Han *et al.*, 2003; Helmke & Weyland, 1984; Maldonado *et al.*, 2005a; Nesterenko *et al.*, 1982; Rainey *et al.*, 1995; Stach *et al.*, 2004). Marine actinobacteria are mostly derived from marine sediments (54%), followed by sponges (21%), marine invertebrates and sea water (Abdelmohsen *et al.*, 2014).

Culture dependent studies are essential approaches for cultivation of natural actinobacterial strains, which can be screened for biological activities of interests and studied for expression of enzymes and bioactive molecules (Vester *et al.*, 2015). However, fastidious growth requirements, including the need of specific nutrients and growth factors limit attempts to cultivate the unculturables (Köpke *et al.*, 2005). Moreover, the dominant species on isolation plates introduce strong interspecies competition for nutrients, which further discourages successful isolation of rare actinobacterial species, causing them to be unculturable. Actinobacteria produced nearly half of the total active microbial metabolites (41%), as compared to fungus (47%) and other bacteria (12%), mainly from *Streptomyces* spp. and *Salinispora* spp. and the rare actinobacteria represent unique sources of novel biologically active compounds (Bérdy,

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2005; Weber *et al.*, 2014; Williams, 2009). Selective isolation techniques using various pre-treatments and incorporation of unusual carbon sources were found to favour the growth of certain taxonomic groups of actinobacteria and encouraged isolation of rare actinobacterial species (Bredholdt *et al.*, 2007; Sun *et al.*, 2010).

Actinobacteria are among saprophytes that exhibit a wide extent of survival and adaptive strategies to persist in natural environments including formation of spores. Members of certain genera such as *Actinoplanes*, *Dactylosporangium*, *Geodermatophilus*, *Planomonospora* and *Spirillospora*, can harbour spores in sporangia as motile flagellated zoospores (Garrity *et al.*, 1996). Some of the non-spore forming actinobacteria such as *Blastococcus* spp. form motile, single flagellated cells. Production of motile spores and cells enables marine actinobacteria to exhibit chemotaxis and access more nutrient sources. Various selective methods can be employed to isolate zoosporic actinobacteria and this will be further explained in the following chapter.

Malaysian waters, located at the Indo-West Pacific region, are well-known to contain high diversity of world marine life species (Shahbudin *et al.*, 2017). Tropical waters in Malaysia provide suitable environment to sustain growth of various marine organisms, in particularly the coral reefs. Malaysia, by its own, covers 3600 square kilometers (1.27%) of the world reef area. Marine parks of the east coast of Peninsular Malaysia reported a total of 323 coral species richness, dominated mainly by shallow fringing reefs, representing about 80% of the coral population identified in the Coral Triangle. Pulau Tioman Marine Parks, located in the state of Pahang, Malaysia is populated by 57 genera from 17 families of hard corals including scleractinian and non- scleractinian corals (Shahbudin *et al.*, 2017). The dominant coral was identified to be members of the *Acroporidae* family with an average total coverage of 31.4%, followed by *Faviidae*

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(21.2%), *Poritidae* (11.1%), and *Alcyoniidae* (8.9%). Corals belonging to the genera *Acropora* and *Montipora* of the *Acroporidae* family had the highest average coverage at Tioman Island with 19.7% and 11.0%, respectively, followed by *Porites* spp. of the *Poritidae* family (10.7%). High diversity of marine organisms and species richness of coral reefs in Tioman Island is believed to associate with great variety of microsymbionts, which could be potential suppliers of clinically important drug lead compounds. Previous study reported marine sponges from Tioman Island remained as an untapped source of rare marine actinobacteria, as evidenced by successful isolation of diverse actinobacteria members of the genera *Actinoplanes*, *Micromonospora*, *Nocardia*, *Polymorphospora*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Salinispora*, *Sprilliplanes* and *Verrucosispora* (Vikineswary *et al.*, 2008). By contrast, marine sediment and corals from Tioman Island are yet to be assessed for actinobacterial species diversity.

The objectives of this study are:

a) To isolate diverse actinobacteria from marine sediments and coral samples collected from the sea bed of Tioman Island, using various selective cultivation techniques.
b) To characterise the novel actinobacterial strains based on polyphasic methods.
c) To screen marine actinobacterial strains for antibacterial activity.
d) To identify antibacterial compound from selected actinobacterial strain.

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CHAPTER 2: LITERATURE REVIEW

2.1 Actinobacteria

The phylum *Actinobacteria* is supported by 16S and 23S rRNA gene analyses, and is separated from other group of bacteria based on the presence of conserved indels in protein including cytochrome- coxidase subunit 1, CTP synthase and glutamyl –tRNA synthase, as well as the characteristic gene arrangements (Ludwig *et al.*, 2012). The phylum consists of bacteria associate with enormous diversity of morphology, physiology and metabolic capabilities, which is further divided into six major classes including *Actinobacteria*, *Acidimicrobiia*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria* and *Thermoleophilia* (Parte, 2018). The class *Actinobacteria* houses 22 orders and 53 families that had been described to date. This study focuses on the isolation and study of antibacterial activity of members of the class *Actinobacteria*.

Actinobacteria are Gram- positive bacteria with cell wall composed of sugars, amino acids and amino sugars. They produce small, compact and soft to leathery, radiating colonies, which are firmly adhering to a wide range of growth media, with the surface being flat or elevated (Muiru *et al.*, 2008). The actinobacterial colonies could also be of smooth surfaces or folded with the absence of aerial mycelia. A single colony of filamentous actinobacteria is a mass of branching filaments, derived from single spore or parts of mycelium. Hence, the filamentous actinobacterial colonies are generally dry, being covered with or without mycelium. This makes them different from the typical bacterial colonies that appear as slimy or glistening, which is an accumulation of cells originating from single cell or several similar cells. When actinobacteria are cultured in stationary broth, cells grow as pellets or surface pellicles and possibly cottony sediment, leaving the broth clear (Bergey & Holt, 1993).

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Actinobacteria are associated with a homologous insertion of about 100 nucleotides between helices 54 and 55 of the 23S rRNA gene and high guanine- plus- cytosine content in the genome, ranging from 51 mol% in corynebacteria to 70 mol% and above as determined for *Streptomyces* and *Frankia* (Ventura *et al.*, 2007). Cell size ranges from 1- 2 μm in diameter. Delineation of actinobacteria at the genus and species levels is often based on morphological classification, chemotaxonomy and molecular taxonomic data. Genetic analyses based on 16S rRNA gene sequences, DNA- DNA hybridization and genome related indices derived from genome sequencing data are necessary to confirm the species novelty. In general, a standard reference work by Shirling and Gottlieb (1966) is always employed for morphological characterisation of actinobacteria and their production of diffusible pigment and carbon cources profiling. In addition, there are also minimal standards, which are useful documents containing guidelines on characterisation of specific groups of microorganisms (Tindall *et al.*, 2010). However, minimal standards are not available for all bacteria and are not all updated. The updated minimal standards that have been published include standards for description of actinobacteria that belonging to the suborder *Micrococcineae* (Schumann *et al.*, 2009), the genus *Mycobacterium* (Levy-Frebault & Portaels, 1992) and the genus *Streptomyces* (Manfio *et al.*, 1995; Shirling & Gottlieb, 1966).

2.1.1 Delineation of *Actinobacteria*: Morphological classification

Morphological heterogeneity is commonly used for specific determination with the presence or absence of substrate or aerial mycelia, the colour of the mycelia, the production of diffusible pigments and the structure and appearance of spores and spore chains as the main determinative factors. Actinobacteria display a wide variety of morphology, extending from coccoid (*Micrococcus* spp.) or rod- coccoid (*Arthrobacter* spp.) through fragmenting hyphae (*Nocardia* spp.) to highly differentiated, permanent branched mycelium (*Streptomyces* spp.) (Barka *et al.*, 2016). Rhodococci produced

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elongated fragments on media with absent of true mycelia and corynebacteria do not produce mycelia (Jones & Goodfellow, 2012). On the other hand, the *Oerskovia* species produced branched substrate mycelia that break up into flagellated motile elements (Stackebrandt & Schumann, 2012a). Members of the family *Micromonospora*ceae members produce extensive substrate mycelia without production of aerial mycelia (Genilloud, 2012).

Development of substrate mycelium begins with outgrowth of a germinating spore. Substrate mycelium penetrates the solid media to absorb nutrients for growth and always appear as shiny and gel-like that varies greatly in terms of sizes, shapes and thickness under microscope (Xu *et al.*, 2008). The substrate mycelia might differentiate into the aerial or sporogenous mycelia when growing on solid media. The aerial mycelia could develop into sporulating hyphae where reproductive spores are differentiated from, a signal that indicates nutrient limitation, growth rate retardation or accumulation of growth inhibitors or extracellular pheromones (Lee & Rho, 1993). Development of mature aerial mycelium with spores usually requires 7- 14 days or even up to one month. The spores could appear as single cells or in chains of different lengths, or they could be harboured in sporangia or endowed with flagella. Formation of spores may also occur on substrate mycelia. Production of motile or non- motile spores had been observed from actinobacteria.

Examples of genera that produce spores on substrate mycelia are *Micromonospora* (Phongsopitanun *et al.*, 2016), *Verrucosispora* (Rheims *et al.*, 1998), *Salinispora* (Maldonado *et al.*, 2005a) and *Jishengella* (Xie *et al.*, 2011) that form single and non-motile spores that are differentiated from the substrate mycelia. Species of the genera *Actinocatenispora*, *Asanoa*, *Catellatospora*, *Longispora* and *Polymorphospora* produce non- motile spores in chains on the surface of substrate mycelia (Vobis *et al.*, 2012). In

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contrast, *Catenuloplanes* spp., *Spirilliplanes* spp. and *Actinoplanes* spp. produce motile spores on substrate mycelia, but spores of *Actinoplanes* spp. were developed within the sporangia. Members of the genera *Couchioplanes*, *Krasilnikovia* and *Pseudosporangium* form spore chains within pseudosporangium- like structures or pseudosporangia on substrate mycelia. By comparison, members of the genus *Intrasporangium* are characterized by the absent of aerial mycelia and intercalary formation of spherical and lemon- shaped vesicles that contain spores or occasionally devoid of spores in the substrate mycelia, instead of forming at the tops of sporangiophores as observed for sporangia- producing actinobacterial species in general (Kalakoutskii *et al.*, 1967).

Representatives of actinobacteria that produce substrate and aerial mycelia include *Actinosynnema* and *Streptomyces.* The *Actinosynnema* spp. produces synnemata from substrate mycelia, from which the aerial mycelia grow and differentiate into chains of spores that are capable of forming flagella in aqueous environment (Hasegawa *et al.*, 1978). The *Streptomyces* spp. produces extensively branching substrate mycelia that differentiate into highly branching aerial mycelia bearing non- motile spores with various length of chains. At the vegetative phase, substrate mycelia of *Streptomyces* spp. are lacking of cross-walls and thus producing a complex tightly woven matrix of mycelia (Anderson & Wellington, 2001). As the colony ages, formation of crosswalls in the multinucleate aerial filaments signifies differentiation of aerial mycelia into spore chains, which is followed by separation of individual cells into spores.

Morphological features of aerial mycelia are significant for characterisation of actinobacterial species. These features include the mode of branching, the configuration of the spore chains, arrangement of the spore- bearing structures and the surface of the spores are often employed for the species separation, in particularly of the *Streptomyces*

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spp. (Shirling & Gottlieb, 1966). The actinobacterial spore chains can be classified as being straight (rectus), flexuous (flexibilis), open loops (relinaculam- apertum), open or closed spirals (spira), or verticillate. Production of diffusible pigment, in particularly of the melanoid pigments that are typically appeared as brown or black, also serves as an important feature for characterisation of actinobacterial species. Actinobacteria are known to produce diffusible pigments include red, yellow, orange, pink, brownish, distinct brown, greenish brown, blue, or black (Shirling & Gottlieb, 1966). Production of diffusible pigments is highly dependent on the actinobacterial strain, the culture media and the age of the culture. Growth phase of a filamentous actinobacteria is summarised in Figure 2.1.

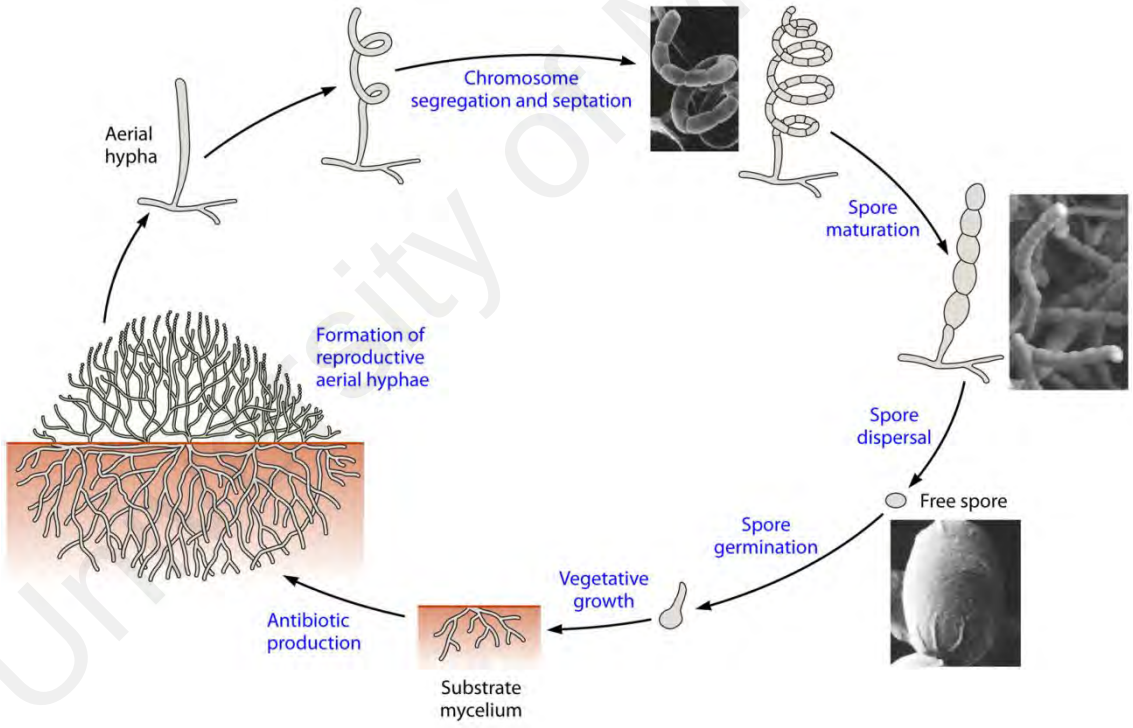

Figure 2.1: The growth cycle of a sporulating actinobacteria that starts with germination of spore (Adapted from Barka *et al.*, 2016).

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2.1.2 Delineation of *Actinobacteria*: Chemotaxanomy

Chemotaxonomy of actinobacteria refers to the study of the distribution of chemical components in the cell walls of actinobacteria, which involves the profiling of cell wall amino acids, lipid, proteins, menaquinones, muramic acid types, sugars and the base composition of DNA. Analysis of the cell wall chemistry of peptidoglycan facilitates grouping of actinobacteria strains up to genus level. The chemotaxonomically important characteristics of cell walls of Gram- positive bacteria include the sugar content in the cell wall peptidoglycan and the presence of specific optical isomers of the chiral nonproteinogenic amino acid, 2, 6- diaminopimelic acid (DAP). The actinobacteria may contain *LL*- DAP or *meso*- DAP as isomer, whereas some actinobacterial genera contain L- diaminobutyric acid (DAB), L- ornithine and L- lysine. Neutral sugars at the cell wall of actinobacteria also serve as important taxonomic markers especially at the suprageneric level. There are five distribution patterns of the major diagnostic sugars. Group A consists of species with cell wall containing arabinose and galactose; group B contains actinobacteria whose cell walls contain madurose; actinobacteria with group C cell wall do not contain diagnostic sugars; the group D cell walls are associated with arabinose and xylose. In general, there are eight distinct classes of cell wall chemotypes of actinobacteria based on presence of different combination of diaminoacid, amino acids and sugars in their cell walls (Table 2.1).

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Table 2.1: The cell wall chemotypes of actinobacteria (Lechevalier & Lechevalier, 1970).

Cell wall type	Major parietal constituents
I	LL- DAP, glycine, no diagnostic sugar
II	*meso*- DAP, glycine, xylose, arabinose
III	*meso*- DAP, madurose or do not contain diagnostic sugar
IV	*meso*- DAP, arabinose, galactose
V	Lysine, ornithine, no diagnostic sugar
VI	Variable presence of lysine, aspartic acid, galactose
VII	DAB, lysine, aspartic acid, glycine, no diagnostic sugar
VIII	Ornithine, no diagnostic sugar

Fatty acids are important building blocks of actinobacterial cellular materials, which present in the cell membranes as acyl constituents of phospholipids. Two major families of membrane fatty acids were identified from bacteria: the straight- chain fatty acid family and the branched- chain fatty acid family (Kaneda, 1991). In general, bacteria are mainly composed of the straight-chain fatty acids in their cellular membrane that include palmitic, stearic, hexadecenoic, octadecenoic, cyclopropanic, 10-methylhexadecanoic and 2- or 3-hydroxyl fatty acids. However, simple combination pattern of the straight chain fatty acids could not provide sufficient variety for proper taxonomy classification. In contrast, branched-chain fatty acids of the *iso* and *anteiso* series with variety degrees of saturation and hydroxylation that are also widely occurred in bacteria, in particularly of the actinobacteria, produce complex combination pattern and therefore provide significant values for bacterial systematic (Kaneda, 1991). Besides that, actinobacterial species could also contain mycolic acids in their cell wall. Variability of the chain lengths and structural complexity of mycolic acids contribute to the definition of actinobacterial genera (Barka *et al.*, 2016). *Corynebacterium* spp. has the simplest corynomycolic acids (22- 36 carbons in length) and *Mycobacterium* spp. has the most complex and species- specific mycolic acids with long branched chains

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(60- 90 carbon atoms). Members of *Rhodococcus* (30- 64 carbon atoms and up to 4 double bonds), *Nocardia* (46- 64 carbon atoms and up to four double bonds) and *Gordonia* (46- 70 carbon atoms and 1–6 double bonds) are associated with intermediate chain- lengths. Fatty acid profiles of actinobacteria are affected by growth phase, incubation temperature, oxygen supply, pH and carbon sources in the culture media (Kaneda, 1991; Tindall *et al.*, 2010). Hence, it is important to ensure identical cultivation conditions if the strains are to be tested for fatty acids analysis. Cultures used for fatty acid profiling are usually harvested in the early stationary phase.

Types of phospholipids in the cell membrane had also been proved to provide useful information for identification and taxonomy classification of actinobacteria. The range of polar lipids that are known to occur in bacteria are phospholipids, glycolipids, phosphoglycolipids, aminophospholipids, amino acid derived lipids, capnines, sphingolipids (glyco- or phosphosphingolipids) and hopanoids (Tindall *et al.*, 2010). Actinobacteria could be classified into five phospholipid groups based on semiquantitative analyses of major phospholipid markers found in the whole- cell extracts (Table 2.2).

Isoprenoid quinones are also important components of the bacterial membranes that participated in cellular electron transport. They can be divided into two major structural groups: the naphthoquinones and the benzoquinones (Collins & Jones, 1981; Tindall *et al.*, 2010). The benzoquinones include ubiquinones and rhodoquinones, which are restricted to members of the classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Betaproteobacteria*. On the other hand, the napthoquinones family houses respiratory lipoquinones synthesize naphthoquinone derivatives that include menaquinones, demethylmenaquinones, monomethylmenaquinones, dimethylmenaquinones and menathioquinones. The *Actinobacteria* members produce menaquinones, which is also

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known as 2-methyl-3-polypreny1-1,4- naphthoquinones (Collins *et al.*, 1981; Tindall *et al.*, 2010). Variations in the length and degree of unsaturation of the C_3- isoprenyl side-chain of menaquinones provide important chemotaxonomic values (Collins *et al.*, 1985). In addition, the actinobacteria also consists of menaquinones with isoprenoid side chains that show different patterns of hydrogenation or menaquinones with terminal ring structures (Tindall *et al.*, 2010).

2.1.3 Delineation of *Actinobacteria*: Molecular taxonomic data

The 16S rRNA gene sequences provide the first indication that a known species or a novel bacterial species has been isolated. The term species is generally defined as a group of closely related strains evolved from a common ancestor that share a degree of phenotypic consistency with a minimum of 98.7% identity between their almost full length 16S rRNA gene sequences (above 1300 bp) and more than 70% pairwise DNA-DNA hybridization (DDH) values and a high mutual phenetic similarity (Stackebrandt *et al*., 2002; Sangal *et al.*, 2016).

The use of gene sequences to infer phylogenetic relationship was first proposed in year 1965 (Zuckerkandl & Pauling, 1965). The 16S rRNA gene that is ubiquitious among bacteria was discovered to be the best candidate gene for phylogenetic study as it permits detection of relatedness among distant species and hence facilitate species identification (Woese & Fox, 1977). In addition, the gene is readily isolated and functionally stable as it is poorly subjected to horizontal gene transfer, and the gene sequence changes slowly with time. In bacterial phylogeny and microbial ecology studies, 16S rRNA gene is well- known as the “gold standard” phylogenetic marker.

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Table 2.2: Types of phospholipids present in the cell wall of actinobacteria. (PIMs, phosphatidylinositolmannosides; PI, phosphatidylinositol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidylmethylethanolamine; APG, Acylphosphatidylglycerol; DPG, diphosphatidylglycerol; GluNU, glucosamine- containing unknowns) (Adapted from Lechevalier *et al.*, 1977).

Phospholipid type	Phospholipids	Example of taxon
PI	PIMs, PI, DPG	*Actinomadura*
	PIMs, PI, PG, APG, DPG	*Corynebacterium*
	PIMs, PI, DPG	*Microtetraspora*
	PI, PG, APG	*Nocardiodes*
PII	PIMs, PI, PE, DPG	*Actinoplanes* spp., *Chainia* spp., *Dactylosporangium* spp., *Microellobosporia* spp., *Micromonospora* spp., *Micropolyspora* spp., *Mycobacterium* spp., *Nocardia* spp., *Streptomyces* spp., *Streptoverticillium* spp.
PIII	PIMs, PI, PG, PC, PME, APG, DPG	*Actinomadura dassonvillei*
	PI, PG, PC, PME, DPG	*Micropolyspora faeni*
	PIMs, PI, PG, PC, PME, DPG	*Nocardia autotrophica*
	PIMs, PI, PC, PE, PME, DPG	*Pseudonocardia thermophila*
PIV	PI, PC, PE, DPG, GluNU and variable presence of PIMs	*Intrasporangium calvum*
	PI, PE, DPG, GlnNU and variable presence of PIMs	*Microbispora* spp.
	PI, PE, PME, DPG, GluNU and variable presence of PIMs	*Streptosporangium roseum*
PV	PI, PG, DPG, GluNU and variable presence of PIMs	*Oerskovia* spp.
	PI, PG, APG, DPG, GluNU and variable presence of PIMs	*Promicromonospora* spp.

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The 16S rRNA gene sequences of type strains of bacterial species with validly published names are available from public databases, including the Genbank® nucleic acid database (NCBI Resource Coordinators, 2013), the EzBioCloud database (Yoon *et al.*, 2017), the SILVA ribosomal RNA gene database (Quast *et al.*, 2013), the all-species living tree project (LTP) database (Yarza *et al.*, 2008), Greengenes database (DeSantis *et al.*, 2006) and the ribosomal database project (RDP) (Cole *et al.*, 2014). The 16S rRNA gene sequences are used in microbial systematic to calculate pairwise sequence similarities and to perform phylogenetic analyses following multiple sequence alignments (Kim *et al.*, 2014a). The 16S rRNA gene sequence similarity between two strains provides a simple yet robust criterion for the identification of novel bacterial species, whereas phylogenetic analyses can be used to elucidate overall evolutionary relationships between related taxa that involve genus or suprageneric classification (Kim *et al.*, 2014a). However, use of 16S rRNA gene as taxonomic marker posted several limitations.

The 16S rRNA gene sequences tend to provide insufficient resolution to distinguish between closely related species as it contains limited number of characters (Nouioui *et al.*, 2018). This is particularly obvious in delineation of the obligate marine actinobacterial genus *Salinispora*. The three *Salinispora* species, including *Salinispora arenicola*, *Salinispora pacifica* and *Salinispora tropica*, displayed low interclade diversity and this placed the entire genus into microdiverse sequence cluster. Pairwise comparison of the 16S rRNA gene sequences of all *Salinispora* species revealed that the strains shared more than 99% of similarity, in which the three strains differed from each other by 6- 12 nucleotides (Jensen & Mafnas, 2006). Classification of these strains into distinct species is totally dependent on characterisation of 16S-23S intergenic spacer regions and DNA- DNA relatedness study as well as phenotypic characterisation data as compared to the members of *Micromonosporaceae*. Moreover, the observation that

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Kitasatospora (Ichikawa *et al.*, 2010) and *Streptacidiphilus* (Kim *et al.*, 2003) fall within the *Streptomyces* 16S rRNA gene tree further questions the resolution power of 16S rRNA gene in species delineation (Nouinoui *et al.*, 2018).

In general, when 16S rRNA gene sequence similarity values are more than 97% when the pairwise comparison is performed over full length 16S rRNA gene sequences, DNA- DNA relatedness study using DDH or analysis of gene sequences with a greater resolution are employed to resolve the taxonomic status, by coupling to characterisation based on phenotypic tests (Tindall *et al.*, 2010). DDH is also performed when the novel taxon contains more than one strain to highlight that all strains share a high degree of hybridization among each other. The hybridization experiment is always performed with all relevant type strains to ensure that there is sufficient dissimilarity to support the classification of the strain as a new taxon (Tindall *et al.*, 2010). The type strain of the genus is the most important reference organism to which a novel species has to be compared when it is identified to be a member of the same genus. Similarly, a species placed in a new genus must be compared with species of closely related taxa, which must include the type species of the genera under study. Melting temperature for hybridization test is calculated based on DNA G+C contents of the strains to be tested (Tindall *et al.*, 2010). Although DDH is believed to produce a higher level of resolution in separation of taxa, it is limited by lacking of reproducibility and compatibility of results between laboratories (Sangal *et al.*, 2016).

Owing to the above limitations of DDH and 16S rRNA gene as mentioned above, the scientific community has urged for a change to a stricter definition for bacterial species. To achieve this, extensive characterisation of larger group of strains at high resolution is needed. Genome sequencing, which can reveal genetic differences between two strains at various resolution levels, is believed to be able to contribute towards this goal

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(Konstantinidis *et al.*, 2006). The average nucleotide identity (ANI; Konstantinidis *et al.*, 2006) and maximal unique matches (MUM; Deloger *et al.*, 2009) indexes have been suggested to replace DDH in defining a bacterial species.

ANI represents a mean of identity/ similarity values between homologous genomic regions shared by two genomes (Kim *et al.*, 2014a). It is demonstrated to correlate with DDH, where the range of 95–96% similarity reflects the current boundary of 70% DDH similarity (Goris *et al.*, 2007; Sangal *et al.*, 2016). The ANI threshold for separating potential genera is 74.8%. ANI estimates genome distances by assessing the list of orthologs and calculate overall divergence of the core genome by averaging percentages of identity at the nucleotide level of all orthologs found (Deloger *et al.*, 2009). Briefly, fixed-length DNA fragments of the first genome are compared to the second genome using BlastN and fragments meeting an identity threshold are kept and used to derive the ANIb, which refers to ANI derived with the BLAST algorithm (Goris *et al.*, 2007). However, ANI is not symmetrical. Lee *et al.* (2016a) showed that the studied genome pairs showed over 0.1- 4.15% discrepancy between reciprocal ANI values obtained using different softwares and this level of discrepancies can cause significant effect on taxonomic interpretation. Hence, a new ANI algorithm that includes the concept of orthology was developed to resolve the problem, in which the reciprocal OrthoANI values are always identical (Lee *et al.*, 2016a).

The ANI that is calculated with the MUMmer algorithm is referred to as ANIm. It performs calculations in a different way from the ANIb, which applies all-against-all BLAST searches for all genes. In the case of ANIb, thousands of searches must be re-run to incorporate the newly sequenced genes and these causes relatively longer processing time. In comparison, the high- speed method MUMmer determines if potential hits are present in the genome that is added to the database (Kurtz *et al.*, 2004).

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If the hit is known before, it would be omitted from subsequent BLAST searches. As such, if there is a large amount of novel proteins present in the new genome, MUMmer reduces processing time that is required to search the genome against the database. However, researchers showed that ANIm provides more robust results when both genomes of compared shared a high degree of similarity, which is more than 90% ANI, as compared to distant genomes, suggesting that the MUM algorithm is sensitive for intraspecies comparisons (Deloger *et al.*, 2009; Richter & Rossello-Mora, 2009).

Another genome related index, the average amino acid identity (AAI), which is derived from the conserved genes has also been proposed to be incorporated into prokaryotic taxonomy as AAI provides robust resolution between distantly related strains. The AAI is calculated based on conserved protein-coding genes between a pair of genomes that are determined by whole-genome pairwise sequence comparison using the BLAST algorithm (Altschul *et al.*, 1997; Thompson *et al.*, 2013). For these comparisons, all protein-coding genes from one genome were searched against all protein-coding genes of the other genome (Thompson *et al.*, 2013). AAI is derived from at least 50 and usually 500 genes in total of the genome and therefore, the effects of horizontal gene transfer and genome sizes are less significant on AAI than on other single-gene-based and gene content-derived approaches (Konstantinidis & Tiedje, 2005). The AAI thresholds for separating potential genera and species are 87.8% and 98.41%, respectively (Sangal *et al.*, 2016).

As a DDH similarity of 70% is still the main criterion for assigning two strains to the same species, the Genome-to-Genome Distance Calculator (GGDC) online tool make possible to calculate DDH values in silico analogues, which are commonly known as digital DDH or dDDH. The calculated dDDH values turned out to correlates well with the wet-lab hybridization results and are able to deal with incomplete genomes or

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genomes with large amount of paralogous genes and large repeats (Thompson *et al.*, 2013). GGDC uses the genome blast distance phylogeny approach to calculate distances between a pair of genomes (Meier-Kolthoff *et al.*, 2013; Thompson *et al.*, 2013). Both genomes of the pair to be compared are locally aligned using BLAST to produce a set of high-scoring segment pairs (HSPs) that are the intergenomic matches. The total number of identical base pairs obtained from the HSPs is transformed into a single genome-to-genome distance value, which is analogous to DDH. Following this, phylogenetic trees are inferred from the distance matrices using neighbour joining. These DDH estimates are based on an empirical reference dataset that consists of real DDH values and genome sequences.

2.2 Marine actinobacteria

Marine bacteria including actinobacteria are either living freely in the ocean or they could also colonise suspended particles to obtain growth substrates, where they constitute up to 20 % of bacterial biomass (Mohit *et al.*, 2014; Overmann *et al.*, 2017; Zhang *et al.*, 2007a). Some of them are also found to associate with sediment and marine organisms. Actinobacteria had been successfully isolated from sea water samples, as shown by isolation of *Aeromicrobium marinum* $T2^T$ and *Serinicoccus marinus* $JC1078^T$ from surface water of German Wadden Sea and East Sea at Korea, respectively, and *Salinibacterium amurskyense* KMM 3673^T from sea water collected at 5 meters depth from Amursky Bay of the Gulf of Peter the Great (Bruns *et al.*, 2003; Han *et al.*, 2003; Yi *et al.*, 2004). Presence of actinobacteria in marine sediments was reported in previously published papers on recovery of actinobacteria from sediment samples collected at various depths, ranging from 0 meter up to 10000 meters (Mincer *et al.*, 2002; Pathom-aree *et al.*, 2006). By using 16S rRNA gene sequences from GenBank, which were then analyzed using the Ribosomal Database Project classifier for actinobacteria that were associated with marine organisms, it was shown that the highest

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number of actinobacterial 16S rRNA gene sequences is associated with sponges and corals, accounted for 63% and 17%, respectively, and followed by ascidian (7%), mollusc (6%), fish and other marine animals (3%), seagrass (2%) and lastly the mangrove and seaweed (each at 1%) (Valliappan *et al.*, 2014).

Marine actinobacteria are able to grow in the presence of sea salt and most of them could tolerate high concentration of sodium chloride. They do not necessarily require salt to live with. However, some actinobacterial strains develop unique adaptation to the marine environment, as characterized by requirement of seawater for growth to occur. Exclusive marine actinobacterial strains had been reported from the genera *Rhodoccocus* and *Streptomyces*, although majority of the members of these genera were isolated from the terrestrial soil. *Rhodococcus marinonascens* 3438W^T and *Streptomyces oceani* SCSIO 02100^T from marine sediment and authigenic carbonate nodule were reported to require supplement of at least 75 % of seawater and 2.5 % of sodium chloride for *in vitro* growth (Helmke & Weyland, 1984; Tian *et al.*, 2012a). Besides that, *Spinactinospora alkalitolerans* CXB-854^T from marine sediment, *Euzebya tangerina* F10^T isolated from the sea cucumber *Holothuria edulis* and *Streptomyces* spp. (*S. tateyamensis* Sp080513SC-30^T, *S. haliclonae* Sp080513SC-31^T and *S. marinus* Sp080513GE-26^T) from marine sponge are also examples of exclusive marine actinobacteria that require at least addition of 1 % of sodium chloride in culture media for growth (Chang *et al.*, 2011; Khan *et al.*, 2010; Kurahashi *et al.*, 2010). The only obligate marine actinobacterial genus described to date is *Salinispora*, which had been shown to be widely distributed in marine sediment samples and sponges (Mincer *et al.*, 2002; Kim *et al.*, 2005; Vidgen *et al.*, 2012).

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The genus *Salinispora* comprised three closely related species, namely *S. arenicola*, *S. tropica* and *S. pacifica*, within the *Micromonosporaceae* family, which share more than 99 % of pairwise similarity of the 16S rRNA gene sequences and the difference of nucleotides falls between six to 12 nucleotides. *S. arenicola* and *S. pacifica* showed the greatest sequence difference, whereas the most similar species were *S. tropica* and *S. pacifica* (Jensen & Mafnas, 2006). The entire genus is thus known as a microdiverse sequence cluster due to lack of interclade diversity within the three *Salinispora* species (Jensen & Mafnas, 2006). Although the conventional 16S rRNA gene phylogeny could not confidently resolve the relationship of *Salinspora* species, species classification was supported by DNA- DNA hybrizdization experiments, in which *S. arenicola* CNH-643^T and *S. tropica* CNB-440^T shared DNA- DNA relatedness value of 44.9 % and *S. pacifica* CNR-114^T shared less than 60 % genomic similarity to *S. arenicola* CNH-643^T and *S. tropica* CNB-440^T (Ahmed *et al.*, 2013; Maldonado *et al.*, 2005a). Recent study that analysed a hundred of genomes of the *Salinispora* strains demonstrated that the genomewide ANI provided a new perspective on *Salinispora* diversity and revealing seven new species in addition to the three decribed species (Millán-Aguinãga *et al.*, 2017).

The phylum *Actinobacteria* contributes to the global carbon cycling via degradation of plant biomass and mediates community dynamics through production of secondary metabolites with diverse biological activities (Lewin *et al.*, 2017). The high cellulolytic ability and diverse chemistry of actinobacteria make them a potential candidate for the bioenergy industry. Moreover, actinobacteria also participate in bioremediation of heavy metals and volatile organic compounds. *Streptomyces* was reported to survive metal contaminated sites through production of metal ion chelators including siderophores and release of extracellular polymeric substances consisting of polysaccharides, proteins, DNA and RNA that alter the mobility of metals, which

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provides them the biosorption and bioaccumulation abilities (Timková *et al.*, 2018). Isoprene or 2-methyl-1,3-butadiene, being the highly reactive biogenic volatile organic compound with detrimental impact on climate and health is mainly produced by terrestrial plants and marine photosynthetic microalgae. Actinobacteria from the genera *Rhodococcus*, *Mycobacterium* and *Gordonia* were able to degrade isoprene through a mechanism that involves initial oxidation of isoprene to 1, 2- epoxy-isoprene, which is subsequently convered to a glutathione conjugate that will be further degraded through enzymatic reactions. Analysis of the *Actinobacteria* assemblages in coastal marine sediments revealed association of actinobacterial genera and species operational taxonomic units with ability to cope with the presence of arsenic, heavy and toxic metals and polycyclic aromatic hydrocarbons (Chen *et al.*, 2016; Duran *et al.*, 2015). In addition, marine actinobacteria was also reported to get involve in the cycling of dissolved protein in the ocean as evidenced by the presence of protein assimilation gene: the TonB- dependent receptor- encoding genes (Orsi *et al.*, 2016).

2.2.1 Actinobacteria associate with sediments in the South China Sea

The South China Sea is one of the largest marginal seas that lies within the West Pacific marine. The three largest rivers in the world, namely the Mekong River, Red and Pearl Rivers shape the detrital fluxes of sediments of South China Sea (Zhu *et al.*, 2013a). The monsoon activity controls the sea surface circulation and cycling of organic carbon and biogenic components of sediments. An average water depth of 1200 meters is recorded with maximum depth to be estimated as 5380 meters.

Microbial abundance in South China Sea was detected to fall between 10^5 and 10^6 cells per gram of wet sediment by analysing the 16S rRNA gene clone library (Zhang *et al.*, 2012a). Actinobacterial species constituted 3.4 % of the bacterial 16S rRNA gene clone library as estimated from marine sediment samples collected off the Xisha

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Trough, China at 3697 meters depth (Li *et al.*, 2008a). Besides that, actinobacteria was also detected from previous studies using various approaches including denaturing gradient gel electrophoresis and cloning of 16S rRNA gene from sediment samples collected at 20- 3888 meters depth from the South China Sea and was found to constitute 2 % of the total operational taxonomic units in the sediment samples (Du *et al*., 2011; Wang *et al.*, 2010a; Wu *et al.*, 2011a; Zhang *et al.*, 2012a). Another bacterial diversity study on marine sediments using pyrosequencing- based analysis of 16S rRNA genes also showed that actinobacteria were detected from samples collected from both shallow and deep sea sediments, however, they were found more abundant in deep sea sediments that were sampled at above 300 meters depth, representing 45.2 % of the total operational taxonomic units (Zhu *et al.*, 2013a). Various culturable actinobacterial species had been isolated from marine sediments of South China Sea and described as novel species belonging to the genera *Amycolatopsis*, *Georgenia*, *Marinactinospora*, *Marininema*, *Nocardioides*, *Prauserella*, *Pseudonocardia*, *Rhodococcus*, *Sciscionella*, *Streptomyces* and *Verrucosispora* (Table 2.3).

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Table 2.3: Examples of actinobacterial species isolated from marine sediments of the South China Sea at various depths. ND denoted as no data is available.

Actinobacteria	Depth (m)	Isolation medium	Salt requirement	NaCl tolerance	Reference
Amycolatopsis marina Ms392A^T	ND	SM1	Yes	0.5- 12 %	Bian *et al.*, 2009
Georgenia sediminis SCSIO 15020^T	141	R2A	No	Up to 5 %	(You *et al.*, 2013)
Marinactinospora thermotolerans SCSIO 00652^T	3865	Raffinose histidine agar	No	Up to 5 %	Tian *et al.*, 2009a
Marininema mesophilum SCSIO 10219^T	2105	R2A	No	Up to 7 %	Li *et al.*, 2012a
Nocardioides nanhaiensis YIM M13091^T	880	DSMZ 621	No	Up to 4 %	Fan *et al.*, 2014
Prauserella marina MS498^T	3602	MOPS-proline agar	No	Up to 10 %	Wang *et al.*, 2010b
Pseudonocardia antitumoralis SCSIO 01299^T	3258	Gauze No. 1	No	Up to 15 %	Tian *et al.*, 2013
Rhodococcus nanhaiensis SCSIO 10197^T	84.5	A1	No	Up to 10 %	Li *et al.*, 2012b
Sciscionella marina SCSIO 00231^T	516	Gauze No. 1	No	Up to 13 %	Tian *et al.*, 2009b
Streptomyces abyssalis YIM M 10400^T	778	Gauze No. 1	No	Up to 6 %	Xu *et al.*, 2012
Streptomyces glycovorans YIM M10366^T	778	Gauze No. 1	No	Up to 7 %	Xu *et al.*, 2012
Streptomyces xishensis YIM M 10378^T	778	Gauze No. 1	No	Up to 7 %	Xu *et al.*, 2012
Streptomyces nanhaiensis SCSIO 01248^T	1632	Humic acid vitamin agar	No	Up to 7.5 %	Tian *et al.*, 2012b
Streptomyces nanshensis SCSIO 01066^T	2015	Gauze No. 1	No	Up to 7 %	Tian *et al.*, 2009c
Streptomyces oceani SCSIO 02100^T	578	Nutrient agar	Yes	2.5- 12.5 %	Tian *et al.*, 2012a
Verrucosispora sediminis MS426^T	3602	Gauze No. 1	No	Up to 6 %	Dai *et al.*, 2010

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2.2.2 Actinobacteria associate with corals in the South China Sea

The South China Sea is located in the tropic zone that lies between the equator and Tropic of Cancer with the climate is associated with daily temperature variation of less than 3 °C and an optimum salinity of 30.0- 34.5 for development of reef- building corals (Guo *et al.*, 1994). Corals from in the water of South China Sea, as identified from Brunei, China (south and southeastern part and Paracel islands at Hainan), West Malaysia (Middle Rocks and eastern Peninsular Malaysia), Philippines (northern Palawan and western Luzon), Singapore, Spratly Islands, Taiwan, Thailand and Vietnam were found to belong to the families of *Acroporidae*, *Agariciidae*, *Caryophylliidae*, *Dendrophylliidae*, *Diploastreidae*, *Euphyllidae*, *Fungiidae*, *Incertaesedis*, *Lobophylliidae*, *Merulinidae*, *Pocilloporidae*, *Poritidae*, *Psammocoridae* and *Siderastreidae* (Huang *et al.*, 2015). The *Acropora* spp. and *Montipora* spp. of the *Acroporidae* family and *Porites* spp. of the *Poritidae* family were found to be the dominant species in most of the studied area, except for Brunei and the Gulf of Thailand where *Montipora* spp. was not detected (Cleary & Hoeksema, 2006; Huang *et al.*, 2009; Huang *et al.*, 2015; Latypov, 2015; Nugroho *et al.*, 2018; Phongsuwan *et al.*, 2013; Shahbudin *et al.*, 2017; Tanaka *et al.*, 2016; Zhao *et al.*, 2008). Table 2.4 listed actinobacterial species that had been reported to associate with corals of the *Acropora* spp., *Porites* spp. and *Montipora* spp. from the South China Sea.

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Table 2.4: Actinobacterial genera that are associated with corals of genera *Acropora*, *Porites* and *Montipora* from various locations including the South China Sea.

Coral	Actinobacteria	Location	Isolate/ Clone	Reference
Acropora gemmifera	*Janibacter*	Palau	Isolate	Kageyama *et al.*, 2007
Acropora digitifera	*Brachybacterium*	Gulf of Myanmar	Isolate	Nithyanand & Pandian, 2009
	Brevibacterium		Isolate	
	Curtobacterium	Gulf of Myanmar	Clone	Nithyanand *et al.*, 2011a
	Micrococcus		Clone	
	Propionibacterium		Clone	
	Streptomyes		Clone	
Acropora millepora	*Brevibacterium*	Luhuitou fringing reef, China	Isolate	Li *et al.*, 2014
	Gordonia		Isolate	
	Jiangella		Isolate	
	Microbacterium		Isolate	
	Micromonospora		Isolate	
	Nocardiopsis		Isolate	
	Pseudonocardia		Isolate	
	Streptomyces		Isolate	
Porites lutea	*Cellulosimicrobium*	Luhuitou fringing reef, China	Isolate	Li *et al.*, 2014
	Gordonia		Isolate	
	Actinopolyspora	Luhuitou fringing reef, China	Clone	Kuang *et al.*, 2015
	Agrococcus		Clone	
	Arthrobacter		Clone	
	Blastococcus		Clone	
	Corynebacterium		Clone	
	Demetria		Clone	
	Dietzia		Clone	
	Fodinicola		Clone	
	Friedmanniella		Clone	
	Geodermatophilus		Clone	
	Iamia		Clone	
	Ilumatobacter		Clone	
	Janibacter		Clone	
	Kocuria		Clone	
	Kytococcus		Clone	
	Microbacterium		Clone	
	Micrococcus		Clone	
	Modestobacter		Clone	

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Table 2.4, continued.

Coral	Actinobacteria	Location	Isolate/ Clone	Reference
Porites lutea	*Nocardioides*	Luhuitou fringing reef, China	Clone	Kuang *et al.*, 2015
	Ornithinimicrobium		Clone	
	Propionibacterium		Clone	
	Pseudonocardia		Clone	
	Rothia		Clone	
	Tersicoccus		Clone	
	Yonghaparkia		Clone	
	Candidatus Aquiluna		Clone	
	Candidatus Microthrix		Clone	
	Brachybacterium	Luhuitou fringing reef, China	Isolate/ Clone	Li *et al.*, 2014; Kuang *et al.*, 2015
	Brevibacterium		Isolate/ Clone	
	Micromonospora		Isolate/ Clone	
	Mycobacterium		Isolate/ Clone	
	Nocardiopsis		Isolate/ Clone	
	Streptomyces		Isolate/ Clone	
Porites harrisoni	*Arthrobacter*	Qit'at Benaya inshore coral reef system north of the Arabian Gulf	Isolate	Mahmoud & Kalendar, 2016
	Brevibacterium		Isolate	
	Kocuria		Isolate	
	Marmoricola		Isolate	
	Microbacterium		Isolate	
	Micrococcus		Isolate	
	Nocardia		Isolate	
	Rhodococcus		Isolate	
	Streptomyces		Isolate	
Montipora capitata	*Kocuria*	Moku O Loe, Kaneohe Bay, Oahu, Hawaii	Isolate	Shore-Maggio *et al.*, 2015
	Micrococcus		Isolate	

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2.3 Cultivation of actinobacteria

In vitro cultivation of microorganisms has enabled description of a vast number of actinobacterial species. Cultivation efforts to yield more of the novel species are still ongoing. However, comparing 16S rRNA gene sequences from cultivated actinobacteria to those determined directly from natural environmental samples disclosed the presence of uncultivable, unculturable or non- culturable actinobacteria, as detected by DGGE or RFLP analyses of 16S rRNA gene fragments (Sun *et al.*, 2010; Yoshida *et al.*, 2008) and metagenomic data analysis that had uncovered a new group of ultrasmall marine actinobacteria with exceptionally low GC content (Ghai *et al.*, 2013). This group of not-to-be-cultured actinobacteria represents a major challenge in microbiology and they deserve attention for discoveries of novel biosynthetic pathways and unique biochemical characterisitics that could offer novel and innovative solutions for biotechnology advancement and development of agricultural sectors as well as public heath related issues.

Cultivation of novel microorganisms is tedious and time consuming and thus the progress had been slow. In comparison, sequencing bacterial genomes and culture-independent studies involving metagenomics or single cell genomics that can rapidly reveal the unexpected physiological capacities of both cultured and uncultured actinobacteria had gained worldwide researchers attention and these approaches are preferred options for environmental actinobacterial diversity studies. Culture-independent approaches cannot replace the cultivation- based studies of phylogenetic and functional novelty. Functional prediction is highly depending on the availability of well- annotated genomes from cultured representatives. Complex functional traits and unusual kinetics or enzyme characteristics cannot be easily deduced from genome sequences alone, instead, culture- based experimentation is required. Besides that, biochemical verification of hypothetical novel pathways and production of secondary

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metabolites also require pure living culture for elucidation of the enzymatic mechanisms and chemical structure of compounds.

In addition, culture- independent studies based on analysis of 16S rRNA gene clone libraries and pyrosequencing were reported to be insufficient to comprehensively describe actinobacterial diversity. Construction of gene clone library is limited by labour intensive nature of library generation and sequencing of a modest number of sequences, which was usually limited to only few hundreds sequences per sample (Duncan *et al.*, 2015). Amplicon pyrosequencing that eliminates the need for cloning and generates large quantities of sequence data to allow for in-depth assessment of bacterial community composition without the bias of culture based methods is soon to replace the previous approach. However, estimation of actinobacterial diversity using pyrosequencing is highly dependent on selection of primers and sequencing depth. Duncan *et al.* (2015) compared culture- independent and culture- dependent approaches to explore actinobacterial diversity in marine sediments. Non- selective primers were selected for amplification of 16S rRNA genes and the authors relied on superior sequencing depth of pyrosequencing to uncover actinobacterial diversity within the background of overall bacterial diversity. The experimental results showed that only a limited number of actinobacterial sequences were obtained despite generation of thousands of sequences per sample. Moreover, *Streptomyces* and *Micromonospora* species that constitute the most commonly isolated population from marine sediments were not detected via pyrosequencing, but were recovered from the studied samples using highly selective isolation techniques (Duncan *et al.*, 2015a). Underestimation of bacterial diversity in environmental DNA suggested that culture- independent study is also limited by the nature of bacteria. The filamentous actinobacteria are commonly exist in sediments as spores that are highly resilient to cell lysis, while the rare actinobacteria could occur at abundancies below detection limits of various culture

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independent methods (Jensen & Lauro, 2008). Nevertheless, highly selective cultivation methods couple to information provided by culture- independent approaches would allow access to diverse biologically novel, not-to-be-cultured actinobacteria. This study will focus on culture- dependent study to assess diversity of actinobacteria in marine sediment and coral samples to match objectives of the grant.

2.3.1 Selective isolation of actinobacteria

Heterotrophic bacterial communities and marine productivity are characterised by concentration and availability of essential nutrients, which refer to carbon and nitrogen substrates, phosphorus and iron (Repeta & Boiteau, 2007). Marine environments are associated with low nutrient concentrations, in which the dissolved organic carbons fall between 40 and 80 μM and dissolved organic nitrogen and phosphorus concentrations are in μM and 10-1000 pM, respectively, and dissolved iron concentrations in the range of 50- 100 pM (Hansell *et al.*, 2009; Torres-Valdés *et al.*, 2009).

Actinobacteria had been documented to live an oligotrophy lifestyle, although they were also reported as copiotrophic (Ho *et al.*, 2017; West *et al.*, 2016). When the environmental conditions are incapable of supporting continued growth such as during nutrient depletion, changes of toxic chemical concentrations and temperature or pressure, the stress response is triggered and actinobacteria in particularly of the copiotrophics would form dormant cells with low metabolic activity to retain viability. The starved or dormant cells are often revivable on growth media with reduced organic carbon content instead of the complex, high- nutrient media (Connon & Giovannoni, 2002; Overmann *et al.*, 2017). In addition, low- nutrient media are also useful to enhance quantitative recovery of bacteria while rich media maximized isolation of bacteria with diverse morphology (Vartoukian *et al.*, 2010; Vishnivetskaya *et al.*, 2000). Since the growth of actinobacteria is also limited by concentration of organic carbon

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and nitrogen, use of polymeric carbohydrates and nitrogen sources including soluble starch, glycerol, raffinose, mannitol, peptone, yeast extract, casein, nitrate, histidine and L-asparagine had resulted in successful isolation, as monomeric substrates are present only in low concentration and are slowly released at the beginning of enrichment, given that extracellular hydrolytic enzymes were present at low abundances (Hameş-Kocabaş & Uzel, 2012; Sait *et al.*, 2002).

The marine habitat has a highly heterogenous chemical seascape due to frequent and pervasive occurrence of microscale chemical gradients and it is also characterised by temporal variability of growth substrates. Marine bacteria have adapted to the challenging conditions through motility and chemotaxis to locate more favorable, spatially localized microenvironments (Stocker & Seymour, 2012). This interesting feature is therefore worth further exploitation for targeting enrichment of novel marine bacteria including actinobacteria. Various selective methods were previously reported to improve isolation of motile actinobacteria, including baiting techniques, centrifugation methods and chemotactic methods that use organic and inorganic nutrients as chemoattractants (Dennis *et al.*, 2013; Garrity *et al.*, 1996; Otugoro *et al.*, 2001).

Actinobacteria are slow growers as compared to bacteria belonging to the other phyla. They generally take few days for development of visible colonies under laboratory conditions. Fastidious actinobacteria, in particularly of the dormant group and the not- to- be- cultured group would require one to three months of incubation for colony formation to become visible (Janssen *et al.*, 2002; Mincer *et al.*, 2002). As such, it is important to avoid repression of the slow growing actinobacteria due to the fast growing bacteria. Pre- treatments of samples prior to isolation in combination with the use of low- nutrient isolation media are the main keys to success isolation of fastidious actinobacteria. Pre- treatment methods that had been reported to successfully recover

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marine actinobacteria from sediment samples are given in Table 2.5. When most of the pre- treatment methods encouraged isolation of actinobacteria belonging to diverse genera, heat treatment at 65 °C and 120 °C for 30 min and 60 min, respectively, were found to enhance growth of *Micromonospora* spp. on ISP 3 and ISP 4 or raffinose-histidine medium supplemented with antibiotics to reduce growth of fungus and fast-growing bacteria (Gärtner *et al.*, 2016; Terahara *et al.*, 2013). By contrast, actinobacteria associated with coral samples were recovered by dilution or direct plating techniques after surface sterilization and pre- treatments are not widely employed (Table 2.6).

2.4 Natural products from marine actinobacteria

Actinobacteria are long recognized to be prolific sources of natural products. Advancement in whole genome sequencing provides insights into their specialized metabolisms. In year 2002, the complete genome sequences of the model actinobacteria *Streptomyces coelicolor* A3(2) revealed complete sequences of the biosynthetic gene clusters coded for secondary metabolites and enabled exploitation of gene clusters encoding the cryptic natural product biosynthetic pathways, which led to disclosure of the potential of this terrestrial origin model organism to produce at least 15 distinct families of specialized metabolites (Bentley *et al.*, 2002; Challis, 2014). Later in year 2007, the genome sequence of *Salinispora tropica* showed that this marine derived model actinobacteria dedicates a large portion of its genome to natural product assembly, which is accounted for about 10 % of its genome, the number that was found to be greater than the *S. coelicolor* A3(2) who devotes 8 % of its genome to the biosynthesis of secondary metabolites (Udwary *et al.*, 2007). Another study showed that 124 distinct biosynthetic gene clusters had been detected from 75 *Salinispora* strains in total and the *Salinispora* species average 14–18 polyketide and non-ribosomal peptide biosynthetic gene clusters per strain (Jensen, 2016; Ziemert *et al.*, 2014).

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Table 2.5: Pre- treatment methods used for the isolation of marine actinobacteria of various genera from sediment samples (modified from Hameş-Kocabaş & Uzel, 2012).

Type	Pre- treatment method	Reference
Mechanic	Shake with glass beads for 60 min in blood tube rotator	Maldonado *et al.*, 2009
Heat treatment	Dry in laminar air flow hood, stamping	Duncan *et al.*, 2015a; Gontang *et al.*, 2007; Jensen *et al.*, 2005; Mincer *et al.*, 2002
	Dry in laminar air flow hood, dilution	Jensen *et al.*, 2005
	Dry sample with speedvac at 30 °C for 16 h and dry heat at 120 °C for 60 min	Bredholt *et al.*, 2008
	Dry at room temperature, heat at 120 °C, 60 min, dilution	Gartner *et al.*, 2016
	Dilution, heat at 50 °C, 60 min	Jensen *et al.*, 1991
	Dilution, heat at 55 °C, 6 min	Duncan *et al.*, 2014; Jensen *et al.*, 2005; Mincer *et al.*, 2002
	Dilution, heat at 55 °C for 6 min and at 60 °C for 10 min	Jensen *et al.*, 2005
	Heat at 45 °C, 55 °C and 65 °C, 30 min	Terahara *et al.*, 2013
Cold- shock treatment	Freeze (-20 °C, 24 h), thawed, dilution	Jensen *et al.*, 2005
	Freeze (-20 °C, 24 h), thawed, dilution, incubation at room temperature for 48 h	Jensen *et al.*, 2005
	Freeze at -18 °C	Bredholdt *et al.*, 2007
Radiation	UV irradiation, 30 s at a distance of 20 cm and 254 nm and 15 W	Bredholdt *et al.*, 2007
	Superhigh frequency radiation in microwave oven for 45 s at 2460 MHz, 80 W	Bredholdt *et al.*, 2007
	Extremely high frequency radiation at 1 kHz within wavelength band of 8- 11.5 mm	Bredholdt *et al.*, 2007
Heat and radiation	Air- dried sediment was heated at 55 °C for 30 min and microwave irradiated at 80 W, 30 s	Eccleston *et al.*, 2008
Centrifugation	Dispersion and differential centrifugation	Maldonado *et al.*, 2005b
Chemical	Phenol (1.5 %) for 30 min at 30 °C	Bredholt *et al.*, 2008
	Dry heat at 120 °C for 60 min and treated with phenol (1.5 %) for 30 min at 30 °C	Bredholt *et al.*, 2008
	Dry heat at 120 °C for 60 min and treated with benzethonium chloride (0.02 %) for 30 min at 30 °C	Bredholt *et al.*, 2008
Pressure	19 ml of wet sediment and 1 ml of 100 µM of N- acetyl- D- glucosamine was incubated in compression- proof steel tubes at 280- 440 bar hydrostatic pressure at13.5 °C for 6 days	Gartner *et al.*, 2016

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Table 2.6: Isolation techniques reported in the previous literatures on isolation of marine actinobacteria from corals. Actinobacterial genera that had been recovered from the respective speciments were also listed.

Isolation technique	Sampling location	Corals	Culturable actinobacterial genera	Reference
Dilution plating	Gulf of Eilat, northern Red Sea, Israel (1- 2 m depth)	*Fungia scutaria*	*Dermatophilus*, *Kocuria*, *Kytococcus*, *Micrococcus*	Lampert *et al.*, 2006
Coral species were incubated in aquaria with sterile sea water for 6 h at 27 °C and 4 liters per hour, homogenized in a commercial Waring blender, spread plate	West Atlantic ocean, 45 m off the coast	*Leptogorgia minimata*, *Iciligorgia schrammi*, *Swiftia exertia*	*Arthrobacter*, *Dietzia*, *Micrococcus*, *Rhodococcus*, *Streptomyces*	Bruck *et al.*, 2007
Dilution plating	Jim Atria wreck site, Pompano Beach, Florida (45 m depth)	*Cirrhipathes lutkeni*	*Propionibacterium*	Santiago-Vazquez *et al.*, 2007
Dilution plating	Angauru Coral Garden, Palau (3- 5 m depth)	*Acropora gemmifera*	*Janibacter*	Kageyama *et al.*, 2007
Dilution plating	Hare Island, Gulf of Myanmar	*Acropora digitifera*	*Brachybacterium*, *Brevibacterium*	Nithyanand & Pandian, 2009
Coral specimen was homogenized in a mortar, suspended in sterile sea water, pass through a filter paper (Schleicher and Schuell, Dassel, Germany), spread plate	Coast of Curacao, Netherland Antilles (2- 30 m depth)	*Palythoa caribaeorum*, *Zoanthus pulchellus*	*Brevibacterium*, *Micrococcus*, *Nocardioides*	Seemann *et al.*, 2009
Dilution plating	Gulf of Eilat, northern Red Sea, Israel (3- 19 m depth)	Corals belonging to genera *Platygyra*, *Porites*, *Favia*, *Stylophora*, *Pocillopora*, *Xenia* and species of *Fungia granulose* and *Rhytisma fulvum*	*Arthrobacter*, *Micrococcus*	Shnit-Orland & Kushmaro, 2009

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Table 2.6, continued.

Isolation technique	Sampling location	Corals	Culturable actinobacterial genera	Reference
Agarsphere culturing technique (encapsulation)	Gulf of Eilat, northern Red Sea, Israel (10- 15 m depth)	*Fungia granulosa*	*Corynebacterium*	Ben-Dov *et al.*, 2009
Direct plating	National Marine Aquarium, Devon, UK	*Sinularia* sp.	*Mycobacterium*	Thomas *et al.*, 2010
Dilution plating	Hare Island, Gulf of Myanmar	*Acropora digitifera*	*Curtobacterium*, *Kocuria*, *Micrococcus*, *Propionibacterium*, *Streptomyces*	Nithyanand *et al.*, 2011b
Dilution plating	Aguja Island, Tayrona National Park, Columbia (15 m depth)	*Siderastrea siderea* with white plague disease	*Brevibacterium*, *Microbacterium*	Cardenas *et al.*, 2012
Dilution plating		*Diploria strigosa*	*Micrococcus*	
Dilution plating		*Diploria strigosa* with white plague disease	*Brevibacterium*, *Micrococcus*	
Vigorous vortex, dilution plating	Hoi Ha Wan Marine Park, Port Shelter, Hong Kong, China	*Platygyra carnosus* with skeletal tissue growth anomalies	*Rothia*	Chiu *et al.*, 2012
Dilution plating	Zhao'an Bay, East China Sea	*Scleronephthya* sp.	*Cellulomonas*, *Dietzia*, *Gordonia*, *Micromonospora*, *Mycobacterium*, *Nocardioides*, *Rhodococcus*, *Streptomyces*	Sun *et al.*, 2012
Dilution plating	Zhao'an Bay, East China Sea (5-10 m depth)	*Antipathes dichotoma*	*Micromonospora*, *Saccharomonospora*, *Streptomyces*	Zhang *et al.*, 2012a
Freeze at -80 C, thawed, dilution plating	Zhao'an Bay, East China Sea (5-10 m depth)	*Alcyonium gracllimum*, *Tubastraea coccinea*	*Brevibacterium*, *Dietzia*, *Kocuria*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Streptomyces*	Yang *et al.*, 2013
Dilution plating	Xisha Island, South China Sea	*Nephthea* sp.	*Dietzia*, *Salinispora*, *Streptomyces*	Ma *et al.*, 2013

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Table 2.6, continued.

Isolation technique	Sampling location	Corals	Culturable actinobacterial genera	Reference
Dilution plating	Sanya coral reef conservation, South China Sea (5- 10 m depth)	*Echinogorgia aurantiaca*, *Melitodes squamata*, *Muricella flexuosa*, *Subergorgia suberosa*, *Verrucella umbraculum*	*Agrococcus*, *Dietzia*, *Micrococcus*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Streptomyces*	Zhang *et al.*, 2013
Dispersion and differential centrifugation, dilution plating	Luhuitou fringing reef, China (3- 5 m depth)	*Porites lutea*, *Acropora millepora*, *Galaxea fascicularis*	*Amycolatopsis*, *Brachybacterium*, *Brevibacterium*, *Cellulosimicrobium*, *Gordonia*, *Jiangella*, *Micrococcus*, *Mycobacterium*	Li *et al.*, 2014
Dilution plating	Red Sea	*Sarcophyton glaucum*	*Streptomyces*	Elahwany *et al.*, 2013
Direct plating	Moku O Loe, Kaneohe Bay, Oahu, Hawaii, USA	*Montipora capitata*	*Kocuria*, *Micrococcus*	Shore-Maggio *et al.*, 2015
Dilution plating	Qit'at Benaya inshore coral reef system, north of the Arabian Gulf	*Coscinaraea columna*	*Arthrobacter*, *Brevibacterium*, *Cellulomonas*, *Dermacoccus*, *Devriesa*, *Kocuria*, *Micrococcus*, *Nocardia*, *Rhodococcus*, *Streptomyces*	Mahmoud & Kalendar, 2016
Dilution plating	Qit'at Benaya inshore coral reef system	*Platygyra daedalea*	*Arthrobacter*, *Brachybacterium*, *Brevibacterium*, *Cellulomonas*, *Kineococcus*, *Kocuria*, *Rhodococcus*	Mahmoud & Kalendar, 2016
Dilution plating	Qit'at Benaya inshore coral reef system	*Porites harrisoni*	*Arthrobacter*, *Brevibacterium*, *Kocuria*, *Marmoricola*, *Microbacterium*, *Micrococcus*, *Nocardia*, *Rhodococcus*, *Streptomyces*	Mahmoud & Kalendar, 2016
Direct plating	Aviles Canyon (1250 - 3000 m depth)	Corals belonging to the Orders of *Scleractinia*, *Gorgonacea* and *Alcyonaea* and *Lophelia pertusa* species	*Micromonospora*, *Myceligenerans*, *Pseudonocardia*, *Streptomyces*	Sarmiento-Vizcaíno *et al.*, 2017

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The unexplored rare or novel strains are believed to be repertoires of novel chemistry and bioactivities. Although *Streptomyces* species is the most versatile producer of bioactive compounds, special attention is also given to isolation, screening, and culturing of rare actinobacteria from rare environmental locations. Rare actinobacteria are characterised as having lower isolation rates as compared to *Streptomyces* species using conventional isolation methods and normal parameters due to the requirement of stringent cultivation conditions (Azman *et al.*, 2015). However, unexplored actinobacterial strains or rare actinobacterial species are not necessarily producing bioactive compounds. Hence, systematic approaches are needed to reveal the true potential of rare actinobacteria in production of bioactive secondary metabolites. Tables 2.7 and 2.8 summarize representative bioactive compounds isolated from marine *Streptomyces* species and marine rare actinobacteria, respectively.

The main focus in natural product discovery is the identification of new bioactive chemical or discovery of previously unreported biological activity with known chemical structure. On the other hand, compound rediscovery due to similar strains replications remains as a major challenge for isolation of novel natural products from actinobacteria. Thus, there is requisite for developments in techniques for bioactivity screening, isolation and separation methods and analytical chemistry (Trindade *et al.*, 2015). High throughput screening of natural product libraries can provide easy means for evaluation of desired bioactivities against an array of natural products (Monciardini *et al.*, 2014). The integrative approaches involving metabolite profiling, bioactivity- and taxonomic-studies have been utilised to characterize different marine actinobacterial strains and biological properties of metabolites produced by them (Betancur *et al.*, 2017). Combination of DNA- and chemical- fingerprinting in de-replication of natural products using robust techniques based on UV absorbance, high pressure liquid chromatography and mass spectrometry had been reported to scrutinize discovery of new compounds

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(Konishi *et al.*, 2007; Larsen *et al.*, 2005). Tables 2.7 and 2.8 summarize approaches that resulted in successful discovery of novel compounds with unique chemical skeletons.

However, compounds with unique physical characteristics but similar mode of action as the known compounds are misclassified. A screening approach built on the basis of chemical-biology fingerprinting had been developed to overcome this challenge by improving accuracy and sensitivity of dereplication. BioMAP, developed by Wong *et al.* (2012), is one of the screening and de-replication technologies that merge LCMS-UV profiling with biological activity evaluation followed by capillary probe NMR spectroscopy and evaluation of NMR database (AntiMarin), based on the hypothesis that antibiotics from the same structural class possess similar biological activity profiles.

Fementation media also played an important role in the discovery of novel secondary metabolites. Biosynthesis of secondary metabolites is associated with differentiation of morphology and biochemical pathways. Expression of biosynthesis genes are regulated by inducers as well as type of nutrients and its concentration present in fermentation media. Carbon source, which is mainly referred to glucose, had been reported to interfere with production of most antibiotics via carbon catabolite repression especially in the *Streptomyces* sp. (Sánchez *et al.*, 2010). Hence, the use of fermentation media supplemented with different species of carbon sources is important to maximize expression of secondary metabolites and its diversity by turning on cryptic pathways and removing carbon catabolite repression pressure.

Systematic enrichment of production is another approach that researchers had employed to improve low titer production of compounds and to induce expression of biosynthesis pathways that are cryptic under normal laboratory conditions. This

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involves transfer of biosynthetic gene clusters into a well-developed heterologous host through biosynthetic engineering (Wenzel & Muller, 2005). Direct cloning and refactoring of silent lipopeptide gene cluster of *Saccharomonospora* sp. CNQ490 have been achieved by heterologous expression in *S. coelicolor* to yield taromycin A (Yamanaka *et al.*, 2014).

Engineering of the precursor pathways can also results in enhancement of secondary metabolites production (Dhakal *et al.*, 2016). Combinatorial biosynthesis that exploits shuffling of anabolic pathways by precursor directed biosynthesis, enzyme level modulations and pathway level recombination had led to discovery of novel natural products (Sun *et al.*, 2015; Winn *et al.*, 2016). The precursor-directed *in- situ* synthesis had successfully enabled generation of new congeners of saccharothriolides from *Saccharothrix* sp. A1506 (Lu *et al.*, 2017). Mutasynthesis that involves modulation of anabolic pathway by generating mutant strain that is lacking of the key aspects of biosynthetic pathway, as well as substitution of natural precursor with analog of precursor had also proven to produce new natural products, as showed in the study on production of fluorinated analog fluorosalinosporamide by *S. tropica*. The halogenase gene salL was inactivated and 5'- fluoro- 5'- deoxyadenosine, which is the fluorinated analog of its natural precursor 5'- chloro- 5'- deoxyadenosine, was used to generate fluorosalinosporamide (Eustaquio & Moore, 2008).

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Table 2.7: An overview of secondary metabolites produced by marine- derived *Streptomyces* species with various biological activities.

Compound	Type/ structure	Isolation source	Activity	Particulars	Reference
Bioxalomycins	Naphthyridinomycin related antibiotic containing oxazolidine ring	Intertidal sediment, *Streptomyces* sp. LL-31F508	Antibacterial	Bioassay- guided	Bernan *et al.*, 1994
Anthranilamides	Anthranilic acid containing amides	Sediment, *Streptomyces* sp. B7747	Anti-microalgae	Bioassay- guided	Biabani *et al.*, 1998
Cyclomarins A- C	Cyclic peptides	Sediment, *Streptomyces* sp.CNB-982	Anti- inflammatory	Bioassay- guided	Renner *et al.*, 1999
6-prenyltryptophol	Indole	Marine invertebrate, *Streptomyces* sp. BL-49-58-005	Cytotoxic	Bioassay- guided	Sanchez-Lopez *et al.*, 2003
Aureoverticillactam	A 22- atom macrocyclic lactam	Sediment, *S. aureoverticillatus*	Cytotoxic	Bioassay- guided	Mitchell *et al.*, 2004
1-Hydroxy-1-norresistomycin	Quinone related antibiotic	Sediment, *S.chibaensis* AUBN1/7	Cytotoxic	Bioassay- guided	Gorajana *et al.*, 2005
Streptokordin	Methylpyridine derivative	Sediment, *Streptomyces* sp. KORDI-3238	Cytotoxic	Bioassay- guided	Jeong *et al.*, 2006
Echinosporins	With tricyclic acetal-lactone structure	Sediment, *S. albogriseolus* A2002	Cell cyle inhibitors, apoptosis inducers	Bioassay- guided	Cui *et al.*, 2007
Marmycins A and B	Pentacyclic C- glycosides	Sediment, *Streptomyces* sp. CNH-990)	Cytotoxic	Bioassay- guided	Martin *et al.*, 2007
Essramycin	Triazolopyrimidine	Sediment, *Streptomyces* sp. Merv8102	Antibacterial	Bioassay- guided	El-Gendy *et al.*, 2008
Albidopyrone	An α- pyrone	Sediment, *Streptomyces* sp. NTK-227	Inhibit protein- tyrosin phosphatise B, regulation of insulin signaling	UV- guided	Hohmann *et al.*, 2009

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Table 2.7, continued.

Compound	Type/ structure	Isolation source	Activity	Particulars	Reference
Tartrolon D	Macrodiolide	Sediment, *Streptomyces* sp. MDG-04-17-069	Cytotoxic	Bioassay- guided	Perez *et al.*, 2009
Benzoxacystol	Benzoxazine type	Sediment, *Streptomyces* sp. NTK-935	Cytotoxic	MS/ MS spectrum pattern- based	Nachtigall *et al.*, 2011
Lobophorins E- F	Spirotetronate antibiotic	Sediment, *Streptomyces* sp. SCSIO 01127	Cytotoxic	Bioassay- guided	Niu *et al.*, 2011
Fradcarbazoles A- C	Indolocarbazoles	Sediment, *Streptomyces* sp. 007M135	Cytotoxic	Bioassay- guided	Fu *et al.*, 2012
Fradimycin A- B, fradic acids A- B	Capoamycin type antibiotic, polyene acids	Sediment, *Streptomyces* sp. PTZ 0025	Antibacterial, cytotoxic	Bioassay- guided	Xin *et al.*, 2012
Napyradiomycins A- F	Meroterpenoids	Sediment, *Streptomyces* spp. CNQ-329 and CNH-070)	Antibacterial, cytotoxic	Bioassay- guided	Cheng *et al.*, 2013
2-hydroxy-5-((6-hydroxy-4-oxo-4H-pyran-2-yl)methyl)-2- propylchroman-4-one	Polyketides	Marine algae, *S. sundarbansensis*	Antibacterial	Bioassay- guided	Djinni *et al.*, 2013
Akaeolide	Carbocyclic polyketide	Sediment, *Streptomyces* sp. NPS554	Antibacterial	UV- guided	Igarashi *et al.*, 2013
Lobophorins H- I	Spirotetronate antibiotic	Sediment, *Streptomyces* sp. 12-A35	Antibacterial	Bioassay- guided	Pan *et al.*, 2013
Champacyclin	Cyclic octapeptide	Sediment, , *Streptomyces* sp. C42	Antibacterial	Bioassay- guided	Pesic *et al.*, 2013
Sungsanpin	Lasso peptide	Sediment, *Streptomyces* sp. SNJ013	Cytotoxic	MS/ MS spectrum pattern- based	Um *et al.*, 2013
4α-dechloronapyradiomycin A1	Napyradiomycin derivatives	Sediment, *Streptomyces* sp. SCSIO 10428	Antibacterial, antioxidant, cytotoxic	Bioassay- guided	Wu *et al.*, 2013

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Table 2.7, continued.

Compound	Type/ structure	Isolation source	Activity	Particulars	Reference
Streptcytosines A- E	Nucleoside antibiotics	Sediment, *Streptomyces* sp. TPU1236A	Antibacterial	Bioassay- guided	Bu *et al.*, 2014
Hyaluromycin	Polyketide, rubromycin family	Sea squirt, *Streptomyces* sp. MB-PO13	Anti- inflammatory	Bioassay- guided	Harunari *et al.*, 2014
Bahamaolide A	Macrocyclic lactone	Sediment, *Streptomyces* sp. CNQ-343	Antifungal	Bioassay- guided	Lee *et al.*, 2014
Rocheicoside A	Cytosine- type nucleosides	Sediment, *S. rochei* 06CM016	Antibacterial, antifungal	Bioassay- guided	Aksoy *et al.*, 2015
Xestostreptin	Dipeptide	Sponge *Xestospongia muta*, *Streptomyces* sp. S.1 and S.2	Antiplasmodial	Bioassay- guided	Rakotondraibe *et al.*, 2015
Marangucycline A- B	C- glycoside angucyclines	Marine sediment, *Streptomyces* sp. SCSIO 11594	Antibacterial, cytotoxic	Bioassay- guided	Song *et al.*, 2015
Monacyclinone F	Angucyclinones	Sponge *Scopalina ruetzleri*, *Streptomyces* sp. M7_15	Antibacterial, cytotoxic	Bioassay- guided	Vicente *et al.*, 2015
1,3-dihydroisobenzofurans	Isobenzofuran, heterocyclic compounds with fused benzene and furan rings	Sediment, *Streptomyces* sp.W007	Cytotoxic	Bioassay- guided	Zhang *et al.*, 2015
Quinomycin G	Echinomycin analogue	Sponge *Gelliodes carnosa*, *Streptomyces* sp. LS298	Antibacterial, cytotoxic	Bioassay- guided	Zhen *et al.*, 2015
Naquihexcin A	S- bridged pyranonaphthoquinone dimer bearing an unsaturated hexuronic acid moiety	Sponge, *Streptomyces* sp. HDN-10-293	Cytotoxic	Bioassay- guided	Che *et al.*, 2016
Streptomyceamide C	A threonine ester	Sediment, *Streptomyces* sp. H74-21	Cytotoxic	Bioassay- guided	Fu *et al.*, 2016

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Table 2.7, continued.

Compound	Type/ structure	Isolation source	Activity	Particulars	Reference
Griseurazin A	A pyrazine- type metabolite	Tidal flat sediment, *S. griseus* subsp. *griseus.*	Anti- inflammatory	Bioassay- guided	Lee *et al.*, 2016b
2(1*H*)- pyrazinones	Pyrazinone derivatives	Sea tunicate, *Streptomyces* sp. Did-27	Cytotoxic	Bioassay- guided	Shaala *et al.*, 2016
Aldgamycins J- O	16- membered macrolides with a branched octose unit	Sediment, *Streptomyces* sp. HK-2006-1	Antibacterial	Bioassay- guided	Wang *et al.*, 2016
Grisemycin	A bridged angucyclinone with a methylsulfinyl moiety	Sediment, *S. griseus* M268	Cytotoxic	UV- guided, bioassay- guided	Xie *et al.*, 2016
Napyradiomycin A1	Meroterpenoids	Intertidal area, *Streptomyces* sp. YP127	Cytotoxic, angiogenesis inhibitor	Bioassay- guided	Hwang *et al.*, 2017
Chalcomycin E	A 16- membered macrolide	Sediment, *Streptomyces* sp. HK-2006-1	Antibacterial	Bioassay- guided	Jiang *et al.*, 2017
Collismycin C	Bipyridines	Unidentified red algae, *Streptomyces* sp. MC025	Biofilm production inhibitor	Bioassay- guided	Lee *et al.*, 2017
Naphthablins B- C	Meroterpenoids	Sediment, *Streptomyces* sp. CP26-58	Cytotoxic	Cytological profiling obtained from high- content screening	Martucci *et al.*, 2017
Fradiamine A	Siderophore	Sediment, *Streptomyces* sp. MM456M-mF7	Antibacterial, cytotoxic	LC-HRESI-MS analysis-based, metabolomics	Takehana *et al.*, 2017
Neo- actinomycins A- B	Actinomycins bearing the 5*H*-oxazolo[4, 5-*b*]phenoxazine chromophore	Sediment, *Streptomyces* sp. IMB-094	Antibacterial, cytotoxic	Biossay- guided	Wang *et al.*, 2017a
12-N-methyl-k252c	Indolocarbazole alkaloid	Sediment, *Streptomyces* sp. A22	Cytotoxic	Bioassay- guided	Cheng *et al.*, 2018

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Table 2.7, continued.

Compound	Type/ structure	Isolation source	Activity	Particulars	Reference
Ala- geninthiocin	Thiopeptide antibiotic	Subtidal sediment, *Streptomyces* sp. ICN19	Antibacterial, cytotoxic	Bioassay- guided	Iniyan *et al.*, 2018
Cyclizidines B- I	Cyclizidine- type alkaloids	Sediment, *Streptomyces* sp. HNA39	Cytotoxic	Bioassay- guided	Jiang *et al.*, 2018
Strepoxepinmycins A- D	Medermycin- type naphthoquinones	Sediment, *Streptomyces* sp. XMA39	Antibacterial, cytotoxic	Bioassay- guided	Jiang *et al.*, 2018
JBIR-150	A 20- membered polyene macrolactam	Sediment, *Streptomyces* sp. OPMA00071	Cytotoxic	Bioassay- guided	Kawahara *et al.*, 2018
Rakicidin F	Cyclic depsipeptide	Unidentified sponge, *Streptomyces* sp. GKU 220	Antibacterial	Bioassay- guided	Kitani *et al.*, 2018
3-Hydroxyquinaldic acid	Hydroxyquinaldic acid derivatives	Scleractinia coral, *Streptomyces* sp. M-157	Cytotoxic	LC- HRMS based	Ortiz-Lopez *et al.*, 2018
Anthracimycin B	A polyketide antibiotic	Gorgonian coral, *Streptomyces* sp. M-169	Antibacterial	LC- HRMS based	Rodriguez *et al.*, 2018
Streptocarbazole E	Indolocarbazoles	Sediment, *Streptomyces* sp. DT-A61	Cytotoxic	Bioassay- guided	Wang *et al.*, 2018a
Tunicamycin E	Nucleoside antibiotic	Sediment, *Streptomyces* sp. SCSIO S15077	Antibacterial, antifungal	Bioassay- guided	Zhang *et al.*, 2018
Staurosporine derivatives	Indolocarbazole alkaloid	Sediment, *Streptomyces* sp. NB-A13	Cytotoxic	Bioassay- guided	Zhou *et al.*, 2019

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Table 2.8: An overview of bioactive secondary metabolites produced by rare actinobacterial species derived from marine environments.

Compound	Actinobacteria	Type/ structure	Isolation source	Activity	Particulars	Reference
Thiocoraline	*Micromonospora* sp.	Depsipeptide	Soft coral	Antibacterial, cytotoxic	Bioassay- guided	Romero *et al.*, 1997
Lomaiviticins A and B	*Micromonospora lomaivitiensis*	Aromatic polyketide	Ascidian (*Polysyncraton lithostrotum*)	Cytotoxic	Biochemical induction assay guided	He *et al.*, 2001
Salinosporamide A	*Salinispora tropica*	γ-lactam-β-lactone	Sediment	Cytotoxic (Proteasome inhibitor)	Bioassay- guided	Feling *et al.*, 2003
Abyssomicin C, G and H, atrop- abyssomicin C	*Verrucosispora* sp.	Polycyclic polyketide	Sediment	Antibacterial	Bioassay- guided; LC-ESI-MS guided	Bister *et al.*, 2004; Keller *et al.*, 2007
Rifamycins	*Salinispora* sp.	Ansamycin type antibiotic	Sponge	Antibacterial	Phylogenetic analysis of biosynthetic gene cluster led to prediction of polyketide antibiotic	Kim *et al.*, 2006a
Marinomycins A- D	*Marinispora* sp.	Macrodiolides	Sediment	Antibacterial, cytotoxic	Bioassay- guided	Kwon *et al.*, 2006
Cyanosporasides A and B	*Salinispora pacifica*	Chloro- and cyanocyclopental[a]-indene- glycosides	Sediment	Cytotoxic	LC- HRMS- UV guided	Oh *et al.*, 2006
Arenicolides A	*Salinispora arenicola*	26- membered ring macrolides	Sediment	Cytotoxic	LC- HRMS based dereplication	Williams *et al.*, 2007a
Saliniketals A and B	*Salinispora arenicola*	Bicyclic polyketides	Sediment	Chemoprevention of cancer	Bioassay- guided	Williams *et al.*, 2007b

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Table 2.8, continued.

Compound	Actinobacteria	Type/ structure	Isolation source	Activity	Particulars	Reference
Salinipyrones A and B	*Salinispora pacifica*	α- pyrones polyketides	Sediment	Anti-inflammatory	LC- HRMS based dereplication	Oh *et al.*, 2008
Pacificanones A and B	*Salinispora pacifica*	Polyketides with 1,3,5,6-tetra-alkyl-substituted cyclohexanone ring	Sediment	Anti-inflammatory	LC- HRMS based dereplication	Oh *et al.*, 2008
Cyclomarin D	*Salinispora arenicola*	Cyclic heptapeptide	Sediment	Cytotoxic	Isotope detection, genome- guided	Schultz *et al.*, 2008
Cyclomarazines A and B		Diketopiperazine dipeptides		Antibacterial		
Arenamides A- C	*Salinispora arenicola*	Cyclodepsipeptides	Sediment	Cytotoxic	LC-MS chemotyping	Asolkar *et al.*, 2009
Lodopyridone	*Saccharomonospora caesia*	Alkaloid	Sediment	Cytotoxic	LC- HRMS- UV guided isolation	Maloney *et al.*, 2009
Antiprotealide	*Salinispora tropica*	A molecular hybrid of salinosporamide A and omuralide	Sediment	Cytotoxic	LC-MS chemotyping	Manam *et al.*, 2009
Salinisporamycin	*Salinispora arenicola*	A rifamycin antibiotic	Sediment	Antibacterial, cytotoxic	Bioassay- guided	Matsuda *et al.*, 2009
Dermacozines A-G	*Dermacoccus abyssi*	Phenazine	Sediment	Cytotoxic	Bioassay- guided	Abdel-Mageed *et al.*, 2010
Arenimycin	*Salinispora arenicola*	Antibiotic of benzo[α]naphthacene quinone class	Sediment	Antibacterial	LC- MS chemotyping	Asolkar *et al.*, 2010
TP-1161	*Nocardiopsis* sp.	Thiopeptide	Sediment	Antibacterial	Bioassay- guided	Engelhardt *et al.*, 2010
Saliniquinones A-F	*Salinispora arenicola*	Anthraquinone- γ-pyrones	Sediment	Cytotoxic	Bioassay- guided	Murphy *et al.*, 2010

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Table 2.8, continued.

Compound	Actinobacteria	Type/ structure	Isolation source	Activity	Particulars	Reference
Fijiolide A	*Nocardiopsis* sp.	A chloroaromatic compound that remains as Bergman cyclization product of enediyne precursors	Sediment	Cytotoxic	LC-MS based dereplication	Nam *et al.*, 2010
JBIR- 66	*Saccharopolyspora* sp.	Diterpene with an N- (4- amino- 2-hydroxybutyryl) acetamide moiety	Unidentified tunicate	Cytotoxic	Selected for chemistry profiling due to strain novelty	Takagi *et al.*, 2010
Salinosporamide K	*Salinispora pacifica*	γ-lactam-β-lactone	Sediment	Cytotoxic	Genome- mining, metabolomics, transcriptomics	Eustáquio *et al.*, 2011
Caerulomycins F- K	*Actinoalloteichus cyanogriseus*	Bipyridine and phenylpyridine alkaloids	Sediment	Antibacterial, antifungal, cytotoxic	LC- HRMS- UV guided isolation, Bioassay- guided	Fu *et al.*, 2011
Levantilides A and B	*Micromonospora* sp.	20- membered macrolides	Sediment	Cytotoxic	LC-MS guided isolation	Gärtner *et al.*, 2011
Marinacarbolines A- D	*Marinactinospora thermotolerans*	β- carboline alkaloids	Sediment	Anti-plasmodial	Bioassay- guided	Huang *et al.*, 2011
Methylpendolmy-cin derivatives		Indolactam alkaloids				
Pseudonocardians A- C	*Pseudonocardia* sp.	Diazaanthraquinone derivatives	Sediment	Cytotoxic	Bioassay- guided	Li *et al.*, 2011
Lymphostins and lymphostinol	*Salinispora* spp.	Pyrroloquinoline alkaloids	Sediment	Cytotoxic, as mammalian target of rapamycin (mTOR) inhibitor	Genome mining of biosynthetic gene clusters	Miyanaga *et al.*, 2011
Bendigoles D- F	*Actinomadura* sp.	Sterols	Sponge	Cytotoxic	High content screening, bioactivity assay- guided	Simmons *et al.*, 2011

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Table 2.8, continued.

Compound	Actinobacteria	Type/ structure	Isolation source	Activity	Particulars	Reference
Neomaclafungins A- I	*Actinoalloteichus* sp.	Oligomycin- class, 26-membered macrolides	Sediment	Antifungal	Bioassay- guided	Sato *et al.*, 2012
Anthracyclinones	*Micromonospora* sp.	Anthracyclinones	Marine tunicate (*Eudistoma vannamei*)	Cytotoxic	Bioassay- guided	Sousa *et al.*, 2012
Peptidolipins B-F	*Nocardia* sp.	Lipopeptides	Marine ascidian (*Tridemnum orbiculatum*)	Antibacterial	LC-MS based dereplication	Wyche *et al.*, 2012
Fluostatins I- K	*Micromonospora rosea*	Fluostatin family members	Sediment	Antibacterial, cytotoxic	Bioassay- guided	Zhang *et al.*, 2012b
Marthiapeptide A	*Marinactinospora thermotolerans*	Polythiazole cyclopeptide	Sediment	Antibacterial, cytotoxic	Genome mining of biosynthetic gene clusters	Zhou *et al.*, 2012
Halomadurones A- D	*Actinomadura* sp.	Chlorinated pyrones	Marine ascidian (*Ecteinascidia turbinata*)	Activator of nuclear factor E2-related factor antioxidant response element in neurodegenerative diseases treatment	Bioassay- guided	Wyche *et al.*, 2013
Sporolides A and B	*Salinispora tropica*	Polycyclic macrolides	Sediment	Anti-viral, as inhibitor of HIV-1 reverse transcriptase	Ligand- based pharmacophore screening	Dineshkumar *et al.*, 2014
Solwaric acids A and B	*Solwaraspora* sp.	Trialkyl-substituted aromatic acids	Ascidian (*Tridemnum orbiculatum*)	Antibacterial	Bioassay- guided	Ellis *et al.*, 2014
Cyanogramide	*Actinoalloteichus cyanogriseus*	Spirocyclic pyrrolo[1,2-c]imidazole alkaloid	Sediment	Cytotoxic	Bioassay- guided	Fu *et al.*, 2014

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Table 2.8, continued.

Compound	Actinobacteria	Type/ structure	Isolation source	Activity	Particulars	Reference
Forazoline A	*Actinomadura* sp.	Polyketide	Marine ascidian (*Ecteinascidia turbinate*)	Antifungal	LCMS- based metabolomics	Wyche *et al.*, 2014
Taromycin A	*Saccharomonospora* sp.	Lipopeptide	Sediment	Antibacterial	Genome mining of biosynthetic gene clusters, heterologous expression	Yamanaka *et al.*, 2014
Retimycin A	*Salinispora* spp.	Quinomycin- type depsipeptide	Sediment	Antibacterial, cytotoxic	LC-MS based dereplication, genome mining	Duncan *et al.*, 2015b
Saccharothrixones A- D	*Saccharothrix* xp.	Tetracenomycin-type polyketides	Sediment	Cytotoxic	PCR- based biosynthetic gene cluster targeting, bioassay- guided	Gan *et al.*, 2015
Nocarimidazoles A and B	*Nocardiopsis* sp.	4-aminoimidazole alkaloids	Sediment	Antibacterial	LC-MS guided fractionation	Leutou *et al.*, 2015
Lagumycin B	*Micromonospora* sp.	Angucyclines	Sediment	Cytotoxic	Bioassay- guided	Mullowney *et al.*, 2015
Sioxanthin	*Salinispora tropica*	Glycosylated carotenoids	Sediment	Anti- oxidant	Genome mining of biosynthetic gene clusters	Richter *et al.*, 2015
Lobosamides A and B	*Micromonospora* sp.	26- membered polyene macrolactams	Sediment	Anti- trypansomal	Genome mining of biosynthetic gene clusters, LC-MS based fractionation, Bioassay- guided	Schulze *et al.*, 2015a
Salinipostins A- K	*Salinispora* sp. strain RLUS08-036-SPS-B	Long chain bicyclic phosphotriesters	Sediment	Anti- malarial	Genome mining of biosynthetic gene clusters, LC-MS based fractionation, Bioassay- guided	Schulze *et al.*, 2015b
Thiolactomycin	*Salinispora pacifica*	Thiotetronic acid antibiotic	Sediment	Bacterial fatty acid synthase inhibitor	Genome mining for antibiotic resistance gene, heterologous expression	Tang *et al.*, 2015

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Table 2.8, continued.

Compound	Actinobacteria	Type/ structure	Isolation source	Activity	Particulars	Reference
Hexaricins A- H	*Streptosporangium* sp.	Pentangular polyphenols, pradimicin- like polyketides	Sediment	Anti- oxidant, as free radicals scavenger	Genome mining for biosynthetic gene clusters, heterologous expression	Gao *et al.*, 2018; Tian *et al.*, 2016
Branimycins B and C	*Pseudonocardia carboxydivorans*	Nargenicins- like macrolide antibiotics	Seawater	Antibacterial	Bioassay- guided	Braña *et al.*, 2017
Isomethoxy-neihumicins	*Nocardiopsis alba*	Lactam- lactim tautomers	Sediment	Cytotoxic	Bioassay- guided	Fukuda *et al.*, 2017
Lodopyridones B and C	*Saccharomonopora* sp.	Lodopyridone congeners	Sediment	Inhibitors of β-site amyloid precursor protein cleaving enzyme 1, useful in neurodegenerative diseases treatment	HPLC- UV guided isolation	Le *et al.*, 2017
Saccharomonopyrones A	*Saccharomonopora* sp.	α- pyrones with ethylbutyl ether moiety	Sediment	Anti-oxidant activity, as free- and catio- radical scavenger	Selected for chemistry profiling due to strain novelty	Yim *et al.*, 2017
Tetrocarcin Q	*Micromonospora carbonacea*	Spirotetronate with glycosyl group	Sponge (*Gelliodes carnosa*)	Antibacterial, cytotoxic	Bioassay- guided	Gong *et al.*, 2018
Salinilactones A- C	*Salinispora arenicola*	Bicyclic lactones	Sediment	Antibacterial	Closed loop stripping analysis using GC/ EI-MS	Schlawis *et al.*, 2018
Albisporachelin	*Amycolatopsis albispora*	Hydroxamate type siderophore	Sediment	Iron chelator	Activity guided isolation	Wu *et al.*, 2018
Nocardiopsistins	*Nocardiopsis* sp.	Angucyclines	Unidentified sponge	Antibacterial	Bioassay- guided	Xu *et al.*, 2018

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CHAPTER 3: MATERIALS AND METHODS

3.1 List of culture media

Culture media used in this study were prepared with 1.3% Bacto-agar and were adjusted to pH 7.5. Isolation media were prepared in 3% ASW, while others were prepared in 2.5% ASW, except for media used for pH and NaCl tolerance tests. ASW was prepared and autoclaved separately to prevent formation of clumps. The pH values for both media and ASW were adjusted to 7.5 with 2.5 M HCl and 2.5 M NaOH. Media were autoclave-sterilized at 121˚C and 15 psi for 15 min. The sterilized isolation media were supplemented with nalidixic acid (15 µg/ml) and nystatin (25 µg/ml) at hand-warmed temperature to reduce growth of Gram-negative bacteria and fungus respectively. The vitamin B complex solution was sterilized through 0.22 µm syringe filter (Minisart® NML with surfactant-free cellulose acetate) and 0.4 ml of the solution was added into autoclave-sterilized HVA medium (100 ml) at hand-warmed temperature. Media formulations (per 100 ml) were listed in tables below.

3.2 Samples collection

Marine environmental samples were collected from Pirate Reef, Tioman Island, Pahang, Malaysia (N: 02˚49'27.1", E: 104˚09'25.0"). The first marine sediment sample (sediment sample Z) was collected on 13 March 2013, at a depth of 7 m by scuba diving. The sediment sample Z consisting of white coarse sands was kept in sterile 50 ml tubes and was stored at 4˚C. Corals including *Porites* sp. and *Montipora* sp. were collected from Renggis Reef (N: 02˚48'33.4", E: 104˚08'8.2"). Coral samples were broken into small pieces with a chisel and a hammer and collected into sterile bags. Marine sediment sample M consisted of brown fine sand was collected during a second visit to Tioman Island in May 2014. Environmental parameters including salinity, pH and temperature of sea water at the sampling sites were recorded for both sampling times.

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Table 3.1: List of culture media for isolation of actinobacteria and culture maintenance.

Culture medium	Formulation (as per 1000 ml)	Reference
Peptone arginine agar (M3)	2 g peptone, 0.1 g L- asparagines, 4 g sodium propionate, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 0.01 g $FeSO_4$, 5 g glycerol	Zhang *et al.*, 2008
Modified mannitol arginine agar (modified M2)	Modified from medium M2: 5 g of D- mannitol, 1 g L-arginine, 1 g K_2HPO_4, 0.5 g $MgSO_4$	Zhang *et al.*, 2008
Humic acid vitamin agar (HVA)	0.5 g humic acid, 0.5 g $MgSO_4$, 1.7 g KCl, 0.02 g $CaCl_2$, 0.5 g Na_2HPO_4, 1 ml/litre trace element solution, 4 ml/litre vitamin B complex solution	Xin *et al.*, 2009
	Trace element solution (in 1 M HCl): 0.01 M $FeCl_3 \cdot 6H_2O$, 0.025 M $MnSO_4H_2O$, 0. 1 M $CaCl_2 \cdot 2H_2O$, 0.01 M $MgSO_4$, 0.001 M $CoCl \cdot 6H_2O$, 0.001 M $Na_2MoO_4 \cdot 2H_2O$	Modified from Xin *et al.* (2009)
	Vitamin B complex solution: 0.25 g each of thiamine-HCl, riboflavin, nicotinic acid, pyridoxine-HCl, inositol, calcium panthothenate and para-aminobenzoate and 0.125 g biotin	Bredholt *et al.*, 2008
MBA	2 g Bacto- casitone, 10 g glycerol, 0.8 g 'Lab-lemco' powder, 1 g yeast extract, 5 g D- mannitol	Tan *et al.*, 2006

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Table 3.2: List of culture media for morphological characterisation of novel marine actinobacterial strains and carbon sources utilisation profiling.

ISP media	Formulation (as per 1000 ml)	Reference
ISP 1	5 g Bacto- tryptone, 3 g Bacto- yeast extract	Shirling & Gottlieb, 1966
ISP 2	4 g Bacto- yeast extract, 10 g Bacto- malt extract, 4 g D-glucose	
ISP 3	20 g Quaker oatmeal, 0.1 ml/ litre of Shirling and Gottlieb trace salts solution Shirling and Gottlieb trace salts solution: 1 g each of $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$	
ISP 4	37 g of Difco™ ISP Medium 4 in 100 ml medium	
ISP 5	1 g L-asparagines, 10 g glycerol, 1 g K_2HPO_4, 0.1 ml/ litre Shirling and Gottlieb trace salts solution	
ISP 6	36 g of Difco™ Bacto-peptone iron agar and 1 g Bacto-yeast extract in 1000 ml medium	
ISP 7	15 g glycerol, 0.5 g L- tyrosine, 1 g L- asparagines, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.1 ml/ litre Shirling and Gottlieb trace salts solution	
ISP 9	The basal mineral salts medium: 2.64 g $(NH_4)_2SO_4$, 2.38 g anhydrous KH_2PO_4, 5.65 g $K_2HPO_4 \cdot 3H_2O$, 1 g $MgSO_4 \cdot 7H_2O$, 10 ml Pridham and Gottlieb trace salts solution, 150 ml of 0.04% bromocresol purple solution Pridham and Gottlieb trace salts solution: 6.4 g $CuSO_4 \cdot 5H_2O$, 1.1 g $FeSO_4 \cdot 7H_2O$, 7.9 g of $MnCl_2 \cdot 4H_2O$, 1.5 g $ZnSO_4 \cdot 7H_2O$	
LM	15 g malt extract broth, 10 g soluble starch, 10 g glucose, 5 g yeast extract, 5 g NaCl, 2 g $CaCO_3$	Urzì *et al.*, 2004
YG-Glyc	5 g yeast extract, 10 g glucose, 100 g glycerol	
YCS- Glc	5 g yeast extract, 15 g casein hydrolysates, 10 g starch, 10 g glucose	
Malt extract agar	48 g Merck malt extract agar	
DSMZ medium 65	10 g malt extract, 4 g glucose, 4 g yeast extract, 2 g $CaCO_3$	Hezbri *et al.*, 2006
DSMZ medium 535	30 g Difco trypticase soy broth	Montero-Calasanz *et al.*, 2013

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Table 3.3: List of media used for hydrolysis tests.

Culture medium	Formulation	Reference
Gelatine medium	8 g Bacto- gelatine in 1 L basal medium	De la Cruz & Torres, 2012
Starch medium	15 g soluble starch in 1 L basal medium	Gordon *et al.*, 1974
Skim milk agar	50 g skim milk in 1 L basal medium	
Urea medium	38.7 g urease test broth, 15 g urea in 1 L basal medium	
Adenine medium	5 g adenine in 1 L basal medium	
Basal medium	4 g Bacto- yeast extract, 10 g Bacto- malt extract per 1 L medium	

Table 3.4: The production media used to screen marine actinobacterial isolates for antibacterial activity

Culture medium	Formulation (per 1000 ml)	Reference
PM3	20 g Quaker oats, 2.5 g glycerol, 1 ml Shirling and Gottlieb trace salts solution	Bredholt *et al.*, 2008
Soybean meal glucose	20 g soluble starch, 15 g soybean flour, 5 g glucose, 2.5 g yeast extract, 1 g $CaCO_3$	Zheng *et al.*, 2000
Micromonospora medium	20 g starch, 15 g glucose, 5 g casein, 4 g yeast extract, 1 g $CaCO_3$, 0.1 g K_2HPO_4, 0.1 g $MgSO_4$	Ismet *et al.*, 2004
ATCC medium 241	20 g glucose, 5 g beef extract, 5 g peptone, 3 g yeast extract, 3 g $CaCO_3$	Atlas, 2004
Starch yeast peptone	10 g soluble starch, 4 g yeast extract, 2 g peptone	Bose *et al.*, 2015

3.3 Pre-treatment of marine sediment samples and isolation and enumeration of marine actinobacteria

Marine sediment sample Z was pre-treated in three different methods prior to isolation: (a) UV irradiation (Bredholdt *et al.*, 2007), (b) flooding in skim milk/HEPES solution (0.1% skim milk in 0.01 M of HEPES), a modified protocol from previous study (Xin *et al.*, 2009), (c) skim milk/HEPES treatment couple to enrichment in HVB. TRhe details of the methods are:

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(a) UV irradiation of sample was performed in a sterile Petri dish using 5 ml of wet sediment suspension at a wavelength of 254 nm. Upon UV exposure, the sediment sample was immediately subjected to serial dilution. The 0.1 ml suspension from each 10- fold dilution was spread on the surface of isolation media in triplicates.

(b) A total of 0.2 ml of wet sediment sample was transferred into 1.8 ml of filter-sterilized skim milk/HEPES solution and incubated at 28˚C with shaking at 200 rpm for 1 h. The suspension was centrifuged at 1000 × g for 10 min and 0.1 ml of the suspension was spread on the surface of isolation media in triplicates without serial dilution.

(c) A total of 0.2 ml of skim milk/HEPES treated suspension from (b) was transferred into 1.8 ml HVB and incubated at 28˚C with shaking at 200 rpm for 24 h. At the end of enrichment, the suspension was serially diluted and spread on the surface of isolation media in triplicates.

Marine sediment sample M was pre-treated in two different methods: (a) UV irradiation (Bredholdt *et al.*, 2007) and (b) heat treatment (Terahara *et al.*, 2013).

(a) Wet sediment sample M was exposed to UV as described above. The sediment sample was serially diluted and spread on surface of isolation media, in triplicates for all dilution tubes.

(b) Dilution tubes containing UV treated suspension was heated at 65˚C for 30 min in a K30 dry bath incubator. A total of 100 µl of heat treated sample from each dilution tube was spread on isolation media.

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A 10-fold serial dilution was performed in sterile 3% ASW (Instant Ocean® Sea Salt, France). Serial dilution up to 10^{-7} was performed for non-treated samples and 10^{-4} for pre-treated samples. A total of 100 µl of sediment suspension from each dilution tube was spread on the surface of isolation media in triplicates. A total of three isolation media were employed in this study (Table 3.1). Isolation plates were incubated at 28˚C for up to eight weeks.

3.4 Isolation of actinobacteria from coral samples

Coral samples were surface sterilized by thorough rinsing with sterile 3% ASW and this step was repeated for five times. Suspension from the final rinse (200 µl) was inoulated on isolation medium. The samples were lyophilized in Scanvac CoolSafe freeze-dryer (Labogene, Denmark) overnight. Lyophilized coral samples were grounded into powder using mortar and pestle. Then, of lyophilized *Montipora* sp. coral sample (0.5 g) was suspended in 5 ml of 3% ASW, whereas 0.6 g of *Porites* sp. coral sample was suspended in 6 ml of 3% ASW. The tubes were centrifuged at 1000 x g for 10 min. Supernatant (2 ml) was transferred into 18 ml liquid broth of isolation media. The enriched culture suspension was subjected to serial dilution in 3% ASW up to 10^{-3} and spread on the surface of isolation media (Table 3.1). All plates were incubated at 28˚C for up to eight weeks.

3.5 Enumeration, purification and primary grouping of marine actinobacteria

Enumeration (CFU/ml) of total bacteria and putative actinobacteria was performed on the 14^{th}, 21^{st} and 28^{th} day during incubation. The estimated CFU count was based on isolation plates having 30-300 colonies. Putative actinobacterial colonies were selected for purification based on colony morphology. Colonies with dry, powdery, compact and raised appearance, as well as colonies with diffusible pigment production were selected.

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Putative actinobacterial colonies were purified by repeated dilution streaking on the respective isolation media, ISP 2 and MBA. Pure colonies were maintained on ISP 2 slants or MBA. Pure actinobacterial isolates were preserved in 20% glycerol suspension at -20˚C and at -80˚C.

Actinobacterial isolates were primarily grouped into two major groups based on colony morphology and colony colour: the *Salinispora*-like strains and the non-*Salinispora*-like strains. The *Salinispora*-like strains were bright orange colonies lacking of aerial mycelium, which is similar to the morphological features of the members of the genus *Salinispora*. The non-*Salinispora*-like strains were dry, powdery or mucoidal and most of them produce aerial mycelium.

3.6 Genomic DNA extraction

Total genomic DNA was extracted from four to five- day- old pure cultures. A total of 3-4 loopfuls of cells were scraped from ISP2 agar plates and transferred aseptically into a 1.5 ml microcentrifuge tube that contained 480 µl of 0.05 M of EDTA-water solution (pH 8) and 120 µl of 10 mg/ml of lysozyme solution. The tube was subsequently incubated at 37˚C for 5-10 minutes to lyse the cell wall. Lysozyme solution was prepared fresh prior to extraction of DNA and stored at 4 ˚C.

Further cell lysis and extraction of DNA was performed with NucleoSpin® Tissue genomic DNA extraction kit (Macherey-Nagel, Germany) according to manufacturer's instruction. The lysozyme solution was removed by centrifugation at 11000 ×g for 5 min and the supernatant was discarded. A total of 180 µl of lysis buffer T1 and 25 µl of Proteinase K were added to the cell pellets. All components were mixed well by gently inverting the tube and incubated at 56˚C for 5-10 min. Cells were further lysed in 200 µl of Buffer B3 at 70˚C for 5 min. For both lysis steps, tubes were gently inverted to homogenize the suspension in between incubation time.

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Cell debris was separated from the DNA-containing solution by centrifugation at 11000 ×g for 5 min. Supernatant was transferred to the column assembly that was prepared by inserting a NucleoSpin® tissue column into a collection tube. A total of 210 µl of molecular biology grade ethanol (Merck) was added to the sample to precipitate DNA and to facilitate binding of DNA to the silica membrane. The column assemble was subjected to centrifuge at 11000 ×g for 1 min. Flow through was collected in collection tube and was discarded. Silica membrane bound with DNA sample was first washed with 500 µl of Buffer BW containing guanidine hydrochloride (36-50%) and isopropanol (20-50%) and flow through was discarded following centrifugation at 11000 ×g for 1 min. The second wash was performed by adding 600 µl of Buffer B5 to the column and centrifuge at 11000 ×g for 1 min. Residual ethanol was removed by centrifuging the empty column at 11000 ×g for 1 min.

The dry NucleoSpin® tissue column was transferred to a sterile 1.5 ml microcentrifuge tube. The slightly alkaline elution buffer BE, which had been pre-warmed at 70 ˚C, was added to the column at a volume of 50 µl. The tube was incubated at room temperature for 1 min. Genomic DNA was eluted by centrifuging at 11000 ×g for 1 min. The column was discarded. Genomic DNA was examined for integrity using 40 ml of 0.8% (w/v) agarose gel, which was pre-stained with 1.5 µl of 20000 × RedSafe™ nucleic acid staining solution (Intron Biotechnology, Korea). Gel electrophoresis was performed in 1 × TAE buffer for 35 min at 100 V. The gel was viewed in a UV transilluminator MUV21 (Major Science, USA). Genomic DNA was stored at -20˚C until further usage.

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3.7 De-replication of non-*Salinispora*-like actinobacterial strains

The 16S rRNA gene and the adjacent 16S-23S ITS region of non-*Salinispora* like actinobacterial strains were amplified with the primer pair pA/ BL235R (Lanoot *et al.*, 2005). Primer pA (5'-AGAGTTTGATCCTGGCTCAG-3') hybridized at position 8- 27 on 16S rRNA and BL235R (5'-GCGCCCTTAAAAACTTGG-3') at position of 3- 20 on 23S rRNA genes, according to the *Escherichia coli* numbering system. The final PCR reaction volume was 50 µL that was consisted of 0.5 – 1.0 µg of genomic DNA, 10 µl of *MyTaq*™ reaction buffer (5×, Bioline, United Kingdom), 0.5 µl *MyTaq*™ DNA polymerase (Bioline, United Kingdom), 0.2 µM of each primer. The PCR reaction was performed in the following steps: initial denaturation at 95˚C for 5 min, subsequently a total of 30 cycles of denaturation at 95˚C for 30 s; annealing at 49˚C for 30 s and extension at 72˚C for 2 min and a final extension at 72˚C for 7 min. Amplicons (approximately 1 µg) were digested using HaeIII (10 U, NEB, USA) at 37˚C for 5 min in a total volume of 25 µl containing 2.5 µl of 10 × NEBuffer and subsequently with BstU1 (10 U, NEB, USA) at 60˚C for 5 min, following the protocol recommended by NEB. Restriction enzyme digested products were resolved on 2% agarose gel containing 1.5 µl of 20000 × RedSafe™ nucleic acid staining solution. The gel was allowed to run for 45 min at 100 V in 1 × TAE buffer.

3.8 De-replication of *Salinispora*-like actinobacterial strains

Salinispora-like actinobacterial strains were subjected to an ITS-RFLP screen using the restriction endonuclease *Ban*1. The 16S-23S ITS region of *Salinispora*-like strains was amplified with the primer pair G1/ L1 (Jensen *et al.*, 1993). Primer G1 (5'-GAAGTCGTAACAAGG-3') targeted the highly conserved region of the adjacent 16S-23S spacer at 30- 40 bp upstream from the spacer boundary. The primer L1 (5'-CAAGGCATCCACCGT-3') hybridized at the most conserved region of 23S gene sequence following the spacer located 20 bp downstream from the spacer boundary.

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Reactions were set up in the same manner as described in section 3.7, except for the annealing temperature was set at 52˚C. The resulted PCR product was subjected to restriction enzyme digestion with 5 U of Ban1 in a total reaction volume of 25 µl (Freel *et al.,* 2012). Each reaction tube contained 2.5 µl of 10 × NEBuffer and 0.5 µg of PCR product. Reaction tubes were incubated at 37˚C for 15 min. Restriction enzyme digested products were examined with 2.5% agarose gel containing 1.5 µl of 20000 × RedSafe™ nucleic acid staining solution in 1 × TAE buffer.

Intra-species variation of *Salinispora*-like strains were examined with Rep-PCR using the primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Vidgen *et al.*, 2012). The 50 µl BOX-PCR reaction mixture contained 0.5 – 1.0 µg of genomic DNA, 10 µl of *MyTaq*™ reaction buffer (5×, Bioline, UK), 0.5 µl *MyTaq*™ DNA polymerase (Bioline, UK), 0.2 µM of BOX-A1R primer. The PCR program consists of steps include initial denaturation at 95˚C for 5 min and 35 cycles of denaturation at 95˚C for 30 s; annealing at 53˚C for 30 s and extension at 68˚C for 5 min and a final extension at 68˚C for 15 min. Amplicons were resolved on a 2% agarose gel containing 1.5 µl of 20000 × RedSafe™ nucleic acid staining solution in 1 × TAE buffer.

3.9 Analysis of RFLP and BOX-PCR banding profiles

Analysis of molecular fingerprinting banding profiles was performed with Bionumerics software package version 7.1 (Applied Maths, Belgium). Gel pictures were imported into the Bionumerics database and converted to 8-bit gray scale TIF images. Spectral analysis was performed for gel images to estimate disc size for background subtraction (background scale) and the cut-off threshold for least-squares filtering (Wiener cutoff scale). Median filter was applied to all images to smooth the densitometric curves. Inter-gel and intra-gel normalizations were achieved by using GeneRuler 100 bp plus DNA ladder (Thermo Fisher Scientific) as the molecular

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marker. Bands were manually assigned. For 16S- ITS- RFLP fingerprinting from section 3.7, bands from 100-1000 bp were included for analysis. On the other hand, bands ranging from 400-3000 bp were considered for analysis of Rep-PCR products obtained from section 3.8. Quantification of similarities between band patterns of isolates was performed using Pearson coefficient. Average band tolerance positions were set at 0.11% for RFLP fingerprinting and 0.0386% for Rep-PCR fingerprinting. The UPGMA dendograms were derived from the resultant similarity matrixes.

3.10 16S rRNA gene sequence analysis

Representative actinobacterial strains of each RFLP group with different morphological characteristics and colony colour were identified by performing phylogenetic analysis of their 16S rRNA gene using the primer pair: 27F (5'-AGAGTTTGATCMTGGCTCAG-3')/ 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Vidgen *et al.*, 2012). The 16S rRNA gene was amplified following the steps consisted of an initial denaturation at 95˚C for 5 min, subsequently a total of 35 cycles of denaturation at 95˚C for 45 s; annealing at 55˚C for 45 s and extension at 72˚C for 45 s and a final extension at 72˚C for 7 min. Sequencing of the 16S rRNA gene was performed by 1st Base, Malaysia using the BigDye® Terminator v3.1 cycle sequencing kit chemistry. 16S rRNA gene sequence chromatograms were examined for evenly-spaced peaks, noise signals and miscalled bases on Sequence Scanner Software version 1.0 (Applied Biosystems, USA).

Closely related phylogenetic neighbours were identified from GenBank databases through EzBioCloud server based on nucleotide similarity values and respective sequences were retrieved (Yoon *et al.*, 2017). Cut-offs for identification of potential novel taxa were based on 16S rRNA gene sequence similarity values of 98.7%, as compared to the closest matches with validly published names (Sangal *et al.*, 2016).

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Almost full length sequence of potentially novel actinobacteria isolate was aligned with multiple sequences of closely related species using CLUSTAL_W (Thompson *et al.*, 1994). Phylogenetic trees were constructed on MEGA 6.0 (Tamura *et al.*, 2013) using neighbour-joining (Saitou & Nei, 1987), maximum likelihood and maximum parsimony based on SPR algorithm (Takahashi & Nei, 2000). In all cases, bootstrap values were calculated based on 1000 re-samplings (Felsenstein, 1985).

3.11 Characterisation of novel marine actinobacterial strains

3.11.1 Morphological characterisation

Description of morphology of novel marine actinobacterial strains was performed on standard media including ISP 2, ISP 3, ISP 4 and ISP 5 (Table 3.2), according to the standard reference work (Shirling & Gottlieb, 1966). Actinobacterial strains were inoculated on plates by dilution streaking. Plates were incubated at 28˚C for 14 days and 21 days. On the 14^{th} and 21^{st} day of incubation, mass colour of aerial mycelia with heavy spore production, substrate mycelium and diffusible pigment were compared with ISCC-NBS colour chart (Kelly, 1958) and recorded from all ISP media. Colour of aerial mycelium and spores were observed from the top view of plates, while substrate mycelium was determined by examination of the reverse side of mass growth. Diffusible pigment production was observed by comparing the colour of agar containing actinobacterial culture with the un-inoculated plates.

Characteristics of spore bearing hyphae and spore chain were examined using cover-slip method. The autoclave-sterilized coverslips were embedded into ISP 2 and ISP 3 at an angle of 45˚ with a sterile forceps (Williams & Cross, 1971). Actinobacteria isolates were inoculated onto the upper surface of the coverslip and plates were incubated at 28˚C for 14 days. Coverslips were removed using a sterile forceps and the uppermost growth surface was examined under a light microscope as unstained preparations at

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magnifications of 100 × or 400 ×. Aerial mycelia were observed as coarse, refractive and phase-bright. Substrate mycelia, in contrast to aerial mycelia, were slender, transparent and phase-dark. In general, aerial mycelia were observed to be darker than substrate mycelium under a light microscope. Spore chains and aerial mycelium were examined for type of morphology and number of spores formed at the tips of aerial hyphae was determined. Presence of globular sporangia, flagellated spores and production of conidia-like spores on the substrate mycelia were examined. Occurrence of sclerotia and fragmentation of substrate mycelium were observed.

Electron micrographs of spore morphology and spore surface were determined on a field emission scanning electron microscopy (JEOL JSM-7001F, Japan) using 14 days or 21 days old culture growing on the best sporulation medium. Agar plugs obtained from the matured culture with spore production (6 mm in diameter) were fixed with one volume of 8% glutaraldehyde in one volume of Sorensen phosphate buffer (pH 7.2) for 60 min. The specimens were washed with one volume of buffer to remove the primary fixative reagent. Secondary fixation was performed by addition of three volumes of 4% osmium tetroxide in water for overnight. Fixation agent was removed by rinsing with water and subjected to dehydration with ethanol in ascending series, started from 10% ethanol in water up to 90% with an increment of 10, followed by 95% ethanol. Each dehydration step was carried out for 15 min. Subsequently, dehydration step with 100% ethanol was repeated twice for 15 min each. Next, the specimen was further dehydrated in three mixtures of ethanol/ acetone in the ratio of 3:1, 1:1 and 1:3 with each step being carried out for 20 min. Final dehydration step was performed in acetone for four times over a course of 80 min. The dehydrated specimen was subjected to critical point drying through carbon dioxide for 1.5 to 2 hours. Dried specimens were mounted onto a stub with carbon adhesive cement and were sputter-coated with 1- 2 nm of gold-palladium

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prior to viewing under scanning electron microscope at magnifications ranged from 5000 × to 10000 ×.

3.11.2 Melanin production

Production of melanin was tested on ISP 1, ISP 6 and ISP 7 media according to Shirling & Gottlieb (1966) (Table 3.2). Culture less than 21 days old was used as the inoculum. All plates were incubated at 28˚C for 14 to 21 days. Melanoid pigment production was characterized by formation of greenish brown to brown and to black diffusible pigment or a distinct brown pigment modified by other colour. Absence of brown to black diffusible pigment formation and total absence of diffusible pigment was regarded as negative for melanin production.

3.11.3 Carbon utilisation

Ability of novel actinobacterial isolates, which were closely related to *Streptomyces* spp., *Nocardiopsis* spp., *Nonomuraea* spp. and *Nocardiopsaceae* members to utilise various carbon sources as sole energy source for growth was determined on modified ISP 9 medium following the standard protocol (Shirling & Gottlieb, 1966). The ISP 9 medium (Table 3.2) was prepared by addition of sterile carbon source into the basal mineral salts medium to give a final concentration of approximately 1%. The pH of basal mineral salts medium was adjusted to 7.0. Bromocresol purple solution was then added into the medium as pH indicator on acid production from carbon sources (Gordon *et al*, 1974).

Carbon sources were prepared as 10% stock in distilled water and sterilized through 0.2 µm surfactant-free cellulose acetate Minisart® NML syringe filter 16534 (Goettingen, Germany). A total of 23 carbon sources were tested, including adonitol, D- arabinose, D- fructose, D- galactose, D- glucose, D- lactose, D- maltose, D- mannitol, D- mannose, D- melezitose, D- melibiose, D- ribose, D- sorbitol, D- trehalose, D- xylose,

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glycine, L- arabinose, L- glutamine, L- lysine, L- methionine, L- sorbose, *meso*- inositol and sucrose. The carbon utilisation testing with ISP 9 was performed in sterile 24- wells polystyrene culture plates SPL#32024. The actinobacterial isolates were inoculated onto the plates in triplicate with D- glucose served as the positive growth control and wells contained basal mineral salts agar without carbon source as negative growth control. Acid production as a result of fermentation was indicated as positive when purple colour of the medium turned into yellow, otherwise, the observation was recorded as negative. Actinobacteria isolates were incubated at 28˚C for 21 days.

Carbon utilisation profile was assessed using API® 20NE (bioMérieux, Inc.) for novel actinobacterial isolates belonging to the genus *Blastococcus*. API® 20NE strips were inoculated with 4- day- old pure culture in duplicates following the steps that were described in the manufacturer's protocol (bioMérieux, USA). Strips were incubated at 28˚C and examined for positive or negative reactions on the 7th and the 14th day (bioMérieux, USA).

3.11.4 Tolerance of pH, temperature and sodium chloride

Novel actinobacterial strains were tested for their ability to tolerate various (a) growth temperature, (b) pH, (c) concentration of sodium chloride. The ISP2 medium was used as the basal medium for all the following tests:

(a) Growth of purified actinobacterial strains was assessed at eleven temperatures: 4˚C, 10˚C, 15˚C, 20˚C, 25˚C, 28˚C, 32˚C, 37˚C, 45˚C, 50˚C and 55˚C and the pH of culture medium was adjusted to 7.5.

(b) The pH range for growth was examined at pH 5.0- 13.0 at intervals of 1 pH unit. The pH of medium was adjusted with buffer systems: 0.1 M citric acid/ 0.1 M sodium citrate was used to adjust pH to 4.0- 5.0; 0.1 M KH_2PO_4/ 0.1 M NaOH for pH 6.0– 8.0;

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0.1 M $NaHCO_3$/ 0.1 M Na_2CO_3 for pH 9.0-10.0; 0.05 M Na_2HPO_4/ 0.1 M NaOH for pH 11.0; 0.2 M KCl/ 0.2 M NaOH for pH 12.0- 13.0 (Xu *et al.*, 2005).

(c) Tolerance to NaCl was tested at concentrations up to 14% (w/v) at intervals of 1%.

Growth of actinobacterial strains was observed and recorded as positive or negative following 14 days of incubation at 28˚C except for plates used for temperature test. In all cases, growth at 28˚C on ISP2 supplemented with 2.5% ASW (pH 7.5) served as the positive control.

3.11.5 Enzyme production assays

(a) Amylase production assay was determined on starch agar (Table 3.3) according to a modified protocol by Gordon *et al.* (1974). A total of 1.5% of soluble starch (Difco) was suspended in cold distilled water and was heated in a microwave until the solution was appeared as transparent. The starch solution was cooled to room temperature and added into the basal medium and sterilized by autoclaving. Actinobacterial strains were inoculated onto the starch agar plates in duplicates and incubated at 28˚C for 4-7 days. Presence of starch was detected by using the standard iodine reagent, which was prepared by dissolving 0.3% of iodine crystal (BDH, UK) and 3% of potassium iodide (Sigma) in distilled water (ZoBell & Hittle, 1969). Presence of starch was indicated by a change of agar colour to blue-black. Hydrolysis of starch was recorded as positive if a clear zone around the growth of actinobacterial colonies was observed. Hydrolysis of starch was recorded as negative if no clear zone was observed around the colonies. *Bacillus subtilis* ATCC 23857 was used as the positive control and *E. coli* ATCC 47076 as the negative control.

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(b) Protease production assay was performed on skim milk agar by detecting decomposition of casein (Table 3.3). Skim milk powder was added into the medium in an amount of 5%. Three bottles contained 40 ml of 5% skim milk, 2.5% of ASW and 1.3% of Bacto- agar, respectively, were autoclaved separately. Skim milk was autoclaved at 116˚C for 10 min whereas ASW and agar were autoclaved at 121˚C for 15 min. All suspensions were cool to approximately 50- 60 °C, mixed and poured into Petri dishes. Actinobacterial strains were inoculated on the skim milk agar plates in duplicates and incubated at 28˚C for 4-7 days. A positive reaction was indicated by the presence of a clearing zone underneath and around the actinobacterial colonies, whereas a negative reaction was recorded when clearing zones were not observed around the colonies. *Bacillus subtilis* ATCC 23857 was used as the positive control and *E. coli* ATCC 47076 as the negative control.

(c) Urease production assay was performed by culturing the strains on a urea medium to detct the ability of novel actinobacterial strains to decompose urea (Table 3.3). Urease test broth and urea solution were filter sterilized through 0.22 µm syringe filter (Minisart® NML with surfactant-free cellulose acetate) and added into autoclave sterilized ASW and agar solution. The medium was prepared in autoclave sterilized screw cap glass test tubes as agar slants. Actinobacteria isolates were inoculated onto the surface of slant and incubated at 28˚C with the cap being loosen. Tubes were examined for growth and changes of colour of the pH indicator phenol red at day 7, 14, 21 and 28 days. Positive hydrolysis reaction was indicated by gradual transition from yellow to bright pink of the medium colour. Slant cultures were compared to an un-inoculated tube that was served as the negative control.

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(d) Decomposition of adenine was tested on basal medium containing 0.5% adenine (Sigma). Adenine was suspended in distilled water and autoclaved separately. Both adenine solution and basal medium bottles were cooled to hand-warmed temperature and homogenized by gentle swirling. Adenine crystals were distributed evenly throughout the solidified agar. Actinobacteria strains were inoculated on the agar in duplicates and incubated at 28˚C. Decomposition of adenine was recorded as positive, as indicated by vanishing of crystals underneath and around the colonies. Plates were examined on the 7^{th} and 14^{th} day of incubation.

(e) The ability of actinobacterial strains to hydrolyze gelatine by producing gelatinase enzyme was determined on the basal medium supplemented with 0.8% (w/v) of Bacto-gelatine. Heavy inoculums were inoculated by streaking across the centre of gelatine plates in duplicates. Plates were incubated at 28˚C. A total of 5 ml of saturated ammonium sulphate solution was added to the plates on the 7^{th} and 14^{th} day of incubation to precipitate non-hydrolyzed gelatine. Positive hydrolysis reaction was indicated by the presence of clear halo zone around the actinobacterial colonies, within 5-10 min upon addition of saturated ammonium sulphate solution (Chapman, 1948). Absence of halo zone around colonies was recorded as negative reaction.

(f) Catalse production was performed for the novel actinobacterial strains. Actinobacterial strains were inoculated on ISP 2. Plates were incubated at 28˚C for 7 days. A clean microscope slide was placed in a sterile Petri dish, which was placed over a dark background. Few colonies of actinobacteria were transferred to the microscope slide with sterile wooden stick. A total of two to three drops of 3% H_2O_2 was added onto the smear. Immediate bubble formation indicated a positive reaction, whereas absent of bubbles or production of a few scattered bubbles indicated a negative reaction (Reiner, 2010).

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(g) The novel actinobacterial strains were also tested for oxidase production. Actinobacterial strains were cultured on TSA supplemented with 2.5% ASW. A strip of filter paper (Whatman No. 1) was moistened with 1% solution of the test reagent, *N, N, N', N'*-tetramethyl-p-phenylenediamine dihydrochloride, which was freshly prepared in distilled water. The 7- day old actinobacterial colonies were rubbed on the wet filter paper with a sterile wooden stick. Formation of intense deep purple hue that appeared within five to ten seconds indicated a positive oxidase reaction. A delayed oxidase positive reaction was indicated by colour changes to purple within 60 to 90 seconds. A negative reaction was indicated by absent of colour changes to purple or colour changes to purple that take longer than 2 minutes to occur (Shields & Cathcart, 2010).

3.11.6 Examination of cell wall composition of novel actinobacteria

3.11.6.1 Analysis of DAP

The protocol for the absence and presence of *meso*- or LL-DAP was modified from Staneck and Roberts (1974). Actinobacterial strains were cultured on ISP2 agar for 7 days at 28˚C. A total of 4 loopfuls of cells were suspended in 200 µl of 6 N HCl in a sterile 1.5 ml microcentrifuge tube. The content was homogenized by vortex. The microcentrifuge tube was subjected to autoclave at 121˚C for 20 min. Tubes were cool to room temperature and centrifuged at 11000 ×g for 15 min. Supernatant was transferred to a clean 1.5 ml microcentrifuge tube and 10 µl of pyridine was added to the supernatant. Cell wall hydrolysates were concentrated by evaporation at 100˚C to dryness. The hydrolysates were resuspended in 30 µl of sterile distilled water and evaporated to dryness at 100˚C again. The cell wall hydrolysates were dissolved in 10 µl of sterile distilled water and applied to the base line of the TLC cellulose plate with aluminium sheet (20 cm × 20 cm, Merck), which was approximately 2 cm from the bottom edge of the chromatogram sheet. Samples were spotted with capillary tube and were at least 1 cm apart. The TLC tank was lined with filter paper at both sides and

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saturated with solvent system for at least 1 hour prior to the run. Upon saturation, the filter paper was thoroughly wet due to migration of solvent.

Ascending TLC was performed to analyse cell wall DAP using a solvent system containing methanol-distilled water-6 N HCl-pyridine that was prepared in a ration of 80: 26: 4: 10 (v/v) for approximately 4 hours. The chromatogram was air dried. Spots were visualized by spraying with 0.2% ninhydrin in acetone, followed by heating at 100˚C for 3 min. DAP spots in cell wall hydrolysates were compared to the DAP standard, which runs concurrently on the same TLC sheet. The DAP standard was prepared as 0.01 M DL-DAP solution that contains both *meso*- and LL-DAP (Sigma) and 1 µl of the DAP standard was spot on the TLC sheet. The DAP spots were observed as gray-green fading to yellow, with the L-isomer migrated ahead of the *meso*- isomer. Amino acids present in the cell wall hydrolysates were observed as purple or red and migrated ahead of the DAP spot.

3.11.6.2 Analysis of whole cell sugars

Actinobacterial strains were inoculated into the ISP 2 broth and incubated for 7 days at 28˚C. Broth was freeze-dried in a Scanvac CoolSafe freeze-dryer. Whole cells sugar was performed and analysed by the Identification Service, DSMZ, Braunschweig, Germany using a previously describe protocol (Staneck & Roberts, 1974). Approximately 25 mg of freeze-dried cells were washed in 1 ml sterile distilled water twice and pellet down by centrifuge at 11000 ×g for 5 min. Cells were disrupted in 1.5 ml of 1 N sulphuric acid (Merck), followed by heating at 121˚C in a dry bath incubator (K30, China) for 20 min and cool to room temperature. Saturated barium hydroxide was prepared in distilled water and added drop wise into the cell wall hydrolysates until the pH was between 5.2-5.5, which was determined with pH meter (Sartorius, Germany). The precipitate was removed by centrifugation at 11000 ×g for 15 min. Supernatant was

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transferred into new 1.5 ml centrifuge tubes and evaporated to dryness at 35˚C. Cell wall hydrolysates were reconstituted in 50 µl of sterile distilled water and spin at 11000 ×g for 2 min to remove the solid residues.

Cell wall sugar was analysed by ascending TLC using the solvent system *n*-butanol-distilled water-pyridine-toluene following the ration 10:6:6:1 (vol/vol). Cell wall hydrolysates were applied to the base line of the TLC cellulose plate with aluminium sheet (20 cm × 20 cm, Merck). Two standard solutions were used: standard 1 and standard 2. Standard 1 contained D-galactose, D-arabinose and D-xylose. Standard 2 was a mixture of L-rhamnose, D-mannose, D-glucose and D-ribose. In both standard solutions, the final concentration of each sugar was 1% (vol/vol). A total of 1 µl of each standard solution was spot on the same TLC plate as cell wall hydrolysates. The TLC plate was allowed to develop for 4 hours. Presence of sugar was detected by spraying the chromatogram with aniline phthalate (Sigma) and R_f values of sugar spots that were present in cell wall hydrolysates were compared with the standards.

3.11.6.3 Analysis of polar lipids

Actinobacterial strains were cultured in ISP 2 broth and freeze-dried. The polar lipid analysis of actinobacterial isolates was performed by the Identification Service, DSMZ, Braunschweig, Germany according to previously described protocols (Minnikin *et al.*, 1977; Tindall *et al.*, 2007). A total of 100 mg of freeze-dried cells were extracted using a solvent system consisted of chloroform-methanol-0.3% aqueous NaCl, which was prepared in a ration of 1:2:0.8 (vol/vol), by stirring overnight. Cell debris was removed by centrifugation. The solvent system contained chloroform-methanol-0.3% aqueous NaCl mixture was adjusted to 1:1:0.9 (vol/vol) to recover polar lipids into the chloroform phase. Polar lipids were separated by two dimensional TLC on an aluminium-backed silica gel plate (10 cm × 10 cm). The glass chromatography tanks

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were lined with filter paper. The first dimension TLC was performed in a solvent system contained chloroform-methanol-water (65:25:4, vol/vol). The plates were air dried at room temperature and developed in the second solvent containing chloroform-methanol-glacial acetic acid-water (80:12:15:4, vol/vol). Plates were air dried and polar lipids were detected using various spraying reagents.

Lipids were detected by molybdophosphoric acid in ethanol and stained blue-black spots. Lipid phosphate was detected with molybedum spray, where lipid compounds containing phosphate ester appeared as blue spots on a white or a light-blue gray background. Lipids containing α-glycols such as phosphatidylglycerol were detected with periodatte-Schiff and stained purple spots. Lipids contains only inositol (phosphatidylinositol) will appear as yellow-brown spots upon spraying with periodatte-Schiff. Sugar-containing lipids such as glycolipids and phosphatidylinositol mannosides were detected with *α*-naphthol in sulphuric acid and producing red-purple spots. Lipids containing free amino group such as phosphatidylethanolamine was detected with ninhydrin in ethanol as red-purple spots. Quaternary nitrogen compounds including phosphatidylcholine were detected as red-orange spot by Dragendorff's reagent.

3.11.6.4 Analysis of menaquinones

Analysis of menaquinones was performed by the Identification Service, DSMZ, Braunschweig, Germany according to a protocol described by Tindall (1990) using 100 mg of freeze-dried cell. Menaquinones were extracted using methanol-hexane in the ration 2:1 (vol/vol) by stirring the material under nitrogen gas for 30 min. Hexane phase was separated from the methanol phase by cooling the extract in an ice bath. Ice-cold hexane was added to produce a biphasic mixture that contained methanol-hexane (1:1, vol/ vol). Hexane phase that constituted the upper phase was removed with a Pasteur pipette and subjected to menaquinones analysis. Menaquinones were separated into

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different classes by TLC on silica gel using a solvent system contained hexane-*tert*-butyl-methylether (9:1, vol/vol). UV absorbing bands corresponding to the different quinone class were eluted from the TLC plate and subjected to analysis on a LDC Analytical (Thermo Separation Products) HPLC. Individual quinones were purified using a reverse phase column (Macherey-Nagel, 2 mm × 125 mm, 3 µm, RP18) and a solvent system containing methanol:heptanes (9:1, vol/vol). Menaquinones were detected at 269 nm.

3.11.6.5Analysis of fatty acids

Fatty acids methyl esters were obtained from the cells by saponification, methylation and extraction using a slight modified method (Miller, 1982; Kuykendall *et al.*, 1988; Sasser, 2001) . Four reagents were prepared as listed in the following to cleave the fatty acids from lipids. The saponification reagent, Reagent 1, was prepared by mixing 45 g of NaOH in 150 ml of methanol and 150 ml distilled water. Reagent 2 was used for methylation of the fatty acids, in which 325 ml of 6 N HCl was diluted in 275 ml methanol. The fatty acid methyl ester was extracted into the organic phase with Reagent 3, which was constituted by 200 ml of hexane and 200 ml of methyl tert-butyl ether. Reagent 4 was prepared by dissolving 10.8 g NaOH in 900 ml distilled water.

Actinobacterial strains were cultured on TSA supplemented with 2.5% ASW for 7-14 days. A total of 40 mg of cells were scraped from Petri dishes and kept in clean test tubes using a nickel loop. A total of 1 ml of Reagent 1 was added to each test tube. Tubes were sealed with Teflon lined caps and homogenized by vortex. The content was heated in a boiling water bath for 30 min. The tube was vigorously vortexed for 5-10 seconds every 5 minutes. Tubes were allowed to cool to room temperature. Fatty acids were subjected to methylation by adding 2 ml of Reagent 2 to the test tubes. The tubes were capped and briefly vortexed, followed by heating at 80 ± 1 ˚C for 10 ± 1 min.

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Tubes were cooled rapidly to room temperature and 1.25 ml of Reagent 3 was added to extract the fatty acids methyl ester into the organic phase and also to separate the organic phase from the aqueous phase. The content was mixed for 10 min by gentle tumbling on a rotator. The aqueous phase, which refers to the bottom layer, was pipette out and discarded. A total of 3 ml of Reagent 4 was added to the organic phase and the tubes were tumbled for 5 min. The organic phase was transferred into a GC vial and separated using Sherlock Microbial Identification System by gas chromatography. Fatty acids were identified and percentages were calculated by the MIS Standard Software (Kampfer & Kroppenstedt, 1996).

3.12 Whole genome analysis of novel actinobacterial strains

Sequencing of the genomes of novel actinobacterial strains was performed by Monash University Malaysia Genomics Facility, Selangor, Malaysia. Genomic DNA was sequenced on the MiSeq Illumina version 2 kit using the configuration of 2 × 250 bp according to the manufacturer's instruction. Paired-end sequencing libraries were prepared using the Nextera XT kit (Illumina). Reads were assembled into contigs using IDBA-UD. Genome sequences of the respective reference strain were retrieved from GenBank database.

Genome annotation was performed using the RAST pipeline. A two-way BLAST based ANI between the genomes of the strains was calculated using OAT (version 0.93.1) based on BLASTn (ANIb) programme, in which nucleotide identity values between fragments of the query strain and genome of the subject strain were calculated using BLASTn program and the mean of these nucleotide identity values was obtained (Lee *et al.*, 2016a). A two-way average AAI between their protein sequences was calculated using the online calculator from the K. Konstantinidis group (http://enve-omics.ce.gatech.edu/aai/) (Rodriguez-R & Konstantinidis, 2014). The dDDH values

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between genomes, genome-to-genome-distance, GC content and differences of GC content between genomes were calculated using GGDC 2.1 (Meier-Kolthoff *et al.*, 2013; Meier-Kolthoff *et al.*, 2014). Biosynthetic gene clusters were predicted using antiSMASH 3.0 (Weber *et al.*, 2015).

DNA-DNA relatedness between strains were carried out by the State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-Sen University, Guangzhou, China, based on a previously described protocol and were recorded as wet lab DDH values (Li *et al.*, 2015). Briefly, DNA-DNA relatedness between novel strains was determined by a fluorometric micro-well method. Determination of the DNA hybridization rate was achieved by measuring fluorescence intensities resulted from enzyme- substrate reaction between streptavidin-inconjugated alkaline phosphatase and 4-methylumbelliferyl phosphate. Fluorescence intensities were measured by a Fluostar optima microplate reader (SPECTRA max GEMINIXPS). The excitation rate and emission rate were measured at a wavelength of 360 nm and 460 nm, respectively. DNA hybridization rate was calculated from quadruplicated hybridization experiments and expressed as mean of the corresponding reciprocal values.

3.13 Assessment of antibacterial activity by agar plug diffusion assay

Antibacterial activity of marine actinobacterial strains were first assayed by agar plug diffusion. Actinobacterial strains were cultured as lawn on five production media supplemented with 2.5% ASW and 1.5% agar as listed in the following: PM3, soybean meal glucose, micromonospora medium, ATCC medium 241 and starch yeast peptone (Table 3.4). Plates were incubated at 28˚C for 14 to 21 days.

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Actinobacteria strains were screened against four test pathogens: *Bacillus subtilis* ATCC 23857, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 47076, *Pseudomonas aeruginosa* ATCC 27853. Pathogens were cultured on MHA (Difco) for 18 h at 37˚C. Colonies were transferred into 10 ml of MHB (Difco) and incubated at 37˚C for 18 h. The concentration of broth culture was standardized according to 0.5 McFarland standards. Turbidity of the pathogen suspension was adjusted to the density of 0.5 McFarland standards using MH broth, by comparing the test and standard against a white background with a contrasting black line. The 0.5 McFarland standards was prepared by mixing 50 µl of 1% barium chloride and 9.95 ml of 1% H_2SO_4 (Andrews, 2001).

The standardized broth culture was inoculated as lawn culture on MHA plates. Agar block containing actinobacteria culture was cut aseptically from the lawn culture on various production media and deposited on the surface of MHA, which was previously inoculated with test pathogens. MHA plates were incubated at 37 ˚C for 18 h. Each actinobacteria was tested in duplicates and not more than six agar plugs were deposited on each MHA plate to avoid overlapping of inhibition zones, which were characterized by clear zone that formed around the agar plug. Diameters of inhibition zones were measured over a black background in millimetre. Positive activity was considered when diameter of inhibition zone was at least 10 mm (Baz *et al.*, 2012).

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3.14 Fermentation and solvent extraction

Selected actinobacterial strain that displayed antibacterial activity against all pathogens was selected for further study on its antibacterial activity profile. The strain was up-scaled in 1 litre of soybean meal glucose medium. First, pure actinobacteria strain was inoculated on ISP 2 plate for 14 days at 28 ˚C. Agar containing actinobacterial culture was aseptically cut into pieces of 1 cm × 1 cm with inoculation loop. A total of 3 agar pieces were transferred to 10 ml ISP 2 broth in screw capped universal bottles. The broth was incubated at 28 ˚C for 4 days at 150 rpm. Then, 4 ml of the ISP 2 culture was transferred to 60 ml of soybean meal glucose broth in a 250 ml screw-capped Erlenmeyer flask. Flasks were incubated in an orbital shaker (New Brunswick™ Innova® 44, Eppendorf) at 28˚C for 4 days at 150 rpm. Large scale fermentation was prepared by transferring 20 ml of the soybean meal glucose culture to 500 ml broth in a 2- litres screw-capped Erlenmeyer flask and incubated at 28˚C at 150 rpm for 21 days. Soybean meal glucose broth was supplemented with 20 g/litre of Amberlite® XAD-16 resins (20-60 mesh, 200 Å mean pore size, Sigma) and sterilized by autoclave at 121 ˚C, 15 psi for 15 min (Gonzalez *et al.*, 2014).

The Amberlite® XAD-16 resins contained excessive salts such as NaCl and Na_2CO_3. Prior to use, resins were first wash with DCM, second wash with methanol and lastly wash twice with distilled water. The resins were poured into a 1 liter Erlenmeyer flask until one-third full and covered with DCM, followed by stirring for 15 minutes. Solvent was removed by filtration through Whatman No. 1 filter paper and resins were transferred back to the Erlenmeyer flask and soaked with methanol. The same step was repeated for methanol and distilled water washing steps. After the final washing step, distilled water was completely removed by filtration and stored at room temperature.

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Biomass and resins were harvested in 50 ml GeneMate® tubes (BioExpress, USA) by centrifugation at 3500 rpm for 15 min after 21 days of incubation. The tubes were lyophilized in the Scanvac CoolSafe freeze-dryer to remove all liquid. The contents were collected in a 500 ml Erlenmeyer flask and subjected to solvent extraction. A solvent mixture containing DCM and methanol in a ration of 50:50 was poured into the flask. Biomass and resins were allowed to soak overnight in the fume hood with the flasks were sealed with aluminium foil. Solvent was collected in a round bottom flask and concentrated in a rotary evaporator. The biomass and resins were extracted with solvent for 3 times in the same manner. Weights of crude extracts were recorded.

Dried crude extract was subjected to solid phase extraction using the reverse phase Discovery® DSC-18 SPE tubes (5 g of bed weight, Sigma) using step gradient elution with 20%, 40%, 60%, 80% methanol in distilled water, 100% methanol and a final flush with 100% ethyl acetate to give six fractions. Prior to use, SPE tubes were washed once with 100% methanol, followed by distilled water wash for twice. Subsequently, the SPE tubes were conditioned with the starting solvent, i.e. 20% methanol in distilled water. For each Discovery® DSC-18 SPE tube with 5 g bed weight, 250 mg of crude extract was loaded into the tube. First, crude extracts were dissolved in 2 ml of 20% methanol in distilled water and the un-dissolved solids were precipitated by centrifugation at 250 ×g for 15 min in a miVac Quattro concentrator (GeneVac, UK). The supernatant was transferred from glass test tube into SPE tube and was eluted with 20% methanol until colour of the extract turn lighter or to colourless using applied pressure from a 20 cc syringe. The pellet was dissolved in 40% methanol, centrifuged and eluted with 40% methanol. This step was repeated with 60%, 80% and 100% methanol as well as 100% ethyl acetate. During the elution step, care was taken to ensure that the SPE packing does not dry before addition of conditioning solvent and sample, by allowing about 1 mm of the solvent to remain above the top tube frit or above the surface of the disk.

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Eluted fractions were collected in glass test tubes and concentrated under vacuum in a speed vac concentrator (Savant SPD2010-230, ThermoFisher Scientific) at room temperature for 10 hours. The SPE fractions were re-constituted in 2 ml of DMSO and subjected for antibacterial screening.

3.15 Screening of SPE fractions for antibacterial activity and BioMap profiling

The experiments described below was carried out in the Linington Laboratory, Department of Chemistry and Biochemistry, University of California Santa Cruz (UCSC), California, USA according to the method described by Wong *et al.* (2012) on 21st August 2014. All six SPE fractions were screened against a panel of 15 pathogens. A total of six Gram-positive pathogens *B. subtilis* ATCC 23857, *Listeria ivanovii* ATCC BAA-139, *Enterococcus faecium* ATCC 6569, *Staphylococcus epidermidis* ATCC 14990^{T}, *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA-44 (MRSA) were used in this screening. Gram-negative pathogens used in the BioMAP screening were *E. coli* ATCC 47076, *Providencia alcalifaciens* ATCC 9886^{T}, *Ochrobactrum anthropi* ATCC 49687, *Enterobacter aerogenes* ATCC 35029, *Acinetobacter baumannii* ATCC 19606^{T}, *P. aeruginosa* ATCC 27853, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* ATCC 700720, *Vibrio cholerae* O1 (biotype EI Tor A1552) and *Yersinia pseudotuberculosis* IP2666 pIBI.

Gram-positive *S. aureus*, MRSA, *L. ivanovii* and *E. faecium* were inoculated in 10 ml TSB. Gram-negative pathogens including *Providencia alcalifaciens* ATCC 9886^{T}, *Ochrobactrum anthropi* ATCC 49687, *Enterobacter aerogenes* ATCC 35029, *Acinetobacter baumannii* ATCC 19606^{T} were cultured in nutrient broth. Other pathogens were inoculated in LB. Pathogens were incubated in a rotating incubator at 30 ˚C overnight at 200 rpm. Overnight culture of pathogens was diluted 1:1000 and seeded in a volume of 30 µl/well in sterile clear propylene 384-wells assay plates. Each

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well was fed with 300 nl of DMSO fractions using a high-throughput pinning robot (Perkin Elmer Janus MDT, USA), which is located at UCSC's Chemical Screening Centre. Growth curves of pathogens were measured at OD600 in an hour interval over 24 hours in an automated plate reader/shaker (Perkin Elmer EnVision, USA).

DMSO fractions that were inhibiting Gram-positive and Gram-negative pathogens were selected for parallel screening of 2-fold dilution series (100 mM – 3 μM) to determine the MIC values. The concentrations (100 mM – 3 μM) were of arbitrary values, i.e. the SPE fraction without any dilution was given an arbitrary concentration of 100 mM, and the fraction was diluted in a 2- fold series down the row of the same column on a 384 multi-wells plate. Pathogens were prepared in the same manner. Each well was fed with 300 nl of fractions and growth curves of pathogens were measured at OD600 in an hour interval over 24 hours.

Data were normalized and BioMAP profile was created and compared to the training set of antibiotics, which contained 44 antibiotics that fall into four classes: the cell wall synthesis inhibitors, DNA synthesis inhibitors, RNA synthesis inhibitors, protein synthesis inhibitor, DNA intercalators and anaerobic DNA inhibitors. The 44 antibiotics that made up the training sets of antibiotics include piperacillin, carbenicillin, ampicillin, penicillin G, cloxacillin, nalidixic acid, ciprofloxacin, levofloxacin, sparfloxacin, norfloxacin, oxytetracycline, doxycycline, tetracycline, demeclocycline, minocycline, erythromycin, clarithromycin, midecamycin, roxithromycin, spiramycin, tobramycin, gentamicin, amikacin, streptomycin, spectinomycin, chloramphenicol, thiamphenicol, florfenicol. tiamulin, clindamycin,lincomycin, ornidazole, furazolidone, nitrofurantoin, rifampicin, rifabutin, rifaximin, fosfomycin, bacitracin, cefadroxil, cefaclor, ceftazidime, polymixin B and actinomycin D. All antibiotics were prepared as 100 μM solution.

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To generate the BioMap profile, data normalization was performed in four steps:

(a) To obtain a concentration-independent ratio of activities, the MIC values obtained for each SPE fraction were divided by the largest value in the series, which was 100 mM;

(b) Value obtained from (a) was transformed into its reciprocal value and then multiplied by 10;

(c) The resulting value from (b) was converted to a $\log_{10}$ value, which is the BioMap value.

(d) An antibacterial activity profile was created by plotting a histogram containing the BioMap values against pathogens.

To obtain a hierarchical clustering heat map, the BioMap values of the same fraction were further normalized. For each extract, the BioMap value was divided by the largest BioMap values for each pathogen that falls within the range 0–1. Hierarchical clustering was created using Pearson correlation similarity metrics and average linkage clustering method with Cluster 3.0 and displayed using TreeView v1.1.6r4. Normalized MIC values that are between 0 to 1 were represented by a red-black colour scheme with a gradient from black to red. Fractions were inactive against pathogens was indicated as black, whereas the most potent fractions were indicated as red.

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3.16 Purification of compounds from fraction with antibacterial activity

The SPE fraction that showed positive antibacterial activity against the panel of pathogens as described in section 3.14 was subjected to Sephadex LH-20 gel filtration chromatography and eluted with 100% methanol. TLC profile of each fraction was obtained by spotting the extract on silica TLC plates (Merck), followed by development of TLC plates in glass TLC jars. A total of three solvent systems were employed: DCM: MeOH (90:10), EtoAc: MeOH (90:10) and ether: MeOH (90:10). The eluted fractions were concentrated to dryness with rotary evaporator, reconstituted in 1 ml DMSO and subsequently subjected to screening for antibacterial activity following the methodology as described in the section 3.14.

Fraction that displayed positive antibacterial activity was subjected to HPLC analysis on Waters HPLC system (USA), by using an analytical HPLC with an ODS column (Phenomenex®, Synergi™, 250 × 4.6 mm, 10 µm). Compounds were eluted at 2 ml/ min with a linear gradient from 10% to 95% methanol in water over the course of 30 min, in which the water used here contained 0.02% formic acid, followed by elution with 100% methanol for 15 min. Fractions were collected in test tubes with the aid of automated time-based fraction collector (Waters SFO system fluidics organizer) and Waters 2998 photodiode array detector. The fractions were concentrated to dryness in a speed vac concentrator (Savant SPD2010-230, ThermoFisher Scientific) at room temperature for 10 hours. Fractions were re-constituted in 10 µl DMSO and screened against the same panel of pathogens as listed in section 3.14.

To obtain enough materials for LCMS and NMR profiling, the selected novel actinobacterial strain was cultured in 30 litres of soybean meal broth and the step-wise up- scaling of the culture was performed as described in the section 3.14. Solvent extraction, fractionation of the crude extracts on SPE and Sephadex LH-20, screening

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for antibacterial activity and purification of the compound of interest (compounds 1 and 2) on HPLC were repeated as described in sections 3.14 and 3.15. Compounds 1 and 2 were purified via isocratic separation using a solvent system containing water/ methanol (30:70) for 20 minutes on an analytical reverse phase HPLC with an ODS column (Phenomenex®, Synergi™, 250 × 4.6 mm, 10 µm). Two fractions containing compounds 1 and 2 were eluted from HPLC and were manually collected in test tubes at minutes 9- 11 for compound 1 and at minutes 14- 15.5 for compound 2. The flow rate was set at 2 ml/ min. The eluted fractions containing both compounds were tested for antibacterial activity. The chemical structure of the compounds were confirmed by NMR spectra which were recorded on Bruker Avance III 400 and 600 spectrometers (USA) at 400 and 600 MHz for ^{1}H nuclei and 100 and 150 MHz for ^{13}C nuclei in $CDCl_3$ with reference to TMS. For compound 2, the 2D NMR experiments including COSY and HSQC were performed to confirm the chemical structure. Mass spectra of the compound of interest including both ESIMS and HRMS were obtained on an Agilent 6530 Q-TOF LC/MS spectrometer (CA, USA), whereas DARTMS were recorded on a JEOL AccuTOF-Dart LC™ time-of-flight mass spectrometer (USA).

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CHAPTER 4: RESULTS

4.1 Isolation and enumeration of marine actinobacteria

The marine sediment samples Z and M, which were collected from the Pirate Reef, Tioman Island, Pahang, Malaysia had a pH of 7.5 and salinity of 34 ppt, which is equivalent to 3.4% (w/v) of NaCl. The underwater temperature was recorded to be 28˚C (the 1st visit in March 2013) and 29˚C (the 2nd visit in May 2014), respectively. Live coral samples of *Porites* sp. and *Montipora* sp. were collected from the Renggis Reef, which the pH, salinity and underwater temperature to be recorded as 7.5, 34 ppt and 28˚C, respectively. Estimated bacterial count was obtained from all plates with the number of colonies falls in the range of 25-250 and recorded as cfu/ml. Examples of isolation plates containing samples that were treated with various pre-treatments on M3, modified M2 and HVA media are shown in Figures 4.1 to 4.11. Isolation plates containing samples without pre- treatment was observed to be dominated by growth of mucoidal bacterial colonies. The modified M2 plates, in particular, were dominated by bacterial colonies that liquefied the agar when sediment samples without pre-treatment were inoculated onto the media as shown in Figure 4.2. By contrast, isolation plates containing pre- treated marine sediment samples were observed to have reduced number of mucoidal bacterial growth and also low number of agar liquefying bacterial growth (Figures 4.7 to 4.10).

The number of bacterial isolated from a range of media using various pre-treatment methods from wet sediment samples was enumerated as CFU/ml and the bacterial counts are given in Table 4.1. The total bacterial count for wet sediment sample Z was estimated to be 1.8×10^5 to 1.3×10^7 cfu/ml. High total bacterial count was recorded from HVB enriched samples and UV treated samples. The highest count was recorded from HVB enriched sample on M3 plate, while the lowest count was estimated from skim milk/HEPES pre-treated sample on M3 plates, with about 100-folds difference in

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cfu count. By contrast, putative actinobacterial count was recorded to be the highest on skim milk/HEPES pre-treated plates on all media. No actinobacteria was recovered from the non-pretreated samples on all isolation plates. In total, 123 putative actinobacteria were successfully isolated from pre-treated marine sediment sample Z. A total 114 putative marine actinobacteria were isolated from the sample pre-treated with skim milk/HEPES solution. The mannitol-based modified M2 and humic acid based HVA plates coupled to skim milk/HEPES treatment were shown to recover the highest number of putative marine actinobacteria from sediment Z. Nine other strains were recovered from the marine sediment sample pre-treated with UV irradiation (n= 2) and HVB enrichment of skim milk/HEPES treated sample (n= 7). Plates pre-treated with UV irradiation led to isolation of only two actinobacteria from modified M2. HVB enrichment of skim milk/HEPES treated sample recovered only four strains on modified M2 plates and three strains on HVA plates.

Estimated bacterial count recorded from sediment M was 3×10^2 cfu/ml to 8.8×10^4 cfu/ml (Table 4.1). M3 medium was found to recover the highest number of total bacteria from marine sediment regardless of pre-treatment method. The highest bacterial count was observed on plates containing non-pretreated sample and the lowest on plates containing heat treated samples, both were from M3 plates. Number of colonies on both modified M2 and HVA plates that contained heat treated samples was below 25, thus no cfu count was calculated and the result was recorded as not determined “nd”. Bacterial count for M3 plates containing heat treated samples was estimated to be 3×10^2 cfu/ml. No actinobacteria was isolated from isolation plates containing non-pretreated sediment sample M. Sixty putative marine actinobacteria was isolated from pre-treated marine sediment M. UV irradiation was shown to recover the highest number of actinobacteria from all three isolation media, which led to isolation of a total of 49 actinobacteria. The highest count was recorded from modified M2 plates (n = 36) and the lowest from M3

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(n = 2). Heat treatment led to isolation of 11 actinobacterial strains. Modified M2 and HVA plates with heat treated samples were observed to have only actinobacterial colonies growing on them, of which three strains were recovered from modified M2 and six strains from HVA. The remaining two strains were isolated on M3.

Bacterial count from coral *Porites* sp. was calculated to be 8×10^2 cfu/ml and 2.45×10^4 cfu/ml on modified M2 and M3, respectively. The highest number of putative actinobacteria was isolated from *Porites* sp. on M3 plates (n = 12) and modified M2 plates (n = 8). No actinobacteria was recovered from HVA. By comparison, the number of bacterial colonies recovered from *Montipora* sp. on all isolation plates was below 25 and only 1 actinobacteria colony was isolated from M3 plate. No actinobacteria was isolated from modified M2 and HVA.

Table 4.1: Estimated counts of bacteria and putative actinobacteria isolated from marine sediment samples using various pre-treatment methods and isolation media. 'nd', not determined.

Sample	Pre-treatment	Bacterial count ($\times 10^5$ cfu/ml)			Putative actinobacterial count		
		M3	Modified M2	HVA	M3	Modified M2	HVA
Z	No treatment	9.7	19.7	40.7	0	0	0
	UV irradiation	9.2	23.3	18.4	0	2	0
	Skim milk/ HEPES	1.8	2.8	14.7	18	51	45
	Skim milk/ HEPES + HVB enrichment	130.0	16.0	15.0	0	4	3
M	No treatment	0.880	0.034	0.008	0	0	0
	UV irradiation	0.055	0.010	0.029	2	36	11
	Heat at 60˚C	0.003	nd	nd	2	3	6
Porites sp.	Broth enrichment	0.245	0.008	0	12	8	0
Montipora sp.	Broth enrichment	nd	0	0	1	0	0

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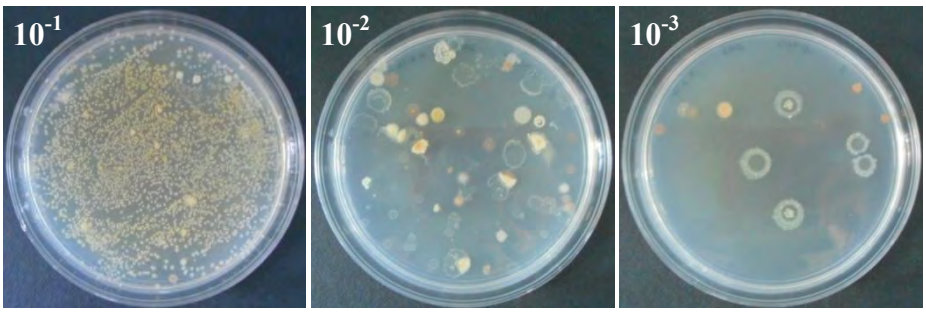

Figure 4.1: M3 isolation plates inoculated with untreated marine sediment sample Z showing colonies isolated at the three different dilutions.

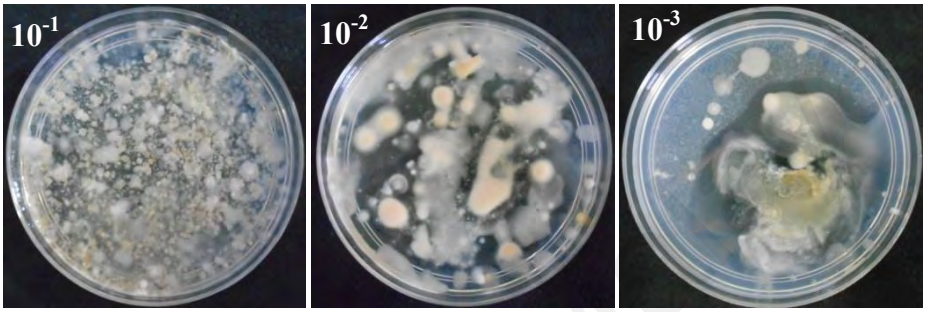

Figure 4.2: Modified M2 isolation plates inoculated with untreated marine sediment sample Z showing colonies isolated at the three different dilutions.

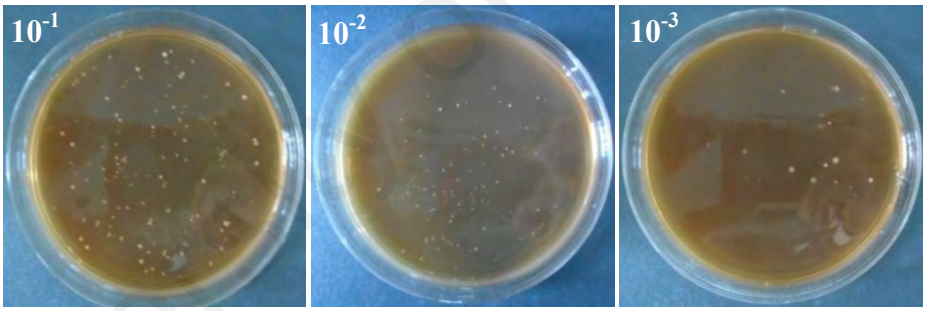

Figure 4.3: HVA isolation plates inoculated with untreated marine sediment sample Z showing colonies isolated at the three different dilutions.

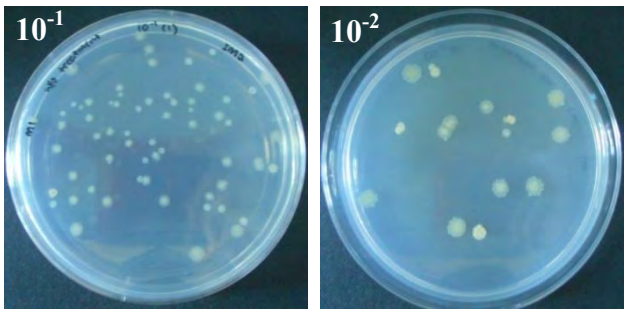

Figure 4.4: M3 isolation plates inoculated with untreated marine sediment sample M showing colonies isolated at the two different dilutions.

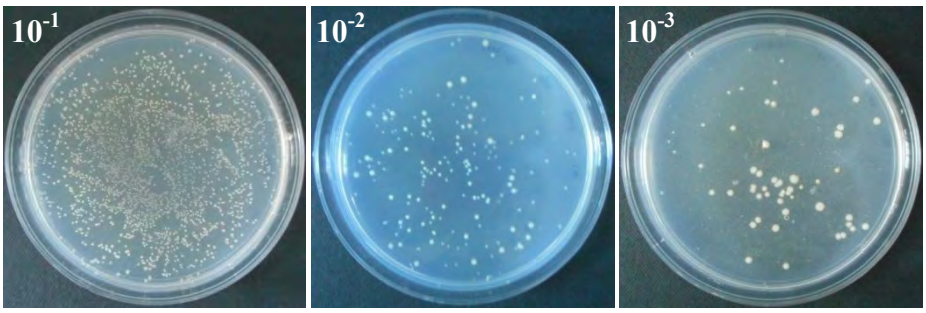

Figure 4.5: Modified M2 isolation plates inoculated with untreated marine sediment sample M showing colonies isolated at the three dilutions.

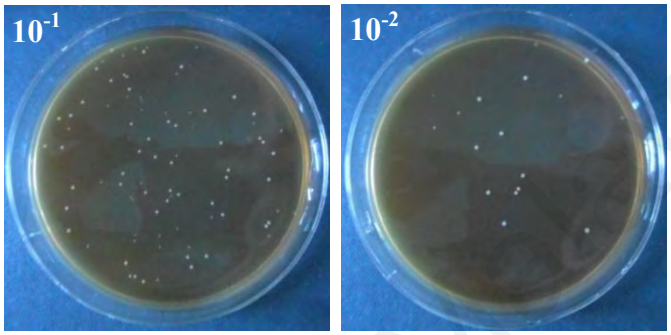

Figure 4.6: HVA isolation plates inoculated with untreated marine sediment sample M showing colonies isolated at the two dilutions.

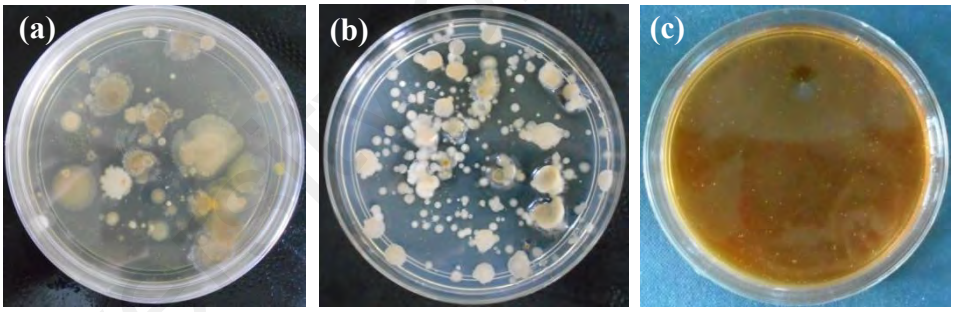

Figure 4.7: Isolation plates inoculated with UV-treated sediment sample at 10^{-1} dilution, showing colonies isolated from (a) M3, (b) modified M2 and (c) HVA.

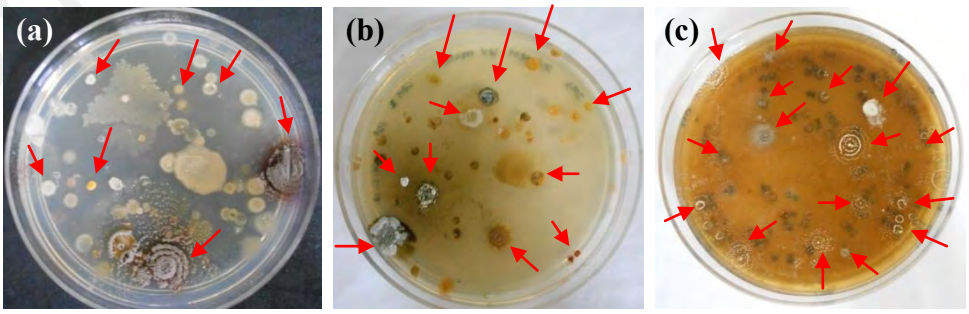

Figure 4.8: Isolation plates inoculated with skim milk/HEPES treated sediment sample (10^{-1} dilution) showing colonies isolated from (a) M3, (b) modified M2 and (c) HVA. The red arrows indicated examples of actinobacterial colonies that grew on the isolation plates.

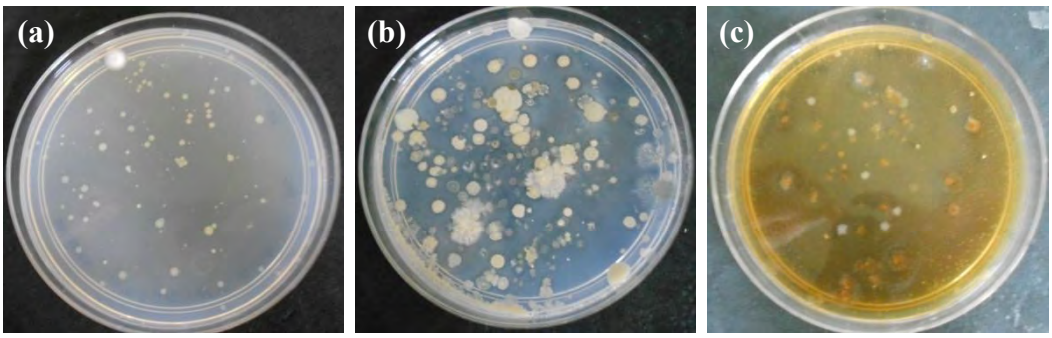

Figure 4.9: Isolation plates inoculated with skim milk/HEPES treated and HVB enriched sediment sample (10^{-3} dilution), showing colonies isolated from (a) M3, (b) modified M2 and (c) HVA.

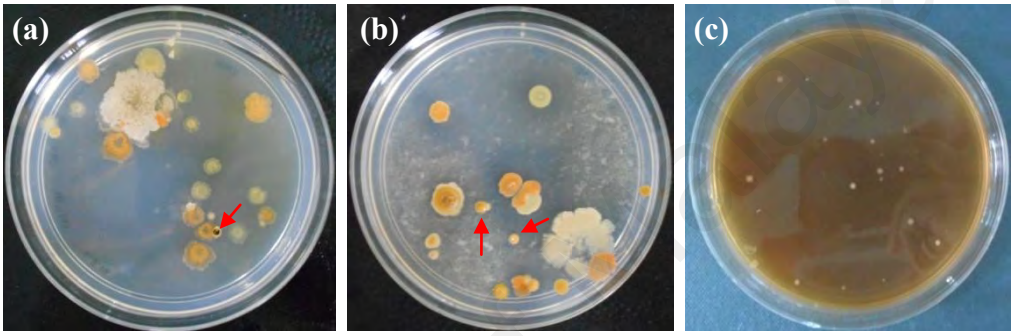

Figure 4.10: Isolation plates inoculated with sediment sample that was diluted to 10^{-1} and pre-heated at 60˚C for 30 min, showing colonies isolated from (a) M3, (b) modified M2 and (c) HVA. The red arrows indicated examples of actinobacterial colonies that grew on the isolation plates.

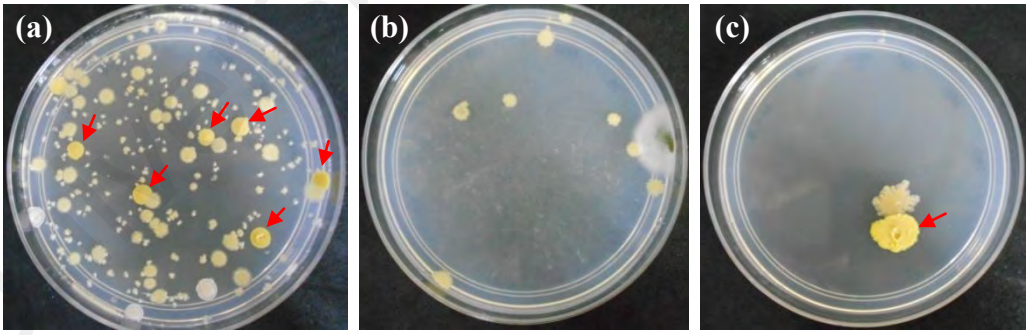

Figure 4.11: Isolation plates inoculated with coral samples showing colonies isolated from (a) *Porites* sp. on M3 at 10^{-1} dilution, (b) *Porites* sp. on modified M2 at 10^{-1} dilution and (c) *Montipora* sp. on M3 (without dilution). The red arrows indicated examples of actinobacterial colonies that grew on the isolation plates.

4.2 Primary grouping of marine actinobacteria

A total of 183 actinobacterial strains isolated from marine sediment samples and coral samples were primarily grouped into *Salinispora*-like and non-*Salinispora*-like strains, based on colony morphology, colour of aerial mycelia and diffusible pigment. The non-*Salinispora* like strains were composed of actinobacteria with blue, brown, gray, red to orange, white, yellow and pink coloured colonies. Actinobacterial colonies of white coloured constituted the largest group of non-*Salinispora*-like strains (Table 4.2). The *Salinispora*-like strains constituted the colour group 8 and were further divided into five sub-groups (Table 4.3). Representative pictures of actinobacterial strains were shown in Figures 4.12 to 4.18. The prefixes TPS and TPM denote strains isolated from sediment samples Z and M, respectively, while TRC denotes actinobacterial strains isolated from coral samples.

A total of 76 actinobacterial strains isolated from sediment sample Z were grouped as non-*Salinispora*-like and 47 as *Salinispora*-like strains. Skim milk/HEPES treatment supported the isolation of higher numbers of non-*Salinispora*-like strains and *Salinispora*-like strains on HVA plates and modified M2 plates, respectively (Table 4.4). UV irradiation was shown to encourage the growth of mucoidal bacteria instead of the actinobacteria. Nevertheless, UV treated samples had shown to recover two non-*Salinispora*-like actinobacterial strains on modified M2. HVB enriched of skim milk/HEPES samples was also shown no effect in improving the isolation of actinobacteria from marine sediment Z. In fact, only four non-*Salinispora*-like strains and three *Salinispora*-like strains were recovered from HVB enriched skim milk/HEPES treated samples using modified M2 and HVA media.

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Out of the 60 actinobacterial isolates recovered from marine sediment sample M, 56 marine actinobacteria strains comprised the *Salinispora*-like group and only four actinobacteria was grouped as non-*Salinispora*-like strains (Table 4.4). UV irradiation was observed to recover the highest number of *Salinispora*-like strains from marine sediment sample M on modified M2 plates (n = 39), and subsequently on HVA (n = 12) and M3 (n = 5). Heat treatment was shown to successfully isolate 11 heat- tolerant *Salinispora*-like strains with the highest count on HVA (n = 6). The non-*Salinispora*-like strains were all isolated from UV treated samples on HVA and were associated with pink or white coloured colonies (Table 4.5).

A total of 20 putative marine actinobacteria was isolated from the coral *Porites* sp. and only 1 actinobacterial strain was isolated from *Montipora* sp. Actinobacteria isolated from both coral samples were non-*Salinispora*-like strains with yellow coloured colonies (Table 4.2). As the actinobacterial colonies isolated from *Porites* sp. were morphologically similar on isolation plates, only one to two colonies of the same morphology were randomly selected for further purification.

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Table 4.2: Colour grouping of non-*Salinispora*-like actinobacterial strains based on colour of aerial mycelium and diffusible pigment on ISP2.

Colour group	Aerial mycelia	Diffusible pigment	Colony surface	Strain
1	Blue	Blue	Powdery	TPS16, TPS81, TPS83
2a	Brown	NA	Wrinkled	TPS114
2b		Olive	Leathery	TPS38, TPS359, TPS445
3a	Gray	NA	Powdery	TPS1, TPS58, TPS61, TPS63, TPS65, TPS66, TPS67, TPS68, TPS209
3b		Olive	Powdery	TPS4, TPS14, TPS27, TPS77, TPS183
3c		Brown	Powdery	TPS11, TPS15, TPS75
3d		Yellow	Powdery	TPS6, TPS53
3e		Dark olive	Powdery	TPS35
4a	Red to orange	NA	Powdery	TPS358a
4b		Yellow	Wrinkled	TPS166
4c		NA	Smooth	TPS357, TPS418, TPS448, TPS459
5a	White	NA	Powdery	TPS3, TPS17, TPS24, TPS41, TPS42, TPS43, TPS44, TPS45, TPS46, TPS47, TPS48, TPS51, TPS89, TPS94, TPS122, TPS125, TPS198, TPS199, TPS201, TPS202, TPS210, TPS211, TPS419, TPM287
5b		Brown	Powdery	TPS10, TPS12, TPS31, TPS60, TPS74, TPS208
5c		Olive	Powdery	TPS5, TPS7, TPS8, TPS143, TPS180, TPS181, TPS364
5d		Orange	Leathery	TPS137
5e		Yellow	Powdery	TPS37, TPS216, TPM81
6a	Yellow	NA	Powdery	TPS2
6b		NA	Smooth	TPS33, TPS92, TPS179, TRC8
6c		NA	Wrinkled	TRC2, TRC15, TRC16
7	Pink	NA	Smooth	TPM87, TPM88

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Table 4.3: Colour grouping of *Salinispora*-like strains with orange coloured colony isolated from marine sediment samples.

Group	Diffusible pigment	Colony surface	Strain
8a	Brown	Leathery	TPS101, TPS102, TPS103, TPS104, TPS105, TPS107, TPS108, TPS109 TPS112, TPS113, TPS115, TPS118, TPS119, TPS120, TPS123, TPS126 TPS127, TPS132, TPS135, TPS142, TPS146, TPS147, TPS148, TPS153, TPS158, TPS167, TPS174, TPS178, TPS335, TPS347, TPS355, TPM91, TPM92, TPM93, TPM95, TPM107, TPM109, TPM114, TPM115, TPM116, TPM117, TPM118, TPM119, TPM120, TPM121, TPM122, TPM123, TPM127, TPM128, TPM130, TPM136, TPM137, TPM138, TPM140, TPM141, TPM144, TPM146, TPM147, TPM148, TPM149, TPM150, TPM151, TPM152, TPM154, TPM155, TPM160, TPM164, TPM165, TPM167, TPM169c, TPM170, TPM171, TPM173, TPM175, TPM176, TPM179, TPM187, TPM199, TPM204, TPM205, TPM206, TPM208, TPM210, TPM227, TPM228
8b	Pink	Folded	TPS32, TPS88, TPS111
8c	NA	Folded	TPS86, TPS106, TPS121, TPS128, TPS175, TPS350, TPS385
8d	NA	Smooth	TPS193, TPS217, TPS409, TPM181
8e	NA	Powdery	TPS90, TPS213, TPS351, TPM84

Table 4.4: Primary grouping of marine sediment-derived actinobacteria based on various pre-treatment methods and isolation media.

Sediment sample	Treatment	Non-*Salinispora*-like (n)			*Salinispora*-like (n)		
		M3	Modified M2	HVA	M3	Modified M2	HVA
Z	UV irradiation	0	2	0	0	0	0
	Skim milk/ HEPES	15	14	41	3	37	4
	Skim milk/ HEPES + HVB enrichment	0	2	2	0	2	1
M	UV irradiation	0	0	4	3	36	6
	Heat at 60˚C	0	0	0	2	3	6

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Table 4.5: Breakdown of the number of non-*Salinispora*-like strains isolated from Tioman marine sediment samples.

Sediment sample	Treatment	Colour group	M3	Modified M2	HVA
Z	UV irradiation	Red to orange	0	2	0
	Skim milk/ HEPES	Blue	0	0	3
		Brown	0	2	1
		Gray	3	2	15
		Red to orange	0	3	0
		White	12	5	20
		Yellow	0	2	2
	Skim milk/ HEPES + HVB enrichment	Red to orange	0	1	0
		Brown	0	0	1
		White	0	1	1
M	UV irradiation	Pink	0	0	2
		White	0	0	2

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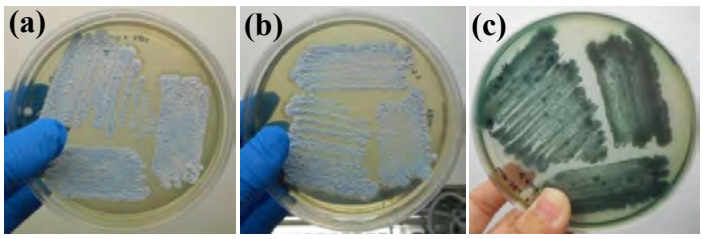

Figure 4.12: Strains (a) TPS81, (b) TPS83, (c) TPS16, which belong to colour group 1 producing blue diffusible pigment and aerial mycelia on ISP2.

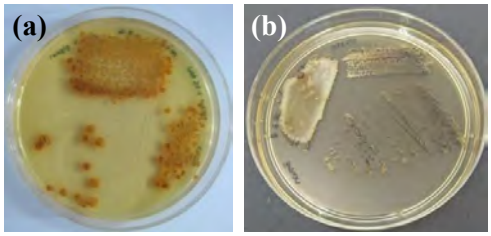

Figure 4.13: Representative strains of colour group 2 (a) TPS114 and (b) TPS359.

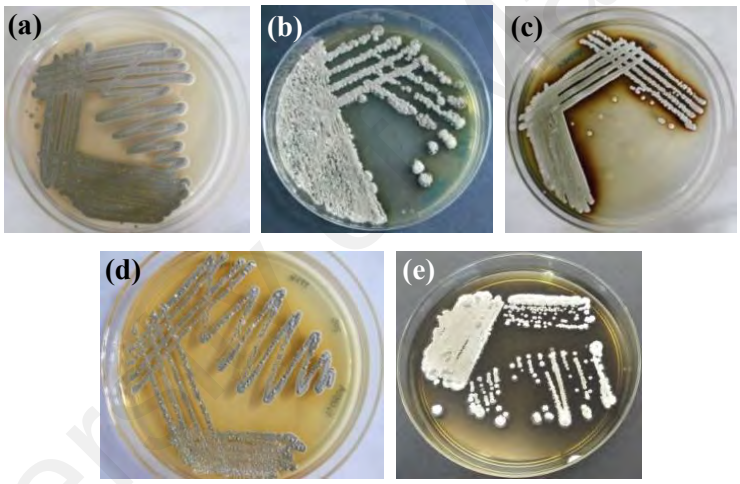

Figure 4.14: Strains (a) TPS1, (b) TPS4, (c) TPS75, (d) TPS53, (e) TPS35 of the colour group 3 produced colonies with gray aerial mycelia on ISP2. Strains TPS4, TPS75, TPS53 and TPS35 produced olive, brown, yellow and dark olive diffusible pigment, respectively.

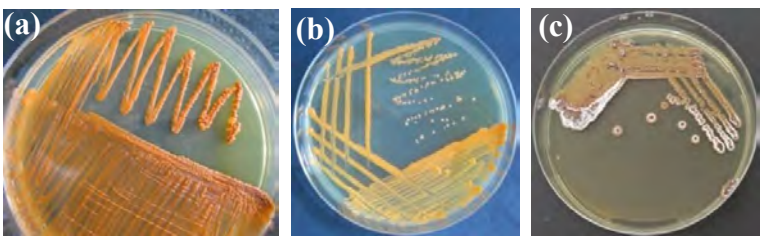

Figure 4.15: Strains (a) TPS166, (b) TPS357, (c) TPS358a of colour group 4 produced red to orange colonies on ISP2. Strains TPS166 produced yellow diffusible pigment. TPS358a is the only strain from colour group 5 with dry surface and producing spores.

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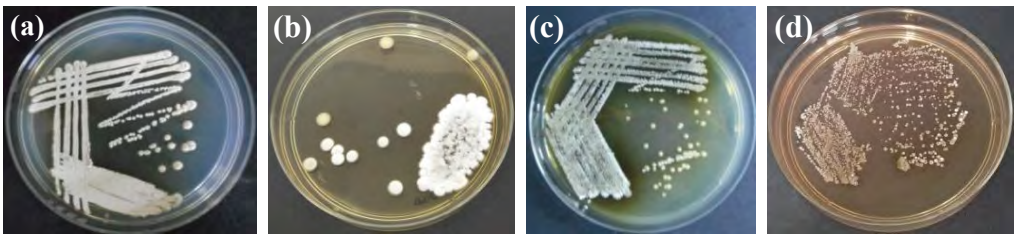

Figure 4.16: Strains (a) TPS42, (b) TPS10, (c) TPS143, (d) TPS137 of colour group 5 produced white colour colonies on ISP2. Strain TPS14 did not produce diffusible pigment, whereas strains TPS10, TPS143 and TPS137 produced brown, olive and orange diffusible pigment, respectively.

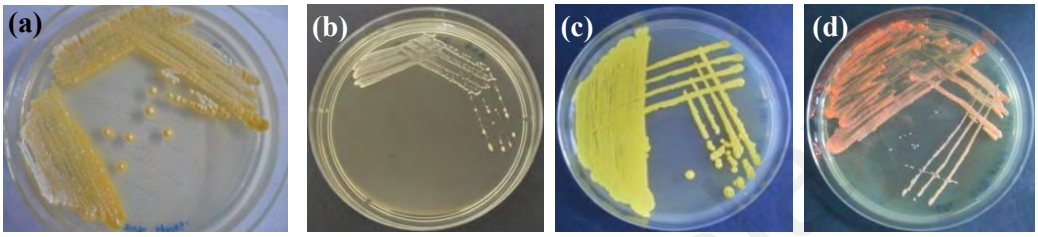

Figure 4.17: Strains (a) TPS2 and (b) TPS179 from colour group 6 that were isolated from marine sediment sample Z, as well as (c) TRC2 isolated from coral *Montipora* sp. represent members of yellow colour group. The representative member of colour group 7 was shown in (d) TPM88.

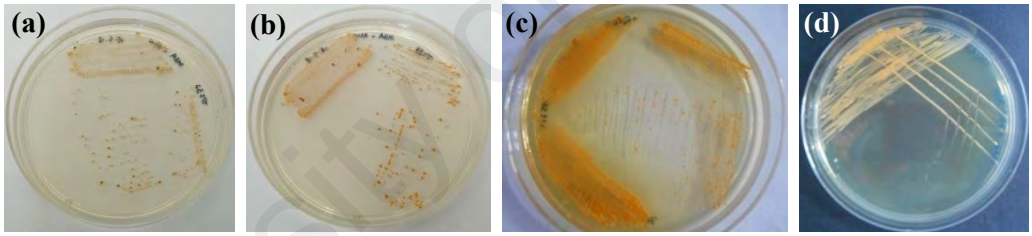

Figure 4.18: Strains (a) TPS32, (b) TPS88, (c) TPS335, (d) TPS409 were members of the group 7, which belong to *Salinispora*-like actinobacterial strains. Strains TPS32 and TPS409 do not produce diffusible pigment on MBA, whereas TPS88 and TPS335 produce light pink and light brown diffusible pigment, respectively.

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4.3 Dereplication of actinobacterial strains using fingerprinting methods and 16S rRNA gene sequencing

Various fingerprinting techniques were performed for both non-*Salinispora*-like and *Salinispora*-like strains derived from marine sediment samples. However, due to low number of isolates, actinobacterial strains that were derived from corals *Porites* sp. and *Montipora* sp. that included all strains from the colour group 6c and strain TRC8 from colour group 6b were sequenced for their 16S rRNA gene using the primer 27F without performing fingerprinting (Table 4.6). Actinobacteria strains isolated from *Porites* sp. were closely related to *Micrococcus* spp. (strain TRC8) and *Kocuria* spp. (strains TRC15 and TRC16). The only strain isolated from *Montipora* sp., TRC2, shared a 99.86% 16S rRNA gene sequence similarities with *Kocuria rhizophila* DSM 11926^{T}.

Fingerprinting was also not performed for non-*Salinispora*-like and two *Salinispora*-like strains isolated from marine sediment sample M, due to low number of isolates (< 10 strains). These include the strains TPM287 (colour group 5a), TPM81 (colour group 5e), TPM87 and TPM88 (colour group 7). The sequence match for these actinobacterial strains based on primer 27F was stated in Table 4.6. These actinobacterial strains were shown to belong to *Streptomyces* spp. (n = 1), *Glycomyces* spp. (n = 1) and *Gordonia* spp. (n = 2) based on 16S rRNA gene similarity.

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Table 4.6: Sequence matches for non-*Salinispor*a-like actinobacterial strains isolated from marine sediment sample M and the coral samples *Porites* sp. and *Montipora* sp.

Colour group	Strain	Closest related species	Family	Similarity (%)
5a	TPM287	*Glycomyces phytohabitans* KLBMP 1483^T (JQ819256)	*Glycomycetaceae*	97.84
5e	TPM81	*Streptomyces abyssalis* YIM M 10400^T (HQ585121)	*Streptomycetaceae*	100.0
6b	TRC8	*Micrococcus yunnanensis* YIM 65004^T (FJ214355)	*Micrococcaceae*	99.25
6c	TRC2	*Kocuria rhizophila* DSM 11926^T (Y16264)	*Micrococcaceae*	96.34
6c	TRC15			99.25
6c	TRC16			99.25
7	TPM87	*Gordonia bronchialis* DSM 43247^T (CP001802)	*Nocardiaceae*	98.80
7	TPM88			98.78

4.3.1 ITS-RFLP analyses of *Salinispora*-like strains and Rep-PCR fingerprinting

A total of 103 *Salinispora*-like strains was screened for ITS gene using L1/ G1 primer pair and the representative gel picture was shown in Figure 4.20. A total of 95 strains produced an amplicon that was about 500 bp. These strains were subsequently digested with restriction enzyme *Ban*1. Eight strains that produced multiple bands or produced amplicons below 400 bp were sequenced for 16S rRNA gene (Table 4.7). They were identified as *Mycobacterium* spp. (n= 3), *Rhodococcus* spp. (n= 1), *Nocardia* spp. (n= 3) and *Pseudonocardia* spp. (n= 1).

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Ban1 digestion of ITS region of the *Salinispora*-like strains revealed that 84 strains out of 95 yielded two RE fragments at 200+ bp and 300+ bp, respectively (Figure 4.20). All 84 strains that shared the same Ban1 profile were subjected for Rep-PCR and genomic fingerprints were generated using BOX-A1R primer. A total of 57 Rep-PCR profiles were obtained (Figure 4.21). Selected strains of different genomic fingerprinting profiles, including strains TPS126, TPS335, TPM91, TPM107, TPM109, TPM128, TPM165, TPM167, TPM169c, TPM187, TPM199, TPM204, TPM205, TPM210, TPM227 and TPM228, were sequenced for their 16S rRNA gene. All strains were identified to be 100% similar to *Salinispora arenicola* CNH-643^{T} based on 16S rRNA gene similarity (Table 4.7). The remaining 11 strains that produced different Ban1 profiles were also sequenced for their 16S rRNA genes. Comparison of 16S rRNA gene sequences with type strains revealed close relationships of these strains with *Plantactinospora* spp. (n= 1), *Micromonospora* spp. (n= 8) and *Jishengella* spp. (n= 2) (Table 4.7).

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Table 4.7: Closest matches for *Salinispora*-like actinobacterial strains based on 16S rRNA gene sequence analyses. Amplicon sizes of ITS region with the use of primer pair L1/ G1 and RE digestion profile are also listed.

Colour group	Strain	Amplicon size (bp)	RE fragments (bp)	Closest match	Family	Similarity (%)
8a	TPS126, TPS335, TPM91, TPM107, TPM109, TPS128, TPM165, TPS167, TPM169c, TPM187, TPM199, TPM204, TPM205, TPM210, TPM227, TPM228	500+	200+, 300+	*Salinispora arenicola* CNH-643^T (AY040619)	*Micromonosporaceae*	100.0
	TPS347	400+	500	*Plantactinospora endophytica* YIM 68255^T (GQ494033)	*Micromonosporaceae*	99.42
8b	TPS32	400+	500+, 300+, 100+	*Micromonospora maritima* D10-9-5^T (HQ704071)	*Micromonosporaceae*	99.91
	TPS88					99.73
	TPS111	400+	300+, 200+	*Micromonospora rosaria* DSM803^T (LRQV01000286)	*Micromonosporaceae*	99.78
8c	TPS86	400+	500+, 200+	*Micromonospora chokoriensis* 2-19/6^T (LT607409)	*Micromonosporaceae*	99.25
	TPS121	400+	500	*Micromonospora peucetia* DSM 43363^T (FMIC01000002)	*Micromonosporaceae*	99.32
	TPS350	400+	300+, 100+	*Micromonospora terminaliae* TMS7^T (KX394339)	*Micromonosporaceae*	99.09
	TPS385					99.19
	TPS175	400+	300+, 100+	*Micromonospora tulbaghiae* DSM 45242^T (EU196562)	*Micromonosporaceae*	99.82
	TPS106	400+	200+	*Jishengella endophytica* 202201^T (EU560726)	*Micromonosporaceae*	99.35
	TPS128					98.97

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Table 4.7, continued.

Colour group	Strain	Amplicon size (bp)	RE fragments (bp)	Closest match	Family	Similarity (%)
8d	TPS193	300+	ND	*Mycobacterium parafortuitum* CCUG 20999^T (MVID01000056)	*Mycobacteriaceae*	98.97
	TPS409	400+	ND	*Mycobacterium rufum* JS14^T (JROA01000001)	*Mycobacteriaceae*	100.0
	TPM181	400+	ND	*Mycobacterium gadium* ATCC 27726^T (X55594)	*Mycobacteriaceae*	97.98
8d	TPS217	200+, 300+	ND	*Rhodococcus ruber* DSM 43338^T (LRRL01000064)	*Nocardiaceae*	99.81
8e	TPS90	400, 400+	ND	*Nocardia elegans* IMMIB N-402^T (AJ854057)	*Nocardiaceae*	99.31
	TPS213					99.13
	TPM84					99.32
8e	TPS351	300+, 500+	ND	*Pseudonocardia carboxydivorans* Y8^T (EF114314)	*Pseudonocardiaceae*	99.53

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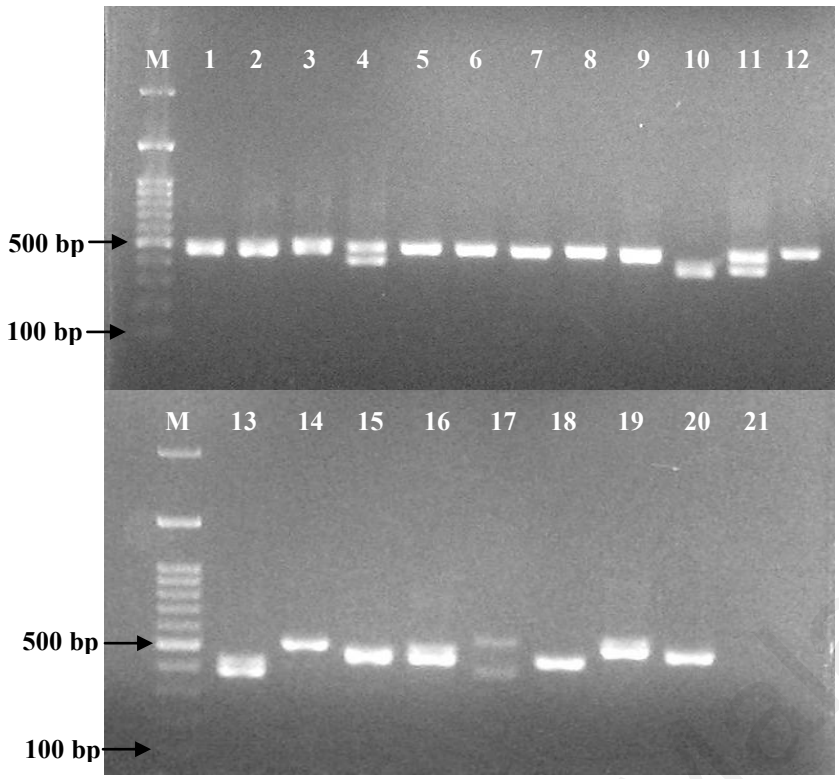
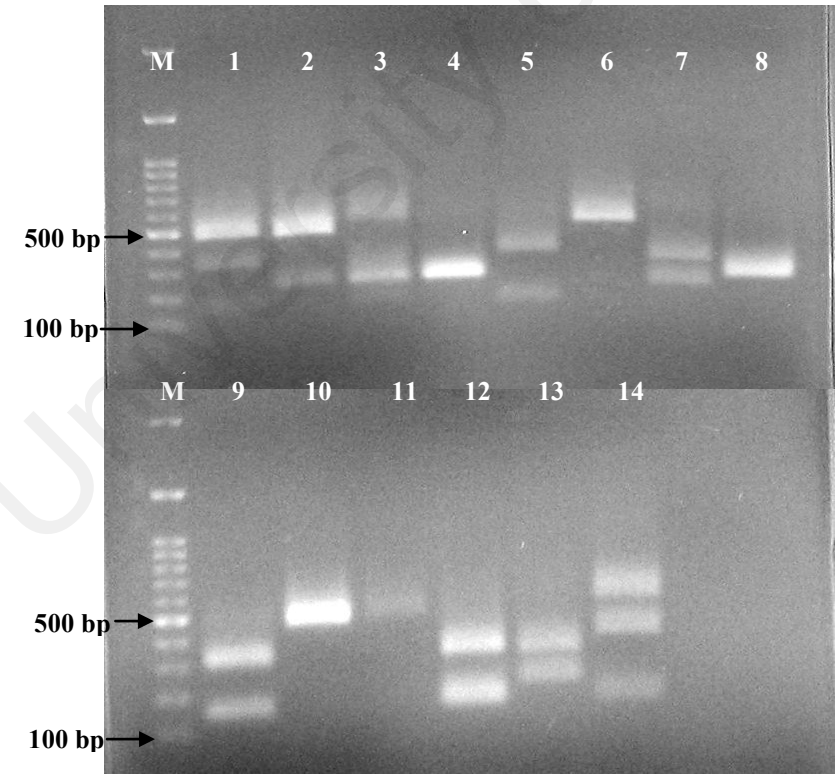


Legend:
M: 100 bp plus DNA ladder
Lane 1: TPS32
Lane 2: TPS86
Lane 3: TPS88
Lane 4: TPS90
Lane 5: TPS106
Lane 6: TPS111
Lane 7: TPS121
Lane 8: TPS128
Lane 9: TPS175
Lane 10: TPS193
Lane 11: TPS213
Lane 12: TPS105
Lane 13: TPS217
Lane 14: TPS335
Lane 15: TPS347
Lane 16: TPS350
Lane 17: TPS351
Lane 18: TPS385
Lane 19: TPS409
Lane 20: TPS126
Lane 21: Negative control

Figure 4.19: Screening of ITS gene of *Salinispora*-like actinobacterial strains using the L1/G1 primer pair. Strains with different banding patterns and amplicon sizes less than 500+ bp were selected for sequencing for 16S rRNA gene.



Legend:
M: 100 bp plus DNA ladder
Lane 1: TPS32
Lane 2: TPS86
Lane 3: TPS88
Lane 4: TPS106
Lane 5: TPS111
Lane 6: TPS121
Lane 7: TPS126
Lane 8: TPS128
Lane 9: TPS175
Lane 10: TPS347
Lane 11: TPS350
Lane 12: TPS385
Lane 13: TPS335
Lane 14: TPS409

Figure 4.20: Representative gel picture on RE digestion of *Salinispora*-like strains was shown here. Strains TPS126 and TPS335 were identified to be *Salinispora arenicola* CNH-643^{T} by sharing 100% similarity of the 16S rRNA gene.

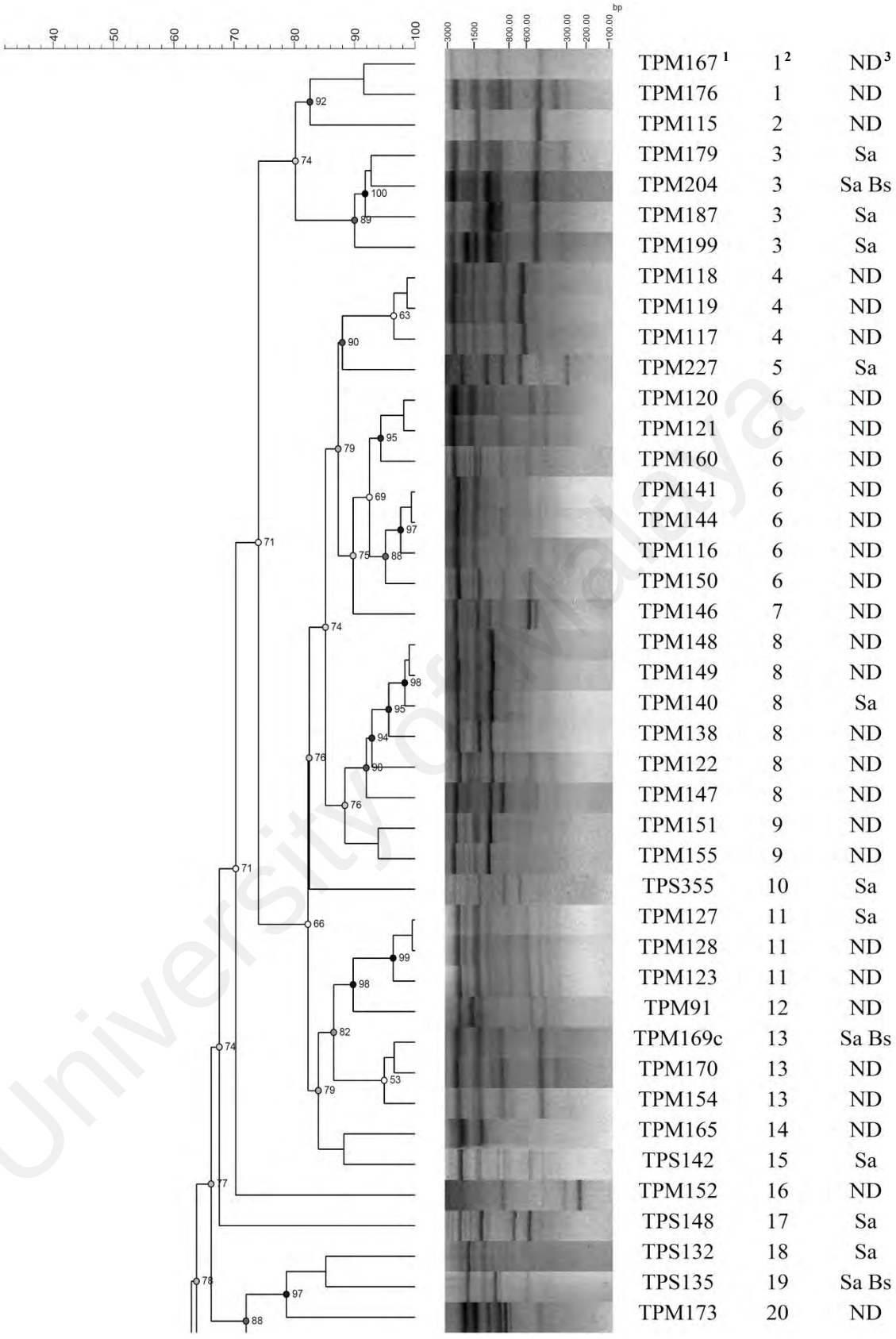


Figure 4.21: Banding profile based on Rep-PCR on 84 *Salinispora*-like actinobacterial strains that produced RE fragments at 200+ bp and 300+ bp using primer BOX-A1R. ([1] denotes the actinobacterial strain, [2] denotes the cluster based on banding pattern, [3] denotes the antibacterial activity profile. Bs, *B. subtilis* ATCC 23857; Sa, *S. aureus* ATCC 29213; Ec, *E. coli* ATCC 47076; Pa, *P. aeruginosa* ATCC 27853. ND, activity was not performed)

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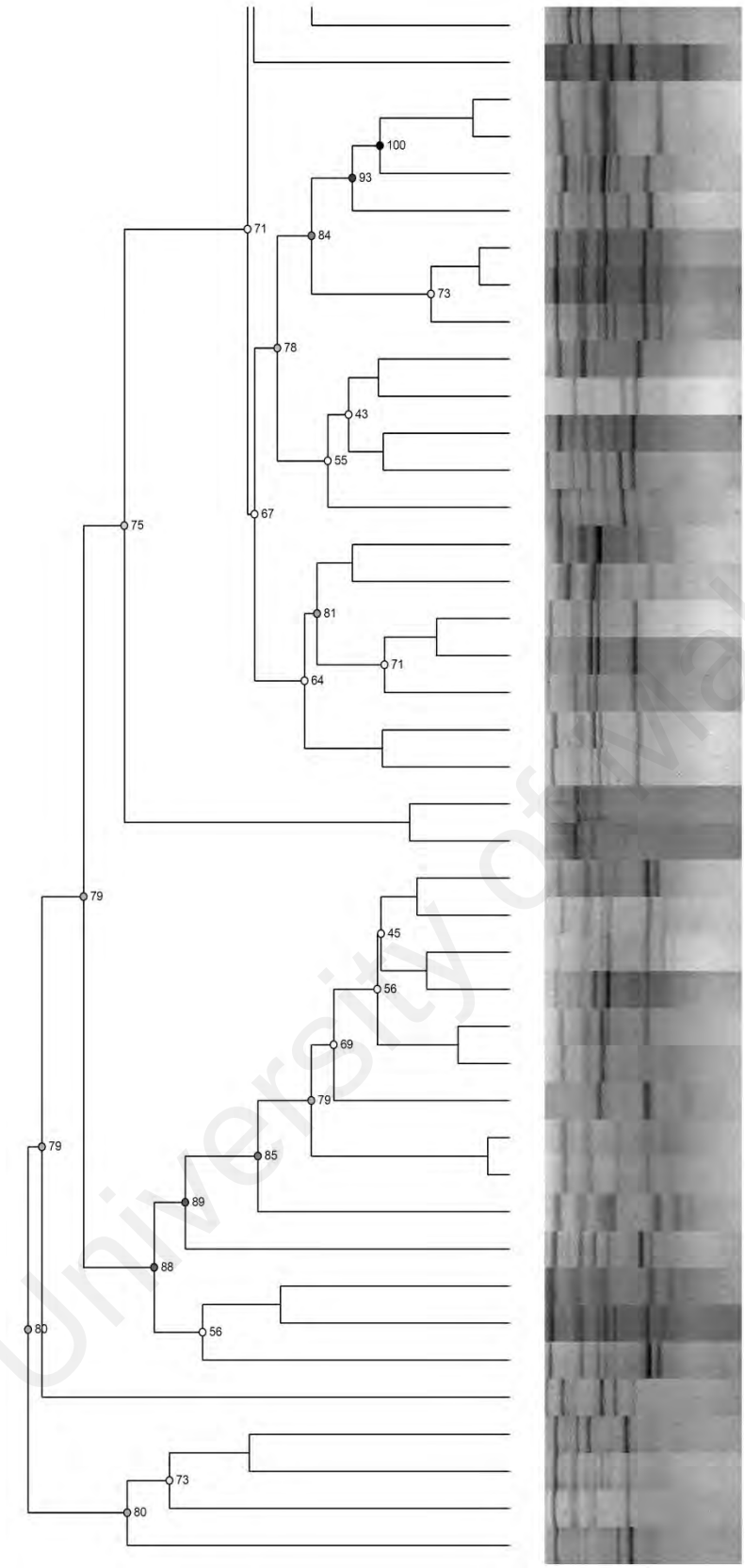

TPS113	21	Sa
TPM228	22	Sa
TPM114	23	ND
TPM136	23	ND
TPS153	24	Sa Bs
TPS174	25	Sa Bs
TPM208	26	Sa
TPM210	26	Sa
TPM206	27	Sa
TPM130	28	ND
TPS104	29	Sa Bs
TPS335	30	Sa Bs
TPS109	31	Sa
TPS112	32	Sa
TPM137	33	ND
TPS167	34	Sa
TPS105	35	Sa
TPS108	35	Sa
TPS107	36	Sa Bs
TPS101	37	Sa Bs
TPS103	38	Sa Bs
TPS126	39	Sa
TPS127	40	Sa
TPM92	41	ND
TPS158	42	Sa
TPS178	43	Sa Pa
TPS147	44	Sa
TPM93	45	ND
TPM95	45	ND
TPM171	46	ND
TPM107	47	ND
TPM109	47	ND
TPM175	48	Sa
TPM164	49	ND
TPM205	50	Sa Bs
TPS123	51	Sa
TPS146	52	Sa
TPS102	53	Sa Bs
TPS118	54	Sa Bs
TPS120	55	Sa Bs
TPS119	56	Sa Bs
TPS115	57	Sa Bs

Figure 4.21, continued.

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4.3.2 Antibacterial activity of *Salinispora*- like strains

Screening of randomly selected 44 strains out of 84 that shared the same Ban1 digestion profile revealed the potential of selected strains to inhibit *S. aureus* ATCC 29213, with diameters of inhibition zones ranged from 10 mm to 21 mm (Table 4.8). A total of 16 strains were also able to inhibit *B. subtilis* ATCC 23857 with diameters of inhibition zones to be above 10 mm. Both strains TPS104 and TPS178 growing on starch yeast peptone agar and ATCC medium 241, respectively, were able to inhibit the Gram- negative *P. aeruginosa* ATCC 27853 with diameters of inhibition zones recorded as 10 mm, in addition to *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213.

A total of 20 strains out of 44 showed positive growth on all production media and positive inhibition against at least one pathogen. Among them, 17 strains were not able to grow on soybean meal glucose. Absent of growth was observed for 12 strains on ATCC medium 241 and one strain on starch yeast peptone medium. Five strains were not able to grow on micromonospora medium and PM3, respectively. On all media with growth, antibacterial activity was also produced in the case of 43 *Salinispora* strains. However, strain TPS142 that showed positive growth on soybean meal glucose medium was not producing any antibacterial activity.

Screening of members of colour group 8b, 8c, 8d and 8e disclosed the potential of TPS88, TPS111 and TPS121 to produce antibacterial activity. Both strains TPS111 and TPS121 inhibited *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857. Strain TPS111 produced activity on four production media (except for ATCC medium 241), whereas strain TPS121 produced activity on all production media. Strain TPS88 produced activity against *S. aureus* ATCC 29213 on Micromonospora medium. Strain TPS347 from colour group 8a did not produce antibacterial activity on all production media.

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Table 4.8: Antibacterial activity profile of *Salinispora*- like strains on five production media using agar plug diffusion assay. Diameters of inhibition zones were recorded in mm. (Bs, *B. subtilis* ATCC 23857; Sa, *S. aureus* ATCC 29213; Ec, *E. coli* ATCC 47076; Pa, *P. aeruginosa* ATCC 27853; ng: no growth of actinobacterial strains on the production medium).

Colour group	Rep-PCR cluster	Genus	Strain	PM3				Soybean meal glucose				Micromonospora medium				ATCC medium 241				Starch yeast peptone			
				Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa
8a	3	*Salinispora*	TPM179	0	0	7	13	0	0	7	19	0	0	6	13	0	0	0	15	0	0	9	21
8a	3	*Salinispora*	TPM204	0	0	8	17	0	0	8.5	17.5	0	0	10.5	20	0	0	7.5	16	0	0	10.5	17.5
8a	3	*Salinispora*	TPM187	0	0	6.5	15.5	0	0	8	20	ng	ng	ng	ng	0	0	8	19.5	0	0	8.5	17.5
8a	3	*Salinispora*	TPM199	0	0	7	15	0	0	8	19	0	0	9.5	20	0	0	7	19.5	0	0	9	17.5
8a	5	*Salinispora*	TPM227	0	0	0	13	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	0	0	0	11
8a	8	*Salinispora*	TPM140	0	0	7	17	0	0	8.5	18.5	ng	ng	ng	ng	0	0	8	14	0	0	9	19
8a	10	*Salinispora*	TPS355	0	0	0	13	ng	ng	ng	ng	0	0	6	13.5	0	0	0	7	0	0	0	12
8a	11	*Salinispora*	TPM127	0	0	0	10	ng	ng	ng	ng	0	0	0	14	ng	ng	ng	ng	0	0	0	11.5
8a	13	*Salinispora*	TPM169c	0	0	7	12	0	0	7	12	0	0	10	20	0	0	8	17	0	0	8.5	18
8a	15	*Salinispora*	TPS142	0	0	0	12.5	0	0	0	0	0	0	8	14	0	0	0	10	0	0	0	13
8a	17	*Salinispora*	TPS148	0	0	0	14.5	0	0	0	10.5	0	0	7.5	15.5	0	0	0	13	0	0	7	12
8a	18	*Salinispora*	TPS132	0	0	0	13.5	ng	ng	ng	ng	0	0	7.5	16	0	0	9	17	0	0	0	13.5
8a	19	*Salinispora*	TPS135	0	0	11	19	0	0	0	13	ng	ng	ng	ng	0	0	9	16	0	0	12	17.5
8a	21	*Salinispora*	TPS113	0	0	0	12.5	0	0	10	17.5	0	0	8.5	12.5	0	0	8.5	16	0	0	0	12
8a	22	*Salinispora*	TPM228	0	0	0	11	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	0	0	0	13.5
8a	24	*Salinispora*	TPS153	0	0	12	18.5	0	0	0	10.5	0	0	8	14	0	0	12	17.5	0	0	0	16
8a	25	*Salinispora*	TPS174	0	0	11	18	0	0	0	13	0	0	10.5	16	0	0	0	15	0	0	12	18

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Table 4.8, continued.

Colour group	Rep-PCR cluster	Genus	Strain	PM3				Soybean meal glucose				Micromonospora medium				ATCC medium 241				Starch yeast peptone			
				Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa
8a	26	*Salinispora*	TPM208	0	0	0	15	0	0	8	19	0	0	7	19	0	0	7	19.5	0	0	10	20
8a	26	*Salinispora*	TPM210	ng	ng	ng	ng	ng	ng	ng	ng	0	0	8	14.5	0	0	7	15.5	0	0	7	13.5
8a	27	*Salinispora*	TPM206	ng	ng	ng	ng	ng	ng	ng	ng	0	0	9	15	0	0	11	21	ng	ng	ng	ng
8a	29	*Salinispora*	TPS104	0	0	9	13.5	0	0	0	11	0	0	9	15	0	7	13	18	0	10	10	16
8a	30	*Salinispora*	TPS335	0	0	7.5	13.5	0	0	6	14.5	0	0	8	19	0	0	0	12	0	0	9.5	20
8a	31	*Salinispora*	TPS109	0	0	0	14	ng	ng	ng	ng	0	0	0	9.5	ng	ng	ng	ng	0	0	0	12
8a	32	*Salinispora*	TPS112	0	0	0	13	ng	ng	ng	ng	0	0	7	9.5	ng	ng	ng	ng	0	0	7.5	11
8a	34	*Salinispora*	TPS167	0	0	0	15	ng	ng	ng	ng	0	0	7.5	15	0	0	0	9.5	0	0	6.5	15
8a	35	*Salinispora*	TPS105	0	0	0	13.5	ng	ng	ng	ng	0	0	0	8.5	ng	ng	ng	ng	0	0	0	9
8a	35	*Salinispora*	TPS108	0	0	0	13.5	ng	ng	ng	ng	0	0	0	8.5	ng	ng	ng	ng	0	0	0	9
8a	36	*Salinispora*	TPS107	0	0	8	12	0	0	9	17	0	0	9.5	21	0	0	7	12	0	0	7	19
8a	37	*Salinispora*	TPS101	0	0	0	15	ng	ng	ng	ng	0	0	9.5	16	ng	ng	ng	ng	0	0	8.5	16.5
8a	38	*Salinispora*	TPS103	0	0	0	15	ng	ng	ng	ng	0	0	9.5	16	ng	ng	ng	ng	0	0	8.5	16.5
8a	39	*Salinispora*	TPS126	0	0	8	16.5	ng	ng	ng	ng	0	0	0	12.5	ng	ng	ng	ng	0	0	6.5	14.5
8a	40	*Salinispora*	TPS127	0	0	8	16	0	0	0	12	0	0	8	19	0	0	0	13	0	0	6.5	14.5
8a	42	*Salinispora*	TPS158	0	0	7	13	0	0	8.5	17	0	7.5	9	17	0	0	12	18.5	0	0	8.5	15.5
8a	43	*Salinispora*	TPS178	0	0	7	12.5	ng	ng	ng	ng	0	7.5	9	17	0	0	10	20	0	0	7.5	12.5
8a	44	*Salinispora*	TPS147	0	0	0	11	0	0	0	11	0	0	6	11	0	8	6.5	14.5	0	0	6	12

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Table 4.8, continued.

Colour group	Rep-PCR cluster	Genus	Strain	PM3				Soybean meal glucose				Micromonospora medium				ATCC medium 241				Starch yeast peptone			
				Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa	Ec	Pa	Bs	Sa
8a	48	*Salinispora*	TPM175	ng	ng	ng	ng	0	0	6	19	0	0	6	13	ng	ng	ng	ng	0	0	8	20
8a	50	*Salinispora*	TPM205	0	0	7	16	0	0	8	18	0	0	9.5	20	ng	ng	ng	ng	0	0	10	19.5
8a	51	*Salinispora*	TPS123	0	0	6	15	0	0	0	9	0	0	7.5	17	0	0	10	17	0	0	0	12.5
8a	52	*Salinispora*	TPS146	0	0	7	15.5	0	0	0	15.5	0	0	6	8	0	8	0	12	0	0	0	11
8a	53	*Salinispora*	TPS102	0	0	9	13.5	0	0	0	11	0	0	9	15	0	0	12.5	16	0	0	7.5	13.5
8a	54	*Salinispora*	TPS118	ng	ng	ng	ng	ng	ng	ng	ng	0	0	14	21	0	0	10	20	0	0	12.5	19.5
8a	55	*Salinispora*	TPS120	0	0	9	13.5	0	0	0	11	0	0	12	18.5	0	0	12	17	0	0	9	14
8a	56	*Salinispora*	TPS119	ng	ng	ng	ng	0	0	7	15	0	7	14	21	0	0	0	9	0	0	11	18
8a	57	*Salinispora*	TPS115	0	0	11	15	0	0	0	12	0	0	8.5	16.5	0	0	13	18.5	0	0	10	16
8b	ND	*Micromonospora*	TPS88	0	0	0	0	0	0	0	0	0	0	8	10	0	0	0	0	0	0	0	0
8b	ND	*Micromonospora*	TPS111	0	0	13	11	0	0	14	12	0	0	15	14	0	0	0	0	0	0	9	10
8c	ND	*Micromonospora*	TPS121	0	0	10	9.5	0	0	11	9	0	0	10	0	0	0	10.5	9	0	0	13.5	11

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4.3.3 Dereplication of non-*Salinispora*-like actinobacterial strains using 16S-ITS RFLP fingerprinting

The size of amplicons resulted from amplification of the 16S rRNA-ITS region using primer pair pA and BL235R was approximately 1.8 kb. Up to ten RFLP fragments were generated from double RE digestion method and the band size fall in the range from 100 bp to 1 kbp for all 76 strains. Only 68 actinobacterial strains from the non-*Salinispora* group yielded RFLP patterns that were clustered into 41 clusters (Figure 4.22). The remaining eight non-*Salinispora*-like actinobacterial strains failed to produce an amplicon. 16S rRNA gene sequence analyses revealed that the eight strains were closely related to *Marinactinospora* spp. (n = 3), *Rhodococcus* spp. (n = 2), *Nocardiopsis* spp. (n = 1), *Agromyces* spp. (n = 1) and *Saccharomonospora* spp. (n = 1) (Table 4.9).

Two major clusters were generated from the RFLP fingerprinting: the *Streptomyces* cluster (Clusters 1 to 38, 63 strains) and the *Blastococcus* cluster (Cluster 39 to 41, 5 strains). Within the *Streptomyces* cluster, four strains TPS35 (Cluster 13, group 3e), TPS37 (Cluster 18, group 5e), TPS89 (Cluster 18, group 5a) and TPS358a (Cluster 36, group 4a) belong to *Streptosporangium* spp., *Saccharopolyspora* spp., *Actinomadura* spp. and *Nonomuraea* spp., respectively. The remaining 59 strains were found to belong to *Streptomyces* spp., which showed 97.30% to 100% similarity to their closest match. Sequencing of the 16S rRNA gene of the members of *Blastococcus* cluster showed that the strains were closely related to *B. saxobsidens* BC448^{T} and *B. endophyticus* YIM 68236^{T}, all below 98.6% of similarity.

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A total of 51 out of 63 strains from the *Streptomyces* cluster and all five strains from the *Blastococcus* cluster were screened for antibacterial activity against *S. aureus* ATCC 29213, *B. subtilis* ATCC 23857, *E. coli* ATCC 47076 and *P. aeruginosa* ATCC 27853 using disc diffusion assay (Figure 4.22). GenBank accession numbers of all sequenced strains listed in the Figure 4.22 was given in Appendix A. Sixteen strains of the *Streptomyces* cluster showed positive antibacterial activity against one or more of the pathogens, whereas all members of the *Blastococcus* cluster did not inhibit any of the pathogens.

Table 4.9: Closest matches for non-*Salinispor*a-like actinobacterial strains that did not produce 16S rRNA- ITS amplicons.

Colour group	Strain	Closest related species	Family	Similarity (%)
1	TPS16	*Marinactinospora thermotolerans* SCSIO00652^T (FUWS01000037)	*Nocardiopsaceae*	97.11
1	TPS81			96.75
1	TPS83			96.91
5a	TPS125	*Saccharomonospora xinjiangensis* XJ-54^T (JH636049)	*Pseudonocardiaceae*	99.70
6a	TPS2	*Nocardiopsis alba* DSM 43377^T (ANAC01000044)	*Nocardiopsaceae*	98.22
6b	TPS33	*Rhodococcus equi* NBRC 101255^T (AF490539)	*Nocardiaceae*	100.0
6b	TPS179			99.78
6b	TPS92	*Agromyces aurantiacus* YIM 21741^T (AF389342)	*Microbacteriaceae*	98.29

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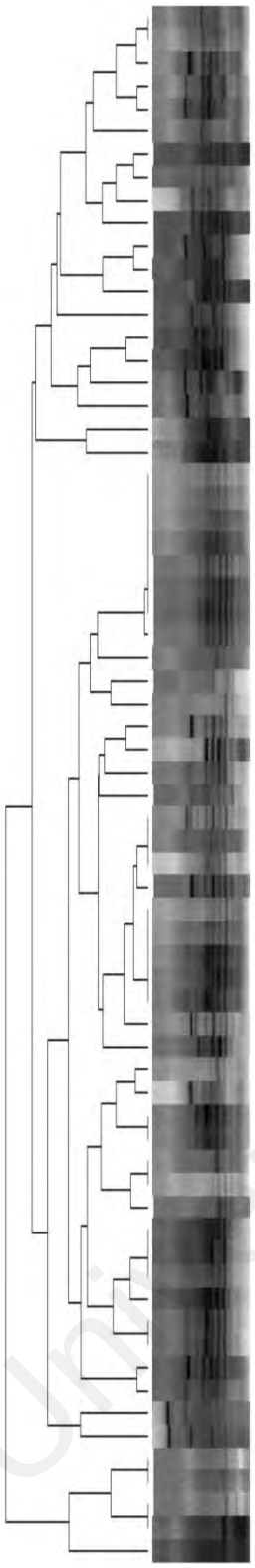

TPS143	1[1]	5c[2]	Bs[3]	*Streptomyces specialis* GW41-1564^T	97.96
TPS94	1	5a	Bs Sa	*Streptomyces aculeolatus* NBRC 14824^T	100.0
TPS74	2	5b	NA	*Streptomyces mayteni* YIM 60475^T	99.10
TPS122	3	5a	NA	*Streptomyces jiujiangensis* JXJ0074^T	99.29
TPS216	4	5e	Bs Sa	*Streptomyces chiangmaiensis* TA4-1^T	98.70
TPS137	5	5d	Bs Sa	*Streptomyces sedi* YIM 65188^T	97.56
TPS14	6	3b	Ec Sa	*Streptomyces intermedius* NBRC 13049^T	99.72
TPS359	7	2b	Bs Sa	*Streptomyces nanshensis* SCSIO01066^T	99.91
TPS38	8	2b	Bs Sa	*Streptomyces nanshensis* SCSIO01066^T	99.31
TPS445	9	2b	Bs Sa	*Streptomyces nanshensis* SCSIO01066^T	99.61
TPS24	10	5a	ND	*Streptomyces jiujiangensis* JXJ0074^T	99.13
TPS41	11	5a	NA		
TPS63	12	3a	NA	*Streptomyces violascens* ISP 5183^T	100.0
TPS35	13	3e	NA	*Streptosporangium amethystogenes* subsp. *fukuiense* JCM 10083^T	99.39
TPS27	14	3b	NA	*Streptomyces hydrogenans* NBRC 13475^T	99.91
TPS42	15	5a	NA		
TPS181	16	5c	Sa	*Streptomyces samsunensis* M1463^T	99.72
TPS180	17	5c	NA	*Streptomyces carpaticus* NBRC 15390^T	99.24
TPS37	18	5e	Bs Sa	*Saccharopolyspora hirsuta subsp. hirsuta* ATCC 27875^T	98.87
TPS89	18	5a	NA	*Actinomadura livida* JCM 3387^T	100.0
TPS198	19	5a		*Streptomyces hydrogenans* NBRC 13475^T	99.91
TPS199	19	5a			
TPS201	19	5a	NA		
TPS202	19	5a			
TPS65	19	3a			
TPS67	19	3a			
TPS68	19	3a			
TPS66	19	3a			
TPS208	20	5b	NA	*Streptomyces thermocarboxydus* DSM 44293^T	99.31
TPS3	21	5a	NA	*Streptomyces caeruleatus* NRRL B-24802^T	97.30
TPS5	21	5c	NA		
TPS11	22	3c	NA	*Streptomyces xiamenensis* MCCC 1A01550^T	99.44
TPS364	22	5c	NA	*Streptomyces xiamenensis* MCCC 1A01550^T	99.36
TPS6	23	3d	Bs Sa Pa	*Streptomyces thermoviolaceus* subsp. *apingens* DSM 41392^T	98.92
TPS15	23	3c	NA	*Streptomyces harbinensis* NEAU-Da3^T	99.47
TPS1	24	3a	Bs Sa	*Streptomyces collinus* NBRC 12759^T	99.01
TPS48	24	5a	NA		
TPS60	24	5b	NA	*Streptomyces somaliensis* DSM 40738^T	100.0
TPS183	24	3b	NA	*Streptomyces harbinensis* NEAU-Da3^T	98.85
TPS43	25	5a			
TPS44	25	5a	NA		
TPS45	25	5a	NA		
TPS46	25	5a			
TPS47	25	5a			
TPS4	26	3b	NA	*Streptomyces carpaticus* NBRC 15390^T	98.12
TPS53	27	3d	NA	*Streptomyces wuyuanensis* CGMCC 4.7042^T	99.10
TPS10	28	5b	Bs Sa Ec	*Streptomyces spongiicola* HNM0071^T	99.51
TPS75	28	3c	NA	*Streptomyces harbinensis* NEAU-Da3^T	99.65
TPS210	29	5a	NA	*Streptomyces albidoflavus* DSM 40455^T	99.82
TPS211	29	5a	NA		
TPS51	30	5a	Bs Sa	*Streptomyces spongiicola* HNM0071^T	99.21
TPS8	30	5c	NA		
TPS209	31	3a	NA	*Streptomyces spongiicola* HNM0071^T	99.30
TPS12	32	5b	Ec Sa	*Streptomyces daghestanicus* NRRL B-5418^T	99.92
TPS17	32	5a	Ec Sa	*Streptomyces jiujangensis* JXJ0074^T	99.56
TPS7	32	5c	NA		
TPS31	33	5b	NA	*Streptomyces daghestanicus* NRRL B-5418^T	99.49
TPS58	34	3a	NA	*Streptomyces daghestanicus* NRRL B-5418^T	100.0
TPS61	34	3a			
TPS114	35	2a	NA	*Streptomyces karpasiensis* K413^T	97.79
TPS358a	36	4a	NA	*Nonomuraea salmonea* DSM 43678^T	98.09
TPS419	37	5a	NA	*Streptomyces koyangensis* VK-A60^T	99.86
TPS77	38	3b	NA	*Streptomyces cheonanensis* VC-A46^T	99.47
TPS357	39	4c	NA	*Blastococcus saxobsidens* BC448^T	97.94
TPS418	39	4c	NA	*Blastococcus saxobsidens* BC448^T	97.97
TPS166	40	4b	NA	*Blastococcus endophyticus* YIM 68236^T	98.31
TPS448	40	4c	NA	*Blastococcus endophyticus* YIM 68236^T	96.19
TPS459	41	4c	NA	*Blastococcus saxobsidens* BC448^T	98.03

Figure 4.22: Banding profiles based on RFLP of 16-23S ITS region of non-*Salinispora*-like strains. Representative strains were selected for 16S rRNA gene sequencing and antibacterial activity screening. The closest related species match and the percentage of similarity (%) are listed. ([1] denotes the fingerprinting cluster group; [2] denotes the colour group based on Table 4.4; [3] denotes pathogens that were inhibited by actinobacterial strain. Bs, *B. subtilis* ATCC 23857; Sa, *S. aureus* ATCC 29213; Ec, *E. coli* ATCC 47076; Pa, *P. aeruginosa* ATCC 27853. NA, not active; ND, no data)

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4.4 Diversity of actinobacterial species from marine sediment samples

In this study, 19 genera from 11 families of the class *Actinobacteria* were successfully isolated from both marine sediment samples, regardless of isolation medium and pre-treatment methods (Table 4.10). They were belonging to the families of *Geodermatophilaceae*, *Glycomycetaceae*, *Microbacteriaceae*, *Micromonosporaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Nocardiopsaceae*, *Pseudonocardiaceae*, *Streptomycetaceae*, *Streptosporangiaceae* and *Thermomonosporaceae*. Seventeen genera were recovered from sediment sample Z. Four genera were co-isolated from both sediment samples, including *Streptomyces* spp., *Salinispora* spp., *Mycobacterium* spp. and *Nocardia* spp. *Gordonia* sp. and *Glycomyces* sp. were only isolated from sediment sample M. Actinobacterial strains from coral samples were belonging to the genera *Micrococcus* and *Kocuria*.

Diversity of actinobacteria was indicated by Shannon-Wiener index (H') (Table 4.10). In the case of sediment sample Z, the diversity indexes calculated for the sample set treated with skim milk/ HEPES couple to the use of modified M2 and HVA, as well as skim milk/HEPES treated sample set with HVB enrichment on modified M2 medium, were the highest among all. This corresponds to successful isolation of eleven and nine genera from skim milk/ HEPES treated sample on modified M2 and HVA plates and four genera from HVB enrichment of skim milk/ HEPES treated sample on modified M2 plates. The rest of the sample sets have *H'* values below 1. Only two strains belong to *Blastococcus* spp. were isolated from sample set pre-treated with UV irradiation using modified M2 medium. On the other hand, the highest *H'* value reported for sediment sample M was the sample set treated with UV irradiation couple to the use of HVA medium, which corresponds to isolation of six genera from the respective sample set. Heat treatment at 60ºC led to isolation of only *Salinispora* spp. from sediment sample M on all three isolation media.

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Table 4.10: Diversity of actinobacteria recovered from Tioman marine sediment samples. Shannon-Wiener index (H') and total number of actinobacterial strains isolated from each medium are listed.

Sediment sample	Pre-treatment	Isolation media	Closest related genera	Number of strains	H'
Z	Skim milk/ HEPES	M3	*Streptomyces*	15	0.6337
			Micromonospora	1	
			Nocardia	1	
			Mycobacterium	1	
		Modified M2	*Streptomyces*	8	1.5278
			Salinispora	29	
			Micromonospora	4	
			Blastococcus	2	
			Rhodococcus	1	
			Nonomuraea	1	
			Saccharomonospora	1	
			Nocardiopsis	1	
			Plantactinospora	1	
			Pseudonocardia	1	
			Jishengella	2	
		HVA	*Streptomyces*	33	1.0961
			Micromonospora	3	
			Marinactinospora	3	
			Actinomadura	1	
			Nocardia	1	
			Rhodococcus	1	
			Streptosporangium	1	
			Saccharopolyspora	1	
			Agromyces	1	
	Skim milk/ HEPES + HVB enrichment	Modified M2	*Streptomyces*	1	1.3863
			Salinispora	1	
			Blastococcus	1	
			Rhodococcus	1	
		HVA	*Streptomyces*	2	0.6365
			Mycobacterium	1	
	UV irradiation	Modified M2	*Blastococcus*	1	0.0000

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Table 4.10, continued.

Sediment sample	Pre-treatment	Isolation media	Closest related genera	Number of strains	H'
M	UV irradiation	M3	*Salinispora*	2	0.0000
		Modified M2	*Salinispora*	36	0.0000
		HVA	*Streptomyces*	1	1.5403
			Salinispora	5	
			Nocardia	1	
			Mycobacterium	1	
			Gordonia	2	
			Glycomyces	1	
	Heat at 60˚C	M3	*Salinispora*	2	0.0000
		Modified M2	*Salinispora*	3	0.0000
		HVA	*Salinispora*	6	0.0000

4.5 Characterisation of novel actinobacterial strains from marine sediment

Actinobacterial strains with a similarity of their 16S rRNA gene sequence lower than 99% to the closest match were subjected to another sequencing using the primer 1492R to obtain an almost full length 16S rRNA gene sequence, which is above 1300 bp. By using 98.6% as the cut-off for delineation of novel bacterial species, a total of 19 non-*Salinispora*-like strains and one *Salinispora*-like strain were identified to be novel actinobacterial species (Table 4.11). All novel actinobacterial strains were isolated from marine sediment samples. The novel actinobacterial species were closely related to members of the genera *Streptomyces* (n= 6), *Blastococcus* (n= 5), *Marinactinospora* (n= 3), *Nocardiopsis* (n= 1), *Nonomuraea* (n= 1), *Agromyces* (n= 1) and *Glycomyces* (n= 1). The novel species of *Salinispora*-like strains was identified to be *Mycobacterium* spp. 16S rRNA gene sequences of the novel actinobacterial strains were deposited into GenBank and accession numbers of the corresponding strains were shown in Table 4.11. Genomes of five novel strains were sequenced, annotated and deposited into GenBank and genome accession numbers were also listed. Strains with genome

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information available on GenBank are TPS16, TPS81, TPS83, TPS2 and TPS3. At the point of writing, only selected novel strains have complete set of description data and whole genome sequencing data due to limited funding. The novel strains TPS3, TPS16, TPS166, TPS357 and TPS418 had been deposited into two culture collection centres: Leibniz Institute DSMZ- German Collection of Microorganisms and Cell Cultures (DSMZ Culture Collection Centre, Braunschweig, Germany) and Thailand Bio-resource Research Centre (TBRC, Pathum Thani, Thailand). The culture collection numbers assigned to the respective strain were listed in Table 4.11.

4.5.1 Characterisation of strains TPS16, TPS81 and TPS83

4.5.1.1 Phylogenetic analysis of 16S rRNA gene

Three actinobacterial strains, including TPS16, TPS81 and TPS83, were identified as novel species (Table 4.11). Strains TPS16, TPS81 and TPS83 were isolated from skim milk/ HEPES treated sediment sample set using HVA. These actinobacterial strains were purified from different replicates of the same isolation medium. All three strains shared 96.63% to 97.07% of 16S rRNA gene similarity to *M. thermotolerans* SCSIO 00652^{T} and could be recognised as a novel genus within the *Nocardiopsaceae* family. The taxonomic position of all three novel strains were confirmed based on phylogenetic trees that were inferred using neighbour joining, maximum likelihood and maximum parsimony methods, by comparing all strains with the closest matches and representative members of the *Nocardiopsaceae* family. The novel strains formed a distinct and stable lineage among members of the family *Nocardiopsaceae* (Figure 4.23), similar observation was also shown in phylogenetic trees established using maximum likelihood and maximum parsimony approaches. All three strains (TPS16, TPS81 and TPS83) were clustered together with a bootstrap value of 100%. The novel strains cluster was separated from the cluster containing *M. thermotolerans* SCSIO 00652^{T} and *M. endophytica* YIM 690053^{T} with a bootstrap value of 62%. Comparing

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the 16S rRNA sequences of all three novel strains revealed that strain TPS16, TPS81 and TPS83 were 100% similar to each other, as indicated by the percent identity matrix as determined by Clustal12.1.

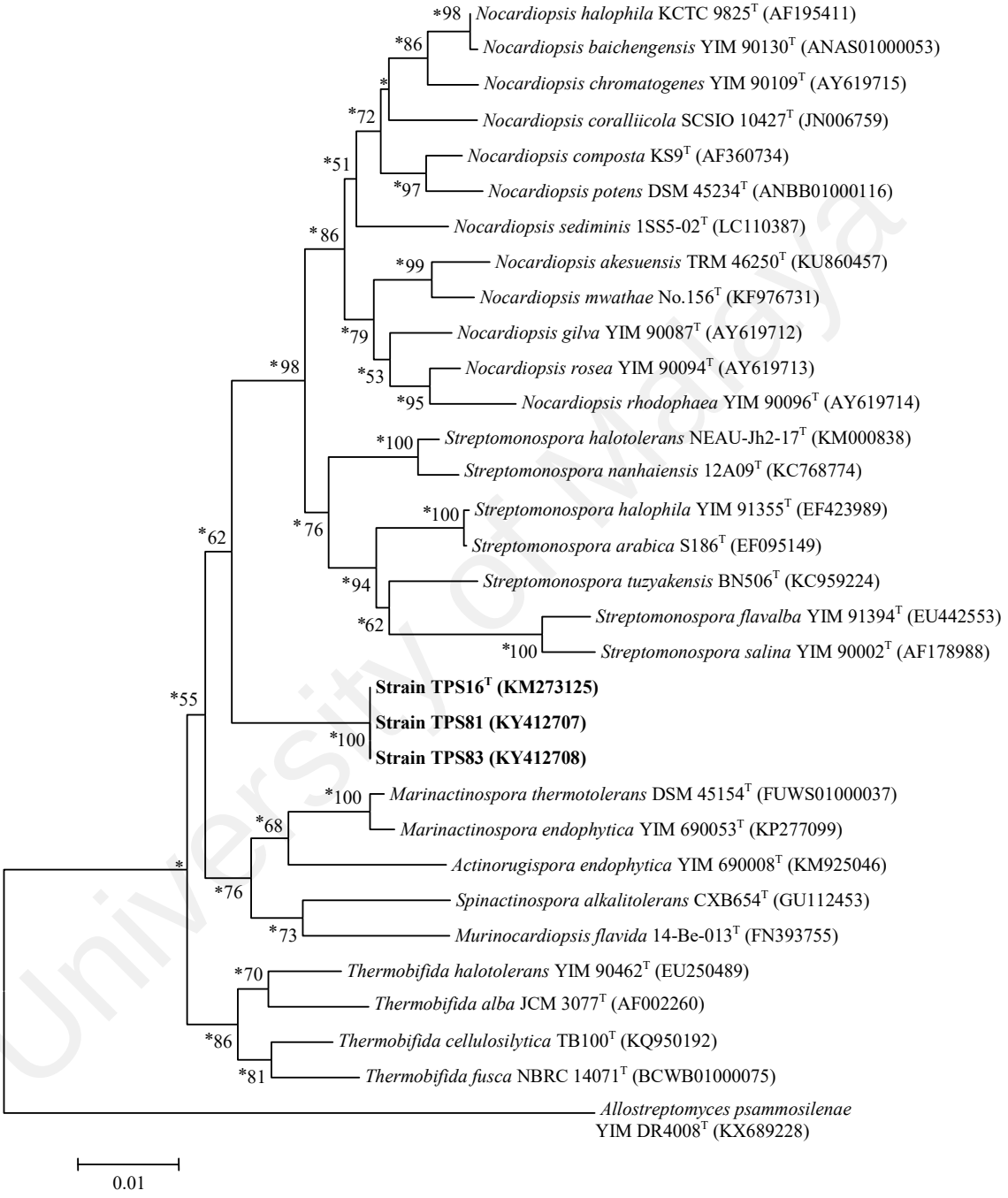

Figure 4.23: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of strains TPS16^T, TPS81 and TPS83 (1406 bp, 1398 bp and 1397 bp) and closely related members in the family *Nocardiopsaceae*. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 1% sequence divergence.

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Table 4.11: Novel actinobacterial strains isolated from Tioman marine sediment sample. Almost full length 16S rRNA gene sequences (>1300 bp) were used to align with corresponding sequences of closely related species using CLUSTAL_W. BLAST searches were performed on the EzBioCloud server. GenBank accession number of the 16S rRNA gene and genome accession numbers of selected strains were listed.

Strain	Pre-treatment, isolation medium	Colour group	Fingerprinting method	Fingerprinting cluster	Family	Closest related match	Similarity (%)	16S rRNA gene accession number	Genome accession number	Culture collection number
TPS16	Skim milk/ HEPES, HVA	1	Direct sequencing	ND	*Nocardiopsaceae*	*Marinactinospora thermotolerans* SCSIO 00652^T (FUWS01000037)	97.60	KM273125	QEIO00000000	TBRC 5138, DSM 46825
						M. endophytica YIM 690053^T (KP277099)	96.87			
TPS81	Skim milk/ HEPES, HVA	1	Direct sequencing	ND	*Nocardiopsaceae*	*Marinactinospora thermotolerans* SCSIO 00652^T (FUWS01000037)	97.28	KY412707	QEIN00000000	To be deposited
						M. endophytica YIM 690053^T (KP277099)	96.57			
TPS83	Skim milk/ HEPES, HVA	1	Direct sequencing	ND	*Nocardiopsaceae*	*Marinactinospora thermotolerans* SCSIO 00652^T (FUWS01000037)	96.63	KY412708	QEIM00000000	To be deposited
						M. endophytica YIM 690053^T (KP277099)	96.56			
TPS2	Skim milk/ HEPES, modified M2	6a	Direct sequencing	ND	*Nocardiopsaceae*	*Nocardiopsis alba* DSM 43377^T (ANAC01000044)	97.08	MG754419	QOCZ01000000	To be deposited
						N. exhalans ES10.1^T (AY036000)	97.06			

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Table 4.11, continued.

Strain	Pre-treatment, isolation medium	Colour group	Fingerprinting method		Fingerprinting cluster	Family	Closest related match	Similarity (%)	16S rRNA gene accession number	Genome accession number	Culture collection number
TPS166	Skim milk/HEPES, modified M2	4b	Double digestion	RE	40	*Geodermatophilaceae*	*Blastococcus capsensis* BMG 804^T (LN626274)	97.42	KM273128	To be sequenced	TBRC 5139, DSM 46827
							B. saxobsidens BC448^T (AJ316571)	97.40			
TPS357	Skim milk/HEPES, modified M2	4c	Double digestion	RE	39	*Geodermatophilaceae*	*Blastococcus endophyticus* DSM 45413^T (NR108608)	97.84	KM273129	To be sequenced	TBRC 5140, DSM 46828
							B. jejuensis KST3-10^T (DQ200983)	97.78			
TPS418	Skim milk/ HEPES + HVB enrichment, modified M2	4c	Double digestion	RE	39	*Geodermatophilaceae*	*Blastococcus jejuensis* KST3-10^T (DQ200983)	97.64	KM364560	To be sequenced	TBRC 5141, DSM 46829
							B. capsensis BMG 804^T (LN626274)	97.57			
TPS448	UV irradiation, modified M2	4c	Double digestion	RE	40	*Geodermatophilaceae*	*Blastococcus saxobsidens* BC448^T (AJ316571)	98.39	MG754426	To be sequenced	To be deposited
							B. jejuensis KST3-10^T (DQ200983)	98.10			
TPS459	UV irradiation, modified M2	4c	Double digestion	RE	41	*Geodermatophilaceae*	*Blastococcus saxobsidens* BC448^T (AJ316571)	98.39	MG754427	To be sequenced	To be deposited
							B. jejuensis KST3-10^T (DQ200983)	98.10			

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Table 4.11, continued.

Strain	Pre-treatment, isolation medium	Colour group	Fingerprinting method	Fingerprinting cluster	Family	Closest related matches	Similarity (%)	16S rRNA gene accession number	Genome accession number	Culture collection number
TPS114	Skim milk/ HEPES, modified M2	2a	Double RE digestion	35	*Streptomycetaceae*	*Streptomyces karpasiensis* K413^T (JQ864430)	98.31	MG754421	To be sequenced	To be deposited
						S. nanshensis SCSIO 01066^T (EU589334)	97.92			
TPS4	Skim milk/ HEPES, modified M2	3b	Double RE digestion	5	*Streptomycetaceae*	*Streptomyces carpaticus* NBRC 15390^T (AB184641)	97.80	MG754420	To be sequenced	To be deposited
						S. harbinensis NEAU-Da3^T (JQ750974)	96.97			
TPS3	Skim milk/ HEPES, modified M2	5a	Double RE digestion	21	*Streptomycetaceae*	*S. spongiae* Sp080513SC-24^T (AB498741)	98.57	KM273126	To be deposited	TBRC 5135, DSM 42167
						S. shaanxiensis CCNWHQ 0031^T (FJ465151)	98.07			
						S. ziwulingensis F22^T (JF957700)	97.34			
TPS143	Skim milk/ HEPES, modified M2	5c	Double RE digestion	1	*Streptomycetaceae*	*Streptomyces specialis* GW 41-1564^T (AM934703)	98.07	MG754423	To be sequenced	To be deposited
						S. hainanensis YIM 41-1564^T (AM398645)	97.22			
TPS137	Skim milk/ HEPES, modified M2	5d	Double RE digestion	5	*Streptomycetaceae*	*Streptomyces sedi* YIM 65188^T (EU925562)	97.65	MG754422	To be sequenced	To be deposited
						S. zhaozhouensis NEAU-LZA-5^T (KC304791)	97.30			

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Table 4.11, continued.

Strain	Pre-treatment, isolation medium	Colour group	Fingerprinting method	Fingerprinting cluster	Family	Closest related match	Similarity (%)	16S rRNA gene accession number	Genome accession number	Culture collection number
TPS183	Skim milk/ HEPES, M3	3b	Double RE digestion	24	*Streptomycetaceae*	*Streptomyces harbinensis* NEAU-Da3^{T} (JQ750974)	97.47	MG754424	To be sequenced	To be deposited
						S. carpaticus NBRC 15390^{T} (AB184641)	97.46			
TPS358a	Skim milk/ HEPES, modified M2	4a	Direct sequencing	ND	*Streptosporangiaceae*	*Nonomuraea salmonea* DSM 43678^{T} (X97892)	98.33	MG754425	To be sequenced	To be deposited
						N. maheshkhaliensis 16-5-14^{T} (AB290014)	98.33			
TPM287	UV irradiation, HVA	5a	Direct sequencing	ND	*Glycomycetaceae*	*Glycomyces phytohabitans* KLBMP 148^{T} (JQ819256)	98.34	MH497601	To be sequenced	To be deposited
						G. arizonensis DSM 44726^{T} (AXWO01000049)	98.20			
TPS92	Skim milk/ HEPES, HVA	6b	Direct sequencing	ND	*Microbacteriaceae*	*Agromyces aurantiacus* YIM 21741^{T} (AF389342)	98.09	KM273127	To be sequenced	To be deposited
						A. binzhouensis OAct353^{T} (KC493987)	98.09			
TPM181	UV irradiation, HVA	8d	ITS gene screening	ND	*Mycobacteriaceae*	*Mycobacterium peregrinum* ATCC 14467^{T} (AF058712)	98.34	MH497598	To be sequenced	To be deposited
						M. longobardum DSM 45394^{T} (JN571166)	98.20			

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4.5.1.2 Morphological characterisation

Cells of the novel strains TPS16, TPS81 and TPS83 were Gram-stained positive. All three strains showed good growth on ISP medium 1, 2, 3, 4, 5, 6 and 7 (Table 4.12). Three strains produce white aerial mycelia and yellowish white to light yellowish brown substrate mycelia on all tested media. Production of white to light blue colour spores was observed for all novel strains on ISP medium 2 (Figure 4.24), PDA and soybean meal agar, along with production of abundant blue diffusible pigment. On ISP medium 3, three strains produce light purplish pink colour spores and moderate yellowish pink diffusible pigment. Growth of the novel strains was compared with their closest related match, *M. thermotolerans* SCSIO 00652^{T}, which produced orange yellow colour substrate mycelia and white to olive- gray colour aerial mycelia on all ISP media (Figure 4.25). No diffusible pigment was produced by the reference strains and three novel strains on ISP 1, ISP 6 and ISP 7.

Micro-morphology of the novel strains was examined by cover slip method, in which the 14 days old culture on ISP 2 was examined under a light compound microscope at 400× magnification without staining, as well as scanning electron microscopy. Aerial mycelia were straight to branching and differentiate into spore chains at the tips with no fragmentation. Spore chains were straight to loop. Formation of spores occurred at the tips of aerial mycelia and they were arranged in a chain of maximum five (Figure 4.26). The rod-shaped spores were of smooth surface with average dimension of 0.6 µm wide and 1.1 µm long (Figure 4.27).

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Table 4.12: Colonies colour of novel actinobacterial strains TPS16, TPS81 and TPS83 on various ISP media supplemented with 2.5% ASW were listed here. Growth of the novel strains was compared to the reference strain, *M. thermotolerans* SCSIO 00652^T.

Culture medium	Strain	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
ISP 1	TPS16, TPS81, TPS83	Powdery	White	Yellowish white	White	Absent
	SCSIO 00652^T	Powdery	White	Strong orange yellow	White	Absent
ISP 2	TPS16, TPS81, TPS83	Powdery	White	Light yellowish brown	Light blue	Light blue
	SCSIO 00652^T	Powdery	White	Vivid orange yellow	White	Absent
ISP 3	TPS16, TPS81, TPS83	Powdery	White	Moderate purplish pink	Light purplish pink	Moderate yellowish pink
	SCSIO 00652^T	Powdery	Light olive gray	Brilliant orange yellow	Light olive gray	Brilliant yellow
ISP 4	TPS16, TPS81, TPS83	Powdery	White	Moderate orange	White	Light yellowish brown
	SCSIO 00652^T	Leathery	White	Pale yellow	Absent	Absent
ISP 5	TPS16, TPS81, TPS83	Powdery	White	Pale yellow	White	Absent
	SCSIO 00652^T	Leathery	White	Pale yellow	Absent	Absent
ISP 6	TPS16, TPS81, TPS83	Powdery	White	Light yellow	White	Absent
	SCSIO 00652^T	Powdery	White	Vivid yellow	White	Absent
ISP 7	TPS16, TPS81, TPS83	Powdery	Pale yellow	Pale orange yellow	White	Absent
	SCSIO 00652^T	Leathery	Absent	Vivid yellow	Absent	Absent

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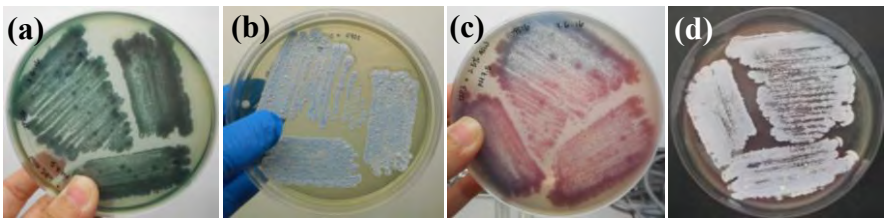

Figure 4.24: Growth of strain TPS16 on ISP 2 (a, b) and ISP 3 (c, d).

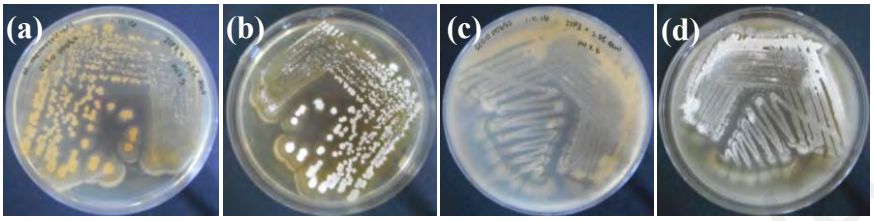

Figure 4.25: Growth of the reference strain *M. thermotolerans* SCSIO 00652^{T} on ISP 2 (a, b) and ISP 3 plates (c, d).

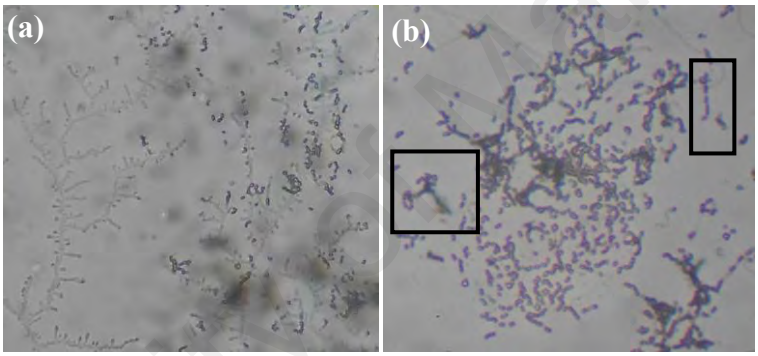

Figure 4.26: (a). Spores of strain TPS16 are formed at the tips of aerial mycelia. (b). The chains of spores are straight to loop as highlighted in the black box frame.). The observations were recorded under a light compound microscope at 400× magnification.

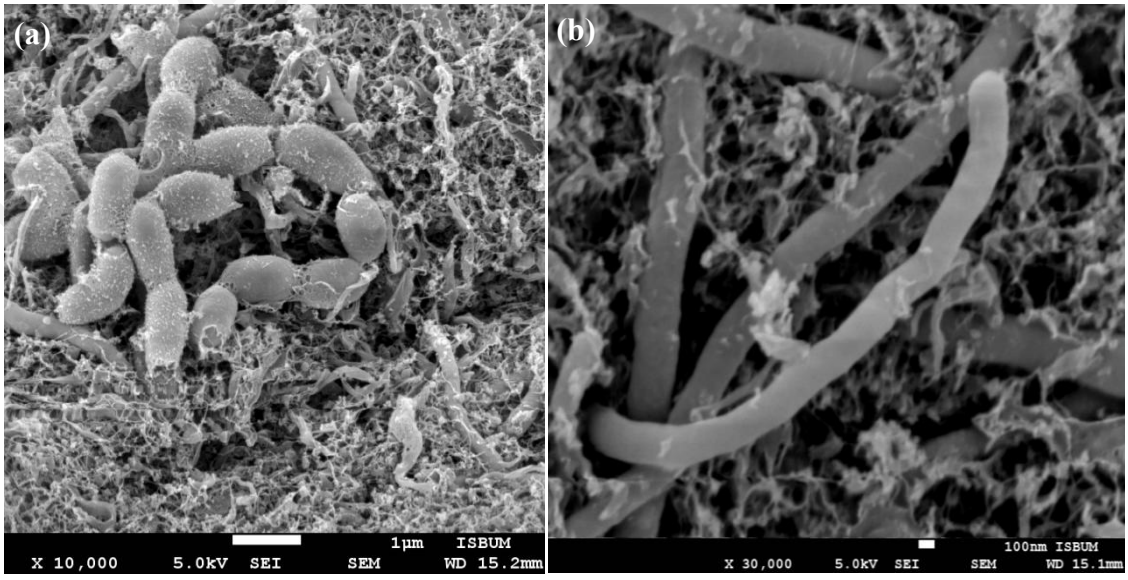

Figure 4.27: Scanning electron micrograph at (a) 10000 × magnification shows the spores (Bar, 1 µm) and (b) at 30000 × magnification that shows aerial hyphae of strain TPS16 cultured on soybean meal glucose agar for 21 days at 28˚C (Bar, 0.1 µm).

4.5.1.3 Carbon utilisation profile

The novel strains TPS16, TPS81 and TPS83 were able to utilise 22 out of 23 carbon sources and produce acid as a product of fermentation of 12 carbon sources (Table 4.13). All three novel strains shared the same carbon utilisation profile. The reference strain showed positive utilisation and fermentation of eight tested carbon sources.

Table 4.13: Carbon utilisation profiling of novel strains TPS16, TPS81 and TPS83 was compared to the reference strain, *M. thermotolerans* SCSIO 00652^T. Positve acid production was given in bracket. (+, positive result; -, negative result; ng, no growth; nd, no data was recorded).

Carbon sources	**Utilisation of carbon source**			
	SCSIO 00652^T	**TPS16**	**TPS81**	**TPS83**
D-glucose	+ (+)	+ (+)	+ (+)	+ (+)
Adonitol	ng	+	+	+
D-arabinose	ng	+	+	+
L-arabinose	+ (+)	+ (+)	+ (+)	+ (+)
D-fructose	+ (+)	+ (+)	+ (+)	+ (+)
D-galactose	ng	+	+	+
Inositol	ng	+ (+)	+ (+)	+ (+)
D-lactose	ng	+	+	+
D-maltose	+	+ (+)	+ (+)	+ (+)
D-mannitol	ng	+ (+)	+ (+)	+ (+)
D-mannose	ng	+ (+)	+ (+)	+ (+)
D-melezitose	ng	+	+	+
D-melibiose	ng	+	+	+
D-ribose	+	+ (+)	+ (+)	+ (+)
D-sorbitol	ng	+	+	+
L-sorbose	ng	+	+	+
Sucrose	+ (+)	+ (+)	+ (+)	+ (+)
D-trehalose	ng	+ (+)	+ (+)	+ (+)
Xylose	+ (+)	+ (+)	+ (+)	+ (+)
Glycine	ng	ng	ng	ng
L-glutamine	ng	+ (+)	+ (+)	+ (+)
L-lysine	ng	+	+	+
L-methionine	ng	+	+	+
Basal medium only	ng	+	+	+

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4.5.1.4 Tolerance of pH, temperature and NaCl

Growth of novel strains TPS16, TPS81 and TPS83 and the reference strain *M. thermotolerans* SCSIO 00652^T at various pH, incubation temperature and NaCl concentrations was listed in Table 4.14. Three novel actinobacterial strains and the reference strain were capable of growing on ISP 2 with pH adjusted to 5- 12. No difference in terms of growth of the novel strains and reference strain at all tested pH as compared to their positive control. The novel strains were able to tolerate growth temperatures at 25ºC, 32ºC, 37 ºC, 45 ºC and 50 ºC. By comparing the growth at different testing temperature to the positive control, which was incubated at 28ºC, the novel strains did not produce spores at 50ºC. Growth was absent at 55ºC for all three novel strains. The reference strain, however, can grow at 55ºC. Interestingly, the novel strains do not produce light blue colour spores when ASW was substituted by NaCl in ISP 2 medium. The reference strain did not produce the white colour aerial mycelia on ISP 2 medium supplemented with NaCl, when compared to growth on ISP 2 supplemented with 2.5% ASW.

4.5.1.5 Hydrolytic enzymes production assays, catalase and oxidase tests and decomposition of adenine

Positive hydrolysis of starch was observed for TPS16, TPS81 and TPS83 through observation of clear zone around the colonies while the agar was stained blue-black upon flooding of the culture with iodine solution. Three novel strains showed positive hydrolysis of gelatine and casein, as indicated by formation of clear halo zones around the colonies on gelatine-ISP 2 and skim milk plates. The novel strains were also able to hydrolyse urea as colour of urea agar changed from yellow to pink. Bubble formation was observed for all three strains when the cells were mixed with 3% H_2O_2 droplet on a glass slide. An intense deep purple hue was observed when the cells were rubbed onto filter papers that were impregnated with oxidase reagent indicated a positive oxidase

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reaction. Decomposition of adenine was indicated by disappearing of adenine crystals around colonies of three novel strains.

4.5.1.6 Cell wall composition of the novel strain TPS16

The diaminopimelic acid isomer present in whole cells hydrolysates of strain TPS16^T was *meso*-diaminopimelic acid, whereas glucose and ribose were present as cell wall sugars. The fatty acid profile of the strain was mainly composed of 26.26% iso-$C_{16:0}$, 16.83% anteiso-$C_{17:0}$, 11.27% iso-$C_{15:0}$, 11.27% $C_{18:1}$w9c, 7.82% anteiso-$C_{15:0}$ and 7.54% iso$C_{17:0}$. The full fatty acid profile was listed in Appendix B. Types of menaquinones present in the cell wall were found to be MK-11(H_2) (27%), MK-10(H_2) (17%), MK-11(H_4) (14%), MK-10(H_4) (10%), MK-11 (9%), MK-12(H_2) (6%), MK-10 (4%), MK-9(H_2) (2%), MK-10(H_6) (2%), MK-11(H_6) (2%), MK-12 (2%) and MK-12(H_4) (2%). Phospholipids present in strain TPS16^T were glycolipids, diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyl-N-methylethanolamine (Appendix C).

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Table 4.14: Growth of three novel strains TPS16, TPS81 and TPS83 and the reference strain at various incubation temperatures, pH and NaCl concentrations. (+, positive growth; -, no growth; w, weak growth)

Parameters	SCSIO 00652^T	TPS16	TPS81	TPS83
pH:				
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	+	+	+	+
Temperature:				
4 ºC	-	-	-	-
15 ºC	-	-	-	-
25 ºC	+	+	+	+
28 ºC	+	+	+	+
32 ºC	+	+	+	+
37 ºC	+	+	+	+
45 ºC	+	+	+	+
50 ºC	+	+	+	+
55 ºC	+	-	-	-
NaCl:				
0%	+	+	+	+
1%	+	+	+	+
2%	+	+	+	+
3%	+	+	+	+
4%	+	+	+	+
5%	+	+	+	+
6%	+	+	+	+
7%	+	+	+	+
8%	+	+	+	+
9%	+	-	-	-
10%	w	-	-	-
15%	-	-	-	-

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4.5.1.7 Antibacterial activity of strains TPS16, TPS81 and TPS83

Three strains displayed positive inhibitory activity against the tester pathogens including *S. aureus* ATCC 29213, *B. subtilis* ATCC 23857, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 47076 by using agar plug diffusion assay (Table 4.15). Examples of screening plates were given in Appendix M. Three strains shared the same inhibitory profile when they were cultured on PM3 and soybean meal glucose media. PM3 culture showed positive inhibitory activity against *B. subtilis* ATCC 23857 only, whereas cultures on soybean meal glucose inhibited the growth of *S. aureus* ATCC 29213, *B. subtilis* ATCC 23857 and *E. coli* ATCC 47076. By growing the strains on the micromonospora medium, TPS16 and TPS83 were able to inhibit the growth of *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213. On the other hand, the strain TPS81 on micromonospora medium was able to inhibit *E. coli* ATCC 47076, in addition to *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213. Strain TPS83 cultured on ATCC medium 241 did not inhibit the growth of any pathogens, whereas TPS16 inhibited the growth of *B. subtilis* ATCC 23857 and TPS81 inhibit growth of both *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213. Strain TPS16 on starch yeast peptone medium did not inhibit the growth of pathogens, while strain TPS83 inhibited the growth of *B. subtilis* ATCC 23857 and strain TPS81 inhibited the growth of *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213.

4.5.1.8 Genome analysis of strains TPS16, TPS81 and TPS83

Genome details of the novel strains were shown in Table 4.16. The GC content of strains TPS16, TPS81 and TPS83 were calculated as 73.8 mol% based on the draft genome assemblies, whereas *M. thermotolerans* SCSIO 00652^{T} has a GC content of 71.6 mol %. Genome sizes of novel strains were estimated to be 6 Mbp. A total of 520, 796 and 635 contigs were produced for strains TPS16, TPS81 and TPS83, respectively.

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Number of coding sequence falls between 5189 and 5245 and number of RNAs were observed to be 62 to 65 for the three novel actinobacterial strains.

Phylogenomic metrics and differences of GC content were calculated (Table 4.16). The dDDH and wet-lab DDH values were calculated to be 22.4 - 22.6% and 21.9-36.9%, respectively. The ANI values of 78.54% were calculated for TPS16 and SCSIO 00652^T, 78.60% for TPS81 and SCSIO 00652^T and 78.40% for TPS83 and SCSIO 00652^T. AAI values were also calculated. Strains TPS16, TPS81 and TPS83 shared AAI values of 60.61%, 60.52% and 60.56% with *M. thermotolerans* SCSIO 00652^T, respectively. The difference of GC content between genomes of strains TPS16 and *M. thermotolerans* SCSIO 00652^T was 2.23%.

Table 4.15: Antibacterial activity profile of novel strains TPS16, TPS81 and TPS83 on five production media using agar plug diffusion assay. Diameters of inhibition zones were recorded in mm. (Bs, *B. subtilis* ATCC 23857; Sa, *S. aureus* ATCC 29213; Ec, *E. coli* ATCC 47076; Pa, *P. aeruginosa* ATCC 27853)

Strain	Tester pathogen	PM3	Soybean meal glucose	Micromonospora medium	ATCC medium 241	Starch yeast peptone
TPS16	Bs	13.5	18.5	15	12	0
	Sa	0	10	9	0	0
	Pa	0	0	0	0	0
	Ec	0	10	0	0	0
TPS81	Bs	13.5	19	20	12.5	10
	Sa	0	12	12.5	10	14.5
	Pa	0	0	0	0	0
	Ec	0	10	11.5	0	0
TPS83	Bs	13.5	16.5	12.5	0	10.5
	Sa	0	10	9	0	0
	Pa	0	0	0	0	0
	Ec	0	10	0	0	0

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Table 4.16: Genome details of strains TPS16, TPS81, TPS83 and *M. thermotolerans* SCSIO 00652^T are listed. Genomic metrics and differences of GC content are also compared between whole genome sequences of strains TPS16^T, TPS81, TPS83 and the reference strain *M. thermotolerans* SCSIO 00652^T.

Strain	**SCSIO 00652^T**	**TPS16**	**TPS81**	**TPS83**
Genome overview:				
G+C content (mol %)	71.61	73.84	73.80	73.81
Estimated genome size (Mbp)	5.66	6.02	6.03	6.05
Number of coding sequences	4992	5189	5200	5245
Number of RNAs	67	63	62	65
dDDH (%):				
SCSIO 00652^T	100.0			
TPS16	22.5 ± 2.35	100.0		
TPS81	22.6 ± 2.35	99.8 ± 0.15	100.0	
TPS83	22.4 ± 2.35	99.7 ± 0.15	99.6 ± 0.20	100.0
Wet-lab DDH (%):				
SCSIO 00652^T	100.0			
TPS16	21.9 ± 0.8	100.0		
TPS81	36.9 ± 0.6	85.3 ± 0.5	100.0	
TPS83	30.5 ± 0.4	88.9 ± 0.3	90.2 ± 0.4	100.0
Differences of GC content (mol %):				
SCSIO 00652^T	0.00			
TPS16	2.23	0.00		
TPS81	2.19	0.04	0.00	
TPS83	2.20	0.03	0.01	0.00
ANI values (%):				
SCSIO 00652^T	100.00			
TPS16	77.64	100.00		
TPS81	77.65	99.96	100.00	
TPS83	77.57	99.95	99.97	100.00
AAI values (%):				
SCSIO 00652^T	100.00			
TPS16	60.61	100.00		
TPS81	60.52	99.91	100.00	
TPS83	60.56	99.91	99.92	100.00

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4.5.2 Characterisation of strain TPS2

4.5.2.1 Phylogenetic analysis of 16S rRNA gene sequence

The actinobacterial strain TPS2 was identified as a novel species belong to the genus *Nocardiopsis*, which was isolated from skim milk/ HEPES treated sediment sample Z using modified M2 medium. Strain TPS2 was closely related to *Nocardiopsis alba* subsp. *alba* DSM 43377^T, *Nocardiopsis terrae* YIM 90022^T and *Nocardiopsis prasina* DSM 43845^T, by sharing 98.96%, 98.90% and 98.83% of 16S rRNA gene similarity with the closely related matches, respectively. Phylogenetic analysis confirmed the taxonomic position of strain TPS2 using neighbour joining, maximum likelihood and maximum parsimony methods. The novel strain formed a separate cluster from the closely related *Nocardiopsis* species (Figure 4.28).

4.5.2.2 Morphological characterisation of strain TPS2

The novel strain TPS2 was Gram-stained positive and showed good growth on ISP medium 1, 2, 3, 4, 5, 6 and 7 (Table 4.17 and Figure 4.29). It produced white aerial mycelia and yellow substrate mycelia on all tested media. Sparse formation of aerial mycelia was observed on ISP 7. No diffusible pigment was produced on all tested ISP media. The novel strain produced brown diffusible pigment on ISP 6 and ISP 7. Micro-morphology of the novel strains was examined by cover slip method. Aerial mycelia of the strain TPS2 were long, branched and straight to flexuous and fragmenting into spores (Figure 4.30). Sporangia and sclerotia were not observed from the novel actinobacterial strain.

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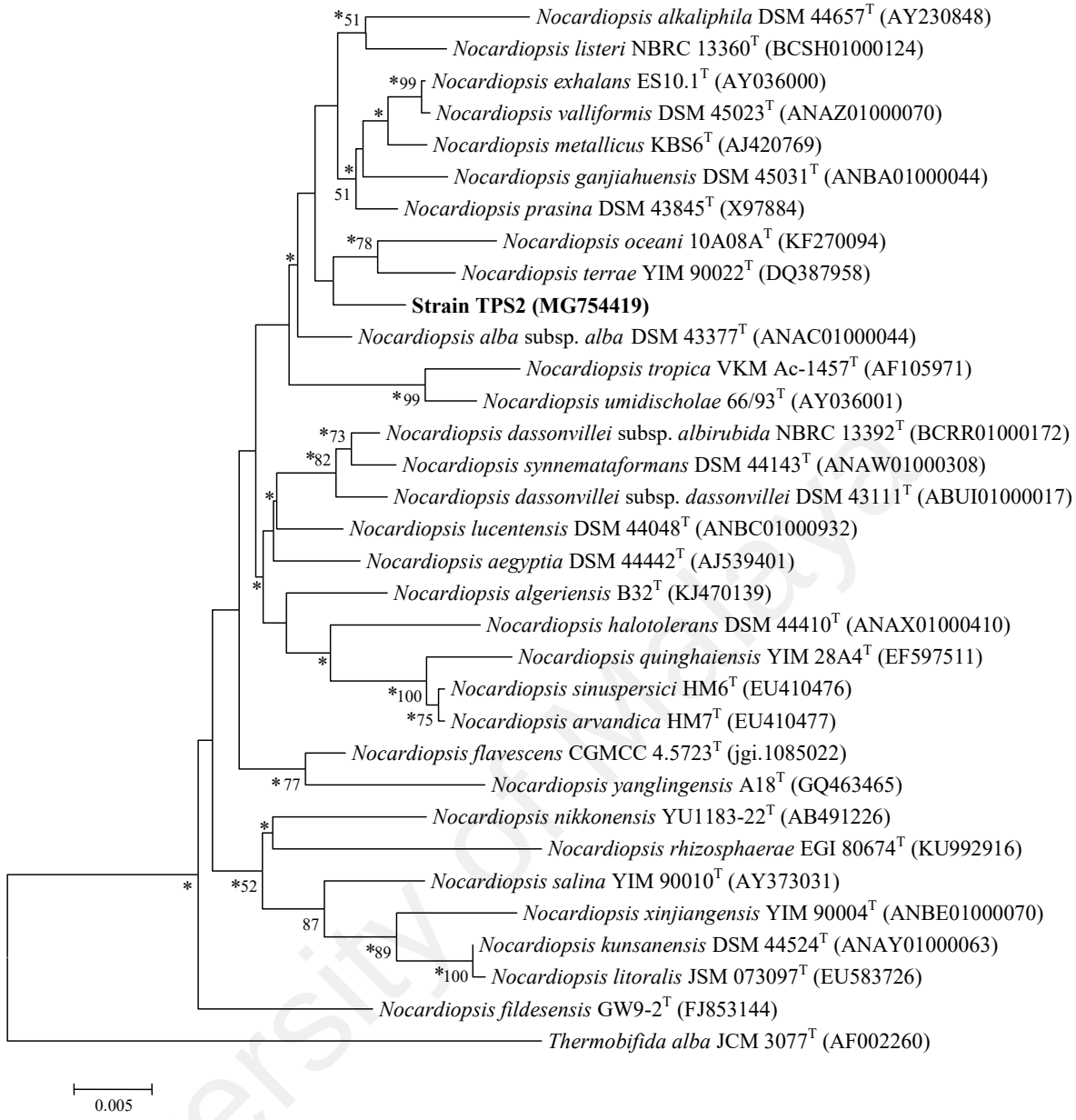

Figure 4.28: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the strain TPS2 and its closely related members in the family *Nocardiopsaceae*. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.5% sequence divergence.

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Table 4.17: Colour of substrate and aerial mycelia, spores and diffusible pigment of strain TPS2 on ISP media supplemented with 2.5% ASW were listed here.

ISP media	Substrate mycelia	Aerial mycelia	Spore colour	Diffusible pigment
ISP 1	Vivid yellow	White	White	Absent
ISP 2	Vivid yellow	White	White	Absent
ISP 3	Brilliant orange yellow	White	White	Absent
ISP 4	Vivid yellow	White	White	Absent
ISP 5	Vivid yellow	White	White	Absent
ISP 6	Deep orange yellow	White	White	Moderate olive brown
ISP 7	Brilliant yellow	White	White	Deep brown

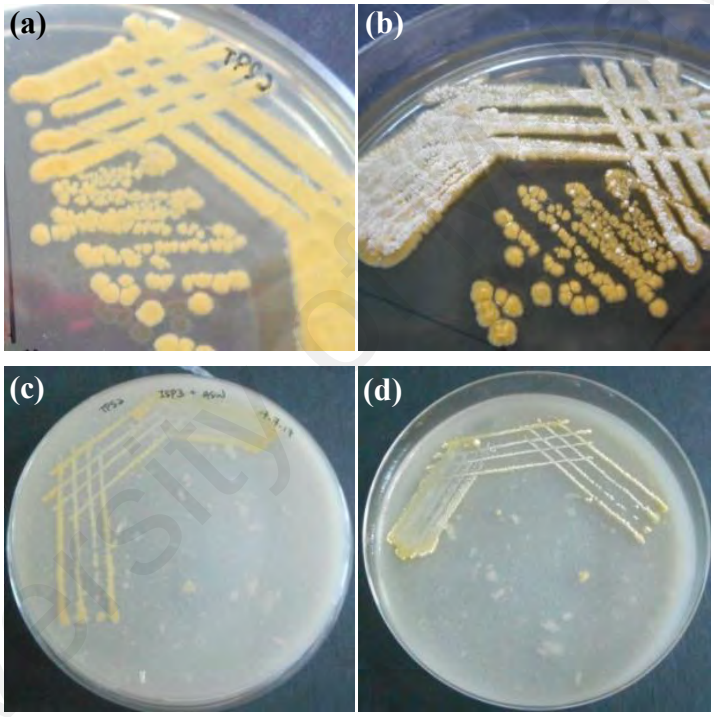

Figure 4.29: Growth of strain TPS2 on ISP 2 (a, b) and ISP 3 (c, d).

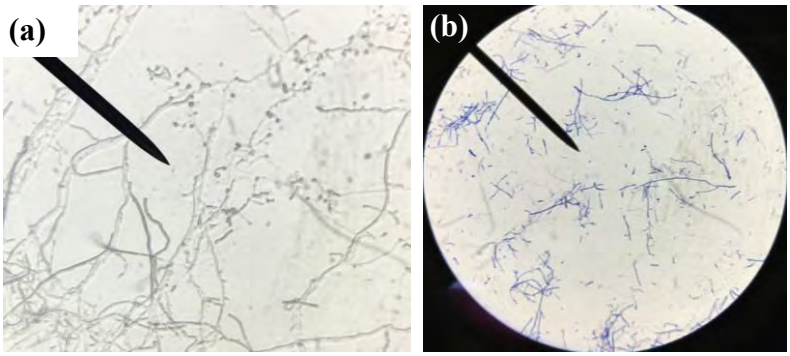

Figure 4.30: Aerial mycelia of TPS2 were long, straight to flexuous and fragmenting into spores (a). The strain was stained purple with Gram-stain (b). Both observations were recorded under a light compound microscope at a magnification of 400×.

4.5.2.3 Carbon utilisation profile

The novel actinobacterial strain TPS2 showed positive utilisation of D- fructose, D-glucose, D- maltose, D- mannitol, D- mannose, D- ribose, D- sorbitol, D- trehalose, glycine, L- arabinose, L- glutamine, L- sorbose, sucrose and xylose. The strain does not show growth when it is inoculated on basal medium without carbon source and media with adonitol, D- arabinose, D- galactose, D- lactose, D- melezitose, D- melibiose, inositol, L- lysine and L- methionine. Colour of the media that were supplemented with 1% of D- fructose, D- maltose, D- mannitol, D- mannose glycine and L- arabinose respectively, were changed from purple to yellow and this indicated acid production.

4.5.2.4 Tolerance of pH, temperature and NaCl

The novel strain TPS2 was able to grow at pH 6, pH 7 and pH 8. No growth was observed for plates adjusted to pH 9 and above. Optimum growth was observed at pH 7, pH 7.5 (positive control) and pH 8. The novel strain was able to grow at 15ºC, 25ºC, 28ºC and 32ºC. No growth at 37ºC and 45ºC. Optimum growth occurred at 28ºC and 32ºC. Good growth occurred on ISP2 plates without supplementation of NaCl and the strain was also able to display good growth on ISP2 supplemented with 1% to 6% of NaCl. Weak growth was observed on ISP2 plate with 7% NaCl as compared to the positive control. No growth was observed from ISP2 plates with 8% NaCl.

4.5.2.5 Hydrolytic enzymes production, catalase and oxidase tests and decomposition of adenine

Hydrolysis of starch was recorded positive for the novel strain TPS2, as clear zone around the colonies on starch- ISP2 plate was observed and presence of starch in the agar was confirmed by changes of agar colour to blue upon flooding with iodine solution. Observation of clear zone around colonies of strain TPS2 on gelatine- ISP2 and skim milk agar plates indicated positive result for hydrolysis of gelatine and casein.

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No colour change was observed on urea agar slant inoculated with strain TPS2. Formation of bubbles was observed for strain TPS2 upon mixing of the cells with 3% H_2O_2 droplet. Oxidase test was recorded as negative as indicated by absent of colour changes to purple of the filter paper impregnated with oxidase reagent. Disappearance of adenine crystals around the actinobacterial colonies indicated that the novel strain was able to decompose adenine.

4.5.2.6 Cell wall composition of the novel strain TPS2

The diaminopimelic acid isomer present in whole cells hydrolysates of strain TPS2 was *meso*-diaminopimelic acid. The cell wall of strain TPS2 was mainly composed of iso-$C_{16:0}$ (36.82%), $C_{18:0}$ 10- methyl (15.63%), anteiso-$C_{17:0}$ (12.06%) $C_{17:0}$ 10- methyl (9.38%), $C_{18:1}$w9c (6.10%) and iso-$C_{15:0}$ (5.63%). The full fatty acid profile was listed in Appendix D. Types of menaquinones present in the cell wall were found to be MK-10(H_2) (13%), MK-10(H_4) (11%), MK-9(H_2) (6%), MK-10 (5%), MK-9(H_4) (4%), MK-9 (3%), MK-10(H_6) (2%), MK-9(H_6) (1%) and traces of MK-9(H_8) (Appendix E). Phospholipids present in strain TPS2 were glycolipids, phosphatidylglycerol, phosphatidylinositol, diphosphatidylglycerol and unknown phospholipids (Appendix E).

4.5.2.7 Antibacterial activity of the novel strain TPS2

The novel strain growing on media including PM3, soybean meal glucose media, micromonospora medium, ATCC medium 241 and starch yeast peptone medium for 14 days and 21 days did not inhibit growth of pathogens as tested by agar plug diffusion assay, including the Gram-positive *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857 and Gram-negative *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 47076.

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4.5.2.8 Genome analysis of the novel strains TPS2

Genome details of the novel strains were shown in Table 4.18. The strain TPS2 was found have a GC content of 70.8 mol% and was sharing the same percentage of GC content with *N. prasina* DSM 43845^T. The other closest related matches including *N. alba* subsp. *alba* DSM 43377^T and *N. listeri* NBRC 13360^T have 69.6 mol% and 68.9 mol% of GC content in their genomes, respectively. The estimated genome size for strain TPS2 was 6.42 Mbp, whereas the closely related matches were reported to have a genome sizes between 5.68 and 5.99 Mbp. Assembly of the genome resulted in 537 contigs. A total of 5590 coding sequences and 64 RNAs were reported to be found from the genome of the strain TPS2. Both closest related matches *N. alba* subsp. *alba* DSM 43377^T and *N. prasina* DSM 43845^T were reported to possess 5237 coding sequences and 69 RNAs, and 5254 coding sequences and 58 RNAs, respectively. *N. listeri* NBRC 13360^T was reported to have 5093 coding sequences and 62 RNAs.

Genomic metrics and differences of GC content were also shown in the Table 4.18. The dDDH values between the whole genome sequences of strain TPS2 and the three type strains were 26.00 ± 2.45%, 25.30 ± 2.40% and 29.10 ± 2.40%, respectively. The calculated ANI values for TPS2 and the type strains were 82.54%, 82.20% and 85.08%, respectively. The novel strain TPS2 shared AAI values of 78.49%, 78.04% and 81.96% with *N. alba* subsp. *alba* DSM 43377^T, *N. prasina* DSM 43845^T and *N. listeri* NBRC 13360^T. Difference of GC content between genomes of strain TPS2 and all three type strains was between 0.01 mol% and 1.93 mol%.

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Table 4.18: Genome details of strain TPS2 and three of its top five closely related matches including *Nocardiopsis alba* subsp. *alba* DSM 43377^T, *Nocardiopsis prasina* DSM 43845^T *and Nocardiopsis listeri* NBRC 13360^T are listed. Genomic metrics and differences of GC content are also compared.

Strain	**DSM 43377^T**	**DSM 43845^T**	**NBRC 13360^T**	**TPS2**
Genome overview:				
G+C content (mol %)	69.6	70.8	68.9	70.8
Estimated genome size (Mbp)	5.84	5.99	5.68	6.42
Number of coding sequences	5237	5254	5093	5590
Number of RNAs	69	58	62	64
dDDH (%):				
DSM 43377^T	100.0			
DSM 43845^T	24.60 ± 2.40	100.0		
NBRC 13360^T	24.90 ± 2.40	24.20 ± 2.35	100.0	
TPS2	26.00 ± 2.45	25.30 ± 2.40	29.10 ± 2.40	100.0
Differences of GC content (mol %):				
DSM 43377^T	0.00			
DSM 43845^T	1.18	0.00		
NBRC 13360^T	0.77	1.95	0.00	
TPS2	1.16	0.01	1.93	0.00
ANI values (%):				
DSM 43377^T	100.0			
DSM 43845^T	81.84	100.0		
NBRC 13360^T	82.03	81.44	100.0	
TPS2	82.54	82.20	85.08	100.0
AAI values (%):				
DSM 43377^T	100.0			
DSM 43845^T	77.19	100.0		
NBRC 13360^T	77.82	76.55	100.0	
TPS2	78.49	78.04	81.96	100.0

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4.5.3 Characterisation of strains TPS3, TPS4, TPS114, TPS137, TPS143 and TPS183

4.5.3.1 Phylogenetic analysis of 16S rRNA gene sequences

A total of six actinobacterial strains were identified to be novel species belong to *Streptomyces*, namely TPS3, TPS4, TPS114, TPS137, TPS143 and TPS183. All six strains were isolated from Tioman marine sediment sample set pre-treated with skim milk/ HEPES. Five of them were isolated on modified M2 plates and one strain, which is the strain TPS183, was isolated on M3 plate (Table 4.11). Among these novel *Streptomyces* spp., the strains TPS4, TPS114 and TPS137 were shown to be the only member in their respective fingerprinting cluster when the non-*Salinispora*-like group was de-replicated using double RE digestion technique (Figure 4.19, Table 4.11).

The almost full length sequences of 16S rRNA genes of the novel strains were compared to the deposited gene sequences of *Streptomyces* type strains in EzBioCloud databases. The taxonomic position of all six novel strains were confirmed based on phylogenetic trees that were inferred using neighbour joining, maximum likelihood and maximum parsimony methods, by comparing the strains with their closest related members of the genus *Streptomyces* (Figures 4.31- 4.37).

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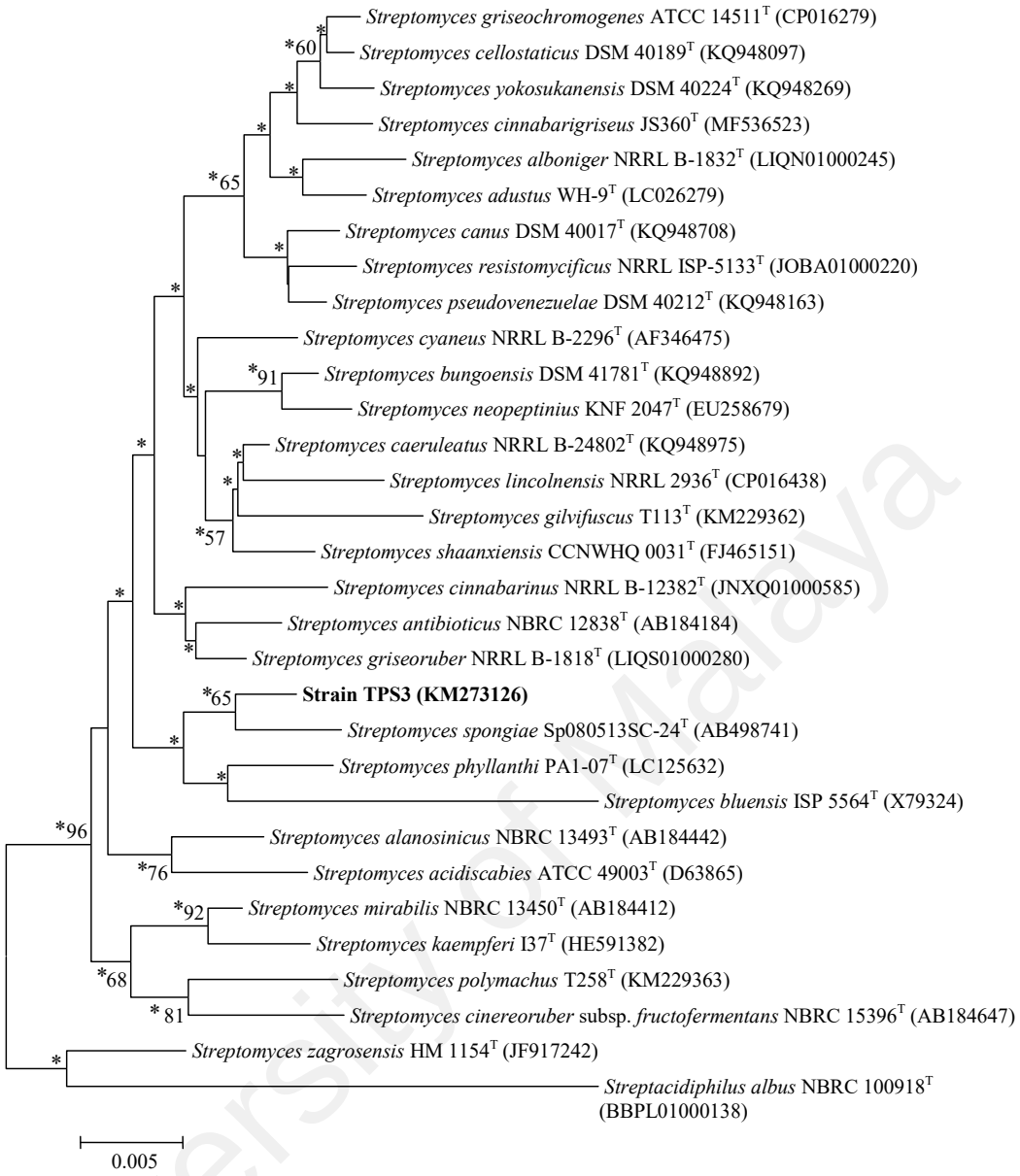

Figure 4.31: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the strain TPS3 and its closely related *Streptomyces* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.5% sequence divergence.

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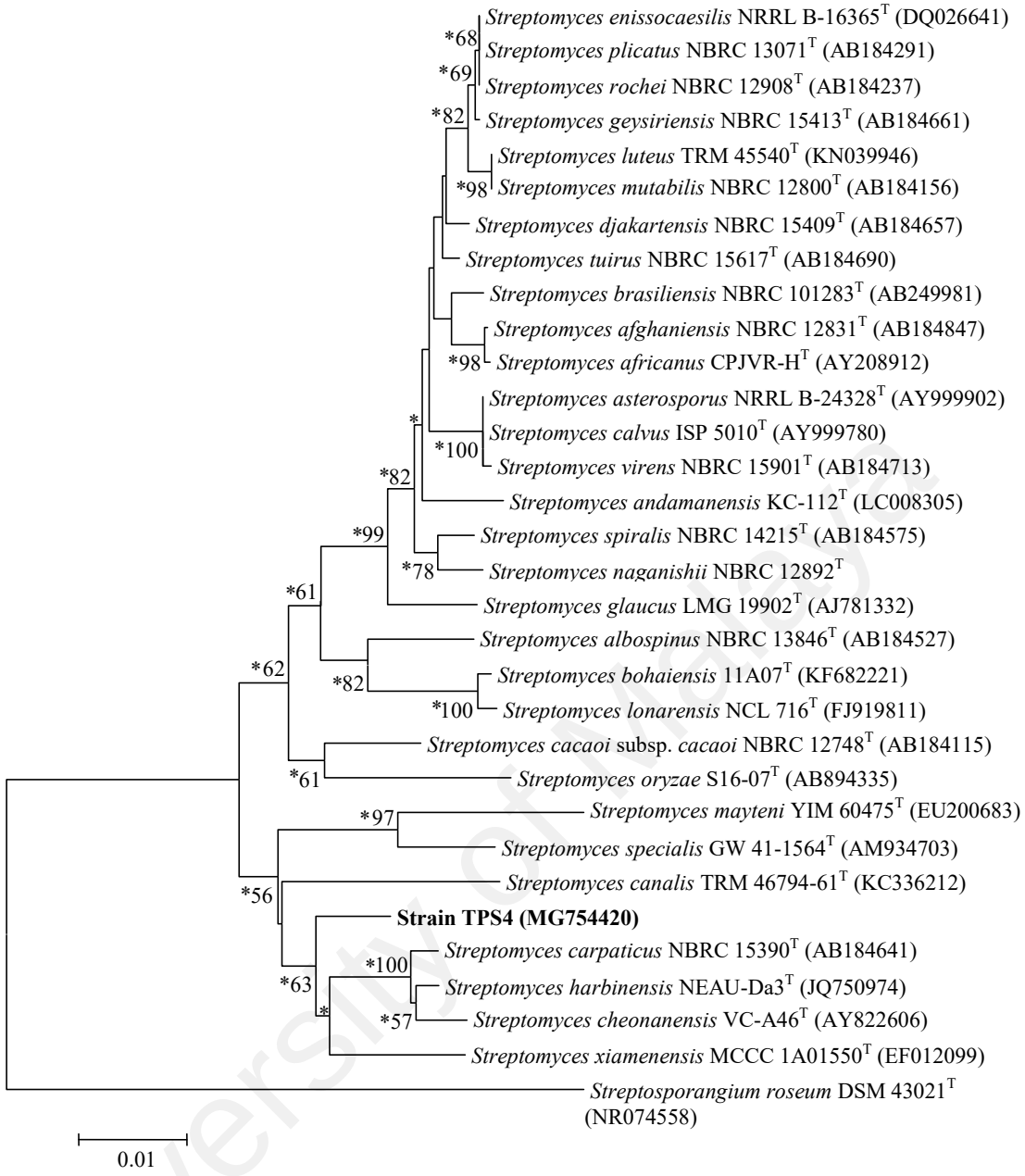

Figure 4.32: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the actinobacterial strain TPS4 and its closely related *Streptomyces* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 1% sequence divergence.

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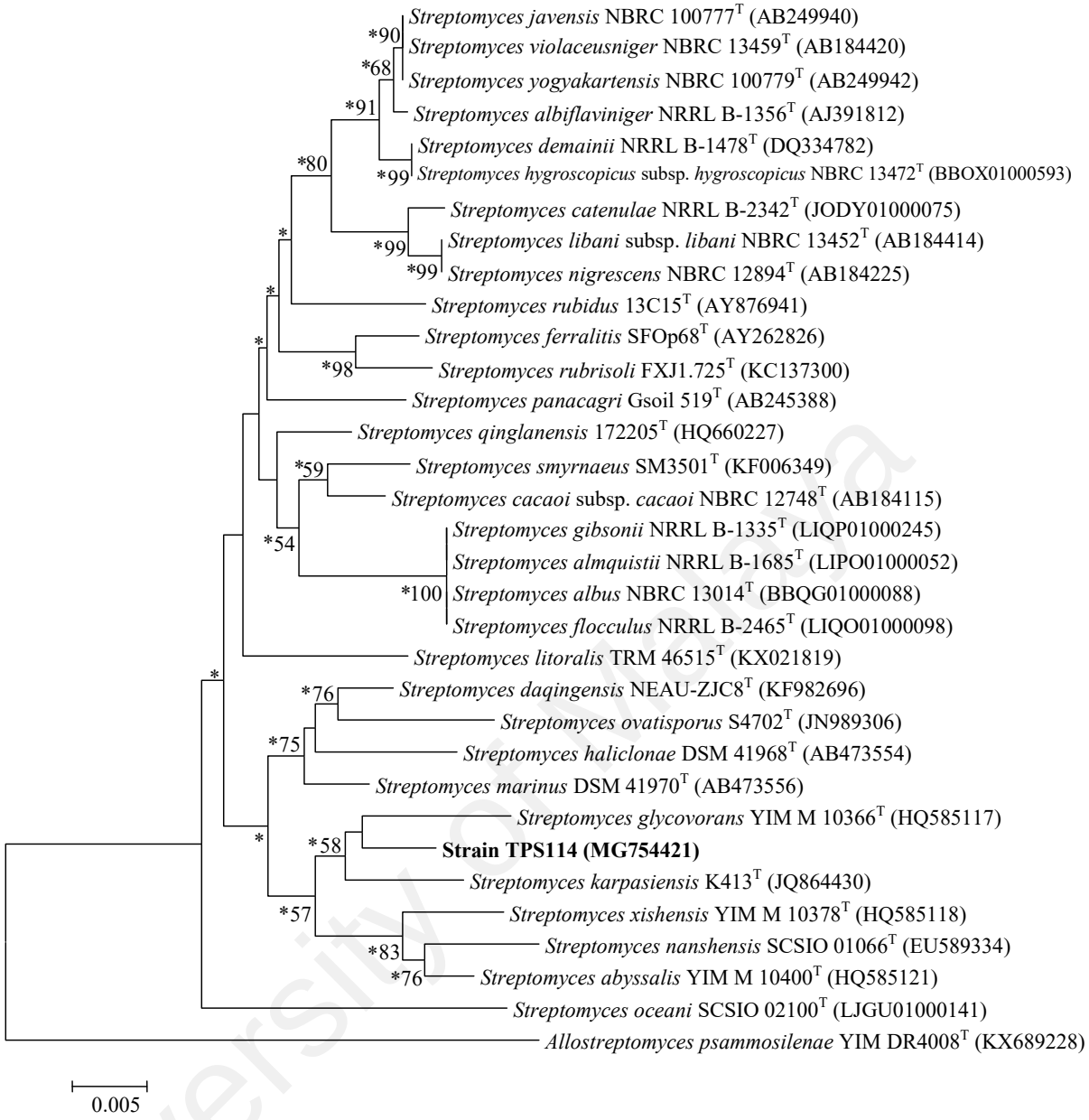

Figure 4.33: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the novel strain TPS114 and its closely related *Streptomyces* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.5% sequence divergence.

University of Pretoria

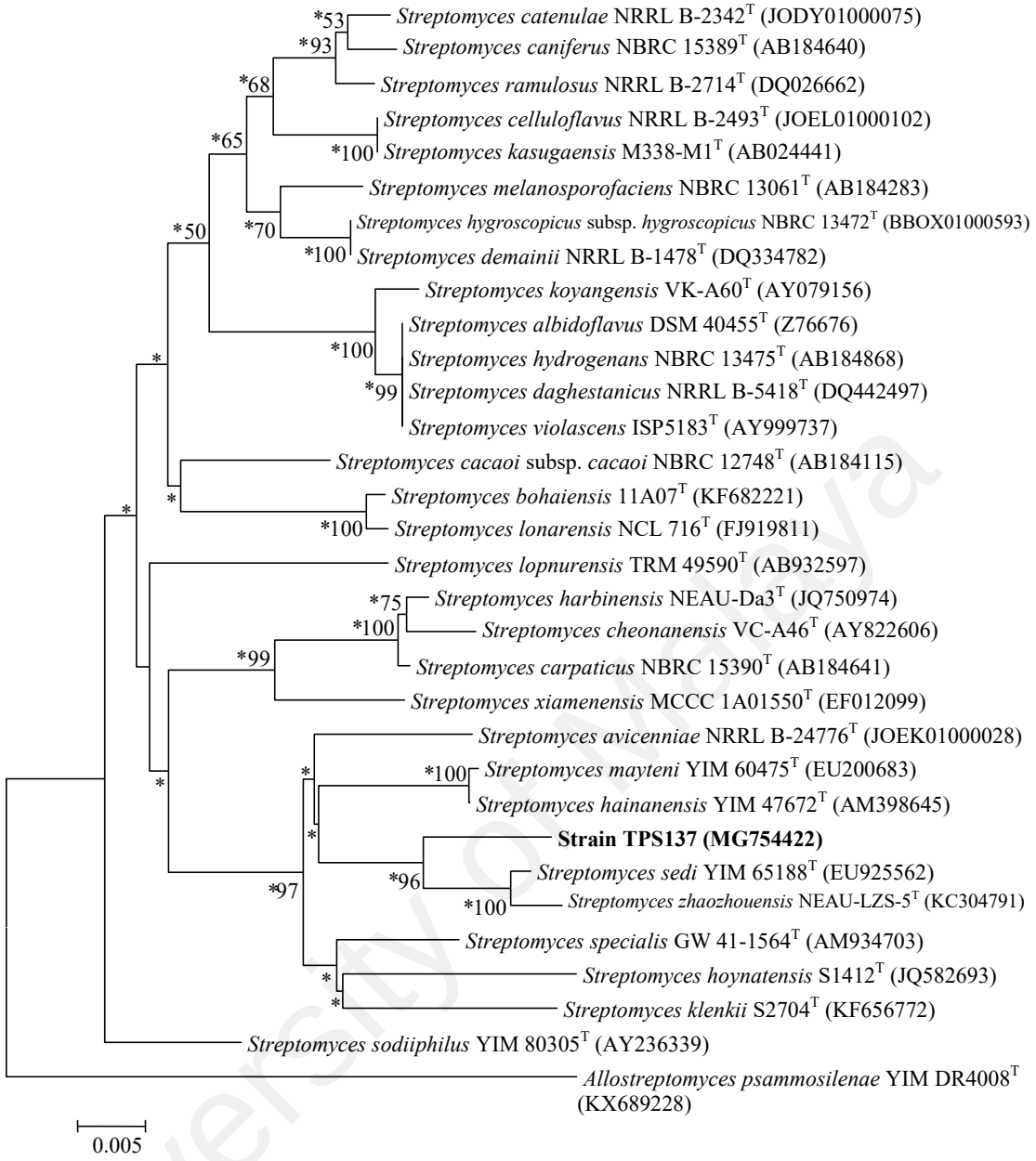

Figure 4.34: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the novel strain TPS137 and its closely related *Streptomyces* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.5% sequence divergence.

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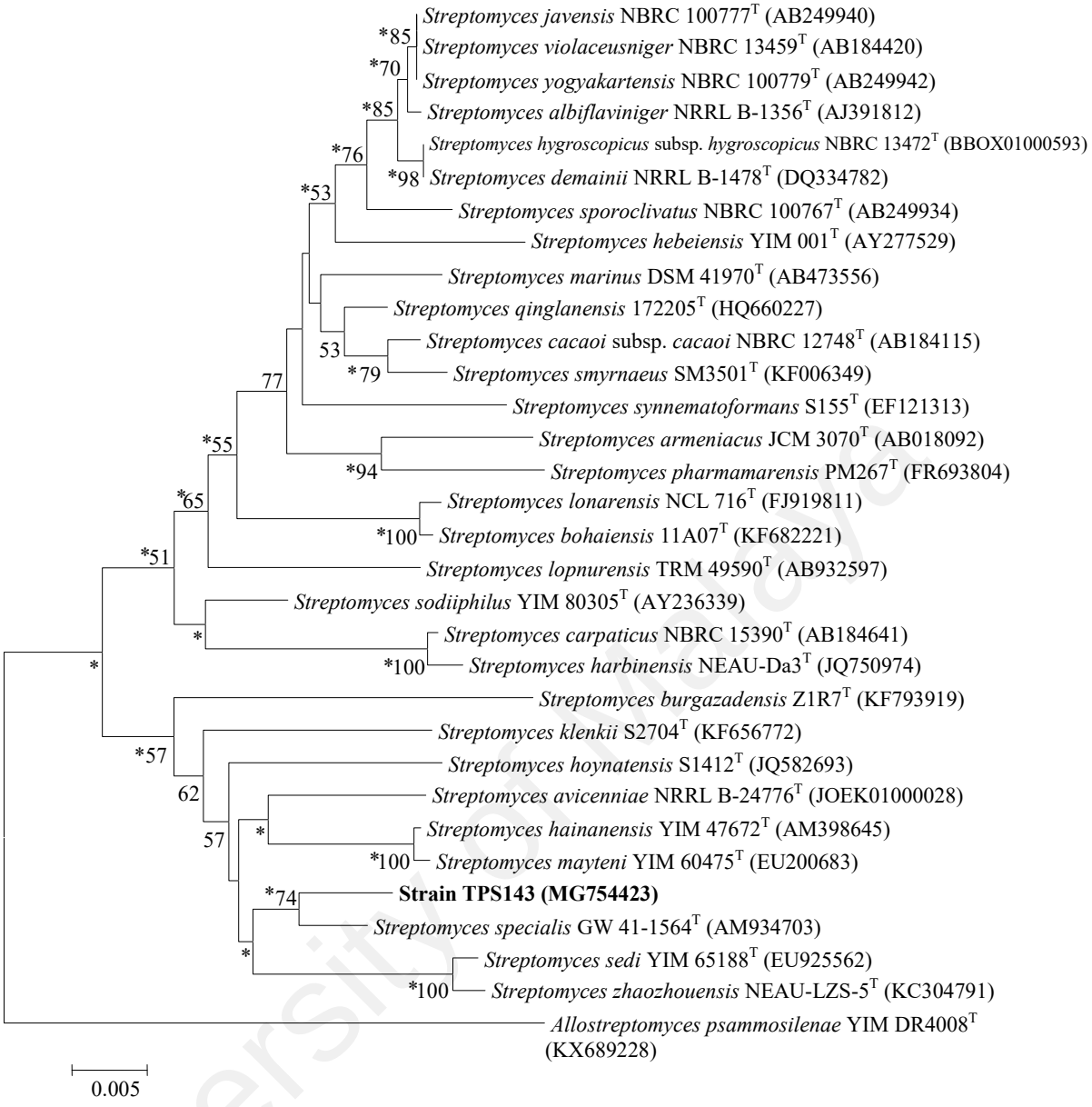

Figure 4.35: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the novel strain TPS143 and its closely related *Streptomyces* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.5% sequence divergence.

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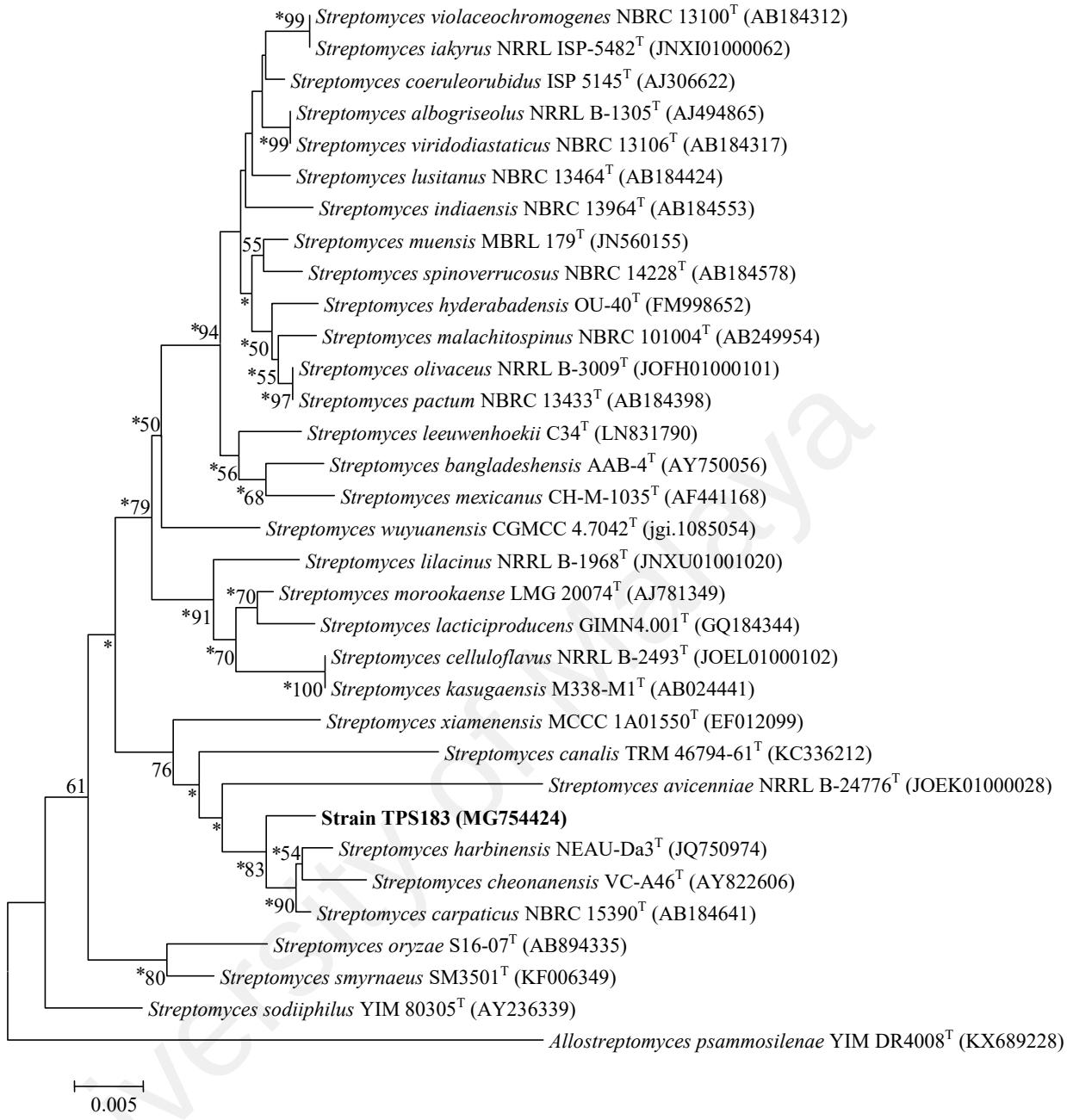

Figure 4.36: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the novel strain TPS183 and its closely related *Streptomyces* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.5% sequence divergence.

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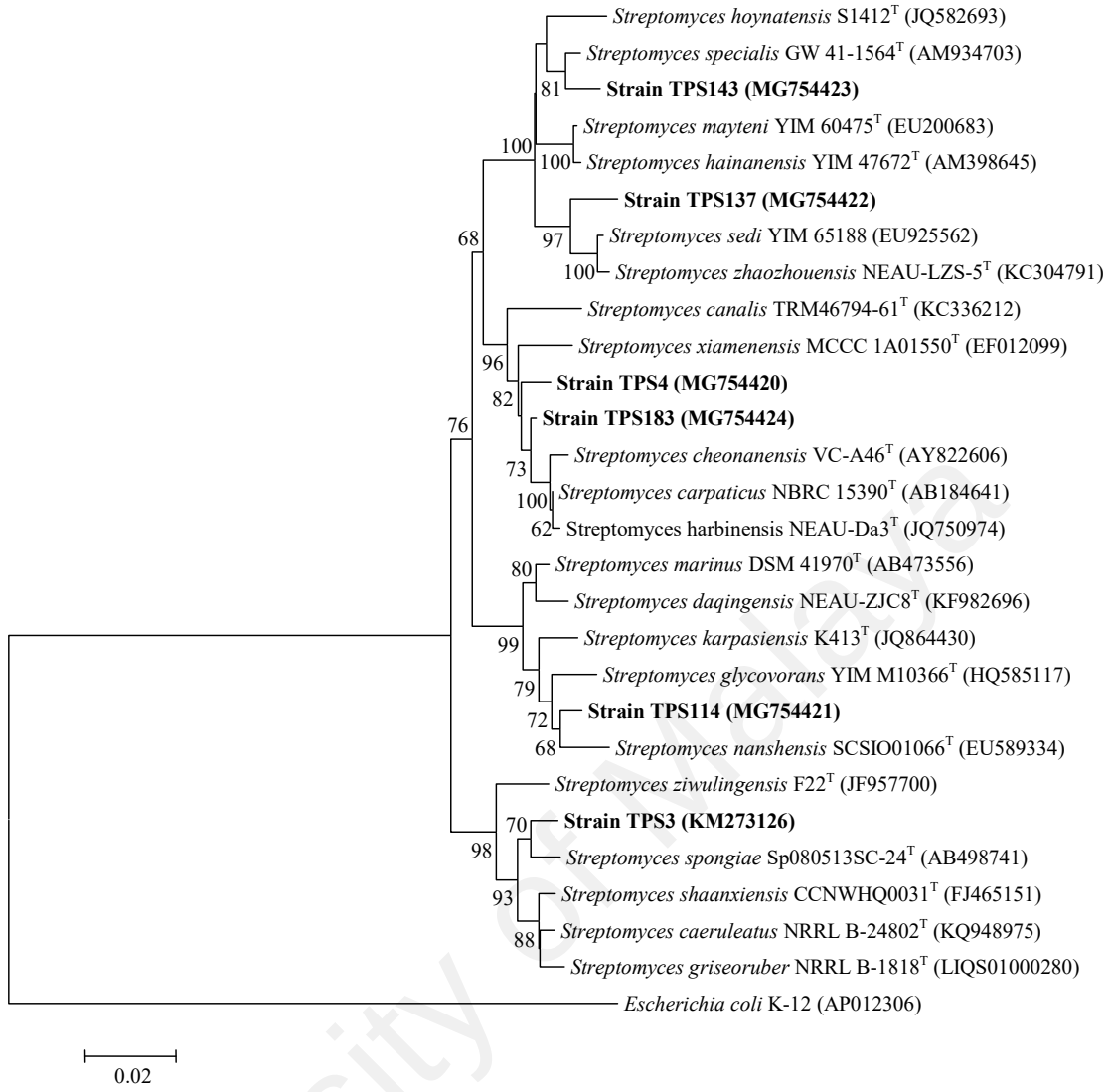

Figure 4.37: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the strains TPS3, TPS4, TPS114, TPS137, TPS143 and TPS183 and their closely related *Streptomyces* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.2% sequence divergence.

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4.5.3.2 Morphological characterisation

Cells of all six novel strains were Gram-stained positive. Growths of the strains TPS4, TPS114, TPS137, TPS143 and TPS183 on various ISP media supplemented with 2.5% ASW were tested (Table 4.19). Growth of the novel strain TPS3 was tested on two sets of culture media, one with supplementation of 2.5% ASW and another one without ASW, and the growth was compared with reference strains *S. ziwulingensis* F22^{T} and *S. shaanxiensis* CCNWHQ 0031^{T} (Table 4.20). Both reference strains were purchased from Japanese Collection of Microorganisms via RIKEN-BRC, Japan.

The strains TPS4, TPS114, TPS137 and TPS183 were able to grow on all ISP media (Figures 4.38, 4.39, 4.40, 4.41 and 4.42). However, spores were not produced when TPS4 was cultured on ISP 4, ISP 5 and ISP 6, and the strain TPS114 did not produce spores on ISP 1, ISP 2 and ISP 3. Spores were also not produced by the strain TPS137 when it was cultured on ISP 5 and ISP 7 media. On ISP 2 medium, the strain TPS137 showed sparse production of spores. The strain TPS143 showed positive growth on ISP 1, ISP 2, ISP 3, ISP 4, ISP 5 and ISP 7, no growth was observed on ISP 6 and sparse production of spores was observed on ISP 1 and ISP 5. Production of green to brown diffusible pigments on ISP 1 was observed for strains TPS4, TPS114, TPS143 and TPS183. Both strains TPS4 and TPS114 also produced brown pigment on ISP 7. None of the novel strains produced diffusible pigment on ISP 6.

The strain TPS3 and both reference strains, *S. ziwulingensis* F22^{T} and *S. shaanxiensis* CCNWHQ 0031^{T}, were able to grow on media supplemented with 2.5% ASW and also media without supplementation of ASW (Figures 4.43 and 4.44 and table 4.20). The culture media used for growth assessment include ISP media 1 to 7, Czapek's solution medium, Gauze's medium, MBA and NA. A point worth to highlight here is that the strain TPS3 produce light blue aerial mycelia when it was cultured on ISP 2, ISP 3 ISP

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4, ISP 5 and ISP 7 without ASW, but white aerial mycelia on the same media supplemented with ASW. It did not produce spores when cultivated on ISP 1, ISP 6, Czapek's solution medium, Gauze's medium and NA, regardless of addition of ASW. No production of spores was also observed for strain TPS3 on ISP 5, ISP 7 and MBA, when ASW was supplemented in these media. Sparse production of spores was observed when TPS3 was cultivated on ISP 3 and ISP 4 supplemented with ASW.

The reference strains *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T were also observed to not producing spores on ISP 1 and ISP 6 media with and without supplementation of ASW, and Czapek's solution medium supplemented with ASW. In addition, the strain *S. ziwulingensis* F22^T did not produce spores on ISP 2 without addition of ASW. Sparse production of spores was observed on NA with ASW for strain *S. ziwulingensis* F22^T. The strain *S. shaanxiensis* CCNWHQ 0031^T did not produce spores on NA with and without addition of ASW, and ASW- supplemented ISP 5 and MBA media. Sparse production of spores by *S. shaanxiensis* CCNWHQ 0031^T was observed on Czapek's solution medium without ASW, ISP 7- ASW and Gauze's-ASW medium.

Examination of the micromorphology of the novel strains by cover slip method and Gram- stain showed that the strains produced long to flexuous and branching aerial mycelia that differentiate into spore chains (Figures 4.45). Strains TPS4 and TPS183 formed spiral spore chains with more than five turns in a chain. Strain TPS114 produce straight aerial mycelia and round spores. The strains TPS137 and TPS143 produced looped spore chains. Sporangium was observed for strain TPS143. The strain TPS3 formed straight aerial mycelia that differentiate into spiral and looped spore chains on ISP 2. Examination of the morphology of spores of strain TPS3 with SEM revealed that the spores have spiny surface and they were arranged in spirals with two to five turns.

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Table 4.19: Colonies colour of novel *Streptomyces* strains on ISP media with ASW. 'ND' denoted data is not available.

Culture medium	Strain	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
ISP 1	TPS4	Powdery	Light grey	Pale greenish yellow	Light grey	Pale greenish yellow
	TPS114	Leathery	Absent	Brilliant yellow	Absent	Light yellow
	TPS137	Powdery	White	Light yellow	White	Absent
	TPS143	Leathery	White	Dark orange brown	White	Moderate olive brown
	TPS183	Powdery	Medium grey	Deep olive green	Medium grey	Deep greenish yellow
ISP 2	TPS4 (Figure 4.33)	Powdery	Light olive grey	Dark olive brown	Light olive grey	Strong greenish yellow
	TPS114 (Figure 4.34)	Leathery	Absent	Brownish orange	Absent	Absent
	TPS137 (Figure 4.35)	Leathery	Deep yellowish brown	Moderate orange	White	Light orange
	TPS143 (Figure 4.36)	Powdery	Greyish greenish yellow	Moderate olive	Greyish greenish yellow	Light olive brown
	TPS183 (Figure 4.37)	Powdery	Dark gray	Brownish black	Dark gray	Dark olive brown
ISP 3	TPS4 (Figure 4.33)	Powdery	Olive gray	Greyish brown	Olive gray	Dark olive
	TPS114 (Figure 4.34)	Leathery	Absent	Strong yellow	Absent	Absent
	TPS137 (Figure 4.35)	Leathery	Absent	Pale yellowish pink	Absent	Light pink
	TPS143 (Figure 4.36)	Powdery	Strong yellowish brown	Strong yellowish brown	White	Dark orange yellow
	TPS183 (Figure 4.37)	Powdery	Olive gray	Greyish olive	Olive gray	Dark greenish yellow
ISP 4	TPS4	Leathery	Light olive gray	Moderate olive brown	Absent	Absent

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Table 4.19, continued.

Culture medium	Strain	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
ISP4	TPS114	Leathery	Strong yellow	Strong yellow	White	Strong yellow
	TPS137	Powdery	White	Light yellowish brown	White	Deep orange brown
	TPS143	Powdery	Light olive grey	Dark brown	Light olive grey	Dark orange yellow
	TPS183	Powdery	Medium grey	Brownish black	Medium grey	Dark olive
ISP 5	TPS4	Leathery	Pale greenish yellow	Pale yellow	Absent	Absent
	TPS114	Leathery	Strong yellow	Strong yellow	White	Absent
	TPS137	Leathery	Absent	Pale yellow green	Absent	Absent
	TPS143	Leathery	Brilliant orange	Moderate orange yellow	White	Absent
	TPS183	Powdery	Yellowish grey	Light olive	Yellowish grey	Light olive
ISP 6	TPS4	Leathery	Pale greenish yellow	Light yellow	Absent	Absent
	TPS114	Leathery	Absent	Strong yellow	Absent	Absent
	TPS137	Powdery	Yellowish grey	Strong brown	Yellowish grey	Absent
	TPS143	No growth	ND	ND	ND	ND
	TPS183	Powdery	Pale violet	Dark brown	Pale violet	Absent
ISP 7	TPS4	Powdery	Yellowish grey	Strong yellowish brown	Yellowish grey	Deep orange brown
	TPS114	Powdery	Dark orange yellow	Dark orange yellow	White	Dark orange yellow
	TPS137	Leathery	Absent	Yellowish white	Absent	Absent
	TPS143	Leathery	Brilliant orange	Moderate orange yellow	White	Absent
	TPS183	Powdery	Light olive grey	Greyish olive	Light olive grey	Absent

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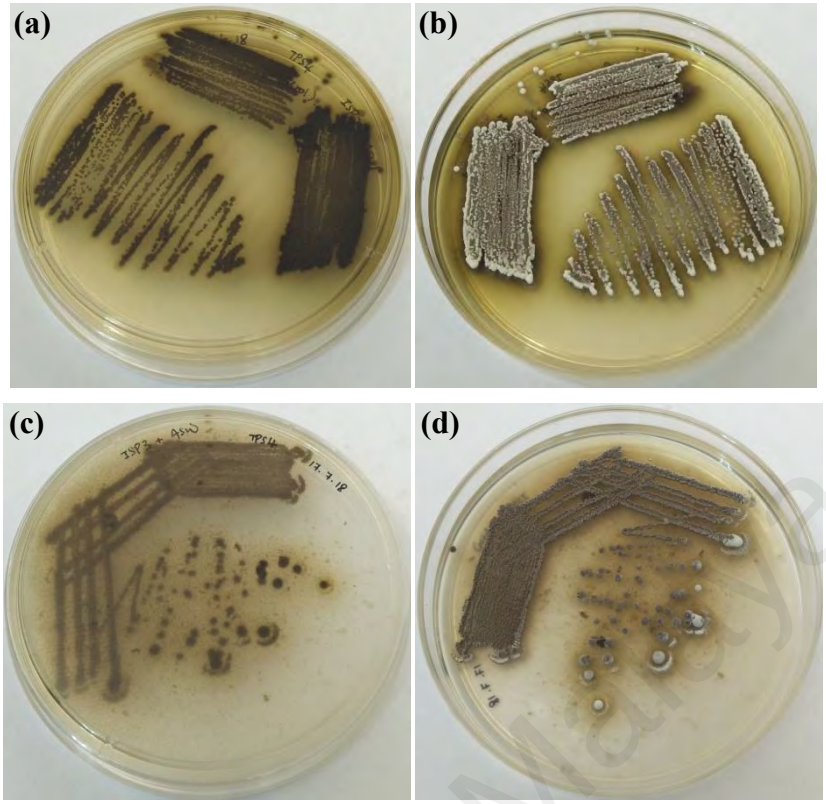

Figure 4.38: Growth of strain TPS4 on ISP 2 (a, b) and ISP 3 (c, d).

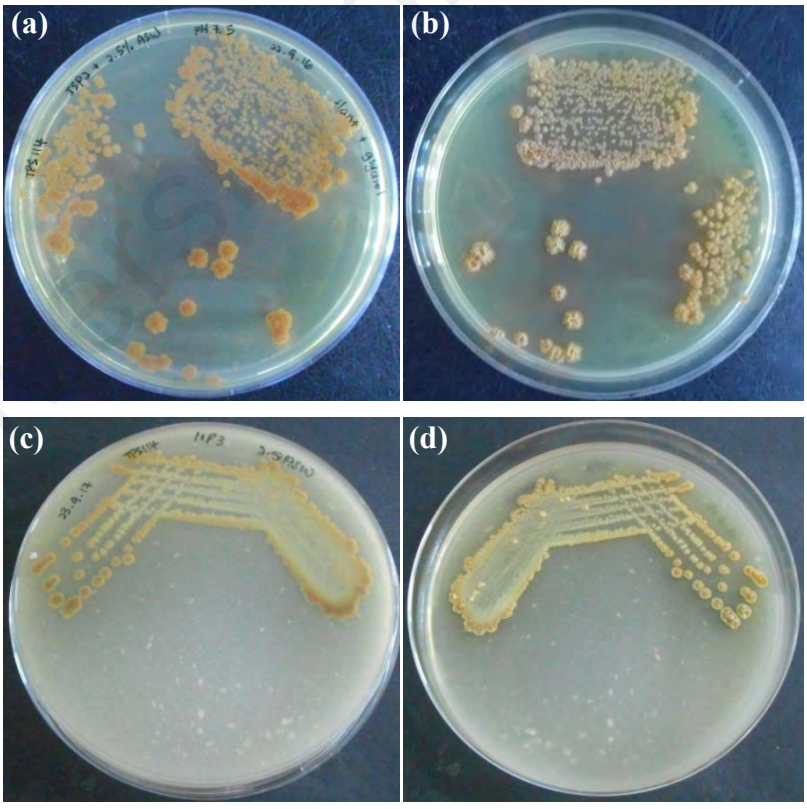

Figure 4.39: Growth of strain TPS114 on ISP 2 (a, b) and ISP 3 (c, d).

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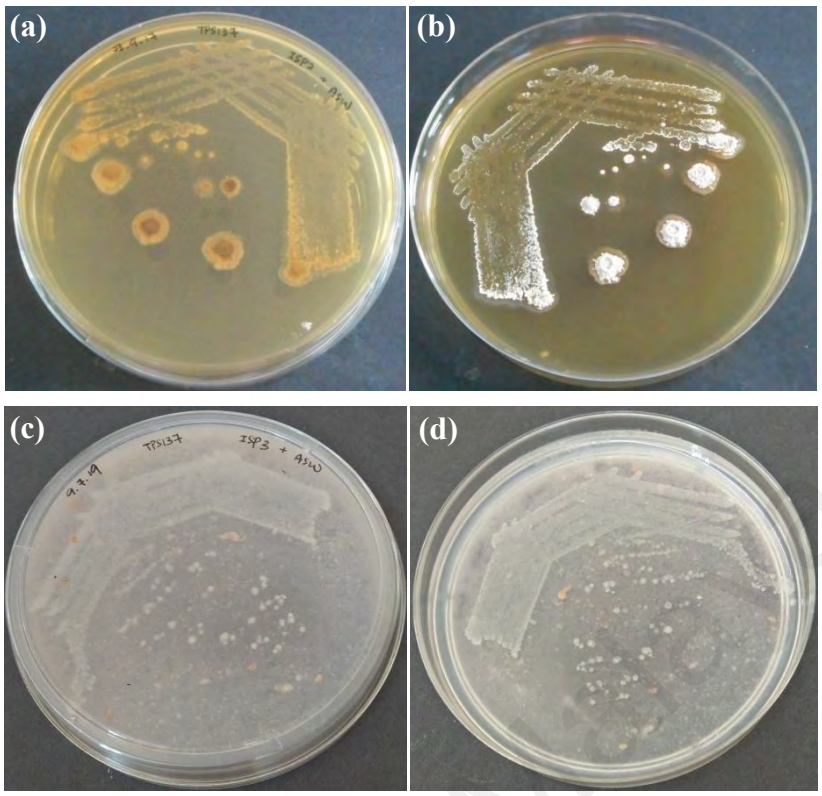

Figure 4.40: Growth of strain TPS137 on ISP 2 (a, b) and ISP 3 (c, d).

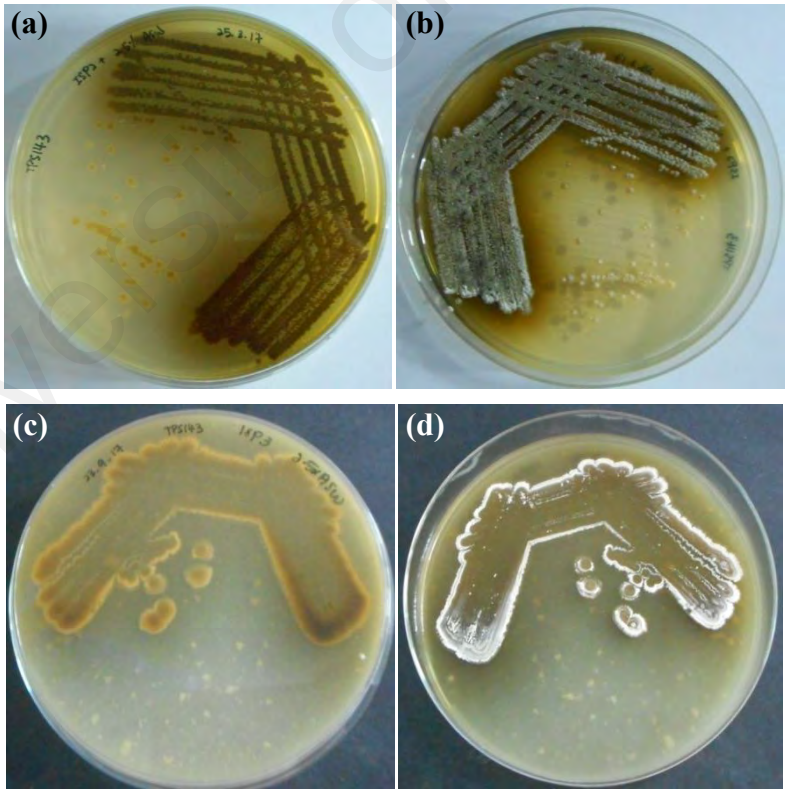

Figure 4.41: Growth of strain TPS143 on ISP 2 (a, b) and ISP 3 (c, d).

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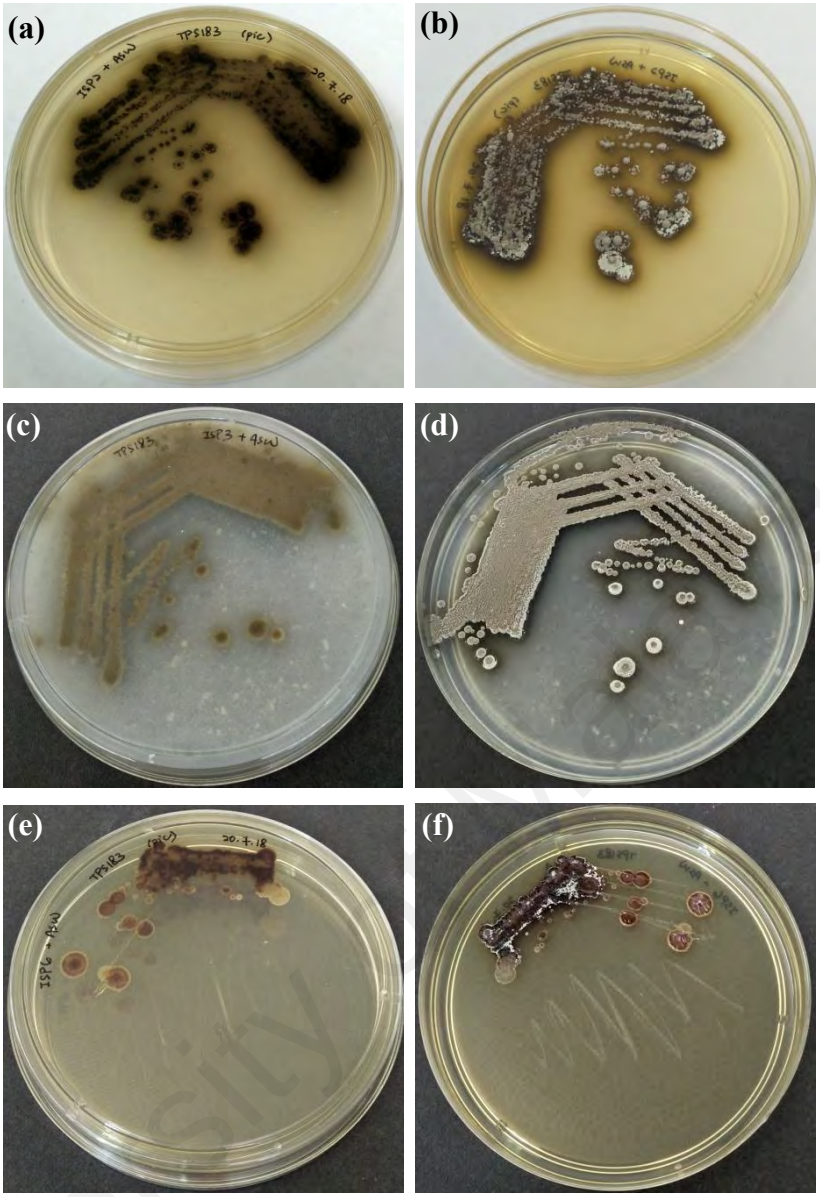

Figure 4.42: Growth of strain TPS183 on ISP 2, ISP 3 (a, b), ISP 3 (c, d) and ISP 6 (e, f).The strain produced reddish brown substrate mycelia on ISP 6 medium as shown in (e), along with production of pale violet aerial mycelia and spores as shown in (f).

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Table 4.20: Growth of the novel strain TPS3, *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T on media with and without supplementation of 2.5% of ASW.

Culture medium	Strain	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
ISP 1	TPS3	Leathery	Absent	Yellowish white	Absent	Absent
	F22^T	Leathery	Absent	Yellowish white	Absent	Absent
	CCNWHQ 0031^T	Leathery	Absent	Light yellow	Absent	Deep yellow
ISP1 + ASW	TPS3	Leathery	Absent	Pale yellow	Absent	Absent
	F22^T	Leathery	Absent	Yellowish white	Absent	Absent
	CCNWHQ 0031^T	Leathery	Absent	Greyish yellow	Absent	Absent
ISP 2	TPS3 (Figure 4.38)	Powdery	Very pale blue	Pale yellow	Very pale blue	Absent
	F22^T	Leathery	Absent	Light yellowish brown	Absent	Absent
	CCNWHQ 0031^T	Powdery	Very pale blue	Light yellow	Very pale blue	Absent
ISP2 + ASW	TPS3 (Figure 4.39)	Powdery	Pale yellow	Pale yellow	White	Light yellow
	F22^T	Powdery	Greyish purplish pink	Strong yellowish brown	Greyish purplish pink	Dark yellowish brown
	CCNWHQ 0031^T	Powdery	White	Strong brown	White	Greyish reddish brown
ISP 3	TPS3	Powdery	Pale purplish blue	Light bluish grey	Pale purplish blue	Absent
	F22^T	Powdery	White	Moderate greenish yellow	White	Dark greenish yellow
	CCNWHQ 0031^T	Powdery	Greyish blue	Light reddish brown	Greyish blue	Greyish reddish purple
ISP3 + ASW	TPS3	Leathery	Very pale blue	Yellowish white	Very pale blue	Absent
	F22^T	Powdery	Greyish greenish yellow	Moderate yellow	White	Light greyish olive
	CCNWHQ 0031^T	Powdery	Pale purplish blue	Deep yellowish brown	Pale purplish blue	Greyish reddish purple

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Table 4.20, continued.

Culture medium	Strain	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
ISP4	TPS3	Powdery	Very pale blue	Pale yellow	Very pale blue	Absent
	F22^T	Powdery	Yellowish white	Light greyish yellowish brown	Yellowish white	Absent
	CCNWHQ 0031^T	Powdery	Pale purplish blue	Yellowish grey	Pale purplish blue	Moderate reddish purple
ISP4 + ASW	TPS3	Leathery	Pale yellow	Pale yellow	White	Absent
	F22^T	Powdery	Greyish pink	Moderate yellow	Greyish pink	Greyish yellowish brown
	CCNWHQ 0031^T	Leathery	Yellowish grey	Yellowish grey	White	Absent
ISP5	TPS3	Powdery	Pale purplish blue	Yellowish white	Pale purplish blue	Absent
	F22^T	Powdery	Yellowish white	Greyish yellow	Yellowish white	Light yellowish brown
	CCNWHQ 0031^T	Powdery	Light yellowish brown	Light yellowish brown	White	Absent
ISP5 + ASW	TPS3	Leathery	Absent	Pale yellow	Absent	Absent
	F22^T	Powdery	Yellowish white	Pale yellow	Yellowish white	Absent
	CCNWHQ 0031^T	Leathery	Absent	Greenish white	Absent	Absent
ISP6	TPS3	Leathery	Absent	Strong yellow	Absent	Absent
	F22^T	Leathery	Absent	Pale yellow	Absent	Absent
	CCNWHQ 0031^T	Leathery	Absent	Strong yellowish brown	Absent	Dark olive brown
ISP6 + ASW	TPS3	Leathery	Absent	Light greenish yellow	Absent	Absent
	F22^T	Leathery	Absent	Pale yellow	Absent	Absent
	CCNWHQ 0031^T	Leathery	Absent	Light reddish brown	Absent	Greyish reddish brown

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Table 4.20, continued.

Culture medium	Strain	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
ISP7	TPS3	Powdery	Very pale blue	Light greyish brown	Very pale blue	Absent
	F22^T	Powdery	Light greyish yellowish brown	Dark yellowish brown	Light greyish yellowish brown	Moderate brown
	CCNWHQ 0031^T	Powdery	Light brown	Brownish black	Pale purplish blue	Brownish black
ISP7 + ASW	TPS3	Leathery	Absent	Pale yellowish green	Absent	Absent
	F22^T	Powdery	Yellowish green	Strong brown	Yellowish green	Light reddish brown
	CCNWHQ 0031^T	Leathery	White	Deep brown	White	Deep yellowish brown
Czapek's solution agar	TPS3	Leathery	Pale yellow	Yellowish white	Absent	Pale orange yellow
	F22^T	Powdery	Pale yellow	Pale yellow	White	Greyish yellow
	CCNWHQ 0031^T	Leathery	Yellowish white	Moderate yellow	Yellowish white	Yellowish grey
Czapek's solution agar + ASW	TPS3	Leathery	Absent	Yellowish white	Absent	Absent
	F22^T	Leathery	Absent	Yellowish white	Absent	Absent
	CCNWHQ 0031^T	Leathery	Absent	Moderate yellow	Absent	Yellowish white
Gauze medium	TPS3	Leathery	Absent	Greenish white	Absent	Absent
	F22^T	Powdery	Greyish yellow	Light yellowish brown	White	Deep yellowish brown
	CCNWHQ 0031^T	Powdery	Greyish yellow	Greyish yellow	White	Moderate blue

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Table 4.20, continued.

Culture medium	Strain	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
Gauze medium + ASW	TPS3	Leathery	Absent	Yellowish white	Absent	Absent
	F22^T	Powdery	Yellowish grey	Light yellow	Yellowish grey	Dark greyish yellow
	CCNWHQ 0031^T	Leathery	Greyish yellow	Greyish yellow	White	Moderate blue
MBA	TPS3	Powdery	Pale yellow	Pale yellow	White	Absent
	F22^T	Powdery	White	Moderate yellow	White	Deep yellowish brown
	CCNWHQ 0031^T	Powdery	Light brown	Deep brown	White	Dark greyish reddish brown
MBA + ASW	TPS3	Leathery	Absent	Pale yellow	Absent	Absent
	F22^T	Powdery	Yellowish white	Strong yellow	Yellowish white	Absent
	CCNWHQ 0031^T	Leathery	Absent	Light yellow	Absent	Absent
NA	TPS3	Leathery	Absent	Yellowish white	Absent	Absent
	F22^T	Powdery	Yellowish white	Pale yellow	Yellowish white	Absent
	CCNWHQ 0031^T	Leathery	Absent	Light yellow	Absent	Absent
NA + ASW	TPS3	Leathery	Absent	Yellowish white	Absent	Absent
	F22^T	Leathery	Yellowish white	Yellowish white	White	Absent
	CCNWHQ 0031^T	Leathery	Absent	Light yellow	Absent	Absent

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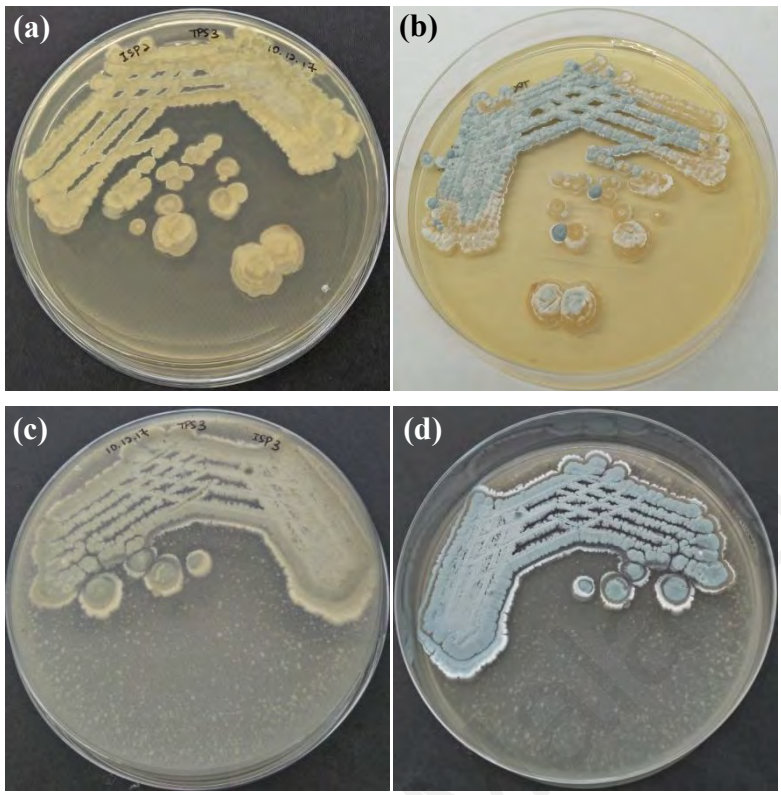

Figure 4.43: Growth of TPS3 on ISP media without supplementation of 2.5% ASW. Colour of substrate and aerial mycelia on ISP 2 (a, b) and ISP 3 (c, d) were shown. Pale blue aerial mycelia/ spores were produced on both media.

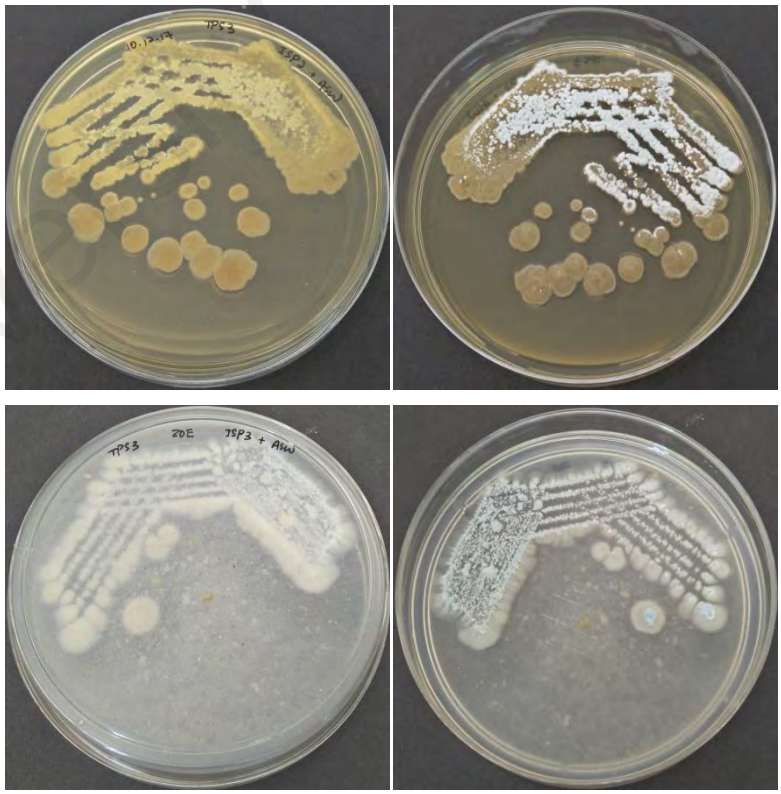

Figure 4.44: Growth of TPS3 on ISP media with 2.5% ASW. Colour of substrate and aerial mycelia on ISP 2 (a, b) and ISP 3 (c, d) were shown. White aerial mycelia/ spores were produced on media ISP 2/ ASW. Aerial mycelia and spores were not produced on ISP 3/ ASW.

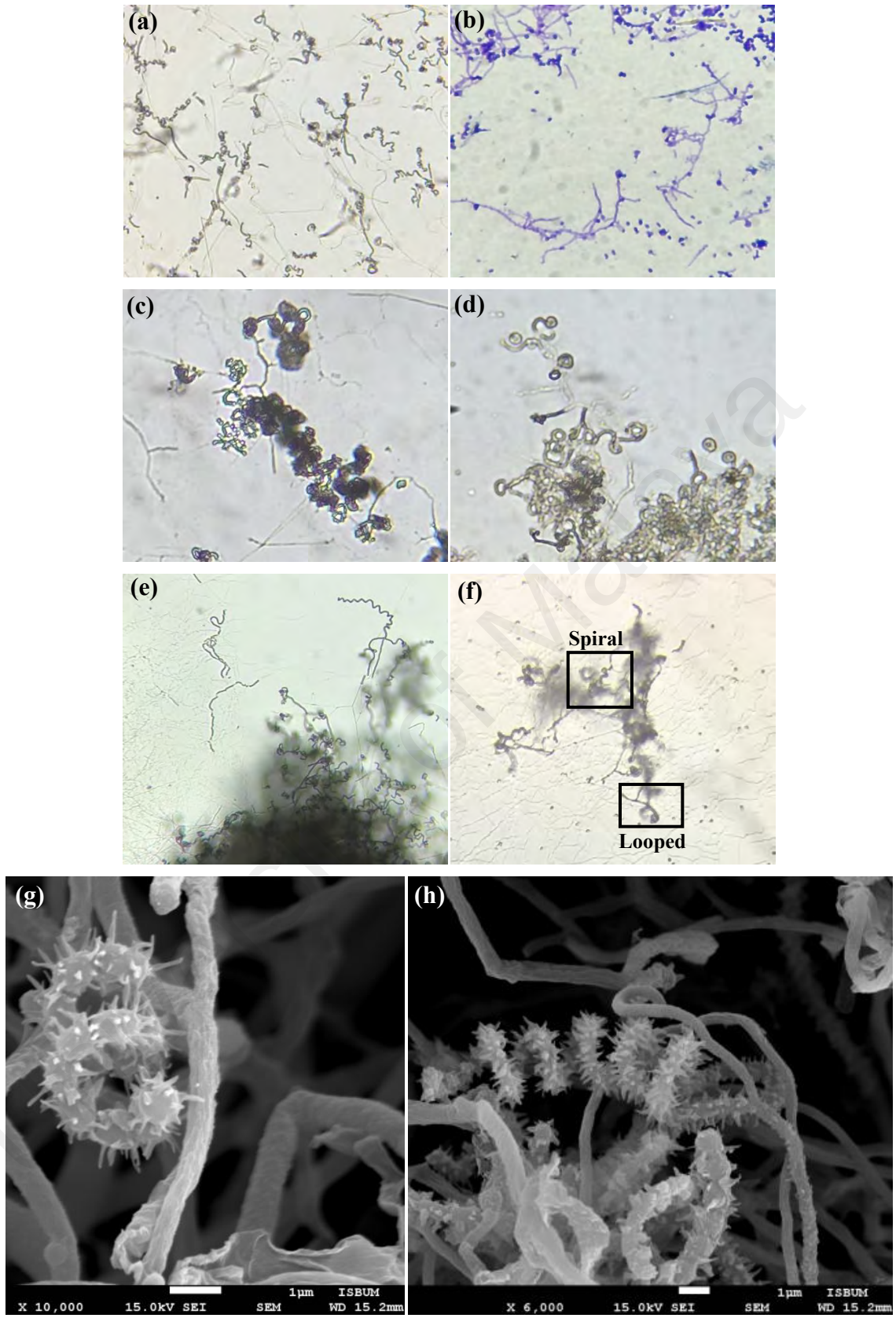

Figure 4.45: Micromorphology of the novel *Streptomyces* spp. ISP 2: (a) TPS4, (b) TPS114, (c) TPS137, (d) TPS143, (e) TPS183 and (f) TPS3. The strain TPS3 formed straight aerial mycelia that differentiate into spiral and looped spore chains, as highlighted in black frames. Images from (a) to (f) were observed and recorded under light microscope at a magnification of 400 ×. Both images (g) and (h) of TPS3 were observed under SEM at a magnification of 10000 × and 6000 ×, respectively, and the bar was set at 1 µm.

4.5.3.3 Carbon utilisation profile

The novel *Streptomyces* strains TPS4, TPS114, TPS137, TPS143 and TPS183 were tested for their ability to utilise carbon sources on ISP9 medium supplemented with 2.5% ASW, whereas TPS3 and its closely related matches *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T were tested on ISP9 without ASW (Table 4.21). Comparing the five strains on ISP9 with ASW, strains TPS4, TPS143 and TPS183 were able to grow on the basal medium without carbon source, however, strains TPS114 and TPS137 did not show any growth. Among the 23 tested carbon sources, four carbon sources including D-sorbitol, L-sorbose, glycine and L-methionine, were utilised by less than three strains out of five. The strain TPS114 was the only strain that showed positive utilisation of glycine and D-sorbitol and TPS183 was able to utilise D-sorbitol, L-sorbose and L-methionine for growth. The strain TPS143 was able to grow on basal medium supplemented with L-sorbose and L-methionine.

The strain TPS3 can utilise 18 carbon sources out of 23, while *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T were able to utilise 15 and 20 types of carbon sources (Table 4.21). All three strains were observed to not be able to grow on D-melezitose and D-sorbitol supplemented media as well as basal medium without carbon source. Strain TPS3 was able to utilise L-lysine, L-methionine and D-arabinose, but *S. ziwulingensis* F22^T was not able to. On the other hand, TPS3 were not able to utilise adonitol, L-sorbose and glycine for growth while *S. shaanxiensis* CCNWHQ 0031^T showed positive growth on media supplemented with these carbon sources. The strain TPS3 was able to grow on L- methionine supplemented medium but no growth was observed for *S. shaanxiensis* CCNWHQ 0031^T on the same medium.

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Table 4.21: Carbon utilisation profile of strains TPS4, TPS114, TPS137, TPS143 and TPS3 and its reference strains *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T. The strains were tested on ISP9 basal medium containing 2.5% ASW, except for the strains TPS3, F22^T and CCNWHQ 0031^T, which were tested on ISP9 basal medium without supplementation of ASW. Carbon source was added into the basal medium as final concentration of 1%. (+, positive result; -, negative result; NG, no growth; ND, no data was recorded).

Carbon sources	**Utilisation of carbon source**							
	TPS4	**TPS114**	**TPS137**	**TPS143**	**TPS183**	**TPS3**	**F22^T**	**CCNWHQ 0031^T**
D-glucose	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+
Adonitol	+ (+)	+ (+)	+	+	+	ng	ng	+ (+)
D-arabinose	+	ng	ng	+ (+)	+	+ (+)	ng	+ (+)
L-arabinose	+	+ (+)	+ (+)	+ (+)	+ (+)	+	+ (+)	+
D-fructose	+	+ (+)	+	+	+ (+)	+	+ (+)	+
D-galactose	+	+	+	+	+	+	+	+
Inositol	+ (+)	+	+	+	+	+	+	+
D-lactose	+	+ (+)	+	+	+	+ (+)	+ (+)	+
D-maltose	+	+ (+)	+ (+)	+	+ (+)	+ (+)	+ (+)	+
D-mannitol	+	+ (+)	+ (+)	+	+ (+)	+	+	+
D-mannose	+	+	+ (+)	+ (+)	+ (+)	+ (+)	+	+
D-melezitose	+	+ (+)	ng	+	+	ng	ng	ng
D-melibiose	+	+ (+)	+ (+)	+ (+)	+	+	+ (+)	+
D-ribose	+	+ (+)	ng	+ (+)	+	+	+ (+)	+
D-sorbitol	ng	+	ng	ng	+	ng	ng	ng
L-sorbose	ng	ng	ng	+	+	ng	ng	+
Sucrose	+	+ (+)	ng	+	+	+ (+)	+	+
D-trehalose	+ (+)	+ (+)	+	+	+ (+)	+ (+)	+ (+)	+
Xylose	+ (+)	+	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	+
Glycine	ng	+	ng	ng	ng	ng	ng	+
L-glutamine	+	ng	+	+	+	+	+	+
L-lysine	ng	+	ng	+	+	+	ng	+
L-methionine	ng	ng	ng	+	+	+	ng	ng
Basal medium only	+	ng	ng	+	+	ng	ng	ng

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4.5.3.4 Tolerance of pH, temperature and NaCl

Abilities of the novel strains to grow at various incubation temperatures and ISP 2 medium adjusted to different pH and supplemented with various NaCl concentrations was shown in Table 4.22. The strain TPS4 was able to grow at pH 6 to pH 12, 15ºC, 25ºC, 28ºC, 32ºC and 37ºC and at 0% to 4% NaCl. However, spores were not produced by TPS4 when incubated at 37ºC and cultivated on ISP 2 plates with 0% and 4% NaCl. The strain TPS114 was able to grow at pH 6 to pH 9 and 15ºC, 28 ºC, 32 ºC and 37ºC and at 0 % to 7 % NaCl. Strains TPS137 and TPS143 were able to grow at pH 6 to pH12 and both strains showed growth at 15ºC, 25ºC, 28ºC, 32ºC and 37ºC. Strain TPS137 was able to tolerate NaCl from 0% to 7%, whereas strain TPS143 was able to grow at NaCl range from 0% to 5%. The strain TPS183, on the other hand, was able to grow at incubation temperature that at 4ºC, 15ºC, 25 ºC, 28ºC, 32ºC, 37ºC and 45ºC, however no growth occurred at 50ºC. Growth of TPS183 also occurred as pH 6 to pH 12 and at NaCl concentration ranged from 0- 7%. Strains TPS3 and *S. ziwulingensis* F22^T were able to grow at pH 6 to pH 11 while strain *S. shaanxiensis* CCNWHQ 0031^T was able to tolerate pH 6 to pH 9. All three strains showed positive growth at incubation temperature at 15ºC, 25ºC, 28ºC, 32ºC and 37ºC. Both TPS3 and *S. ziwulingensis* F22^T were able to tolerate NaCl concentration from 0% to 5% and strain CCNWHQ 0031^T was able to tolerate 0% to 6% NaCl.

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Table 4.22: Growth of novel strains, *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T at various incubation temperatures, pH and NaCl concentrations. (+, positive growth; -, no growth; ND, no data).

Parameters	**TPS4**	**TPS114**	**TPS137**	**TPS143**	**TPS183**	**TPS3**	**F22^T**	**CCNWHQ 0031^T**
pH:								
6	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+
10	+	-	+	+	+	+	+	-
11	+	-	+	+	+	+	+	-
12	+	-	+	+	+	-	-	-
Temperature:								
4 ºC	-	-	-	-	+	-	-	-
15 ºC	+	+	+	+	+	+	+	+
25 ºC	+	+	+	+	+	+	+	+
28 ºC	+	+	+	+	+	+	+	+
32 ºC	+	+	+	+	+	+	+	+
37 ºC	+	+	+	+	+	+	+	+
45 ºC	-	-	-	-	+	-	-	-
50 ºC	nd	nd	nd	nd	-	nd	nd	nd
NaCl:								
0%	+	+	+	+	+	+	+	+
1%	nd	+	+	nd	nd	+	+	+
2%	+	+	+	+	+	+	+	+
3%	+	+	+	nd	nd	+	+	+
4%	+	+	+	+	+	+	+	+
5%	+	+	+	+	nd	+	+	+
6%	-	+	+	-	+	-	-	+
7%	nd	+	+	nd	+	-	-	-
8%	nd	-	-	nd	-	nd	nd	nd

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4.5.3.5 Hydrolytic enzymes production, catalase and oxidase tests and decomposition of adenine

The result of hydrolysis tests for all six novel strains and the reference strains *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T were recorded in Table 4.23. Hydrolysis of starch was recorded positive for all novel strains belong to the genus *Streptomyces*, as indicated by formation of clear zone around the colonies on starch-ISP2 plate while presence of starch in the agar was confirmed by changes of agar colour to blue upon flooding with iodine solution. Formation of clear zone around colonies of all novel *Streptomyces* strains was also observed on gelatine- ISP2 and skim milk agar plates indicated positive result for hydrolysis of gelatine and casein, respectively. Changes of yellow colour of the urea agar slants to pink colour were observed for strains TPS114, TPS137, TPS3, *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T indicated positive hydrolysis of urea, whereas the colour of urea agar slants remained unchanged as yellow colour for TPS4, TPS143 and TPS183. Formation of bubbles upon addition of 3% H_2O_2 droplet to the cells were observed for all the strains, indicated positive catalase test. Oxidation test was observed to be negative for all strains, as indicated by absent of formation of purple colour of the filter paper impregnated with oxidase reagent. Disappearance of adenine crystals around the actinobacterial colonies indicated that the novel strain TPS3 and its reference strains, *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T were able to decompose adenine. Decomposition of adenine was recorded negative for strains TPS4, TPS114, TPS137, TPS143 and TPS183.

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Table 4.23: Testing of the novel actinobacterial strains, *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T to hydrolyse various substrates and ability to decompose adenine. (+, positive test result; -, negative test result).

Substrate/ Test	TPS4	TPS114	TPS137	TPS143	TPS183	TPS3	F22^T	CCNWHQ 0031^T
Starch	+	+	+	+	+	+	+	+
Skim milk	+	+	+	+	+	+	+	+
Gelatine	+	+	+	+	+	+	+	+
Urea	-	+	+	-	-	+	+	+
Catalase	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-
Decomposition of adenine	-	-	-	-	-	+	+	+

4.5.3.6 Cell wall composition of the novel strain TPS3

The diaminopimelic acid isomer present in whole cells hydrolysates of strain TPS3 was *LL*-diaminopimelic acid. The major types of fatty acids in the cell wall were iso-$C_{16:0}$ (33.19%), anteiso-$C_{15:0}$ (8.90%), iso-$C_{14:0}$ (6.40%) and iso-$C_{16:1}$ H (5.35%). The full fatty acid profile was summarised in Appendix F. Types of menaquinones present in the cell wall were MK-9(H_8) (61%), MK-9(H_6) (24%), MK-9(H_4) (2%) and 1% of each of the following: MK-9(H_2), MK-10, MK-10(H_2) and MK-10(H_6) (Appendix G). Strain TPS3 contained phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, glycolipids, phosphoglycolipids as well as unknown lipids, phospholipid and aminolipid (Appendix G).

4.5.3.7 Antibacterial activities of *Streptomyces* strains

A total of 60 actinobacterial strains identified to be *Streptomyces* spp. were isolated from sediment samples: 59 strains, inclusive of novel strains TPS3, TPS4, TPS114, TPS137, TPS143 and TPS183, were isolated from sediment Z and one strain from sediment sample M (Table 4.10, under the sub-chapter 4.4). A total of 15 strains including the three of the novel strains TPS114, TPS137 and TPS143 showed inhibitory

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activity against at least one of the tested pathogens. Antibacterial activity profiles of the strains were summarized in Table 4.24.

4.5.3.8 Genome analysis of the novel strain TPS3

Genome of the novel *Streptomyces* strain TPS3 was compared with the closely related matches *S. kanasensis* ZX01^T, *S. caeruleatus* NRRL B-24802^T and *S. griseoruber* NRRL B-1818^T, as genome data of both reference strains *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T are not available (Table 4.25). The strain TPS3 shared 16S rRNA gene similarities of 96.47%, 95.83% and 95.55% with *S. kanasensis* ZX01^T ZX01^T, *S. caeruleatus* NRRL B-24802^T and *S. griseoruber* NRRL B-1818^T, respectively. A total of 956 contigs were generated from the genome of strain TPS3, whereas the genome of its related matches were reported to have total contig number of 225, 116 and 963 for *S. kanasensis* ZX01^T, *S. caeruleatus* NRRL B-24802^T and *S. griseoruber* NRRL B-1818^T, respectively.

The novel strain TPS3 had a GC content of 70.3 mol% while the closest related matches *S. kanasensis* ZX01^T, *S. caeruleatus* NRRL B-24802^T and *S. griseoruber* NRRL B-1818^T were reported to have 73.9 mol%, 70.8 mol% and 71.5 mol% of GC content in their genomes, respectively. The estimated genome size for strain TPS3 was 10.04 Mbp, whereas the genome seizes of its closely related matches were 7.02 Mbp for *S. kanasensis* ZX01^T, 10.27 Mbp for *S. caeruleatus* NRRL B-24802^T and 9.87 Mbp for *S. griseoruber* NRRL B-1818^T. A total of 8652 coding sequences and 200 RNAs were identified from the genome assembly of the strain TPS3. The closest related matches *S. kanasensis* ZX01^T was identified to have 6195 coding sequences and 73 RNAs, *S. caeruleatus* NRRL B-24802^T was found to possess 9104 coding sequences and 72 RNAs while *S. griseoruber* NRRL B-1818^T had 8742 coding sequences and 65 RNAs.

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Table 4.24: Antibacterial activity profile of the *Streptomyces* strains on five production media using agar plug diffusion assay. Diameters of inhibition zones were recorded in mm. (Bs, *B. subtilis* ATCC 23857; Sa, *S. aureus* ATCC 29213; Ec, *E. coli* ATCC 47076; Pa, *P. aeruginosa* ATCC 27853).

Strain	Tester pathogen	PM3	Soybean meal glucose	Micromonospora medium	ATCC medium 241	Starch yeast peptone
TPS114	Bs	11	13	15	15	15
	Sa	11	10	17	15	15
TPS137	Bs	0	0	9	11	0
	Sa	0	0	13	9	0
TPS143	Bs	13	9	14	0	7
	Sa	0	0	7	0	7
TPS1	Bs	0	8	10	13	10
	Sa	0	7	7.5	11	8
TPS6	Bs	0	0	14.5	0	0
	Sa	0	7	12	0	12
	Pa	0	7	8	0	0
TPS10	Bs	0	10	0	0	0
	Sa	9	10	0	19	0
	Ec	0	0	0	15.5	14
TPS12	Sa	0	0	0	19	15
	Ec	0	0	0	17	18.5
TPS17	Sa	0	0	0	23	24
	Ec	0	0	0	27	27
TPS14	Bs	16	0	0	0	0
	Sa	10	0	0	0	0
TPS38	Sa	0	0	0	10	0
TPS51	Bs	0	0	0	13	10
	Sa	0	7	0	11	8
TPS94	Bs	0	14.5	11	12	11
	Sa	0	16	12	12.5	11.5
TPS181	Sa	0	10	0	11	7
TPS216	Bs	0	9	0	11	0
	Sa	0	0	0	11	0
TPS359	Bs	9	0	8	7	10
	Sa	9	0	10	7	7
TPS445	Bs	0	12.5	11.5	8	9
	Sa	0	9.5	11	7.5	0

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Table 4.25: Genome details of the novel *Streptomyces* strain TPS3 and closely related matches: *S. kanasensis* ZX01^T, *S. caeruleatus* NRRL B-24802^T and *S. griseoruber* NRRL B-1818^T. Genomic metrics and differences of GC content are also compared between the genome sequences.

Strain	TPS3	ZX01^T	NRRL B-24802^T	NRRL B-1818^T
Genome overview:				
G+C content (mol %)	70.30	73.90	70.80	71.50
Estimated genome size (Mbp)	10.04	7.02	10.27	9.87
Number of coding sequences	8652	6195	9104	8742
Number of RNAs	200	73	72	65
dDDH (%):				
TPS3	100.0			
ZX01^T	23.50 ± 2.40	100.0		
NRRL B-24802^T	25.90 ± 2.40	23.20 ± 2.40	100.0	
NRRL B-1818^T	25.50 ± 2.40	23.60 ± 2.40	26.90 ± 2.45	100.0
Differences of GC content (mol %):				
TPS3	0.00			
ZX01^T	3.62	0.00		
NRRL B-24802^T	0.52	3.10	0.00	
NRRL B-1818^T	1.21	2.41	0.69	0.00
ANI values (%):				
TPS3	100.00			
ZX01^T	77.42	100.00		
NRRL B-24802^T	80.98	77.51	100.00	
NRRL B-1818^T	80.31	77.98	81.89	100.00
AAI values (%):				
TPS3	100.00			
ZX01^T	68.89	100.00		
NRRL B-24802^T	75.55	69.09	100.00	
NRRL B-1818^T	73.99	68.60	76.39	100.00

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4.5.4 Characterisation of strains TPS166, TPS357, TPS418, TPS448 and TPS449

4.5.4.1 Phylogenetic analysis of 16S rRNA gene sequences

Five actinobacterial strains were identified as novel *Blastococcus* species, including TPS166, TPS357, TPS418, TPS448 and TPS459. The strains were isolated on modified M2 medium from skim milk/ HEPES and UV treated sediment samples. Pair- wise comparison of 16S rRNA gene sequences showed that they shared the same closely related matches with different similarity values (Table 4.11). Multiple sequence alignment of 16S rRNA gene sequences of the novel strains using Clustal 2.1 program revealed that five strains shared similarity between 96.94% and 99.63% to each other (Table 4.26). Phylogenetic analysis confirmed the taxonomic position of five novel strains using neighbour joining, maximum likelihood and maximum parsimony methods. Five strains formed a separate cluster from the closely related *Blastococcus* species. The strain TPS166 formed a separate sub- cluster from the other four strains (Figure 4.46).

Table 4.26: Percent identity matrix created by Clustal 2.1 programme revealed similarity of the 16S rRNA gene sequences of five novel *Blastococcus* strains to each other. Values are given as percentage.

Strain	TPS166	TPS357	TPS418	TPS448	TPS459
TPS166	100.0				
TPS357	97.94	100.0			
TPS418	96.74	98.01	100.0		
TPS448	98.60	99.63	99.27	100.0	
TPS459	97.37	98.39	98.61	99.26	100.0

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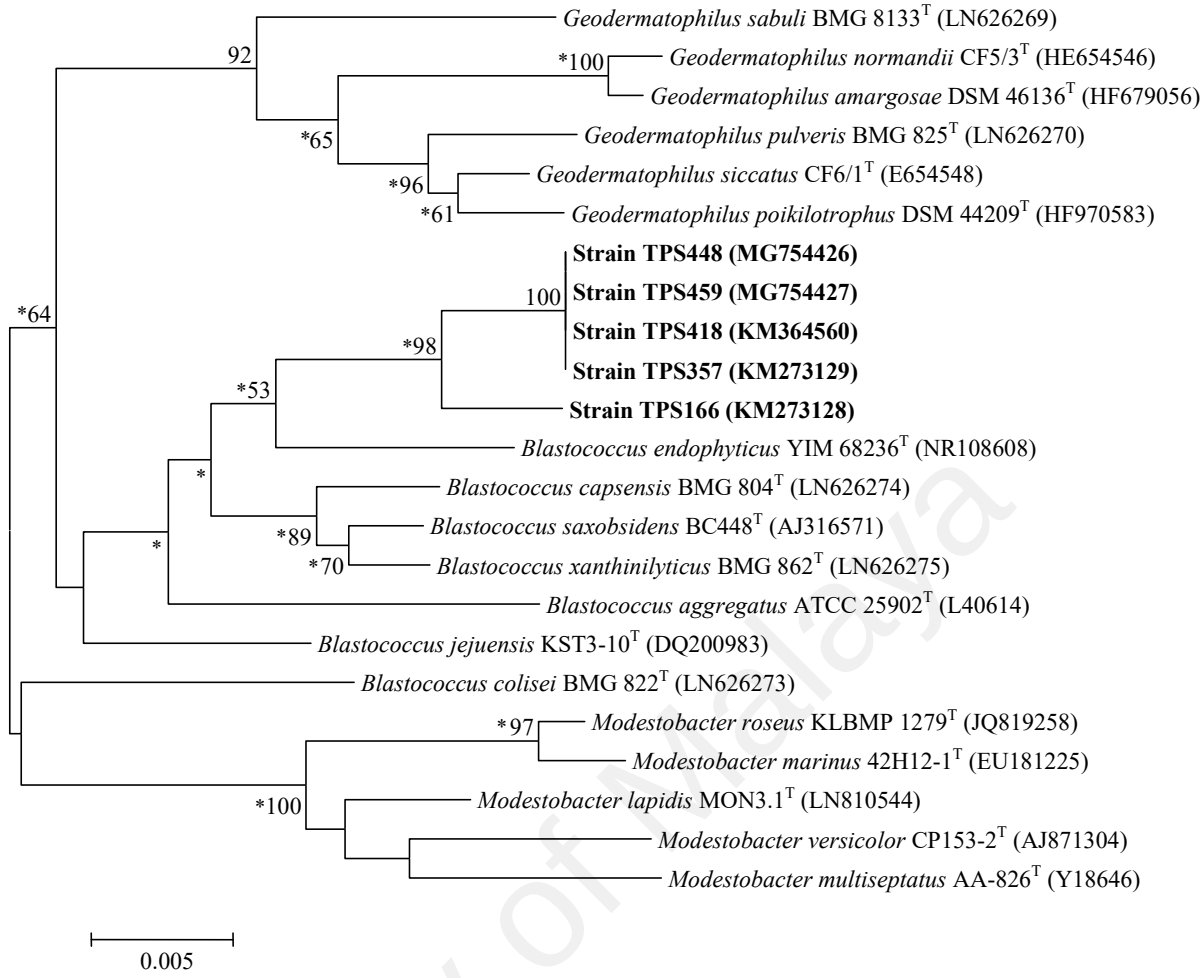

Figure 4.46: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the novel strains TPS166, TPS357, TPS418, TPS448 and TPS459 together with their closely related *Blastococcus* species. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with maximum likelihood and maximum parsimony approaches. Bar represents 0.5% sequence divergence.

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4.5.4.2 Morphological characterisation

Cells of the TPS166, TPS357, TPS418, TPS448 and TPS459 were Gram-stained positive. Microscopic examination of the four days old cultures under light microscope at a magnification of 1000 × showed that the strains produced variety of cell forms. They were cocci to elongated rods and form aggregates (Figure 4.47).

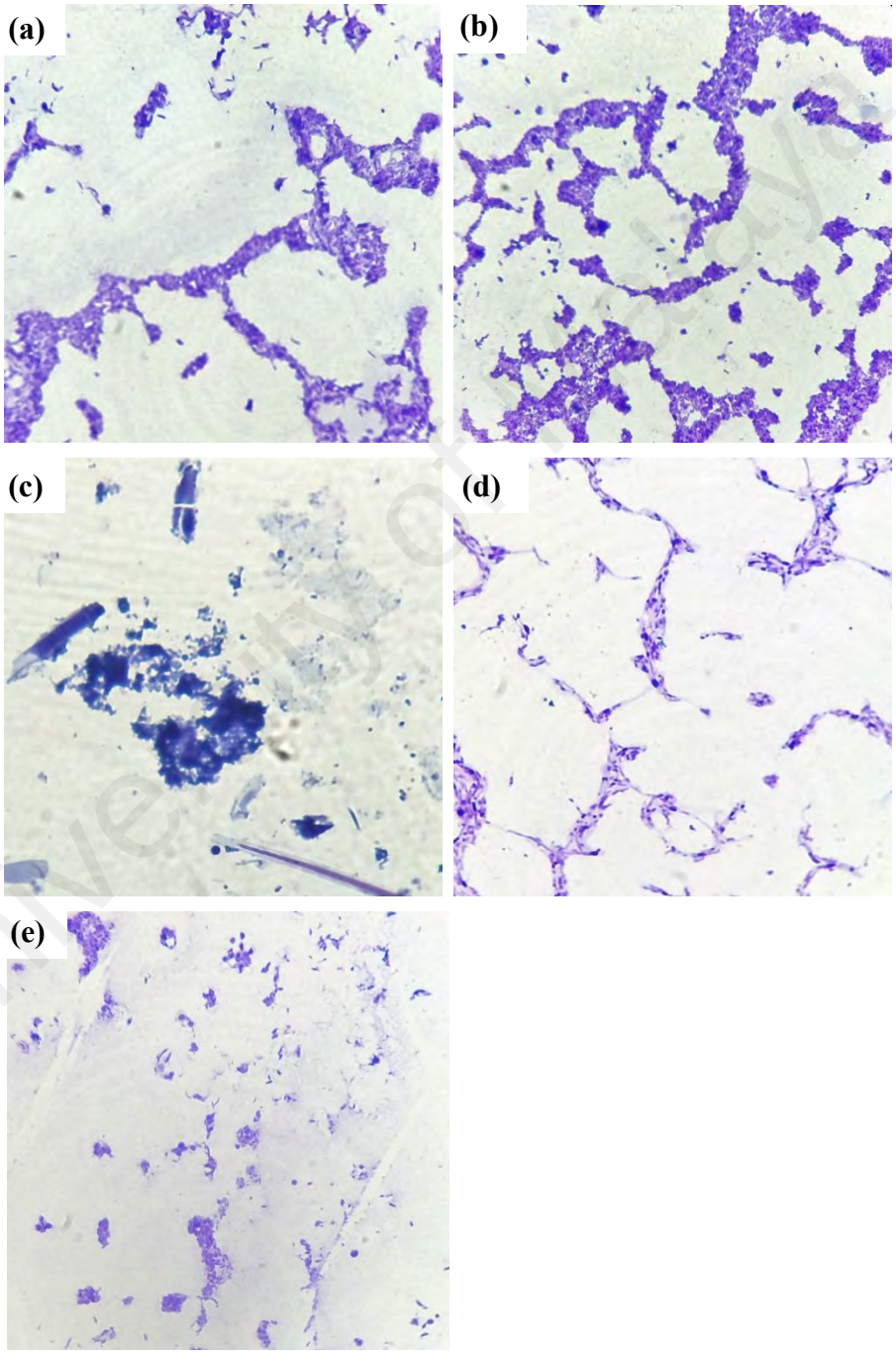

Figure 4.47: Gram- stain of cells for strains (a) TPS166, (b) TPS357, (c) TPS418, (d) TPS448 and (e) TPS459. Microscopic examination was performed under light compound microscope at 1000 × magnification.

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The novel strains showed positive growth on LM, DSMZ medium 65, DSMZ medium 535, YG- Glyc, YCS- Glc and all ISP media (Table 4.27). Strains TPS166 and TPS357 showed positive growth on malt extract agar, but strains TPS418, TPS448 and TPS459 did not grow on malt extract agar. Strains TPS357, TPS418, TPS448 and TPS459 produced orange or pink colonies on all tested media. Diffusible pigment production was not observed for strains TPS448 and TPS459 on all media. The strains TPS357 and TPS418 produced light orange yellow diffusible pigment on DSMZ medium 535. The strain TPS166 produced reddish brown colonies on ISP 2, LM, YCS-Glc and DSMZ medium 65, and orange colonies on the other media. Yellow diffusible pigment was produced by TPS166 on ISP 2, LM and DSMZ medium 535. Colonies of strains TPS357, TPS448 and TPS459 were circular and smooth on all media with an entire margin and moist surface. Strains TPS166 and TPS418 were observed to have wrinkled surface on ISP 2 and LM, respectively. Colonies of both TPS166 and TPS418 were circular and smooth on other culture media. Growth of the novel strains on LM, ISP 2 and DSMZ medium 535 was shown in figures 4.48, 4.49, 4.50, 4.51 and 4.52.

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Table 4.27: Colonies colour of novel strains TPS166, TPS357, TPS418, TPS448 and TPS459 on media supplemented with 2.5% ASW. (ng, No growth; nd, No data)

Culture medium	Strain	Colony colour	Diffusible pigment
LM	TPS166	Dark reddish orange	Strong orange yellow
	TPS357	Deep yellowish pink	Absent
	TPS418	Deep yellowish pink	Absent
	TPS448	Deep yellowish pink	Absent
	TPS459	Deep yellowish pink	Absent
Malt extract agar	TPS166	Strong orange	Absent
	TPS357	Light orange	Absent
	TPS418	ng	nd
	TPS448	ng	nd
	TPS459	ng	nd
YCS- Glc	TPS166	Vivid reddish orange	Absent
	TPS357	Brilliant orange	Absent
	TPS418	Brilliant orange	Absent
	TPS448	Brilliant orange	Absent
	TPS459	Brilliant orange	Absent
YG- Glyc	TPS166	Vivid orange	Absent
	TPS357	Brilliant orange	Absent
	TPS418	Brilliant orange	Absent
	TPS448	Brilliant orange	Absent
	TPS459	Brilliant orange	Absent
DSMZ medium 65	TPS166	Strong reddish brown	Absent
	TPS357	Deep yellowish pink	Absent
	TPS418	Deep yellowish pink	Absent
	TPS448	Deep yellowish pink	Absent
	TPS459	Deep yellowish pink	Absent
DSMZ medium 535	TPS166	Vivid pink	Light yellow
	TPS357	Vivid yellowish pink	Light orange yellow
	TPS418	Vivid yellowish pink	Light orange yellow
	TPS448	Vivid yellowish pink	Absent
	TPS459	Vivid yellowish pink	Absent
ISP 1	TPS166	Brilliant orange	Absent
	TPS357	Moderate orange	Absent
	TPS418	Brilliant orange	Absent
	TPS448	Brilliant orange	Absent
	TPS459	Brilliant orange	Absent

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Table 4.27, continued.

Culture medium	Strain	Colony colour	Diffusible pigment
ISP 2	TPS166	Deep reddish orange	Strong yellow
	TPS357	Vivid reddish orange	Absent
	TPS418	Vivid reddish orange	Absent
	TPS448	Vivid reddish orange	Absent
	TPS459	Vivid reddish orange	Absent
ISP 3	TPS166	Deep yellowish pink	Absent
	TPS357	Light orange	Absent
	TPS418	Light orange	Absent
	TPS448	Brilliant orange	Absent
	TPS459	Brilliant orange	Absent
ISP 4	TPS166	Moderate orange yellow	Absent
	TPS357	Light orange yellow	Absent
	TPS418	Light orange yellow	Absent
	TPS448	Light orange yellow	Absent
	TPS459	Light orange	Absent
ISP 5	TPS166	Pale pink	Absent
	TPS357	Pale pink	Absent
	TPS418	Pale pink	Absent
	TPS448	Pale pink	Absent
	TPS459	Pale pink	Absent
ISP 6	TPS166	Strong orange	Absent
	TPS357	Brilliant orange	Absent
	TPS418	Brilliant orange	Absent
	TPS448	Brilliant orange	Absent
	TPS459	Brilliant orange	Absent
ISP 7	TPS166	Light orange	Absent
	TPS357	Light orange	Absent
	TPS418	Light orange	Absent
	TPS448	Light orange yellow	Absent
	TPS459	Light orange yellow	Absent

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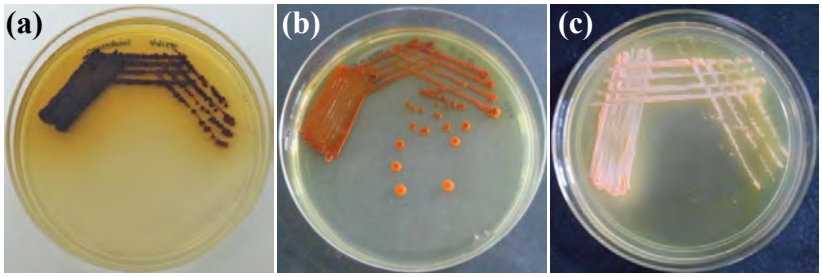

Figure 4.48: Growth of TPS166 on (a) LM medium, (b) ISP 2, (c) DSMZ medium 535.

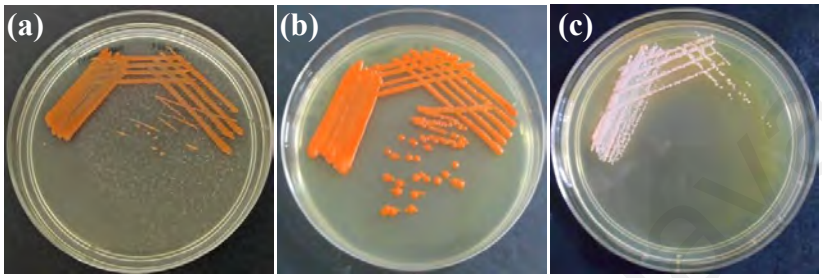

Figure 4.49: Growth of TPS357 on (a) LM medium, (b) ISP 2, (c) DSMZ medium 535.

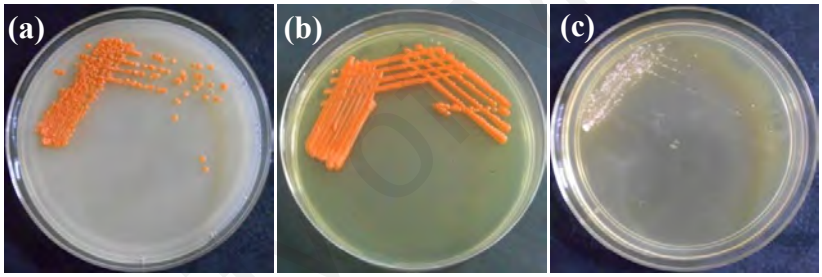

Figure 4.50: Growth of TPS418 on (a) LM medium, (b) ISP 2, (c) DSMZ medium 535.

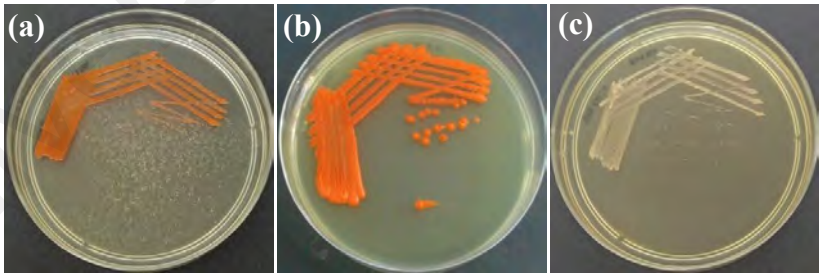

Figure 4.51: Growth of TPS448 on (a) LM medium, (b) ISP 2, (c) DSMZ medium 535.

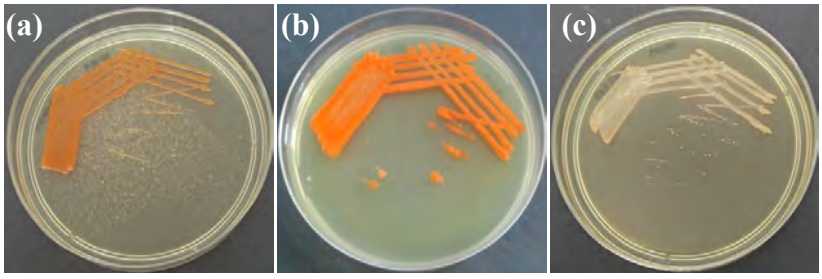

Figure 4.52: Growth of TPS459 on (a) LM medium, (b) ISP 2, (c) DSMZ medium 535.

4.5.4.3 Carbon utilisation profile

Carbon utilisation profiles of the novel *Blastococcus* strains TPS166, TPS357, TPS418, TPS448 and TPS459 were obtained on ISP 9 as basal medium (Table 4.28). All five strains showed positive utilisation of 23 carbon sources on ISP 9. All five strains showed positive utilisation of D- glucose, D- fructose, D- mannose and xylose and produced acid from these carbon sources, as indicated by colour changes of medium from purple to yellow. By contrast, D- galactose, inositol and D- arabinose were utilised by all five strains but none of the strains produced acid from these carbon sources. Acid production from D- lactose, D- mannitol, L- sorbose, glycine and L- glutamine were only observed for strain TPS166.

Abilities of strains TPS166, TPS357 and TPS418 to utilise carbon sources were also tested using API® 20 NE kit (Table 4.31). API test result indicated that strains TPS166, TPS357 and TPS418 were able to assimilate D- glucose, L- arabinose, D- mannose, D- mannitol, N- acetyl- glucosamine, D- maltose, potassium gluconate, adipic acid and phenylacetic acid. Strain TPS418 was able to assimilate capric acid and trisodium citrate whereas the other two strains were not able to. Strains TPS357 and TPS418 showed positive assimilation of malic acid, whereas TPS166 showed negative result of assimilation of malic acid.

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Table 4.28: Carbon utilisation profiling of novel strains TPS166, TPS357, TPS418, TPS448 and TPS459. (+, positive result; -, negative result).

Carbon sources	Utilisation of carbon source				
	TPS166	**TPS357**	**TPS418**	**TPS448**	**TPS459**
D-glucose	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
Adonitol	+ (+)	+	+	+	+
D-arabinose	+	+	+	+	+
L-arabinose	+ (+)	+ (+)	+ (+)	+ (+)	+
D-fructose	+	+	+	+	+
D-galactose	+	+	+	+	+
Inositol	+	+	+	+	+
D-lactose	+ (+)	+	+	+	+
D-maltose	+ (+)	+	+ (+)	+ (+)	+ (+)
D-mannitol	+ (+)	+	+	+	+
D-mannose	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
D-melezitose	+ (+)	+	+	+	+ (+)
D-melibiose	+	+	+	+	+
D-ribose	+ (+)	+ (+)	+ (+)	+ (+)	+
D-sorbitol	+ (+)	+	+	+	+
L-sorbose	+ (+)	+	+	+	+
Sucrose	+ (+)	+	+	+	+ (+)
D-trehalose	+ (+)	+	+	+	+ (+)
Xylose	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)
Glycine	+ (+)	+	+	+	+
L-glutamine	+ (+)	+	+	+	+
L-lysine	+	+	+	+	+
L-methionine	+	+	+	+	+
Basal medium only	+	+	+	+	+

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4.5.4.4 Tolerance of pH, temperature and NaCl

Growth of novel strains at different incubation temperature and ISP 2 medium adjusted to different pH and supplemented with various NaCl concentrations was shown in Table 4.29. Strains TPS166, TPS357, TPS418, TPS448 and TPS459 were able to grow at pH 6- 12, with optimum at pH 7 to pH 9. All five strains were able to grow at 15ºC, 25ºC, 28ºC, 32ºC and 37ºC. No growth was observed at 4ºC for all strains. Weak growth was observed for strain TPS166 at 15ºC, whereas TPS357 and TPS418 showed good growth at 15ºC. Good growth was observed for all strains at 25ºC, 28ºC, 32ºC and 37ºC. Strains TPS166, TPS357 and TPS418 were able to grow at 45ºC, while TPS448 and TPS459 were not able to grow at 45ºC. All five strains were able to show good growth at ISP 2 without supplementation of NaCl. They were also able to develop growth in the presence of 1% to 6% NaCl. Strains TPS166, TPS357 and TPS418 showed good growth at 7% NaCl and weak growth at 8% NaCl.

4.5.4.5 Hydrolytic enzyme production, catalase and oxidase tests and decomposition of adenine

The result of hydrolysis tests for the novel strains was recorded in Table 4.30. Hydrolysis of starch was recorded negative as indicated by absent of clear zone around the colonies on starch- ISP2 plate upon flooding with iodine solution. No clear zone was observed around the colonies on skim milk plates for all novel strains, indicated negative result for hydrolysis of casein. Hydrolysis of gelatine on gelatine- ISP 2 plates was recorded positive for strains TPS357, TPS418 and TPS448, as indicated by the presence of clear hydrolysing zone around the colonies. Weak positive hydrolysis of gelatine was observed for TPS166. Hydrolysis of gelatine was negative for strain TPS459, as clear zone was not observed around the colonies. All strains did not change the colour of urea agar from yellow to pink, indicated negative result for hydrolysis of urea. Formation of bubbles upon addition of 3% H_2O_2 droplet to the cells were observed

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for all the strains, indicated positive catalase test. Oxidation test was observed to be negative for all strains, as indicated by absent of formation of purple colour of the filter paper impregnated with oxidase reagent. Decomposition of adenine was also recorded negative for all five strains, as adenine crystals around the colonies of all strains were not disappeared.

Hydrolysis test was also carried out using API® 20 NE kit on strains TPS166, TPS357 and TPS418 (Table 4.31). The strain TPS166 showed positive results for reduction of nitrates to nitrites, but negative result for reduction of nitrates to nitrogen, while both TPS357 and TPS418 showed negative for reduction of nitrates to nitrites and reduction of nitrates to nitrogen. All strains reported to show negative results for indole production, fermentation of glucose and production of oxidase. All three strains showed positive results for hydrolysis of arginine, urea, esculin and 4- nitrophenyl- βD-galactopyranoside. Strain TPS357 showed positive hydrolysis of gelatine whereas TPS166 and TPS418 showed negative result for hydrolysis of gelatine.

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Table 4.29: Growth of the novel strains including TPS166, TPS357, TPS418, TPS448 and TPS459 on ISP 2 medium at different incubation temperatures, pH and NaCl concentrations. (+, positive growth; -, no growth; w, weak growth, nd: not determined)

Parameters	TPS166	TPS357	TPS418	TPS448	TPS459
pH:					
6	+	+	+	+	+
7	+	+	+	+	+
8	+	+	+	+	+
9	+	+	+	+	+
10	+	+	+	+	+
11	+	+	+	+	+
12	+	+	+	+	+
Temperature:					
4 ºC	-	-	-	-	-
15 ºC	+	+	+	+	+
25 ºC	+	+	+	+	+
28 ºC	+	+	+	+	+
32 ºC	+	+	+	+	+
37 ºC	+	+	+	+	+
45 ºC	+	+	+	-	-
50 ºC	-	-	-	-	-
NaCl:					
0%	+	+	+	+	+
1%	+	+	+	+	+
2%	+	+	+	+	+
3%	+	+	+	+	+
4%	+	+	+	+	+
5%	+	+	+	+	+
6%	+	+	+	+	+
7%	+	+	+	+	+
8%	w	w	w	-	-
9%	-	-	-	nd	nd

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Table 4.30: Testing of strains TPS166, TPS357, TPS418, TPS448 and TPS459 to hydrolyse various substrates and ability to decompose adenine. (+, positive test result; -, negative test result).

Substrate/ Test	TPS166	TPS357	TPS418	TPS448	TPS459
Starch	-	-	-	-	-
Skim milk	-	-	-	-	-
Gelatine	w	+	+	+	-
Urea	-	-	-	-	-
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Decomposition of adenine	-	-	-	-	-

4.5.4.6 Antibacterial activity

Strains TPS166, TPS357, TPS418, TPS448 and TPS459 that cultured on PM3, soybean meal glucose, micromonospora medium, ATCC medium 241 and starch yeast peptone medium, did not display inhibitory activity against tested pathogens including *B. subtilis* ATCC 23857, *S. aureus* ATCC 29213, *E. coli* ATCC 47076 and *P. aeruginosa* ATCC 27853 when the strains were screened by agar plug diffusion assay.

4.5.4.7 Cell wall composition of strains TPS166 and TPS459

The diaminopimelic acid isomer present in whole cells hydrolysates of strains TPS166 and TPS459 were *meso*-diaminopimelic acid. The major types of fatty acids in the cell wall of strain TPS166 were iso- $C_{16:0}$ (36.06%), $C_{18:1}\omega9c$ (9.79%), $C_{17:0}$ (8.24%), and $C_{16:0}$ (5.71%). On the other hand, major fatty acids detected for the strain TPS459 were iso- $C_{16:0}$ (26.14%), $C_{17:1}\omega8c$ (19.15%), $C_{18:1}\omega9c$ (15.33%), $C_{17:0}$ (7.54%) and iso- $C_{15:0}$ (6.52%),. The full fatty acid profiles of both strains were summarised in Appendix H. Menaquinones detected in the cell wall of strain TPS166 include MK-9(H_8) (66%), MK-9(H_6) (27%) and MK-9(H_4) (5%), whereas MK-9(H_4) (46%), MK-9(H_6) (16%), MK-9 (6%), MK-9(H_2) (1%) and traces of MK-9(H_8) were detected in the cell wall of strain TPS459 (Appendix I). Polar lipids present in the cell walls of strain TPS166 were phosphoglycolipid, phosphatidylcholine, phosphatidylglycerol,

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Table 4.31: The API® 20 NE test result for strains TPS166, TPS357 and TPS418 after 14 days of incubation at 28ºC. (+, positive result; -, negative result)

Tests	Active ingredients	Reactions/ Enzymes	TPS166	TPS357	TPS418
NO_2	Potassium nitrate	Reduction of nitrates to nitrites	Red (+)	Colourless (-)	Colourless (-)
N_2		Reduction of nitrates to nitrogen	Red (-)	Pink (-)	Pink (-)
TRP	L- tryptophane	Indole production	Yellow (-)	Yellow (-)	Colourless (-)
GLU	D- glucose	Fermentation	Blue (-)	Blue (-)	Blue (-)
ADH	L- arginine	Arginine dihydrolase	Red (+)	Red (+)	Red (+)
URE	Urea	Urease	Red (+)	Red (+)	Red (+)
ESC	Esculin ferric citrate	Hydrolysis (β-glucosidase)	Black (+)	Black (+)	Black (+)
GEL	Gelatine (bovine origin)	Hydrolysis (protease)	No pigment diffusion (-)	Diffusion of black pigment (+)	No pigment diffusion (-)
PNPG	4- nitrophenyl- βD-galactopyranoside	β- galactosidase (para- nitrophenyl- βD-galactopyranosidase)	Yellow (+)	Yellow (+)	Yellow (+)
GLU	D- glucose	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
ARA	L- arabinose	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
MNE	D- mannose	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
MAN	D- mannitol	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
NAG	N- acetyl-glucosamine	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
MAL	D- maltose	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
GNT	Potassium gluconate	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
CAP	Capric acid	Assimilation	Transparent (-)	Transparent (-)	Opaque (+)
ADI	Adipic acid	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
MLT	Malic acid	Assimilation	Transparent (-)	Opaque (+)	Opaque (+)
CIT	Trisodium citrate	Assimilation	Transparent (-)	Transparent (-)	Opaque (+)
PAC	Phenylacetic acid	Assimilation	Opaque (+)	Opaque (+)	Opaque (+)
OX	1% tetramethyl-p-phenylenediamine dihydrochloride	Cytochrome oxidase	Light purple (-)	Light purple (-)	Light purple (-)

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diphosphatidylglycerol and unknown lipid and unknown phospholipids (Appendix I). Strain TPS459 contained phosphoglycolipid, phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol and unknown phospholipids (Appendix I).

4.5.5 Characterisation of strain TPS358a

4.5.5.1 Phylogenetic analysis of 16S rRNA gene sequence

The strain TPS358a was identified to be a novel species of the genus *Nonomuraea*. Comparison of the 16S rRNA gene sequences showed that TPS358a was closely related to *Nonomuraea salmonea* DSM 43678^T (98.33%), *Nonomuraea maheshkhaliensis* 16-5-14^T (98.33%) and *Nonomuraea kuesteri* NRRL B-24325^T (98.19%). The actinobacterial strain was isolated from skim milk/ HEPES treated sediment sample Z on modified M2 medium (Table 4.11). The taxonomic position of strain TPS358a was confirmed by phylogenetic analysis using neighbour joining, maximum likelihood and maximum parsimony methods. The novel strain formed a separate cluster from the closely related *Nocardiopsis* species (Figure 4.53).

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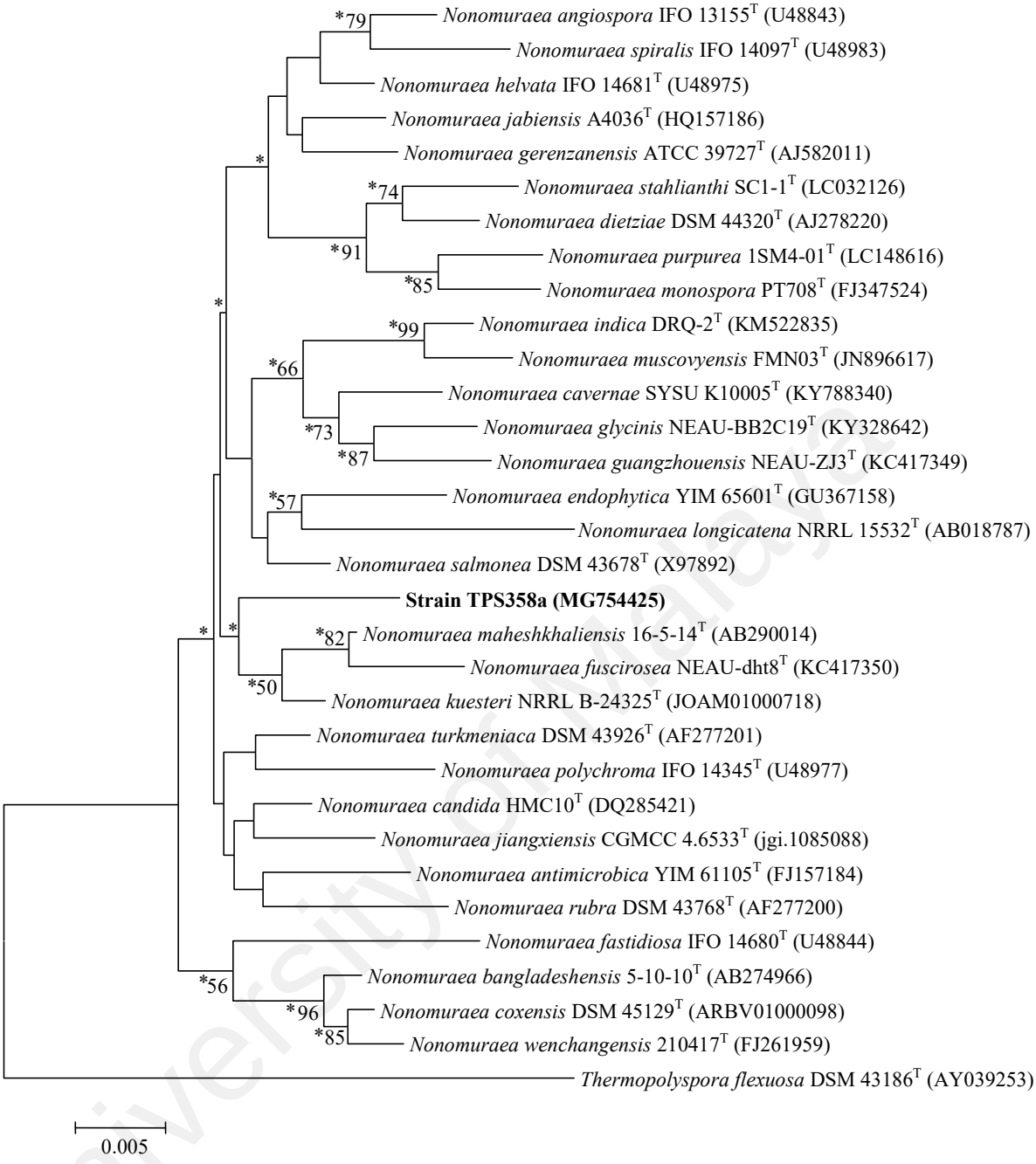

Figure 4.53: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of the strain TPS358a and its closely related members in the family *Streptosporangiaceae*. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 0.5% sequence divergence.

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4.5.5.2 Morphological characterisation of strain TPS358a

The novel strain TPS358a was Gram-stained positive and showed good growth on ISP medium 1, 2, 3, 4, 5, 6 and 7 (Table 4.32 and figure 4.54). The strain produced spores on ISP 2 and ISP 3 media, but not producing spores on other media. It produced white and yellow aerial mycelia on ISP 2 and ISP 3, respectively, and dark reddish brown substrate mycelia on both media. Aerial mycelia were not produced on ISP 4, ISP 5 and ISP 7 media. No diffusible pigment was produced on all tested ISP media. The novel strain produced deep reddish orange diffusible pigment on ISP 6. Aerial mycelia of the strain TPS358a were long and branching. Long chain of spores was observed to be produced on the aerial mycelia (Figure 4.54).

Table 4.32: Colour of substrate and aerial mycelia, spores and diffusible pigment of strain TPS358a on ISP media supplemented with 2.5% ASW were listed here.

Culture medium	Colony surface	Aerial mycelia	Substrate mycelia	Spores	Diffusible pigment
ISP 1	Leathery	Dark reddish orange	Dark reddish orange	Absent	Absent
ISP 2	Powdery	White	Dark reddish orange	White	Absent
ISP 3	Powdery	White and brilliant orange yellow	Moderate orange yellow	White and brilliant orange yellow	Absent
ISP 4	Leathery	Absent	Light orange yellow	Absent	Absent
ISP 5	Leathery	Absent	Yellowish white	Absent	Absent
ISP 6	Wrinkles	Deep reddish brown	Deep reddish brown	Absent	Deep reddish orange
ISP 7	Leathery	Absent	Yellowish white	Absent	Absent

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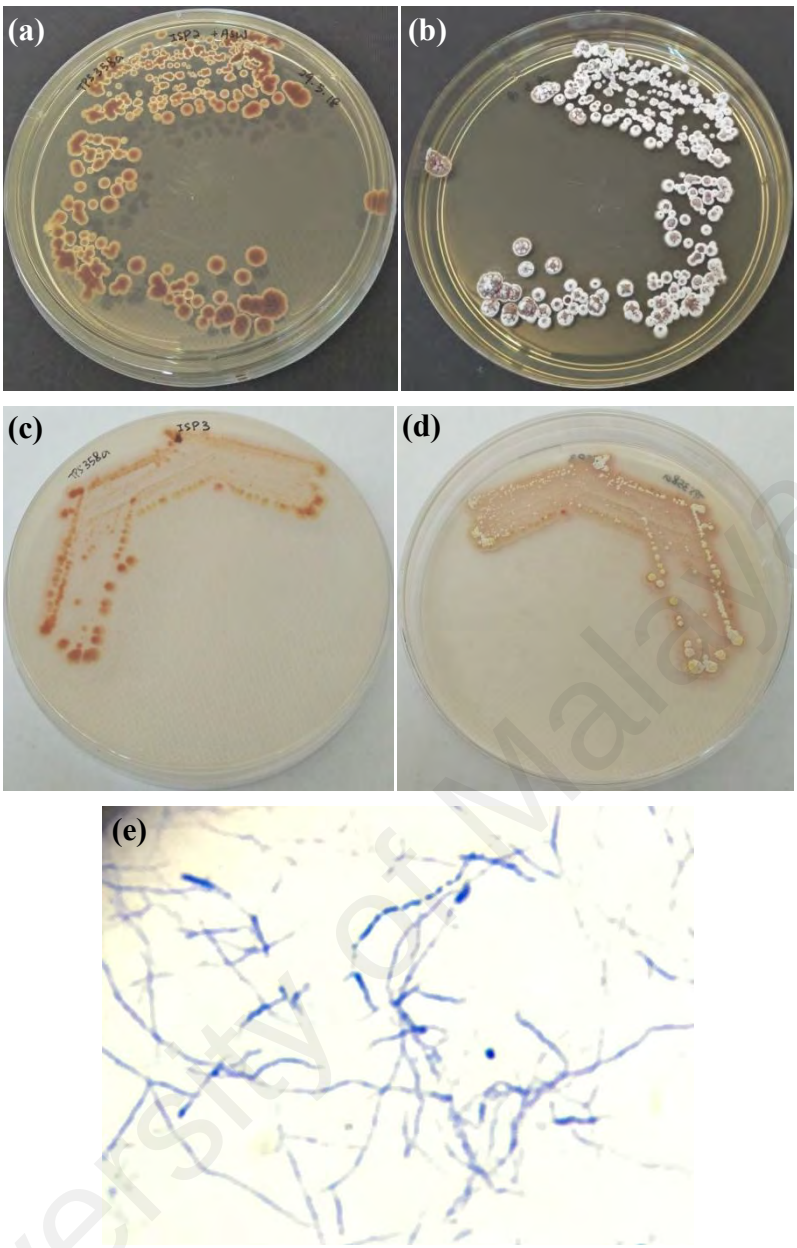

Figure 4.54: Growth of strain TPS358a on ISP 2 (a, b) and ISP 3 (c, d). Cells were Gram- stained positive as shown in (e), long spore chain was observed to grow on aerial mycelia, which were observed to be long and branching, under a light microscope at 400 × magnification.

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4.5.5.3 Carbon utilisation profile

Strain TPS358a showed positive growth on ISP 9 supplemented with 18 types of carbon sources, including Adonitol, D- arabinose, D- fructose, D- galactose, D- glucose, D-lactose, D- maltose, D- mannitol, D- mannose, D- melezitose, D- melibiose, D- ribose, D-trehalose, inositol, L- arabinose, sucrose, xylose and the amino acid L- glutamine. The strain does not show growth when it is inoculated on basal medium without carbon source and also on media with D-sorbitol, glycine, L-lysine, L-methionine and L-sorbose. The novel strain produced acid from adonitol, L-arabinose, D-fructose and D-ribose, as indicated by changes of media colour from purple to yellow. Weak acid production by TPS358a was observed on ISP 9 supplemented with D-glucose, D-arabinose and D-mannitol.

4.5.5.4 Tolerance of pH, temperature and NaCl

The novel strain TPS358a showed good growth on ISP 2 medium adjusted to pH range from 6- 12. The novel strain was also able to grow at incubation temperature set at 15ºC, 25ºC, 28ºC, 32ºC and 37ºC. No growth was observed at 4ºC, 45ºC and 50ºC. Good growth occurred on ISP 2 plates without supplementation of NaCl and the strain was also able to display good growth on ISP2 supplemented with 1% to 4% of NaCl. Weak growth on 5% NaCl ISP 2 plates as compared to the positive control.

4.5.5.5 Hydrolytic enzymes production, catalase and oxidase tests and decomposition of adenine

Hydrolysis of starch was recorded positive for strain TPS358a, as clear zone around colonies on starch- ISP2 plate was observed and the presence of starch in the agar was confirmed by changes of agar colour from yellow to blue upon flooding with iodine solution. Observation of clear zone around colonies of strain TPS358a on skim milk agar plate and gelatine agar plate indicated positive result for hydrolysis of casein and

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gelatine. The urea agar slant that was inoculated with TPS358a was observed to have colour changes from yellow to purple that indicated positive urea hydrolysis reaction. Formation of bubbles was observed for strain TPS358a upon mixing of the cells with 3% H_2O_2 droplet. Oxidase test was recorded as positive as indicated changes of colour of the filter paper impregnated with oxidase reagent from colourless to purple. Disappearance of adenine crystals around the actinobacterial colonies indicated that the novel strain TPS358a was able to decompose adenine.

4.5.5.6 Cell wall composition of the novel strain TPS358a

The diaminopimelic acid isomer present in whole cells hydrolysates of strains TPS358a was *meso*-diaminopimelic acid. The major types of fatty acids in the cell wall of strain TPS358a were iso- $C_{16:0}$ (37.20%), $C_{17:0}$ 10- methyl (16.67%), iso- $C_{16:1}$ G (6.81%) and $C_{17:1}\omega6c$ (5.22%). The full fatty acid profile of strain TPS358a was given in Appendix J. Menaquinones detected in the cell wall of strain TPS358a include MK-9(H_4) (61%), MK-9(H_6) (18%) and MK-9(H_2) (16%) (Appendix K). The cell wall polar lipids were detected as phosphatidylinositol with unknown lipids and glycolipids as well as phosphoaminolipid (Appendix K).

4.5.5.7 Antibacterial activity

The novel strain TPS358a growing on media including PM3, soybean meal glucose media, micromonospora medium, ATCC medium 241 and starch yeast peptone medium for 14 days and 21 days did not inhibit growth of pathogens as tested by agar plug diffusion assay, including the Gram-positive *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857 and Gram-negative *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 47076.

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4.5.6 Characterisation of strain TPM287

4.5.6.1 Phylogenetic analysis of 16S rRNA gene sequence

The strain TPM287 was identified to be a novel species of the genus *Glycomyces*. The 16S rRNA gene sequence of TPM287 shared a 98.34%, 97.20% and 96.27% with *Glycomyces phytohabitans* KLBMP 1483^T, *Glycomyces arizonensis* DSM 44726^T and *Glycomyces tenuis* DSM 44171^T, respectively. The actinobacterial strain was isolated from UV treated sediment sample M on HVA (Table 4.11). The taxonomic position of strain TPM287 was confirmed by phylogenetic analysis using neighbour joining, maximum likelihood and maximum parsimony methods. The novel strain formed a separate cluster from the closely related *Glycomyces* species (Figure 4.55).

4.5.6.2 Morphological characterisation of strain TPM287

The novel strain TPM287 was Gram-stained positive. Growth occurred on ISP medium 1, 2, 3, 4, 5, 6 and 7 (Table 4.33 and Figure 4.56). Only vegetative mycelium was produced on ISP medium 1, 4, 5, 6 and 7. The strain produced aerial mycelia and spores on ISP 2 and ISP 3. It produced dark reddish brown substrate mycelia on ISP 2 and ISP 3, along with white aerial mycelia on ISP 2 and yellow aerial mycelia on ISP 3. No diffusible pigment was produced on all ISP media. Fetid odour was produced on ISP 2 medium. Deep reddish orange diffusible pigment was produced on ISP 6. Substrate mycelia of TPM287 on ISP 2 were long and straight, as observed under light microscope at 400 × magnification.

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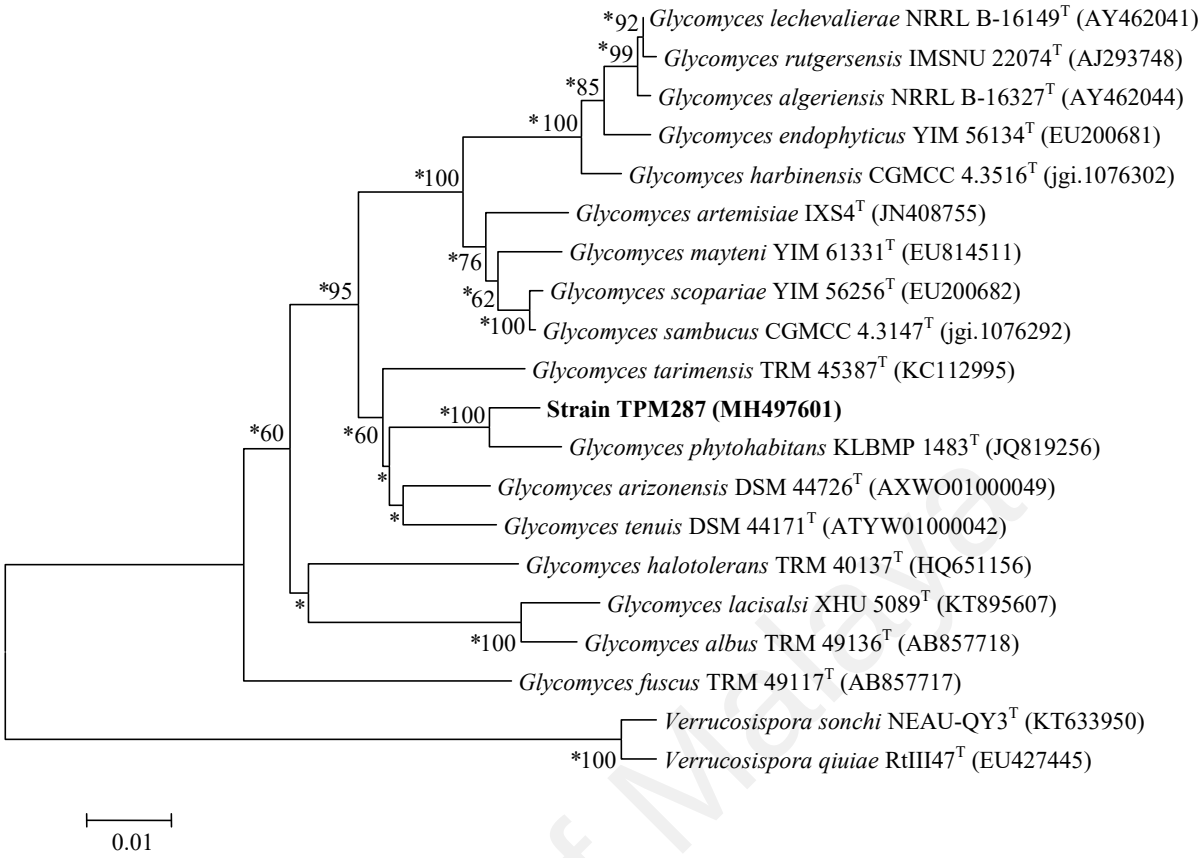

Figure 4.55: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of strains TPM287 and closely related members of the genus *Glycomyces*. *Verrucosispora sonchii* NEAU-QY3^T and *Verrucosispora qiuiae* RtIII47^T were outgroup. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 1% sequence divergence.

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Table 4.33: Colour of substrate and aerial mycelia, spores and diffusible pigment of strain TPM287 on ISP media supplemented with 2.5% ASW were listed here.

Culture medium	Colony surface	Aerial mycelia	Substrate mycelia	Spore colour	Diffusible pigment
ISP 1	Leathery	Absent	Pale yellow	Absent	Absent
ISP 2	Leathery	Absent	Light yellow	Absent	Absent
ISP 3	Leathery/ Powdery	White	Pale yellow	White	Absent
ISP 4	Leathery	Absent	Pale yellow	Absent	Absent
ISP 5	Powdery	White	Pale yellow	White	Absent
ISP 6	Leathery	Absent	Light yellow	Absent	Absent
ISP 7	Powdery	White	Brilliant orange yellow	White	Brownish orange

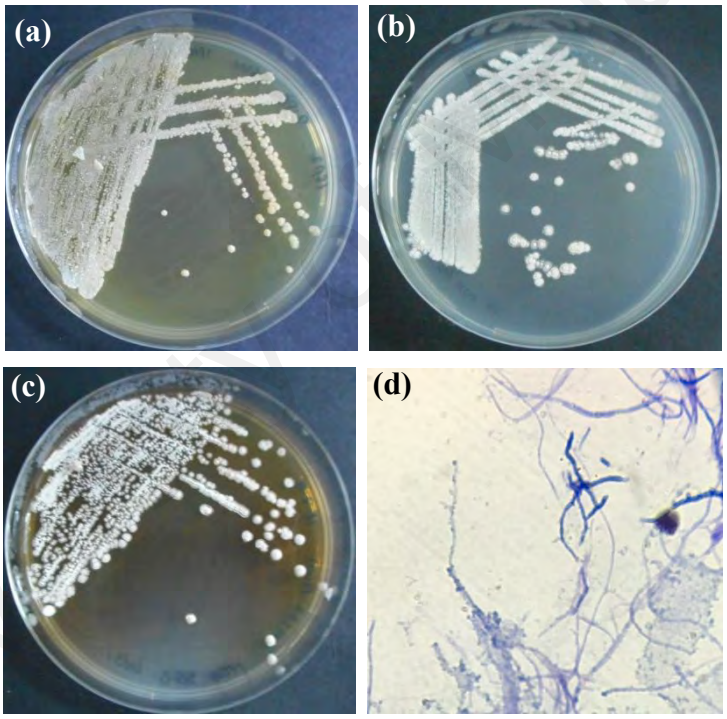

Figure 4.56: Growth of TPM287 on (a) ISP 2, (b) ISP 5 and (c) ISP 7. Microscopic examination of the culture on ISP 2 medium revealed the presence of long and straight vegetative mycelia at 400 × magnification as shown in (d).

4.5.6.3 Carbon utilisation profile

The strain TPM287 showed good growth on ISP 9 supplemented with 15 types of carbon sources and these include D- arabinose, D- glucose, D- galactose, D- lactose, D-mannitol, D- mannose, D- melezitose, D- ribose, sucrose, D- trehalose and the amino acids L- arabinose. Sparse growth was observed on ISP 9 with D-maltose, D-melibiose, xylose and L- glutamine. The strain also showed positive growth when it was inoculated on basal medium without carbon source. Acid production from D- glucose, D- galactose, D-maltose, D-melibiose, D-ribose, D-trehalose, sucrose, xylose and L- arabinose was observed. White aerial and spores production was observed on ISP 9 medium supplemented with D-lactose, D-mannitol, D-mannose and D-trehalose. Acid production was observed on ISP 9 supplemented with D-glucose, L-arabinose, D-galactose and D-mannose, as indicated by changes of media colour from purple to yellow.

4.5.6.4 Tolerance of pH, temperature and NaCl

The novel strain TPM287 showed good growth on ISP 2 medium adjusted to pH range from 7- 11. No growth was observed at pH 6 and weak growth of the strain occurred at pH 12. At pH below 9, the strain was not producing aerial mycelia and spores. At pH 9, 10 and 11, the strain produced white aerial mycelia and spores. Good growth of TPM287 was observed at 25ºC, 28ºC, 32ºC, 37ºC and 45ºC. No growth at 4ºC and 50ºC. Weak growth of TPM287 was observed at 15ºC. At all tested incubation temperatures, the strain TPM287 did not produce aerial mycelia and spores. Strain TPM287 did not grow on ISP 2 medium without NaCl supplementation. Growth was also not observed on ISP2 medium with 4% NaCl and above. Weak growth on 1% NaCl plate and good growth occurred on ISP 2 plates with 2% and 3% NaCl.

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4.5.6.5 Hydrolytic enzymes production, catalase and oxidase tests and decomposition of adenine

Hydrolysis of starch was recorded positive for strain TPM287, as clear zone around the colonies on starch- ISP2 plate was observed and presence of starch in the agar was confirmed by changes of agar colour to blue upon flooding with iodine solution. Observation of clear zone around colonies of strain TPM287 on skim milk agar plate indicated positive result for hydrolysis of casein. Gelatine test was also recorded positive for TPM287 as clear zone around the colonies was observed upon flooding the gelatine- ISP 2 plate with ammonium sulphate. No colour change of the urea agar slant that was inoculated with strain TPM287. The urea agar slant remained yellow and this indicated a negative urea hydrolysis reaction. Formation of bubbles was observed for strain TPM287 upon mixing of the cells with 3% H_2O_2 droplet. Disappearance of adenine crystals around the actinobacterial colonies indicated that strain TPM287 was able to decompose adenine. Oxidase test was recorded negative, as indicated by no changes of colour of the filter paper impregnated with oxidase reagent.

4.5.6.6 Antibacterial activity

The novel strain TPM287 growing on media including PM3, soybean meal glucose media, micromonospora medium, ATCC medium 241 and starch yeast peptone medium for 14 days and 21 days did not inhibit growth of pathogens as tested by agar plug diffusion assay, including the Gram-positive *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857 and Gram-negative *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 47076.

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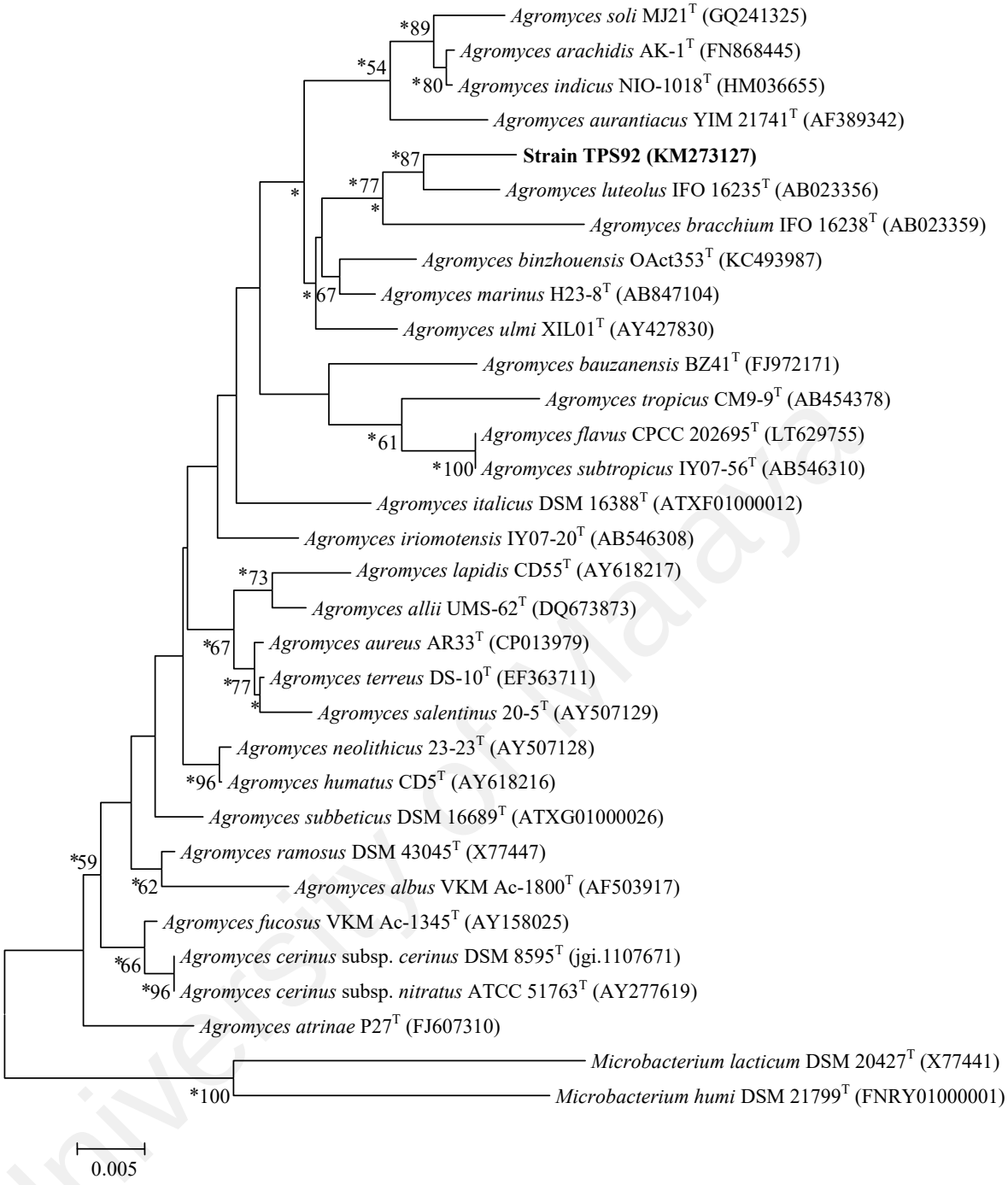

Figure 4.57: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of strain TPS92 and closely related members of the genus *Agromyces*. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 5% sequence divergence.

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4.5.7 Characterisation of strain TPS92

4.5.7.1 Phylogenetic analysis of 16S rRNA gene sequence

The strain TPS92 was identified to be a novel species of the genus *Glycomyces*. The 16S rRNA gene sequence of TPS92 was 98.09%, 98.09% and 97.94% similar to *Agromyces aurantiacus* YIM 21741^T, *Agromyces binzhouensis* OAct353^T and *Agromyces luteolus* IFO 16235^T, respectively. The actinobacterial strain was isolated from skim milk/ HEPES treated sediment sample Z on HVA medium (Table 4.11). The taxonomic position of TPS92 was confirmed by phylogenetic analysis using neighbour joining, maximum likelihood and maximum parsimony methods. The novel strain formed a separate cluster from the closely related *Agromyces* species (Figure 4.57).

4.5.7.2 Morphological characterisation of strain TPS92

The novel strain TPS92 was Gram-stained positive. Growth occurred on ISP medium 1, 2, 3, 4, 5, 6 and 7 (Figure 4.58). Vegetative mycelia that penetrated into agar were produced on all media but aerial mycelia were absent on all culture media. The strain produced circular, smooth, yellow colonies on all media. No diffusible pigment was produced on all ISP media. The strain produced light yellow colour substrate mycelia on ISP 1 and ISP 2, yellowish white colour substrate mycelia on ISP 3, ISP 4 and ISP 5, and pale yellow coloured substrate mycelia on ISP 6 and ISP 7. Microscopic examination of strain TPS92 at 1000 × magnification showed that the cells were straight to slightly curve rods and aerial mycelia were not observed.

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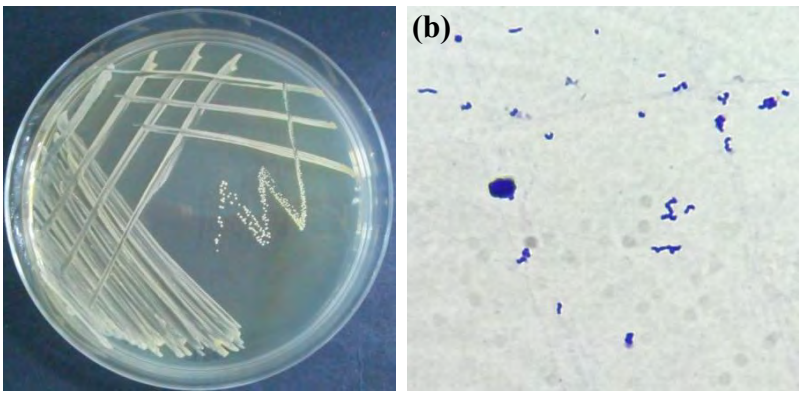

Figure 4.58: Growth of TPS92 on ISP 2 medium: (a) strain TPS92 produced circular colonies with smooth surface and substrate mycelia that penetrate into the agar. (b) Microscopic examination of strain TPS92 under a light compound microscope at 1000 × magnification.

4.5.7.3 Carbon utilisation profile

The strain TPS92 showed positive growth on ISP 9 supplemented with 17 types of carbon sources as listed in the following: D- glucose, Adonitol, D- arabinose, L-arabinose, D- fructose, D- maltose, D- mannitol, D- mannose, D- melezitose, L- sorbose, D- ribose, Sucrose, D- trehalose, Xylose, L-glutamine, L-lysine and L-methionine. Sparse growth on basal medium without carbon source was observed. Acid production was observed on ISP 9 supplemented with D-mannose, D-melezitose and sucrose, as indicated by changes of media colour from purple to yellow. Weak acid production from L- arabinose and xylose was observed.

4.5.7.4 Tolerance of pH, temperature and NaCl

Weak growth of TPS92 was observed on ISP 2 with pH 6 and good growth was observed on medium with pH 7 to pH 12. Good growth of TPS92 was observed at 25ºC, 28ºC, 32ºC, 37ºC and 45ºC. No growth at 4ºC and 50ºC. Weak growth of TPS92 was observed at 15ºC. At all tested incubation temperatures, the strain TPS92 did not produce aerial mycelia and spores. Positive growth was observed on ISP 2 plates supplemented with 0- 6% NaCl. Growth was not observed on ISP 2 medium with supplementation of 7% and 8% NaCl.

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4.5.7.5 Hydrolytic enzymes production, catalase and oxidase tests and decomposition of adenine

Hydrolysis of starch was recorded positive for strain TPS92, as clear zone around the colonies on starch- ISP2 plate was observed and presence of starch in the agar was confirmed by changes of agar colour to blue upon flooding with iodine solution. Absence of clear zone around colonies of strain TPS92 on skim milk agar plate indicated negative result for hydrolysis of casein. Gelatine test was also recorded negative for TPS92 as clear zone around the colonies was not observed upon flooding the gelatine- ISP 2 plate with ammonium sulphate. Colour of the urea agar slant that was inoculated with strain TPS92 did not change from yellow to pink, thus indicated a negative urea hydrolysis reaction. Formation of bubbles was observed for strain TPS92 upon mixing of the cells with 3% H_2O_2 droplet. The strain TPS92 was unable to decompose adenine as disappearance of adenine crystals around the colonies was not observed. Oxidase test was recorded negative, as indicated by no changes of colour of the filter paper impregnated with oxidase reagent.

4.5.7.6 Cell wall composition of the novel strain TPS92

The major types of fatty acids in the cell wall of strain TP92 were iso- $C_{16:0}$ (29.13%), anteiso- $C_{15:0}$ (28.01%), anteiso- $C_{17:0}$ (26.56%) and iso- $C_{15:0}$ (8.13%), The full fatty acid profile of strain TPS92 was given in Appendix L. Menaquinones detected in the cell wall of strain TPS92 include MK-11 (78%), MK-12 (20%) and MK-10 (3%) (Appendix L). The cell wall polar lipids were detected as diphosphatidylglycerol, phosphatidylglycerol, unknown lipids and glycolipids as well as phosphoglycolipid (Appendix L).

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4.5.7.7 Antibacterial activity

The novel strain TPS92 growing on media including PM3, soybean meal glucose media, micromonospora medium, ATCC medium 241 and starch yeast peptone medium for 14 days and 21 days did not inhibit growth of pathogens as tested by agar plug diffusion assay, including the Gram-positive *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857 and Gram-negative *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 47076.

4.5.8 Characterisation of strain TPM181

4.5.8.1 Phylogenetic analysis of 16S rRNA gene sequence

The strain TPM181 was identified to be a novel species of the genus *Mycobacterium*. The almost full length 16S rRNA gene sequence of TPM181 was closely related to *Mycobacterium peregrinum* ATCC 14467^T (98.34%), *Mycobacterium longobardum* DSM 45394^T (98.20%) and *Mycobacterium mageritense* DSM 44476^T (98.20%). The actinobacterial strain was isolated from UV treated sediment sample M on HVA (Table 4.11). The taxonomic position of strain TPM181 was confirmed by phylogenetic analysis using neighbour joining, maximum likelihood and maximum parsimony methods. The novel strain formed a separate cluster from the closely related *Mycobacterium* species (Figure 4.59).

4.5.8.2 Growth of TPM181 on ISP 2 medium and Gram-stain

The novel strain TPM181 was Gram-stain and acid- fast positive bacterium and the cells were observed to be straight rods at a magnification of 1000 ×. Growth occurred on ISP 2 followed by 7 days of incubation was shown in Figure 4.60. The strain produced circular, smooth, orange colonies on ISP 2. Colonies were convex, round and entire- edged and aerial mycelia were absent. No diffusible pigment was produced.

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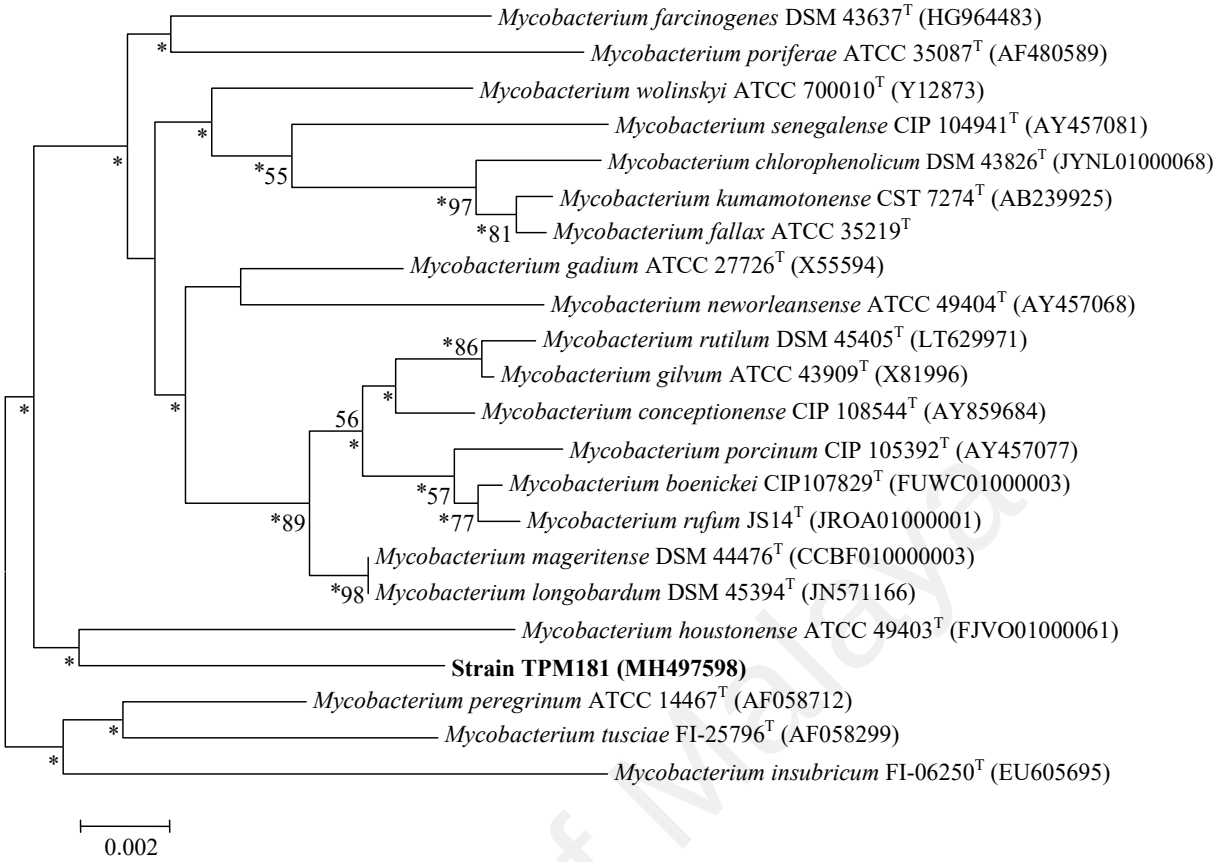

Figure 4.59: Neighbour-joining tree based on almost full length 16S rRNA gene sequences of strain TPM181 and closely related members of the genus *Mycobacterium*. Bootstrap values are denoted at nodes on branches based on 1000 re-sampling values, only values higher or equal to 50% are indicated here. Asterisks denote branches that are also recovered with both maximum likelihood and maximum-parsimony approaches. Bar represents 2% sequence divergence.

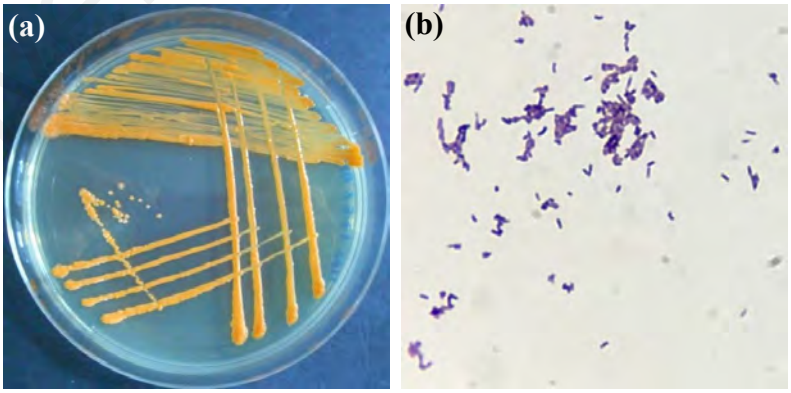

Figure 4.60: Growth of strain TPM181 on ISP 2 medium was shown in (a). The novel strain produced orange circular colonies with smooth and moist surface. Straight rod cells were observed under the light compound microscope at 1000 × magnification, as shown in (b).

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4.5.8.3 Carbon utilisation profile

The strain TPM181 showed positive growth on ISP 9 supplemented with D- glucose, adonitol, D- fructose, D- galactose, inositol, D- lactose, D- maltose, D- mannitol, D- mannose, D- melezitose, D- melibiose, D- ribose, D- sorbitol, D- trehalose, D- xylose, L- arabinose, sucrose, glycine, L- glutamine, L- lysine. No growth on basal medium without carbon source. Acid production was observed on ISP 9 supplemented with D- fructose, and mannitol, as indicated by changes of media colour from purple to yellow.

4.5.8.4 Tolerance of pH, temperature and NaCl

Good growth of TPM181 was observed on ISP 2 with pH 6 up to pH 10 and weak growth was observed on ISP 2 with pH 11 and pH 12. Good growth was also observed at 25ºC, 28ºC, 32ºC and 37ºC. No growth at 4ºC and 45ºC. Weak growth was observed at 15ºC. The strain grows well on ISP 2 medium supplemented with 1% to 8% NaCl. Weak growth was observed on ISP 2 without NaCl. Growth was not observed on ISP 2 with 9% NaCl.

4.5.8.5 Hydrolytic enzymes production, catalase and oxidase tests and decomposition of adenine

Hydrolysis of starch was recorded negative for strain TPM181, as clear zone around the colonies on starch- ISP2 plate was not observed and presence of starch in the agar was confirmed by changes of agar colour to blue upon flooding with iodine solution. Hydrolysis of casein and gelatine was also recorded negative as indicated by absence of clear zone around colonies on skim milk agar plate and gelatine plate. Hydrolysis of urea was recorded as positive as colour of the urea agar slant that was inoculated with the strain turned pink. Formation of bubbles was observed for strain TPM181 upon mixing of the cells with 3% H_2O_2 droplet. Oxidase test was recorded negative, as indicated by no changes of colour of the filter paper impregnated with oxidase reagent.

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The strain was unable to decompose adenine as disappearance of adenine crystals around the colonies was not observed.

4.5.8.6 Antibacterial activity

The novel strain TPM181 growing on media including PM3, soybean meal glucose media, micromonospora medium, ATCC medium 241 and starch yeast peptone medium for 14 days and 21 days did not inhibit growth of pathogens as tested by agar plug diffusion assay, including the Gram-positive *S. aureus* ATCC 29213 and *B. subtilis* ATCC 23857 and Gram-negative *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 47076.

4.6 Draft genome sequences of selected novel strains

4.6.1 RAST functional annotation of the predicted protein- coding genes

RAST functional annotation of the predicted protein- coding genes from the genomes of all five novel strains including three novel *Nocardiopsaceae* actinobacteria (TPS16, TPS81 and TPS83), *Nocardiopsis* sp. (TPS2) and *Streptomyces* sp. (TPS3) revealed that the highest number of genes (more than 200 genes) was designated to metabolism of carbohydrates, amino acids and their derivatives, protein, fatty acids, lipids and isoprenoids, production of cofactors, vitamins, prosthetic groups and pigments (Table 4.34). The functional annotation of protein- coding genes also unveiled the presence of large number of genes that are related to stress response in the genomes, including genes that are associated with oxidative stress, coding genes for cold shock and heat shock proteins, as well as genes that were responsible for detoxification, osmoregulation, uptake of choline and betaine and biosynthesis of betaine and ectoine. Genes related to metabolism of DNA, RNA, sulphur, potassium, phosphorus, nitrogen, iron and aromatic compounds were also linked to the genomes.

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Table 4.34: Number of genes in various subsystem categories for the novel strains TPS16, TPS81, TPS83, TPS2 and TPS3 that were annotated using the RAST server.

Subsystem categories	TPS16	TPS81	TPS83	TPS2	TPS3
Carbohydrates	480	443	467	500	821
Amino acids and derivatives	448	434	427	443	724
Protein metabolism	279	273	288	265	323
Cofactors, vitamins, prosthetic groups, pigments	239	234	232	279	377
Fatty acids, lipids and isoprenoids	187	174	179	191	324
Nucleosides and nucleotides	125	119	124	123	149
Membrane transport	131	119	121	103	132
Stress response	122	124	113	128	208
Cell wall and capsule	123	116	117	97	159
DNA metabolism	118	121	120	101	132
Respiration	105	103	104	99	166
RNA metabolism	71	72	72	76	115
Sulfur metabolism	51	50	51	51	103
Metabolism of aromatic compounds	36	35	34	65	102
Virulence, disease and defense	59	56	57	48	86
Regulation and cell signaling	41	43	42	45	70
Phosphorus metabolism	35	36	37	38	40
Cell division and cell cycle	34	33	32	34	32
Miscellaneous	32	32	32	36	56
Nitrogen metabolism	29	29	28	29	39
Iron acquisition and metabolism	19	19	18	51	39
Potassium metabolism	17	17	17	15	18
Motility and chemotaxis	5	5	5	5	12
Phages, prophages, transposable elements, plasmids	3	8	8	0	3
Dormancy and sporulation	2	2	2	2	16
Scondary metabolism	0	0	0	21	13

The five novel strains were also found to have genes related to copper homeostasis, which coded for copper resistance and homeostasis proteins, mercuric reductase and cytochrome c- type biogenesis and synthesis protein. Heavy metal and drug resistance genes related to cobalt- zinc- cadmium resistance, fluoroquinolones resistance were also present in their genomes. Moreover, the detoxification genes found in the genome also

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include genes responsible for production of beta- lactamase. Genomes of the *Nocardiopsaceae* strains including TPS2, TPS16, TPS81 and TPS83 were found to have genes related to copper tolerance. In addition, the three strains TPS16, TPS81 and TPS83 also possess genes related to arsenic resistance. Strain *Nocardiopsis* sp. TPS2, on the other hand, also showed the presence of invasion and intracellular resistance genes related to *Mycobacterium* virulence operon. The *Streptomyces* sp. TPS3 also possesses resistance genes to various heavy metals or antibiotics including zinc, tellurite and tetracycline, in addition to genes responsible for production of aminoglycoside-, streptomycin- and spectinomycin- adenyltransferases.

4.6.2 Prediction of BGCs for secondary metabolites production

In total, 59 BGCs were predicted by antiSMASH from the novel strain TPS16 (Table 4.35). The types of predicted BGC include saccharide, indole, terpene, type- I polyketide synthases (T1-PKS), type- II polyketide synthase (T2-PKS), non- ribosomal polypeptide synthetases (NRPS), fatty acids, siderophore, ectoine and lassopeptide (Table 4.35). Similarity of the predicted biosynthetic gene clusters to the known biosynthetic gene cluster was given in percentage. Out of these 59 BGCs, 30 of them were not identified and not related to the known BGCs: 22 putative type gene clusters, four saccharide type clusters, an indole cluster, a terpene cluster, a T1-PKS cluster and a T2-PKS cluster. The sister strains TPS81 and TPS83 were predicted to possess 16 and 14 BGCs, respectively, including T1-PKS and T2-PKS clusters, NRPS clusters, butyrolactone-other KS cluster, lassopeptide cluster, ectoine cluster, indole cluster, siderophore cluster, terpene clusters (Table 4.35 and Table 4.36). Among these BGCs, one BGC from each of the following type was remained unidentified and not related to the known BGCs, including indole, terpene, T1-PKS, T2-PKS, NRPS clusters. Among these BGCs detected with antiSMASH, the methylpendolmycin gene cluster (BGC0000391) is of particular interest as the compound was also isolated from crude

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extract of strain TPS83, which is further discussed in the section 4.7. This BGC was found to be present in the genome of strain TPS16 with accession number QEIO01000080 and in strain TPS81 with accession number QEIN01000045.

Prediction of BGC from the genome of the *Nocardiopsis* sp. strain TPS2 by antiSMASH revealed the presence of 14 BGCs, namely the thiopeptide- trans- AT PKS cluster, ectoine, siderophore, lantipeptide, T1 PKS, NRPS and terpene (Table 4.37). The three lantipeptide clusters located at 23110 nt to 46531 nt, 4375 nt to 42312 nt and 1 nt to 13563 nt in the genome remained not related to any known BGC.

The antiSMASH server predicted there were 29 BGCs present in the genome of strain TPS3, including bacteriocin, T1 and T2 PKS, NRPS, terpene, *trans*- AT PKS, siderophore, ectoine, butyrolactone and borrelidin (Table 4.38). In addition to the BGC listed in Table 4.37, there are nine BGCs that were remained not identified and related to the known BGC: three siderophore type BGCs (located at 1 nt to 3067 nt, 1 nt to 10047 nt and 10817 nt to 23950 nt), two terpene type BGCs (located at 27883 nt to 48893 nt and 10172 nt to 32382 nt), two T1 PKS BGC (located at 1 nt to 14403 nt and 1 nt to 1401 nt), one bacteriocin type BGC (located at 9986 nt to 21335 nt) and one *trans*-AT PKS type BGC (located at 1 nt to 5850 nt).

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Table 4.35: Total number of BGC predicted from the draft genomes of strains TPS16, TPS81 and TPS83.

Biosynthetic gene cluster	Number of gene cluster related to known BGC			Number of gene cluster not related to known BGC			Total number of BGC		
	TPS16	TPS81	TPS83	TPS16	TPS81	TPS83	TPS16	TPS81	TPS83
Putative	14	0	0	22	0	0	36	0	0
Saccharide	1	0	0	5	0	0	6	0	0
Indole	0	0	0	1	1	1	1	1	1
Terpene	1	1	1	1	1	0	2	2	1
T1-PKS	1	1	1	1	1	1	2	2	2
T2-PKS	1	1	1	1	1	1	2	2	2
NRPS	2	2	2	0	1	0	2	3	2
Fatty acids	1	0	0	0	0	0	1	0	0
Fatty acid-Butyrolactone-Other KS	1	1	1	0	0	0	1	1	1
Fatty acids- NRPS	1	0	0	0	0	0	1	0	0
Siderophore	1	1	1	0	0	0	1	1	1
Ectoine	1	1	1	0	0	0	1	1	1
Lassopeptide	1	1	1	0	0	0	1	1	1
Other	1	1	1	1	1	1	2	2	2
Total BGCs	27	10	10	32	6	4	59	16	14

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Table 4.36: List of biosynthetic gene clusters predicted from the novel strains TPS16, TPS81 and TPS83 using antiSMASH server.

Strain	Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
TPS16		41434	66699	Isofuranonaphthoquinone	8	BGC0001386
TPS81	T2 PKS	1	22215	R1128	8	BGC0000261
TPS83		1	9723			
TPS16		24489	63370	Neocarzilin	14	BGC0000111
TPS81	T1 PKS	7019	45900	Tiacumicin B	14	BGC0000165
TPS83		1	32778	Pristinamycin	14	BGC0000952
				Griseoviridin/ viridogrisein	14	BGC0000459
TPS16		882	23587			
TPS81	Lassopeptide	1	19792	SSV-2083	25	BGC0000579
TPS83		1	17463			
TPS16		4260	16098	Desferrioxamine B	80	BGC0000941
TPS81	Siderophore	1000	12838	Desferrioxamine B	80	BGC0000940
TPS83		2001	13839			
TPS16		5055	15459	Ectoine	100	BGC0000853
TPS81	Ectoine	882	11286	Ectoine	50	BGC0000852
TPS83		931	11335	Ectoine	50	BGC0000858
				Ectoine	50	BGC0000854
TPS16		1	8917			
TPS81	Other	1	6995	Paromomycin	5	BGC0000712
TPS83		1	11103			

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Table 4.36, continued.

Strain	Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
TPS16	Fatty acid-butyrolactone- other KS	1	15013			
TPS81		1	24809	Colabomycin	4	BGC0000213
TPS83		1	15577			
				Isorenieratene	85	BGC0000664
TPS16	Terpene	1	15782	Carotenoid	85	BGC0000649
TPS81		1	15782	Carotenoid	85	BGC0000633
				Sioxanthin	28	BGC0001087
				Mannopeptimycin	7	BGC0000388
				Desotamide	7	BGC0001196
TPS81	NRPS	1	14134	Marfomycins	7	BGC0001214
TPS83		1	14186	Laspartomycin	7	BGC0000379
				Friulimicin	7	BGC0000354
TPS16	Putative	44895	61621	D-cycloserine	27	BGC0000896
TPS16	Putative	81316	86582	Lasalocid	3	BGC0000087
TPS16	Putative	46696	65870	Bottromycin A2	9	BGC0000469
TPS16	Putative	496	9197	Enduracidin	4	BGC0000341
				Gobichelin	16	BGC0000366
TPS16	Putative	23746	37073	Coelichelin	11	BGC0000325
				Scabichelin	11	BGC0000423

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Table 4.36, continued.

Strain	Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
TPS16	Putative	54618	62098	Congocidine	9	BGC0000327
				Distamycin	9	BGC0001147
				Salinomycin	6	BGC0000144
				Nocathiacin	4	BGC0000609
TPS16	Putative	337	34679	Glycopeptidolipid	4	BGC0000365
				Fluostatin	4	BGC0000223
				Sporolide	4	BGC0000150
				A54145	4	BGC0000291
				Kanamycin	1	BGC0000703
				SF2575	1	BGC0000269
				Pentalenolactone	1	BGC0000678
TPS16	Putative	75	30972	SW-163	1	BGC0000434
				Kedarcidin	1	BGC0000081
				C-1027	1	BGC0000965
				Lidamycin	1	BGC0001397
				Hedamycin	1	BGC0000233
				Himastatin	8	BGC0000117
TPS16	Putative	4201	29006	Laspartomycin	8	BGC0000379
				Phenalinolactone	12	BGC0000654

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Table 4.36, continued.

Strain	Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
TPS16	Putative	75	30972	Teicoplanin	1	BGC0000441
				Teicoplanin	1	BGC0000440
TPS16	Putative	8591	28683	Paromomycin	5	BGC0000712
				Frankiamicin	5	BGC0001197
TPS16	Putative	4	8797	Staurosporine	17	BGC0000827
				Staurosporine	17	BGC0000825
				Avermectin	17	BGC0000025
				Vancomycin	17	BGC0000455
				Ristocetin	17	BGC0000418
				Ristomycin A	17	BGC0000419
				Staurosporine	17	BGC0000826
				Lomaiviticin	17	BGC0000241
				Chloroeremomycin	17	BGC0000322
				Lomaiviticin	17	BGC0000240
TPS16	Putative	810	9538	Herboxidiene	2	BGC0000165
TPS16	Putative	1282	8782	Distamycin	14	BGC0001147
				Congocidine	9	BGC0000327
TPS16	Putative	149	4190	Echosides	11	BGC0000340

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Table 4.36, continued.

Strain	Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
				Cinerubin B	17	BGC0000212
				Aclacinomycin	11	BGC0000191
				Arimetamycin	11	BGC0000199
TPS16	Putative	573	3796	Nogalamycin	8	BGC0000250
				Kosinostatin	5	BGC0001073
				Cosmomycin	8	BGC0001074
				Aclacinomycin	5	BGC0000193
				Chartreusin	5	BGC0000206
TPS16	Fatty acid	1	17913	Chlorizidine A	7	BGC0001172
TPS16	Saccharide	6387	46542	Phosphonoglycans	12	BGC0000807
				Avermectin	33	BGC0000025
				Calicheamicin	33	BGC0000033
				Lankamycin	33	BGC0000085
				Elaiophylin	33	BGC0000053
TPS16	Fatty acid-NRPS	1	28585	SF2575	22	BGC0000269
				AT2433	22	BGC0000809
				Pradimicin	22	BGC0000256
				Versipelostatin	22	BGC0001204
				Lipomycin	22	BGC0001003

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Table 4.36, continued.

Strain	Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
TPS16	NRPS	1	25344	Methylpendolmycin/ pendolmycin	28	BGC0000391
				Lyngbyatoxin	28	BGC0000384
				Friulimicin	57	BGC0000354
				WS9326	42	BGC0001297
				Laspartomycin	57	BGC0000379
				Teleocidin B	28	BGC0001085
				Salinilactam	42	BGC0000142
				Lobosamide	42	BGC0001303
				Desotamide	28	BGC0001196
				Micromonolactam	28	BGC0000095
TPS16	NRPS	1	18956	Mannopeptimycin	7	BGC0000388
				Desotamide	7	BGC0001196
				Marfomycins	7	BGC0001214
				Laspartomycin	7	BGC0000379
				Friulimicin	7	BGC0000354
				Skyllamycin	7	BGC0000429
TPS81	NRPS	1	25236	Methylpendolmycin/ pendolmycin	28	BGC0000391
				Lyngbyatoxin	28	BGC0000384
				Friulimicin	57	BGC0000354

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Table 4.36, continued.

Strain	Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
TPS81	NRPS	1	25236	Laspartomycin	57	BGC0000379
				A54145	57	BGC0000291
				Teleocidin B	28	BGC0001085
				Salinilactam	42	BGC0000142
				Lobosamide	42	BGC0001303
				Desotamide	28	BGC0001196
TPS81	Fatty acid-butyrolactone- other KS	1	24809	Simocyclinone	4	BGC0001072
				Skyllamycin	4	BGC0000429
				Auricin	4	BGC0000727
				Coelimycin	4	BGC0000038
				Colabomycin	4	BGC0000213
TPS83	NRPS	1	29448	Marformycins	12	BGC0001214
				Microbisporicin	12	BGC0000529
				Planosporicin	12	BGC0000544
TPS83	Terpene	1	23716	Isorenieratene	100	BGC0000664
				Carotenoid	100	BGC0000649
				Carotenoid	100	BGC0000633
				Isorenieratene	100	BGC0001227
				Sioxanthin	28	BGC0001087

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Table 4.37: List of biosynthetic gene clusters identified from the genome of the novel strain TPS2 using antiSMASH server.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
Thiopeptide- *trans*- AT PKS	1	71396	Dorrigocin/ migrastatin	54	BGC0000177
			Lactimidomycin	45	BGC0000083
			Desotamide	27	BGC0001196
			9-methylstreptimidone	27	BGC0000171
			Cycloheximide/ actiphenol	27	BGC0000175
			Sorangicin	18	BGC0000184
			Etnangien	18	BGC0000179
Trans- AT PKS	1	22344	Dorrigocin/ migrastatin	45	BGC0000177
			Lactimidomycin	36	BGC0000083
			Bongkrekic acid	18	BGC0000173
T1 PKS	1	36450	Leucanicidin	100	BGC0001232
			Simocyclinone	100	BGC0001072
			Lasalocid	175	BGC0000087
			Lasalocid	175	BGC0000086
			Elaiophylin	125	BGC0000053
			Filipin	125	BGC0000059
			Tirandamycin	75	BGC0001052
			Streptolydigin	75	BGC0001046
			Spinosad	125	BGC0000148
			Midecamycin	125	BGC0000096

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Table 4.37, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
NRPS	1	23625	Scabichelin	20	BGC0000423
			Griseoviridin/ viridogrisein	20	BGC0000459
			Jawsamycin	20	BGC0001002
			Pristinamycin	20	BGC0000952
			Cremimycin	20	BGC0000042
			SW-163	20	BGC0000434
			Triostin A	20	BGC0000450
			Thiocoraline	20	BGC0000445
			Skyllamycin	20	BGC0000429
			Incednine	20	BGC0000078
NRPS	1	9861	Marformycins	12	BGC0001214
			Laspartomycin	16	BGC0000379
			Friulimicin	16	BGC0000354
			Telomycin	16	BGC0001406
			Skyllamycin	16	BGC0000429
			Lipopeptide 8D1-1/ lipopeptide 8D1-2	16	BGC0001370
			Griselimycin	12	BGC0001414
			Calcium-dependent antibiotic	16	BGC0000315
			Feglymycin	16	BGC0001233
			Enduracidin	16	BGC0000341

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Table 4.37, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
Ectoine	19552	29953	Ectoine	75	BGC0000853
			Ectoine	50	BGC0000852
			Ectoine	50	BGC0000858
			Ectoine	50	BGC0000860
Siderophore	29274	41199	Desferrioxamine B	80	BGC0000941
			Desferrioxamine B	80	BGC0000940
Terpene	1	12847	Isorenieratene	85	BGC0000664
			Carotenoid	85	BGC0000649
			Isorenieratene	85	BGC0001227
			Carotenoid	85	BGC0000633
			Sioxanthin	28	BGC0001087
Terpene	1	7277	2-methylisoborneol	75	BGC0000657

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Table 4.37, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
Other	1	24074	Avermectin oleandrose	37	AB032523_1_c1
			Vancomycin glucose/ vancosamine	37	HE589771_4_c4
			Balhimycin glucose/ vancosamine	37	Y16952_4_c4
			Staurosporine deoxysugar	37	AB088119_2_c2
			Polyketomycin beta-D-amicetose/ alpha-L-axenose	37	FJ483966_3c3
			Granaticin deoxysugar	37	GU233672_2_c2
			Rubradirin nitrosugar	25	AJ871581_2_c2
			Chlorothricin D-olivose	25	DQ116941_2_c2
			Nogalamycin nogalamine	25	AF187532_1_c1
			Streptolydigin L-rhodinose	25	FN433113_2_c2
Other	1	4737	Kutznerides	6	BGC0000378

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Table 4.38: Prediction of biosynthetic gene clusters predicted from the novel strain TPS3 using antiSMASH server.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
trans-AT PKS	1522	51373	Dorrigocin/ migrastatin	45	BGC0000177
			Lactimidomycin	45	BGC0000083
			Viguiepinol	27	BGC0000286
			9- methylstreptimidone	27	BGC0000171
			Pactamycin	18	BGC0000119
			Pristinamycin	18	BGC0000952
			Cycloheximide/ actiphenol	18	BGC0000175
			Sorangicin	18	BGC0000184
			Etnangien	18	BGC0000179
T1 PKS-Butyrolactone – NRPS	1	44208	RK-682	45	BGC0000140
			Friulimicin	27	BGC0000354
			Maklamicin	45	BGC0001288
			Chlorothricin	45	BGC0000036
			Kijanimicin	36	BGC0000082
			Lobophorin	36	BGC0001004
			Lobophorin	36	BGC0001183
			Abyssomicin	45	BGC0000001
			Quartromicin	36	BGC0000133
			Tetronomycin	36	BGC0000164

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Table 4.38, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
T2 PKS- Other KS	1	33437	Granaticin T2 PKS	50	GU233672_1_c1
			Medermycin T2 PKS	33	AB103463_1_c1
			TLN-05220	66	BGC0001062
			Lysolipin	43	BGC0000242
			Hexaricin	43	BGC0001376
			FD-594	43	BGC0000222
			Rubromycin	43	BGC0000266
			A-74528	40	BGC0000190
			Xantholipin	50	BGC0000279
			Griseorhodin	36	BGC0000230
			Pradimicin	30	BGC0000256
			Fredericamycin	36	BGC0000224
T2 PKS – T1 PKS	1	39379	Simocyclinone T2 PKS	50	AF324838_1_c1
			Landomycin T2 PKS	50	AF080235_1_c1
			Polyketomycin T2 PKS	33	FJ483966_1_c1
			Curamycin	58	BGC0000215
			Spore pigment	41	BGC0000272
			Xantholipin	41	BGC0000279
			Pradimicin	41	BGC0000256

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Table 4.38, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
			Rubromycin	41	BGC0000266
			Hexaricin	41	BGC0001376
T2 PKS – T1 PKS	1	39379	Griseorhodin	41	BGC0000230
			Frankiamicin	41	BGC0001197
			Arixanthomycin	41	BGC0000200
			Stambomycin	32	BGC0000151
			Meridamycin	12	BGC0001011
			Meridamycin	12	BGC0001013
			Nocardiopsin	12	BGC0001195
			JBIR-100	8	BGC0001348
T1 PKS	1	36828	Pladienolide	12	BGC0000126
			Aculeximycin	20	BGC0000002
			Nystatin-like Pseudonocardia polyene	16	BGC0000116
			PM100117/ PM100118	20	BGC0001359
			ECO-02301	24	BGC0000052
			Bafilomycin	11	BGC0000028
T1 PKS	1	10541	Indanomycin	22	BGC0000079
			Midecamycin	9	BGC0000096

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Table 4.38, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
T1 PKS	1	26390	Angolamycin	80	BGC0000018
			Nigericin	180	BGC0000114
			Nemadectin	100	BGC0000109
			Pimaricin	100	BGC0000125
			Leucanicidin	80	BGC0001232
			Rifamycin	80	BGC0000137
			Meridamycin	60	BGC0001012
			Macbecin	60	BGC0000090
			Rifamycin	80	BGC0000136
			Lankamycin	60	BGC0000085
			Candicidin	23	BGC0000034
			FR-008	23	BGC0000061
			ML-449	23	BGC0000097
T1 PKS	1	16911	Maklamicin	19	BGC0001288
			Napthomycin	23	BGC0000106
			Streptazone E	14	BGC0001296
			Thuggacin	14	BGC0001342
			Monensin	19	BGC0000100
			Phoslactomycin B	14	BGC0000123

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Table 4.38, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
Butyrolactone	1	6007	Granaticin	8	BGC0000227
			Erdacin	5	BGC0000221
			Alnumycin	5	BGC0000195
			Aranciamycin	5	BGC0000197
			Steffimycin	5	BGC0000273
			Pyralomicin	5	BGC0001038
Ectoine	1	8444	Ectoine	100	BGC0000853
			Ectoine	50	BGC0000858
			Ectoine	50	BGC0000852
			Ectoine	50	BGC0000854
NRPS	24012	56403	Mithramycin	5	BGC0000247
			Saframycin A	5	BGC0000422
			Lankamycin	5	BGC0000085
			Lactonamycin	5	BGC0000238
			Xantholipin	5	BGC0000279
NRPS	1	20251	Scabichelin	30	BGC0000423
			Albachelin	30	BGC0001211
			Griseoviridin/ viridogrisein	20	BGC0000459
			Lipopeptide 8D1-1/ lipopeptide 8D1-2	30	BGC0001370

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Table 4.38, continued.

Type	From (nt)	To (nt)	Most similar known biosynthetic gene cluster	Similarity (%)	MIBiG BGC-ID
NRPS	1	20251	Telomycin	30	BGC0001406
			Griselimycin	20	BGC0001414
			Marfomycins	20	BGC0001214
			Skyllamycin	20	BGC0000429
			Laspartomycin	20	BGC0000379
Terpene	1	15997	Kanamycin	8	BGC0000705
			Kanamycin	8	BGC0000704
Terpene	30249	46145	Kanamycin	3	BGC0000703
Terpene	1	10930	2-methylisoborneol	100	BGC0000658
			Rishirilide B	100	BGC0001179
Terpene	4147	23175	Hopene	53	BGC0000663
			Phosphonoglycans	15	BGC0000807
Terpene	1	16160	Lipopeptide 8D1-1/ lipopeptide 8D1-2	4	BGC0001370
Bacteriocin	235083	245298	Informatipeptin	28	BGC0000518
Siderophore	2823	14592	Desferrrioxamine B	100	BGC0000940
			Desferiioxamine B	83	BGC0000941
Other	1	26053	Borrelidin	9	BGC0000031

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4.7 Characterisation of antibacterial compound from strain TPS83

The strains TPS16, TPS81 and TPS83 are putatively identified as a novel genus of the family *Nocardiopsaceae* (see sub-chapter 4.5.1). These strains were the only actinobacterial isolates derived from marine sediment samples in this study that exhibited significant antibacterial activity against the Gram- negative *E. coli* ATCC 47076 as tested by agar plug diffusion assay, in addition to *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213, when grown on soybean meal glucose medium (Table 4.15). Based on novelty and spectrum of activities, strains TPS16, TPS81 and TPS83 were of particularly interest for further antibacterial activity study. Since the three novel strains that were cultured on soybean meal agar medium inhibited the same number and same type of pathogens during agar plug diffusion assay, the strain TPS83 was randomly selected over the other two strains for further chemistry study as they are clonal strains and thus they should share an identical chemistry profile.

The strains TPS83 was cultured in 1 L SBM broth for 7 to 28 days. The biomass was harvested on 7-days basis. The 7 days culture did not produce antibacterial activity against all 15 pathogens. The 14- and 21- days culture produce antibacterial activity against *B. subtilis* ATCC 23857, *E. faecium* ATCC 6569, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA-44 (MRSA), *S. epidermidis* ATCC 14990^T and *P. alcalifaciens* ATCC 9886^T. The crude extract from different period of culture was standardized to 20 mg/ ml and 20 µl of each extract was injected into HPLC. Chromatograms obtained from three batches of extracts were compared by using major peaks 1, 2 and 3 as reference (Figure 4.61). The 21 days culture was thus selected based on AUC.

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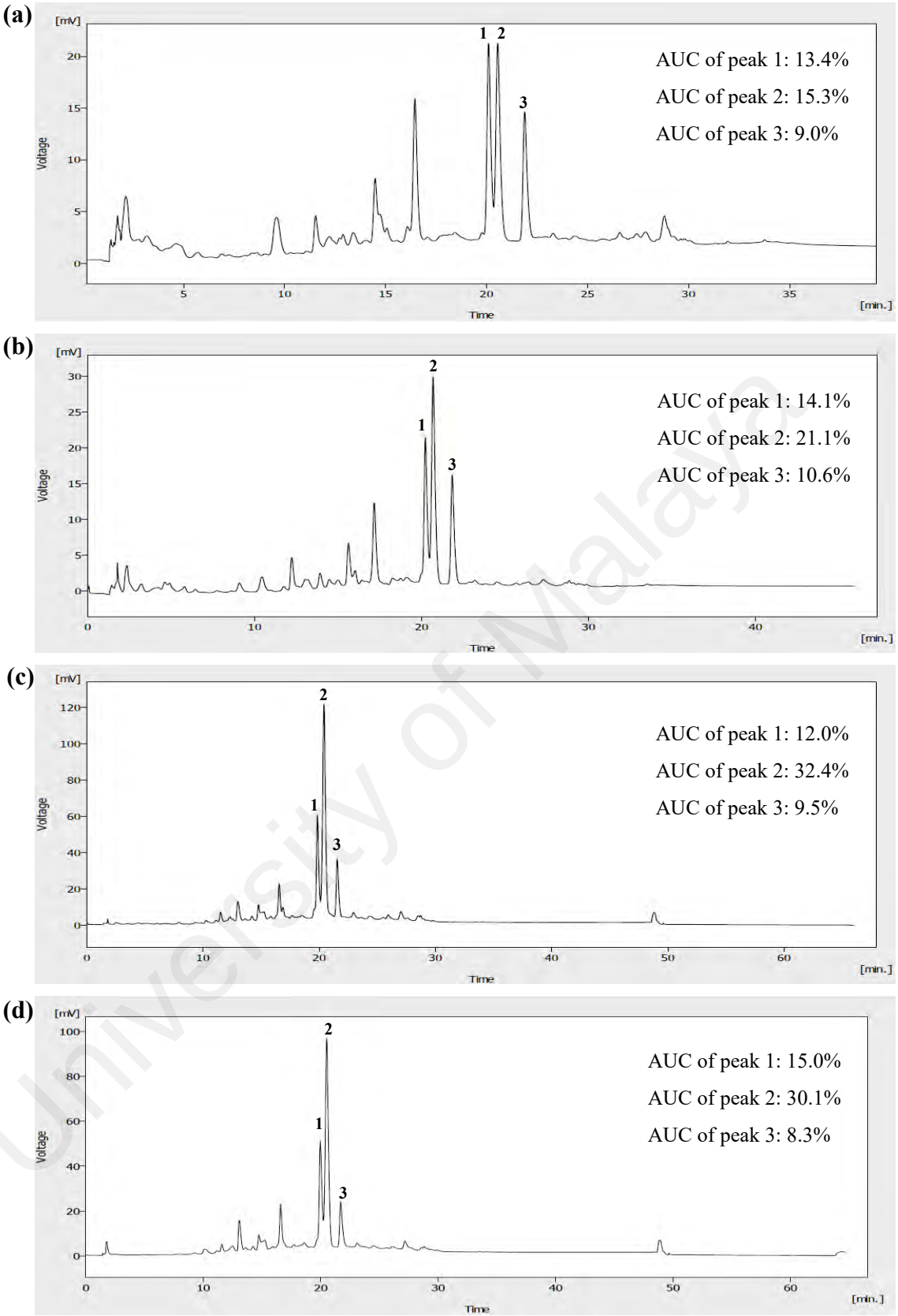

Figure 4.61: HPLC profiles of crude extracts of strain TPS83 at different period of incubation: (a) 7 days, (b) 14 days, (c) 21 days and (d) 28 days. AUC of major compounds 1, 2 and 3 were stated for extracts obtained from 7 days, 14 days, 21 days and 28 days old broth.

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A total of 30 L of soybean meal broth (21 days old) was extracted with DCM: MeOH in 50:50 ratio, which gave 42.93 g of crude extract. A portion of the crude extract, which was weighed 22.01 g was subjected for SPE fractionation. Fractionation of crude extract with SPE tubes using various MeOH concentration resulted in five fractions: Fraction A, B, C, D and E, which were eluted with 20%, 40%, 60%, 80% and 100% MeOH, respectively, yielded SPE fractions with 3.50 g, 4.50 g, 3.70 g. 3.10 g and 2.42 g. Final elution was performed using 100% EtoAc and resulted in 1.79 g of fraction F. Total extract eluted from SPE was 19.01 g. The remaining 3 g of extracts were solids that do not dissolve in 20% and above of MeOH solution.

The SPE fractions were tested for antibacterial activity. The MIC values of each fraction were listed in Table 4.39. All six fractions showed positive antibacterial activity against the Gram-positive *S. epidermidis* ATCC 14990^{T} and the Gram- negative *P. alcalifaciens* ATCC 9886^{T}. Fraction D showed positive antibacterial activity against *B. subtilis* ATCC 23857, *E. faecium* ATCC 6569, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA-44 (MRSA), *S. epidermidis* ATCC 14990^{T} and *P. alcalifaciens* ATCC 9886^{T}. The antibacterial activity profile of Fraction D was presented as histogram that contained BioMap values against pathogens (Figure 4.62). The activity profile of Fraction D was aligned with reference antibiotics based on the normalized MIC values, using Pearson correlation similarity metrics and average linkage clustering. Fractions A, B, C, D, E and F and fosfomycin produced a separate cluster from reference antibiotics. Fraction D formed a separate sub- cluster from fraction A, B, C, E and F and fosfomycin (Figure 4.63). The MIC and BioMAP values of reference antibiotics were given in Appendix N and O, respectively.

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Fraction D (1.423 g) was divided into three batches (B1, B2 and B3) and subjected for purification on Sephadex LH-20 using MeOH (Table 4.40). Purification of B1 resulted in 25 sub- fractions. All fractions were screened for antibacterial activity against *S. aureus* ATCC 29213, MRSA and *P. alcalifaciens* ATCC 9886^T. Six fractions (Fr. 7- 12) were combined based on TLC profile and antibacterial activity against *S. aureus* ATCC 29213 and MRSA. Purification of B2 led to production of 33 sub-fractions. Fraction 7 was able to inhibit the growth of *P. alcalifaciens* ATCC 9886^T. Five fractions (Fr. 8- 12) showed positive inhibitory activity against *S. aureus* ATCC 29213 and MRSA. A total of 27 sub- fractions were produced from B3, in which Fr. 7 inhibited the growth of *P. alcalifaciens* ATCC 9886^T and Fr. 8- 9 inhibited the growth of *S. aureus* ATCC 29213 and MRSA.

Fractions including Fr. 7- 12 from B1, Fr. 9- 12 from B2 and Fr. 8- 9 from B3 were combined as one fraction, which was labelled as 4_C1 (Figure 4.64, Appnedix P). Further purification of Fr. 4_C1 results in nine sub- fractions. Fr. 4_C1_3, 4_C1_4 and 4_C1_5 were combined and fractionated to give 10 fractions. Fr. 3-5_3, Fr. 3-5_4, Fr. 3-5_5 and Fr. 3-5_6 were combined and purified on Sephadex LH-20 eluted with MeOH. Three fractions: Fr. 3-6_2, Fr. 3-6_3 and Fr. 3-6_4 were combined and purified to obtain seven sub- fractions including fractions Fr. 2-4_2, Fr. 2-4_3, Fr. 2-4_4 and Fr. 2-4_5, which were combined and purified to give 13 fractions. A total of five fractions including Fr. 2-5_7, Fr. 2-5_8, Fr. 2-5_9, Fr. 2-5_10 and Fr. 2-5_11 were combined and purified to give nine fractions. The 4th and 5th fraction were combined as Fr. 7-11_4-5 and the 6th and 7th fraction were combined as Fr. 7-11_6-7. Both fractions, Fr. 7-11_4-5 and Fr. 7-11_6-7, were purified on analytical HPLC to give compound 1 (3.0 mg) and compound 2 (8.8 mg) via isocratic separation on HPLC as described in section 3.15. The eluted fractions containing both compounds were tested for antibacterial activity. The fraction containing compound 1 was tested negative for antibacterial activity, by

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contrast, the fraction containing compound 2 was observed to exhibit antibacterial activity against *B. subtilis* ATCC 23857, *E. faecium* ATCC 6569, *S. aureus* ATCC 29213, MRSA and *S. epidermidis* ATCC 14990^{T}.

Table 4.39: The MIC values of the TPS83 SPE fractions against *P. alcalifaciens* ATCC 9886^{T}, *B. subtilis* ATCC 23857, *E. faecium* ATCC 6569, *S. aureus* ATCC 29213 and *S. aureus* ATCC BAA-44 (MRSA) and *S. epidermidis* ATCC 14990^{T}. Fraction without activity towards tester pathogens was indicated as MIC value > 100 mM.

Fractions/ antibiotics	**MIC (mM)**					
	***P. alcalifaciens* ATCC 9886^{T}**	***B. subtilis* ATCC 23857**	***E. faecium* ATCC 6569**	***S. aureus* ATCC 29213**	**MRSA**	***S. epidermidis* ATCC 14990^{T}**
A	25.0	> 100	> 100	> 100	> 100	6.25
B	0.78	> 100	> 100	> 100	> 100	6.25
C	3.12	> 100	> 100	> 100	> 100	3.12
D	6.25	100.0	100.0	100.0	100.0	25.0
E	6.25	> 100	> 100	> 100	> 100	50.0
F	25.0	> 100	> 100	> 100	> 100	100.0

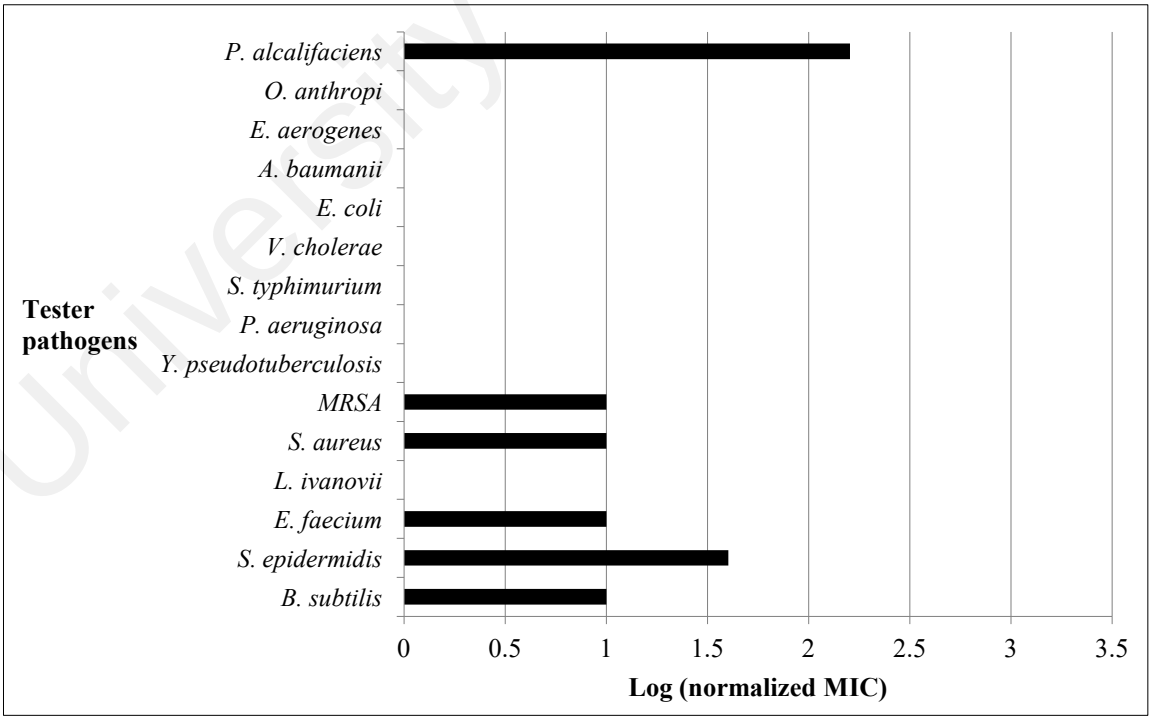

Figure 4.62: The antibiotic profile of fraction D, eluted with 80% MeOH through 5 g SPE tube. The log normalized MIC values were plotted against tester pathogens.

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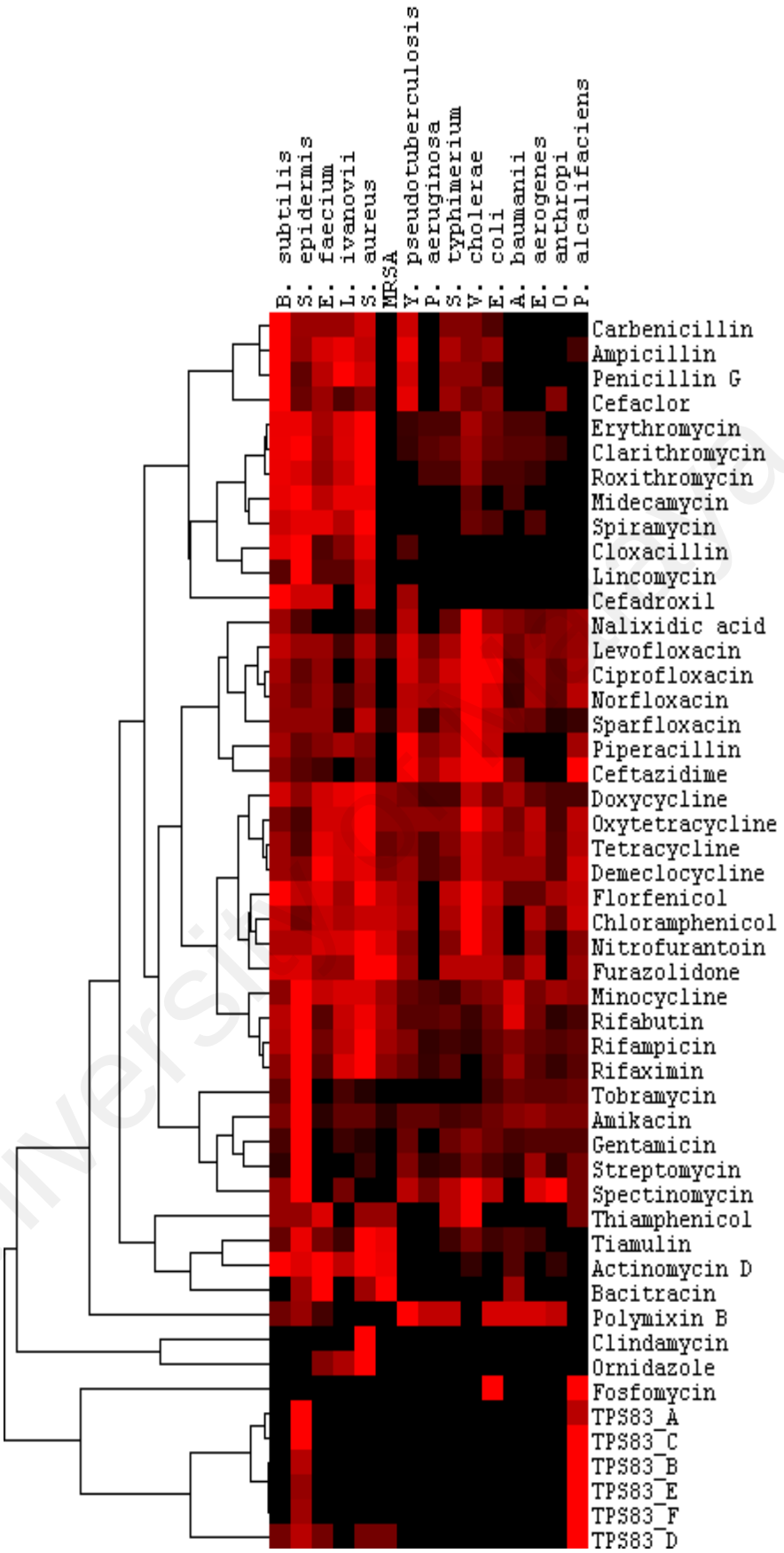

Figure 4.63: Hierarchical clustering of active fractions with reference antibiotics based on normalized MIC values revealed a single and distinct cluster of all TPS83 crude fractions, which was closely related to fosfomycin based on the activity profile. Potency of fractions was represented by a red–black colour scheme: inactive (black) and most active (red).

Table 4.40: Antibacterial activity of strain TPS83 of sub- fractions derived from three batches of Fraction D, eluted with MeOH through Sephadex LH-20 column.

Batch number	Fractions (Fr.)	Weight (mg)	Targeted pathogens
B1	1	1.2	No activity
	2	66.9	No activity
	3	23.3	No activity
	4	10.0	No activity
	5	58.0	No activity
	6	38.3	*S. aureus* ATCC 29213, MRSA, *P. alcalifaciens* ATCC 9886^T
	7 - 12	54.5	*S. aureus* ATCC 29213, MRSA
	13	40.2	No activity
	14- 18	18.3	No activity
	19	-	No activity
	20	-	No activity
	21	-	No activity
	22	0.1	No activity
	23	-	No activity
	24- 25	-	No activity
B2	1	71.8	No activity
	2	19.4	No activity
	3	23	No activity
	4	10.2	No activity
	5	11.1	No activity
	6	25.2	No activity
	7	16.3	*P. alcalifaciens* ATCC 9886^T
	8	23.3	*S. aureus* ATCC 29213, MRSA
	9 – 12	62.2	*S. aureus* ATCC 29213, MRSA
	13 – 14	7.3	No activity
	15 – 17	33.7	No activity
	18	16.2	No activity
	19 – 20	12.2	No activity
	21	2.2	No activity
	22- 23	3.1	No activity
	24	3.4	No activity
	25 – 26	1.8	No activity
	27 – 28	2.5	No activity
	29 – 30	2.5	No activity

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Table 4.40, continued.

Batch number	Fractions (Fr.)	Weight (mg)	Targeted pathogens
B2	31	0.9	No activity
	32 – 33	-	No activity
B3	1	95.9	No activity
	2	30.3	No activity
	3	22.4	No activity
	4 - 5	43.1	No activity
	6	52.1	No activity
	7	65.4	*P. alcalifaciens* ATCC 9886^T
	8 – 9	64.7	*P. alcalifaciens* ATCC 9886^T, *S. aureus* ATCC 29213, MRSA
	10	5.2	No activity
	11	2.9	No activity
	12 – 14	73.5	No activity
	15	28.9	No activity
	16	8.2	No activity
	17 – 18	12.3	No activity
	19 – 20	3.7	No activity
	21	1.9	No activity
	22	1.7	No activity
	23	1.6	No activity
	24	0.8	No activity
	25	0.9	No activity
	26	0.8	No activity
	27	1.2	No activity

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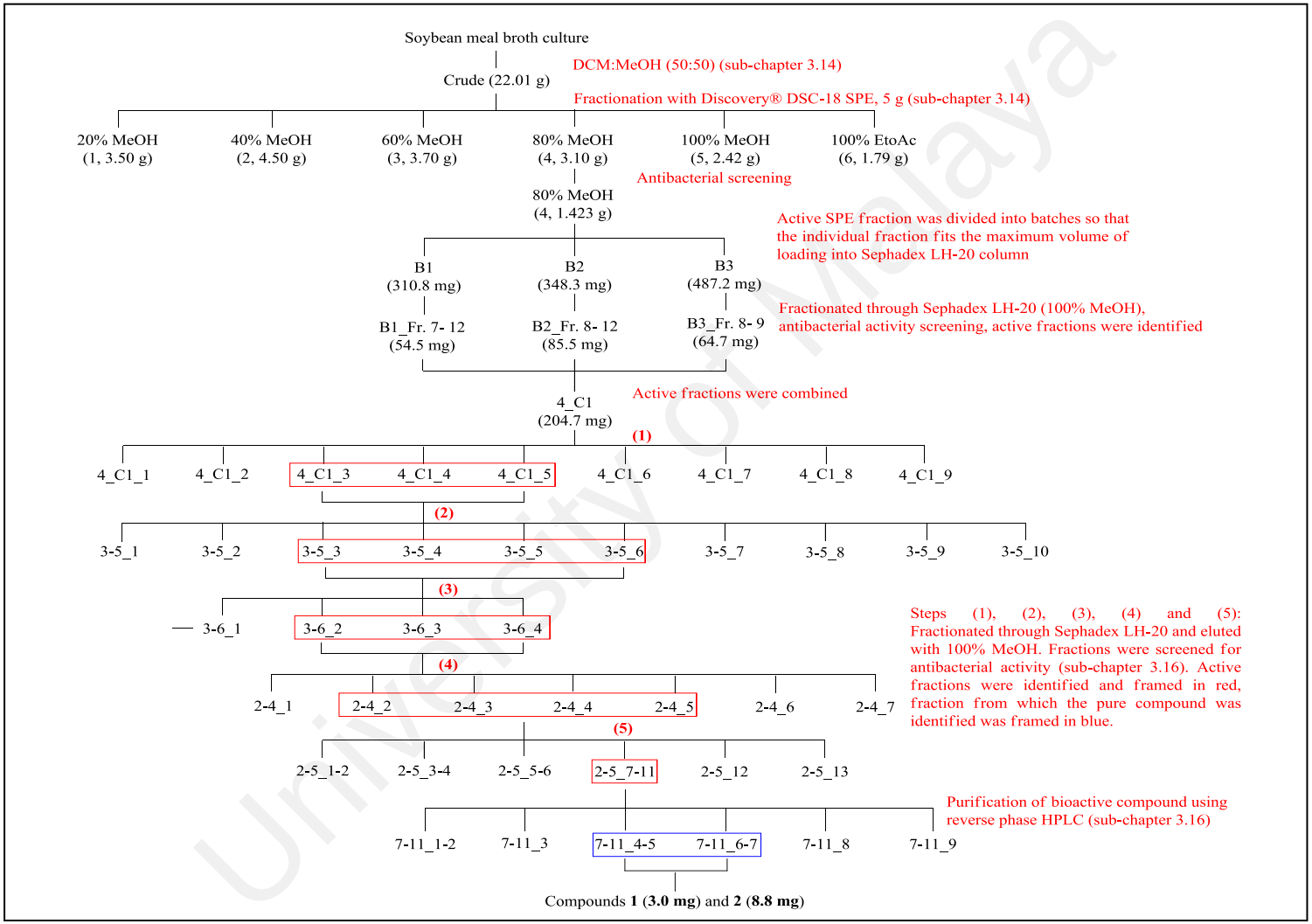

Figure 4.64: Summary chart on purification of compounds 1 and 2 from Fraction D on Sephadex LH-20 and subsequent HPLC- UV guided fractionation.

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Two compounds: nocapyrone R (1) and methylpendolmycin (2) were identified. Both compounds were purified from the fractions 7-11_4-5 and 7-11_6-7 on HPLC. The two major peaks indicated the presence of compounds 1 and 2 as shown in Figure 4.65 were collected manually in test tubes and subjected for NMR and HRDARTMS analyses. The HRDARTMS of compound 1 from TPS83 obtained on JEOL-AccuTOF-DART mass spectrometer showed the presence of pseudomolecular ion at m/z 253.1918 $[M + H]^+$, where an $[M + H]^+$ peak was shown at m/z 384.2761 for compound 2. The retention time for compound 1 (nocapyrone R) and compound 2 (methylpendolmycin) was 10.44 minutes and 14.46 minutes, respectively (Figure 4.65). The UV absorption of both compounds 1 and 2 were measured at a wavelength of 229 nm (Figure 4.65). Details on the spectroscopic data of both compounds were presented in Tables 4.41 and 4.42. The ^{1}H NMR spectra were given in Figures 4.66 and 4.67. The ^{13}C NMR spectra including the extended spectra of both compounds were given in appendices Q and R, whereas appendices S and T contained the extended spectra of ^{1}H NMR of compounds 1 and 2. Confirmation of the chemical structure of compound 2 was also performed with 2D NMR experiments including COSY and HSQC (Appendices U and V).

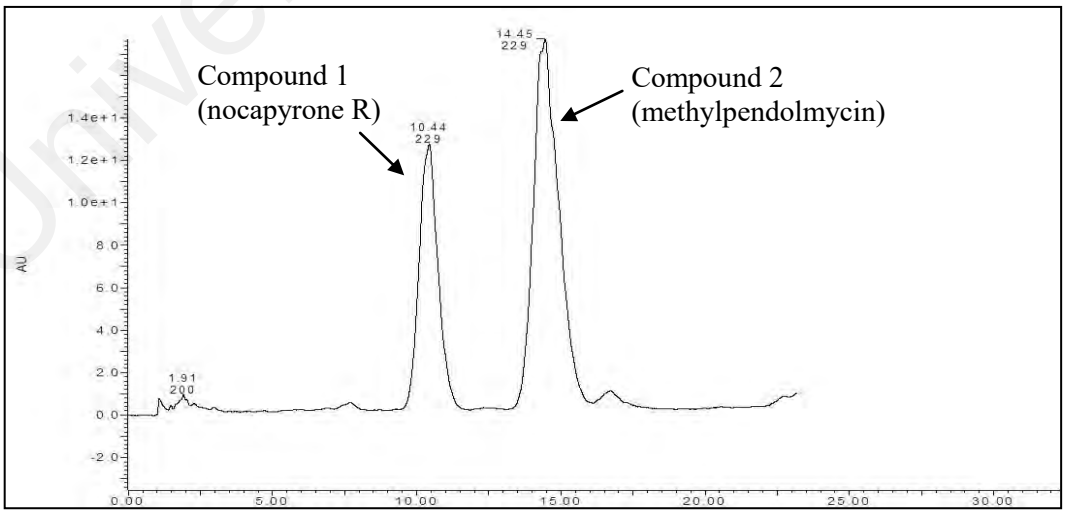

Figure 4.65: The HPLC profiles of compound 1 (nocapyrone R) and compound 2 (methylpendolmycin). Measurement of the intensity of absorbance (AU) was plotted against time (minutes). This is an isocratic separation using a solvent system containing water/ methanol (30:70) for 20 minutes on an analytical reverse phase HPLC with an ODS column (Phenomenex®, Synergi™, 250 × 4.6 mm, 10 µm).

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Table 4.41: The ^{1}H and ^{13}C NMR spectroscopic data for compound 1 (Nocapyrone R).

Position	**δ_H, mult. (*J* in Hz)**	**δ_C type**
1		166.6, C
2		109.2, C
3		168.5, C
4		109.1, C
5		159.3, C
6	2.49, triplet (7.7)	30.9, CH_2
7	1.62, multiplet	27.5, CH_2
8	1.30, multiplet	26.9, CH_2
9	1.19, multiplet	38.5, CH_2
10	1.52, multiplet	27.8, CH
11	0.86, doublet (6.5)	22.6, CH_3
12	0.86, doublet (6.5)	22.6, CH_3
13	2.03, singlet	10.0, CH_3
14	3.80, singlet	60.2, CH_3
15	1.92, singlet	9.9, CH_3

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Table 4.42: The ^{1}H and ^{13}C NMR spectroscopic data for compound 2 (Methylpendolmycin).

Position	δ_H, mult. (*J* in Hz)	δ_C, type
2	6.83, singlet	121.08, CH
3		114.09, C
3a		118.63, C
4		146.52, C
5	6.69, doublet (8.0)	106.20, CH
6	7.00, doublet (8.0)	119.12, CH
7		122.74, C
7a		137.46, C
8	3.17, multiplets	33.97, CH_2
	3.02, multiplets	
9	4.32, multiplets	55.63, CH
11		174.01, C
12	4.45, doublet (10.3)	69.42, CH
14	3.74, doublet of doublets (11.2, 3.6)	65.16 CH_2
	3.56, multiplets	
15	2.40, multiplets	34.31, CH
16	1.42, multiplets	24.57, CH_2
	0.65, multiplets	
17	0.60, triplet (7.0)	10.34, CH_3
18	0.92, doublet (6.5)	17.26, CH_3
19		40.14, C
20	6.22, doublet of doublets (17.5, 10.6)	149.53, CH
21	5.31, doublet (17.5)	111.31, CH_2
	5.22, doublet (10.6)	
22	1.48, singlet	27.22, CH_3
23	1.52, singlet	26.79, CH_3
24	2.90, singlet	33.17, CH_3

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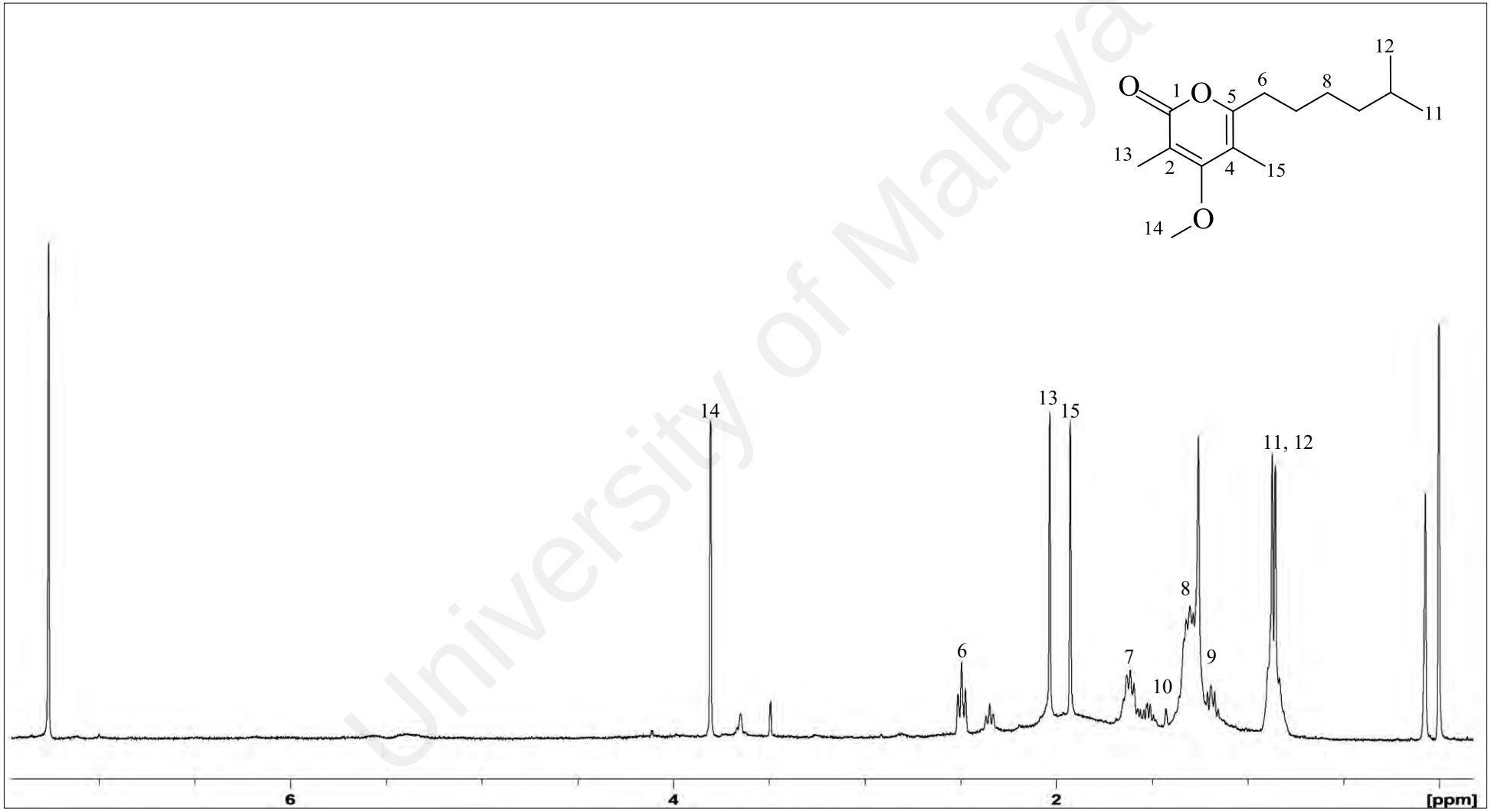

Figure 4.66: ^{1}H NMR Spectrum ($CDCl_3$, 400 MHz) of compound 1 (nocapyrone R).

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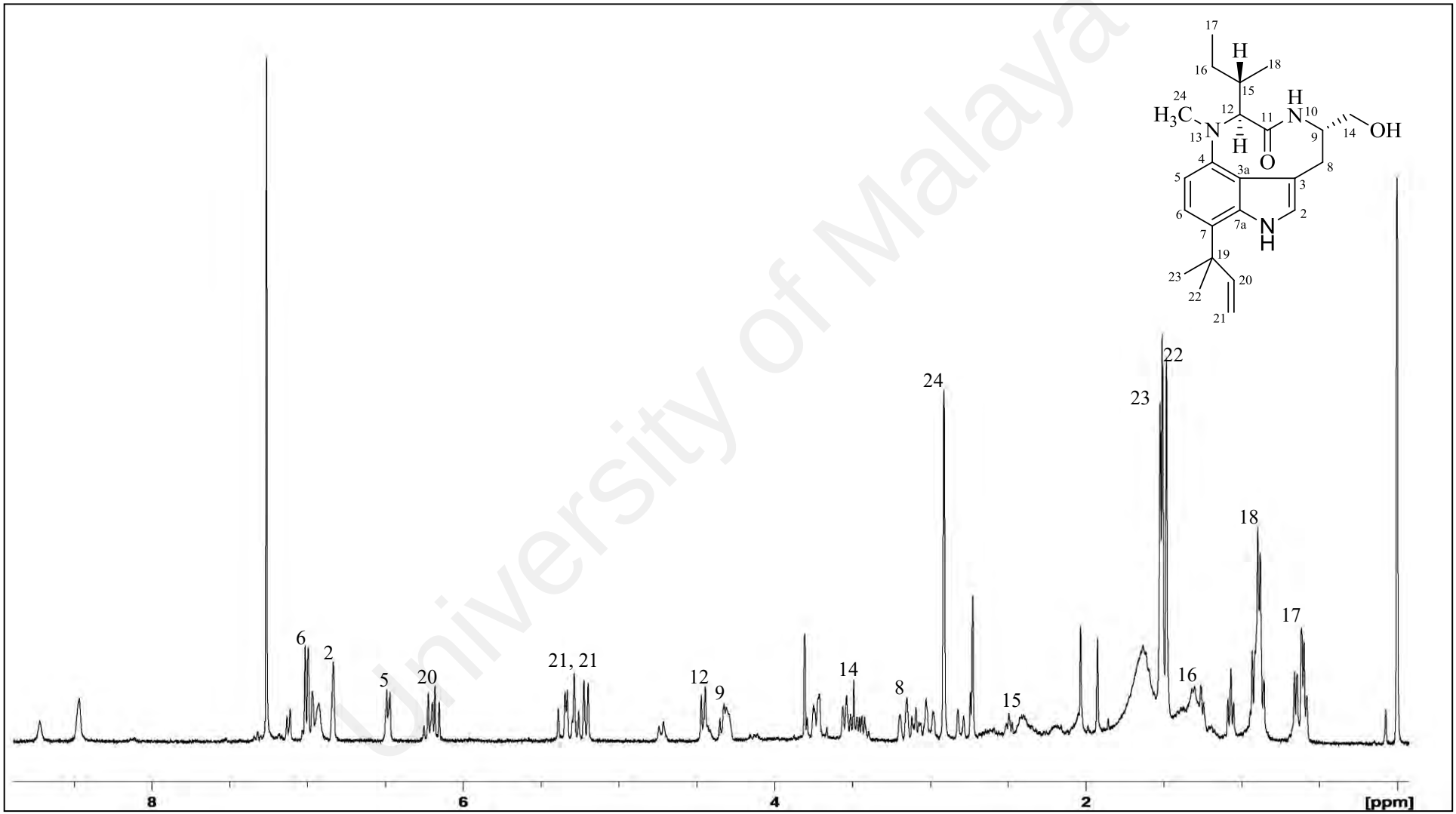

Figure 4.67: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of compound 2 (methylpendolmycin).

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CHAPTER 5: DISCUSSION

5.1 Pre-treatments and selective isolation media

Actinobacteria had been reported to be isolated from wet marine sediment samples using various pre-treatment methods (Mincer *et al.*, 2002; Maldonado *et al.*, 2005b; Bredholdt *et al.*, 2007). The obligate marine actinobacteria *Salinispora* spp. were first isolated from wet marine sediment samples using the dilution-and-heat-shock method and stamping method, in addition to *Micromonospora* spp. (Mincer *et al.*, 2002). Later reports also demonstrated successful isolation of actinobacteria from wet marine sediment samples including members of *Actinomadura*, *Dietzia*, *Gordonia*, *Microbacterium*, *Mycobacterium*, *Nocardiopsis*, *Pseudonocardia*, *Saccharopolyspora*, *Streptosporangium*, *Nonomuraea*, *Verrucosispora*, *Williamsia, Rhodococcus*, *Nocardia*, *Knoellia* and *Glycomyces* (Bredholdt *et al.*, 2007; Maldonado *et al.*, 2005b). Presence of actinobacteria in wet sediment was also evidenced by the isolation of *Streptomyces* spp., *Nocardiopsis* spp., *Promicromonospora* spp. and *Actinoalloteichus* spp. from temperate marine sediment of Newfoundland, Canada (Duncan *et al.*, 2014).

In this study, diverse marine actinobacteria were isolated from marine sediment samples. High putative actinobacterial count for marine sediment sample Z was recorded from HVA medium and modified M2 medium coupled to skim milk/HEPES treatment. HVA medium was shown to improve isolation of non-*Salinispora*-like actinobacterial strains. Humic acid is a sole carbon and nitrogen source that had been proved to activate spore germination by actinobacteria including *Streptomyces* spp. and various rare actinobacteria, while reducing growth of non-filamentous bacteria colonies (Hayakawa, 2008). Successful isolation of actinobacteria with antibacterial activity from marine sediment samples using humic acid vitamin agar had been demonstrated previously (Xiong *et al.*, 2015). By comparison, most of the *Salinispora*-like actinobacterial strains were isolated using modified M2, a mannitol-based medium

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designed for this study. Isolation medium with low concentrations of mannitol had been reported to produce high percentage of sea water requiring strains including *Salinispora* spp. from marine sediment (Jensen *et al.*, 2005).

On all isolation media, skim milk/HEPES treatment couple to centrifugation at 1000 × g was shown to improve isolation of marine actinobacteria from wet sediment sample Z. Low total bacterial count but high actinobacterial count was observed for plates containing skim milk treated sediment samples (Table 4.1). Skim milk was shown to be an effective agent for selective isolation of zoosporic actinobacteria. Suzuki *et al.* (1999) demonstrated that skim milk as flooding solution recovered high percentage of zoosporic actinobacteria. Flooding solution containing 0.1% skim milk stimulated motility of certain zoosporic actinobacteria including *Actinoplanes* spp., *Planobispora* spp. and *Planomonospora* spp. By coupling skim milk treatment to centrifugation at 1000 to 1500 × g, non-motile actinobacteria and *Streptomyces* spp. were eliminated and therefore facilitating isolation of rare actinobacteria (Hayakawa, 2008; Suzuki *et al.*, 1999). HVB enrichment of skim milk/HEPES treated sediment sample Z was observed to encourage the growth of higher population of fast growing non-actinobacterial strains. Thus, the recovery rate of actinobacteria was much lower compared to the skim milk/HEPES pre-treatment without enrichment in HVB.

UV treatment recovered low number of actinobacterial isolates from wet sediment Z, in which only two actinobacterial isolates were recovered and fast growing bacteria were found to dominate isolation plates that contained UV treated sediment sample Z. Both of the actinobacterial isolates from UV treated sediment sample Z were observed as irregularly shaped coccoid cell aggregates when examined under a light microscope. Highly UV resistant actinobacteria belong to genera *Arthrobacter*, *Curtobacterium*, *Geodermatophilus* and *Cellulomonas* had been isolated from desert rock varnish

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samples treated with UV irradiation, which were found to appear in clusters or as aggregates (Kuhlman *et al.*, 2005). Aggregated cells of *Geodermatophilus* species was reported to require larger doses of UV-C to fully inactivate the cells (Paulino-Lima *et al.*, 2016). Although actinobacteria was known to be resistant to UV irradiation, *Gammaproteobacteria* including *Pseudomonas* sp., *Vibrio* sp., *Spongiibacter* sp., *Pseudoalteromonas* sp. and *Halomonas* sp. was also reported to be UV- resistant group in aquatic environments (Kviatkovski *et al.*, 2018; Santos *et al.*, 2013). In addition, *Alphaproteobacteria* including *Sphingomonas* sp., *Erythrobacter* sp. and *Phaeobacter* sp. from waters of estuarine system and marine environment also showed high resistance to UV irradiation.

In contrast, UV treatment was observed to eliminate fast growing bacteria and facilitated isolation of actinobacteria from wet sediment M. High actinobacterial count, especially of the *Salinispora*- like isolates, was observed from UV treated sediment sample M couple to the use of modified M2 as compared to heat treatment and samples without treatment. UV irradiation at 254 nm, or UV-C irradiation had been reported to inhibit growth of genera that are sensitive to radiation and thus facilitated isolation of actinobacteria (Bredholdt *et al.*, 2007). UV-C radiation causes oxidative damage to lipids and proteins, as indicated by the presence of high level of thio- barbituric acid reactive substances and carbonyls in the previous report (Santos *et al.*, 2013). In addition, DNA strand breakage was also induced by UV-C irradiation among radiation sensitive strains (Santos *et al.*, 2013). Actinobacteria survived UV radiation via few mechanisms including (a) production of mycosporines and mycosporine-like amino acids, which are the UV absorbing compounds as observed in *Pseudonocardia* sp. and *Actinosynnema* sp. (Miyamoto *et al.*, 2014), (b) accumulation of triacylglycerol, which in turn contributes to increasing production of cellular NADPH to maintain effectiveness of oxidative stress protecting enzymes, as reported for *Rhodococcus* sp.

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(Bequer Urbano *et al.*, 2013), (c) accumulation of high ration of intracellular Mn^{2+}/ Fe, where Mn^{2+} ions interact synergistically with small molecule metabolites present in cells such as orthophosphates to boost protein protection as demonstrated by *Geodermatophilus* sp. (Paulino-Lima *et al.*, 2016).

Only *Salinispora*-like actinobacterial isolates were successfully recovered from wet sediment sample M by heat treatment. Actinobacterial counts of *Salinispora*- like isolates on HVA plates were 2- folds and 3- folds higher than modified M2 and M3 plates, respectively. Growth of *Salinispora arenicola* colonies from heat- treated spores at 45- 55ºC for 10 min had been reported, and the authors reported no growth of colonies when spores were heat treated at 58- 70ºC (Ng *et al.*, 2013). In this study, *Salinispora*- like colonies were isolated from sediment samples treated at 65ºC for 30 min. Heat treatment was well- known to be an effective way to isolate *Micromonospora* spp. from soil and sediment samples, which belong to the *Micromonosporaceae* family as *Salinispora* spp. Previous study demonstrated that treatments of estuarine sediment samples at 65ºC drastically reduced the number of *Streptomyces* spp. colonies and increased the number of *Micromonospora* spp. colonies on isolation plates (Terahara *et al.*, 2013). Treatment of spores at 70ºC for 10 min in phosphate buffer had been reported to increase the number of culturable *Micromonospora* spp. colonies by five-folds, suggesting that a large proportion of *Micromonospora* spp. spores were constitutively dormant (Hoskisson *et al.*, 2000). Although details of mechanism are still remained unknown, heat treatment at 70ºC for as short as 10 min was found to stimulate spore respiration by utilizing endogenous storage compounds, especially when an external energy- yielding substrate was absent (Hoskisson *et al.*, 2000).

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Both sediment samples Z and M were collected at different depth but at the same location (Pirate Reef, Tioman Island), differed in that the former sediment contained white coarse sand, while the latter was mainly composed of brown fine sand. In addition, sediment samples Z and M were collected at two different expeditions. Total bacterial count of sediment Z was 10 × to 5000 × higher than the count of sediment M for untreated and UV- treated samples, depending on the pre-treatment methods and isolation media. Despite treating both sediment samples Z and M with UV in the same manner and isolation of actinobacteria was carried out on the same media, higher actinobacterial count was recorded from sediment M than sediment Z. Bredholdt *et al.* (2007) reported the difference in relative and absolute numbers of non- *Streptomyces* and non- *Micromonospora* actinomycetes isolated from two samples that were collected at approximately the same depth but few tens of meters apart at the same location, at which one of the sediments contained fine mud and clay, while the other was mainly composed of fine mud and sand, despites identical isolation techniques was employed for both samples. This observation suggests that knowledge on the logistics of sampling site is crucial for bioprospecting focused on actinobacterial counts and species.

Actinobacterial counts from both of the coral samples *Montipora* sp. and *Porites* sp. were far lower than the sediment samples Z and M. Only yellow colonies of non-*Salinispora*-like isolates were able to be recovered from the coral samples. On all isolation plates, the number of colony was less than 25, far more lesser than the minimum number of colonies to perform a CFU count, which in this study, the range of 25 to 250 colonies per plate was used. This suggested that the actinobacteria present in the both coral samples appears to be in low abundancy or at least more difficult to recover. Different isolation media and pre-treatment methods should be carried out for the isolation of actinobacteria from these coral samples.

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In this study, the actinobacterial strains isolated from *Porites* sp. on glycerol-arginine based M3 medium were identified to be closely related to *Kocuria rhizophila* DSM 11926^{T} and *Micrococcus yunnanensis* YIM 65004^{T}. Actinobacteria had been isolated from *Porites lutea* using marine agar, proline trehalose agar, yeast extract agar, raffinose histidine agar and pyruvic acid sodium asparagine agar, in which *Brevibacterium* spp., *Brachybacterium* spp., *Micromonospora* spp., *Mycobacterium* spp., *Gordonia* spp., *Nocardiopsis* spp., *Cellulosimicrobium* spp. and *Streptomyces* spp. were recovered from the coral tissue fragments (Li *et al.*, 2014). Actinobacteria including the *Kocuria* sp., *Brevibacterium* sp. and *Marmoricola* sp. were also isolated from *Porites harrisoni* using Reasoner's 2A agar, starch casein agar, M2 agar containing glycerol and arginine as carbon sources and M4 medium containing fucise and proline as carbon sources (Mahmoud & Kalendar, 2016).

The only actinobacteria isolated from coral *Montipora* sp. was closely related to *Kocuria rhizophila*, which was also the only bacterial isolate recovered from this coral using M3 medium. According to literatures, bacterial community in *Montipora capitata* corals was dominated by *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria* and *Firmicutes*, whereas *Actinobacteria* was one of the the minor phyla that represented <1% of sequences per fragment, according to the analsyis of high throughput sequencing results of the V3 hypervariable region of the 16S rRNA genes (Shore-Maggio *et al.*, 2015). The authors also worked on isolation of actinobacteria from the coral *M. capitata* using glycerol artificial sea water agar and the actinobacteria isolates were closely related to *Kocuria rosea* and *Micrococcus luteus*. An earlier report on diazotrophic bacteria associtae with *Montipora capitata* and *Montipora flabellata* also showed that *Gammaproteobacteria* was the dominant bacterial class and *Vibrio* spp. were the prevailing group in both coral species, as revealed by phylogenetic analysis of partial sequences of the nitrogenase gene *nifH* (Olson *et al.*, 2009). These

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previous reports supported the fact that actinobacterial community associate with *Montipora* corals was of low diversity and thus lead to recovery of low numbers of actinobacterial colonies.

5.2 Diversity of isolated actinobacteria of marine sediment samples

Analyses of fingerprinting profiles and 16S rRNA gene sequences of actinobacterial isolates from marine sediment sample Z revealed close relationships to members of 18 genera: *Actinomadura*, *Agromyces*, *Blastococcus*, *Jishengella*, *Marinactinospora*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardiopsis*, *Nonomuraea*, *Plantactinospora*, *Pseudonocardia*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Salinispora*, *Streptomyces*, and *Streptosporangium*. Almost half and a quarter of the total actinobacteria isolated were *Streptomyces* spp. (47.97%) and *Salinispora* spp. (23.58%), respectively. Shannon–Wiener index (H') for the skim milk/ HEPES pre-treated sample indicated that modified M2 medium and HVA recovered greater diversity of actinobacteria than M3. *Streptomyces* species were isolated in a high number on modified M2 and HVA media. Strains of *Salinispora* spp. and *Blastococcus* spp. were recovered exclusively from modified M2 medium regardless of pre-treatments. Genera that were also recovered from modified M2 medium included *Nonomuraea*, *Saccharomonospora*, *Nocardiopsis*, *Plantactinospora* and *Pseudonocardia*.

The fingerprinting profile was able to dereplicate actinobacterial isolates from sediment sample Z to the genus and species level. Two major clusters were obtained for the non-*Salinispora*-like group of actinobacterial isolates by de-replicating the actinobacterial isolates via double digestion approach. *Streptomyces* spp. was found to dominate the non-*Salinispora* group of isolates and they share more than 30 banding patterns, suggesting the high diversity between the actinobacterial isolates. Previous

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finding reported that the primer pair pA and BL235R worked well in targeting the highly variable 16S- 23S ITS region of 16S rRNA gene (Lanoot *et al.*, 2005). Double digestion of 16S-23S ITS region with tetra-cutter restriction enzymes BstU1 and HaeIII had been showed to work effectively in dereplication of *Streptomyces* spp. as both enzymes are rich in GC recognition sequence. The authors had successfully delineated 463 actinobacterial isolates that belonging to *Streptomyces* and *Kitasatospora* genera by performing 16S ITS fingerprinting with BstU1 and HaeIII, in which the non-related *Streptomyces* species were digested into unique band patterns with least overlapping of banding profile (Lanoot *et al.*, 2005).

Novel *Streptomyces* species were identified by the unique fingerprinting profile as shown by strains TPS137 (Cluster 5), TPS3 (Cluster 21), TPS4 (Cluster 26), TPS114 (Cluster 35) and confirmed by sequencing of the 16S rRNA gene. These strains were grouped as single-member cluster. In addition, TPS358a (Cluster 36) was also identified as novel *Nonomuraea* species that clustered closely with strain TPS114. Interestingly, members of the *Blastococcus* cluster were all identified to belong to novel *Blastococcus* species. The five known *Blastococcus* species validly described to date, including *B. endophyticus*, *B. capsensis*, *B. saxobsidens*, *B. jejuensis* and *B. aggregatus*, were isolated from medicinal plant leaves (Zhu *et al.*, 2013b), archaeological Roman pool (Hezbri *et al.*, 2016), monument stones (Urzì *et al.*, 2004), beach sediment (Lee, 2006) or brackish water (Ahrens & Moll, 1970), respectively. This study demonstrated the first successful isolation of members of *Blastococcus* from marine sediment collected off the ocean floor.

In total, 17 novel species of putative actinobacteria were isolated from Tioman marine sediment sample Z, constituted 21.5% of the total number of non-*Salinispora* isolates and 13.5% of the total actinobacteria isolates, suggesting that marine sediment

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of Tioman Island, Pahang, Malaysia is a potential source of novel secondary metabolites. All new taxons were recovered from mannitol- based IM2 and humic acid-based IM3 suggesting the crucial role of isolation media in recovering potential novel species. Skim milk/ HEPES treatment remained the most effective way for high recovery rate of novel actinobacterial species.

For marine sediment sample M, analyses of 16S rRNA gene sequence of the actinobacterial strains indicated close relationships of the isolates to members of genera *Salinispora*, *Streptomyces*, *Nocardia*, *Mycobacterium*, *Gordonia* and *Glycomyces*. *Salinispora* spp. accounted for 90% of total actinobacteria isolated from marine sediment sample M. High $H^{'}$ value for UV irradiated sample showed that HVA medium recovered greater diversity of actinobacteria than modified M2 and M3. Despites that, the highest actinobacterial count was obtained from UV irradiated sample on modified M2 medium, all the isolates, however, were identified to be closely related to *Salinispora* spp.

For *Salinispora*-like actinobacterial strains from both sediment samples Z and M, restriction analysis was performed using the six-cutter endonuclease BanI. The BanI restriction enzyme has a recognition site in the *S. arenicola* ITS region, but absent recognition site in both *S. pacifica* and *S. tropica*. Freel *et al.* (2012) showed successful dereplication of large number of *Salinispora* spp. strains via ITS RFLP screening using BanI and consistent banding patterns were observed for 219 strains that were closely related to *S. arenicola*. The genus *Salinispora* is known to be micro-diverse sequence cluster. Low interspecies and intraspecies diversity within the genus is largely due to common horizontal gene transfer (Jensen & Mafnas, 2006). The genome analysis method, BOX-PCR was proved to be useful in detecting intra- and inter-species variation among the *Salinispora* spp. isolated from sponges (Vidgen *et al.*, 2012).

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Vidgen *et al.* (2012) showed that 20 *Salinispora* strains out of 42 strains with varying BOX- PCR profile are identical in their 16S rRNA gene and also to the *S. arenicola* CNH-643^T. The same observation was obtained in this study, in which the BOX- PCR profile show intra-species variations in *Salinispora* strains isolated from Tioman Island, although these strains were 100% similar to each other and also to *S. arenicola* CNH-643^T of their 16S rRNA gene sequences. Actinobacterial isolates that are similar to *S. pacifica* or *S. tropica* were not detected in this study.

5.3 Characterisation of novel actinobacterial strains

At the time of writing this thesis, the reference strains were purchased and tested in parallel with the novel strains, including TPS16, TPS81 and TPS83 that belonging to a novel genus within the *Nocardiopsaceae* family and the *Streptomyces* sp. strain TPS3, on various tests such as the colony morphology, growth tolerance, carbon source utilisation profiles and production of hydrolytic enzymes. Reference strains were not available in the laboratory for other novel strains. Hence, these novel actinobacterial strains were compared to their reference strains using the previously published data.

5.3.1 Characterisation of novel actinobacterial strains from the *Nocardiopsaceae* family

Members within the family of *Nocardiopsaceae* are known to be halophiles or halotolerant species that tolerated 10% NaCl or above, as showed by members of the genera *Haloactinospora* and *Salinactinospora* (Chang *et al.*, 2012; Tang *et al.*, 2018). The type genus *Nocardiopsis* also contains alkaliphilic members such as *Nocardiopsis valliformis* and *Nocardiopsis dassonvillei* subsp. *prasina* that tolerate up to pH 13 (Miyashita *et al.*, 1984; Yang *et al.*, 2008a). Members of the family *Nocardiopsaceae* are commonly present in terrestrial soil, however, the genus *Spinactinospora* was only discovered from marine sediments and the type species *Spinactinospora alkalitolerans*

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is known to be alkaliphilic (Chang *et al.*, 2012). *M. thermotolerans* was isolated from marine sediment collected from the South China Sea at a depth of 3865 m.

Members of the family *Nocardiopsaceae* are aerobic, Gram-positive and non acid-fast. They form extensively branched substrate mycelium with occasional observations of fragmented substrate mycelium (Meyer, 1976). In terms of chemotaxonomy, all members of the family *Nocardiopsaceae* contain *meso*-diaminopimelic acid in the cell wall hydrolysates (Kroppenstedt & Evtushenko, 2006). The menaquinone and fatty acid profiles are diverse among the members. Nevertheless, fatty acid profiles of *Nocardiopsaceae* family members are characterized by the presence of 14-methylpentadecanoic ($C_{16:0}$ iso) and 14-methylhexadecanoic acid ($C_{17:0}$ anteiso) (Kroppenstedt & Evtushenko, 2006).

5.3.1.1 Characterisation of strains TPS16, TPS81 and TPS83

The strains TPS16, TPS81 and TPS83 differ from the reference strain *M. thermotolerans* SCSIO 00652^T by producing abundant of pigment on ISP 2. Diffusible pigments were not produced on ISP 2 by reference strain SCSIO 00652^T. Moreover, the type strain TPS16 was observed to have spores with smooth surface. In contrast, the spore surface of *M. thermotolerans* SCSIO 00652^T was reported to associate with wrinkled surface (Tian *et al.*, 2009a). Presence of *meso*-DAP and iso-$C_{16:0}$ as a major fatty acid confirmed the taxonomic position of strain TPS16 within the family of *Nocardiopsaceae*. The predominant menaquinones in cell wall hydrolysate of strain TPS16^T are MK-11(H_2), MK-10(H_2), MK-11(H_4) and MK-10(H_4), whereas the reference strain SCSIO 00652^T consists of MK-10(H_8), MK-11(H_8) and MK-11(H_{10}) (Tian *et al.*, 2009a). Besides that, the novel strain TPS16 was also found to have phosphatidylethanolamine as one of the polar lipids, which had not been reported for the reference strain (Tian *et al.*, 2009a). Cell wall chemistry profile of the novel strain

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TPS16 is compared with all validly described genera within the family of *Nocardiopsaceae*, as shown in Table 5.1. Cell wall hydrolysates of the novel strain TPS16 revealed the presences of *meso*- DAP, glucose and ribose as cell wall sugar and presence of iso-C16:0 as the predominnat fatty acids, which were also preent in the other genera within the *Nocardiopsaceae* family. The most striking differences between novel strain TPS16 and other genera of the *Nocardiopsaceae* family are characterised by the presence of MK-11(H_2) as the major menaquinone that was not detected in other genera (Table 5.1).

The result Table 4.16 shows a comparison of dDDH and wet-lab DDH values, ANI and AAI values between the novel strains and *M. thermotolerans* SCSIO 00652^T. Both dDDH and wet-lab DDH values were well below the recommended cut-off values of 70% for establishment of novel species (Sangal *et al.*, 2016). The ANI values of 78.53% were calculated for TPS16^T and SCSIO 00652^T, 78.60% for TPS81 and SCSIO 00652^T and 78.40% for TPS83 and SCSIO 00652^T. Although the ANIb values calculated for the novel strains were slightly higher than the threshold value of 74.8% for novel genus delineation, the AAI values calculated were much lower than the threshold value 87.8% (Sangal *et al.*, 2016). The relatively low ANI and AAI values suggested that strains TPS16, TPS81 and TPS83 belong to a new genomic species and should be described as a novel genus within the *Nocardiopsaceae* family.

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Table 5.1: Comparison of chemotaxonomic properties of strain TPS16 and recognized genera within the *Nocardiopsaceae* family. Phospholipids were abbreviated in the following: DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; PME, phosphatidylmethylethanolamine; GL, unknown glycolipid(s); PL, unknown phospholipid(s). For fatty acids, "ai" denoted anteiso and "i" denoted iso. 1, TPS16; 2, *Marinactinospora* (Tian *et al.*, 2009a); 3, *Thermobifida* (Yang *et al.*, 2008b; Zhang *et al.*, 1998); 4, *Nocardiopsis* (Meyer, 1976); 5, *Murinocardiopsis* (Kämpfer *et al.*, 2010); 6, *Haloactinospora* (Tang *et al.*, 2008); 7, *Spinactinospora* (Chang *et al.*, 2011); 8, *Salinactinospora* (Chang *et al.*, 2012); 9, *Streptomonospora* (Cai *et al.*, 2008); 10, *Allosalinactinospora* (Guo *et al.*, 2015); 11, *Actinorugispora* (Liu *et al.*, 2015); 12, *Lipingzhangella* (Zhang *et al.*, 2016a).

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12
GC mol%	73.8	70.5– 72.0	67.5– 70.9	67.1-76.8	72.4	68	71.1	60.1	70.7– 74.4	69.6	63.1	67.6
Cell wall sugars	Glucose, ribose	Glucose	Glucose, galactose, xylose	Ribose, glucose, mannose	None	Galactose, ribose, glucose	Ribose, glucose, galactose, fructose	Glucose, xylose, ribose, arabinose	Galactose	None	Glucose, mannose, ribose, galactose, rhamnose	Mannose, galactose
Predominant menaquinones	MK-11(H_2), MK-10(H_2), MK-11(H_4), MK-10(H_4)	MK-11(H_8), MK-11(H_{10}), MK-10(H_8)	MK-10(H_4), MK-10(H_6), MK-10(H_8)	MK-10(H_6), MK-10(H_8)	MK-10(H_4), MK-11(H_4), MK-12(H_2), MK-10(H_8)	MK-10(H_8), MK-11(H_4), MK-11(H_6), MK-11(H_8)	MK-10(H_8), MK-10(H_6), MK-9(H_8)	MK-10(H_8), MK-9(H_8), MK-10(H_2), MK-10(H_6)	MK-10(H_8), MK-10(H_6), MK-11(H_8)	MK-9(H_8), MK-10(H_8), MK-10(H_6)	MK-10 (H_4), MK-10 (H_6), MK-10 (H_8), MK-10 (H_2)	MK-10(H_8), MK-10(H_6)
Diagnostic phospholipids	GL, DPG, PC, PE, PG, PME PL	DPG, PG, PC, PI, PIM, PL	DPG, PME, PC, PI, PG, PE, PL	DPG, PME, PE, PG, PC, PL	PC, DPG, PG, PI, PL	DPG, PG, PC, PIM, PL	DPG, PC, PG, PI, PL	DPG, PG, PL, GL, PL	DPG, PG, PIM, PC, PI, PL	DPG, PG, PC, PME, PE, GL, PL	DPG, PC, PI, PIM, GL, PL	PG, PC, DPG, PE, GL, PL
Major fatty acids (> 10%)	i-$C_{16:0}$, ai-$C_{17:0}$, i-$C_{15:0}$, $C_{18:1}$ ω9c	10-methyl-$C_{18:0}$, i-$C_{16:0}$, i-$C_{16:1}$	i-$C_{16:0}$, ai-$C_{17:0}$	iso-$C_{16:0}$, ai-$C_{17:0}$, $C_{17:1}$ω8c, $C_{18:1}$ω9c	i-$C_{16:0}$, ai-$C_{17:0}$, $C_{18:1}$ ω9c	ai-$C_{17:0}$, i-$C_{16:0}$	i-$C_{16:0}$, ai-$C_{17:0}$, $C_{18:0}$	i-$C_{16:0}$, ai-$C_{17:0}$, $C_{16:0}$	i-$C_{16:0}$, ai-$C_{17:0}$, 10-methyl $C_{17:0}$, 10-methyl $C_{18:0}$	i-$C_{16:0}$, $C_{16:0}$	i-$C_{16:0}$, ai-$C_{17:0}$, $C_{18:1}$ ω9c	i-$C_{16:0}$, ai-$C_{17:0}$, 10-methyl-$C_{18:0}$

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In addition, the novel strains TPS16, TPS81 and TPS83 also differ from the reference strain by absence of growth at 55ºC, while the reference strain showed positive growth. All strains managed to tolerate a wide range of temperature (25 ˚C to 50 ˚C, optimum at 25-28 ˚C), pH (equally well at 6-12) and NaCl (up to 8%, optimum at a range of 0-4%). All three strains were also found to share the same carbon utilisation profile. The strains can utilise adonitol, D- arabinose, D- galactose, inositol, D- lactose, D- mannitol, D-mannose, D-melezitose, D- melibiose, D- sorbitol, L- sorbose and D- trehalose but *M. thermotolerans* SCSIO 00652^T, cannot. Furthermore, the novel strains could utilise the amino acids L- glutamine, L- lysine and L- methionine as carbon sources while the reference strain showed negative utilisation.

Hence, a new genus, *Marinitenerispora* gen. nov., is proposed within the family *Nocardiopsaceae* based on polyphasic data and the type species is *Marinitenerispora sediminis* gen. nov., sp. nov. The type strain is TPS16^T (=DSM 46825^T = TBRC 5138^T). *Marinitenerispora* (*Ma.ri.ni.te.ne.ri.spo'ra*. L. adj. *marinus* of the sea; L. adj. *tener* delicate; Gr. n. *spora* a seed; N.L. fem. n. *Marinitenerispora* a marine organism bearing delicate spores). The novel genus *Marinitenerispora* contains aerobic, Gram-positive, moderately thermotolerant, filamentous actinomycetes. Substrate mycelia are straight without fragmentation. Aerial mycelia are branching and not fragmented. *Meso*-DAP is present in cell wall hydrolysates, with glucose and ribose as the characteristic sugars. Predominant menaquinones are MK- 11 (H_2), MK- 10 (H_2), MK- 11 (H_4) and MK- 10 (H_4). Major fatty acids include *iso*- $C_{16:0}$, *anteiso*- $C_{17:0}$, *iso*- $C_{15:0}$ and $C_{18:1}$ *ω9c*. Polar lipid profile mainly consists of diphosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyl-N-methylethanolamine, also consists of unknown polar lipids and glycolipids. The type species is *Marinitenerispora sediminis* (se.di'mi.nis. L. gen. n. sediminis of sediment).

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The type species *Marinitenerispora sediminis* contains aerial mycelia that are yellow to white on all culture media after 14 days of incubation at 28 ˚C, followed by formation of blue colour spores only after 21 days of incubation. Powdery colonies were produced on ISP 1, ISP 2, ISP 3, ISP 4, ISP 5, ISP 6 and ISP 7. A noticeably increase in production of blue diffusible pigment was observed on soybean meal agar and ISP2 after incubation for 21 days. Grows equally well at a pH range from 6- 12. Grows at temperature from 25- 50 ˚C (optimum at 28 ˚C) and concentration of sodium chloride range from 0- 8 % with optimum growth between 0- 4 %. Various carbon sources are utilized for growth, including adonitol, amylase, cellulose, D- fructose, D- glucose, D- lactose, D- mannitol, D- mannose, D- melezitose, D- melibiose, D- sorbitol, D- trehalose, D- xylose, glycerol, inositol, L- arabinose, L- rhamnose, maltose, raffinose and sucrose. The strain produces acid from, D- trehalose, L- arabinose and sucrose. However, the strain is not able to utilize citrate as sole carbon source. Hydrolysis of adenine, casein, gelatine and starch are positive, whereas hydrolysis of Tween 80 is not observed. Positive for catalase, oxidase and urease and negative for nitrate reduction, milk coagulation and milk peptonization. The in silico G+C content of the draft genome of type strain TPS16^T is 73.8 mol%.

The type strain TPS16^T (=DSM 46825^T = TBRC 5138^T) and the strains TPS81 and TPS83 were isolated from marine sediment collected from the Pirate Reef, Tioman Island, Pahang, Malaysia. The Taxonumbers generated in the digital protologue for strain TPS16^T are GA00024 and TA00546 for genus and species, respectively. The accession numbers for 16S rRNA gene and genome sequences of the type strain TPS16^T are KM273125 and QEIO00000000, respectively.

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5.3.1.2 Characterisation of *Nocardiopsis* sp. TPS2

The *Nocardiopsis* species are aerobic, Gram- positive and catalase positive filamentous actinobacteria (Bennur *et al.*, 2015). They produced wrinkled or folded colonies on solid media and well- developed substrate mycelium and abundant aerial mycelium that fragment into spores. Cell walls components are characterized by the presence of *meso*- DAP, a characteristic of all members within the *Nocardiopsaceae* family, but without the presence of diagnostic cell wall sugar (Bennur *et al.*, 2015; Kroppenstedt & Evtushenko, 2006).

Strain TPS2 was compared to its closest related matches including *Nocardiopsis terrae* YIM 90022^T, *Nocardiopsis alba* subsp. *alba* DSM 43377^T and *Nocardiopsis prasina* DSM 43845^T. The reference strains were selected based on the phylogenetic analysis of 16S rRNA gene sequences and the BLAST result. *N. terrae* YIM 90022^T was isolated from saline soil collected from the Qaidam Basin, north- west China (Chen *et al.*, 2010). *N. alba* subsp. *alba* DSM 43377^T was isolated from drainage of a hip (Peltola *et al.*, 2001). *N. prasina* DSM 43845^T was recovered from a soil sample from Japan (Miyashita *et al.*, 1984).

Both strains TPS2 and *N. terrae* YIM 90022^T showed positive growth on ISP 2 and ISP 3, however, strain TPS2 was unable to produce diffusible pigment and *N. terrae* YIM 90022^T produced deep brown diffusible pigment on both media according to literature (Chen *et al.*, 2010). On ISP 4 and ISP 5, strain TPS2 produced yellow substrate mycelia while *N. terrae* YIM 90022^T produced light gray substrate mycelia. The second reference strain, *N. alba* subsp. *alba* DSM 43377^T produced the light yellow substrate mycelium and white aerial mycelium without diffusible pigment as reported previously and this observation was similar to strain TPS2, of which that produced yellow- to orange- yellow coloured substrate mycelium and white aerial mycelium on

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all ISP media (Grund & Kroppenstedt, 1990). Growth and formation of aerial mycelia by *N. prasina* DSM 43845^T was reported to appear at pH 9 and above (Yassin *et al.*, 1997). By comparison, formation of aerial mycelia by strain TPS2 was observed at pH 7- 8.

Strain TPS2 also showed a difference in terms of growth tolerance to NaCl, pH and incubation temperature. Optimum growth of strain TPS2 was observed at 28°C and 32°C on media with a NaCl concentration of 1- 6% and with pH 7- 8. No growth was recorded at pH 9 and above for strain TPS2. In contrast, *N. alba* subsp. *alba* DSM 43377^T showed optimum growth at pH 9 and above and *N. terrae* YIM 90022^T also showed optimum growth at pH above 8 (Chen *et al*., 2010; Grund & Kroppenstedt, 1990;). Among the tested carbon source, strain TPS2 differs from *N. terrae* YIM 90022^T in terms of utilisation of D- mannitol and L- arabinose, where TPS2 showed positive utilisation and *N. terrae* YIM 90022^T was unable to utilise them (Chen *et al*., 2010). Strain TPS2 and *N. terrae* YIM 90022^T showed negative utilisation of D-galactose, but *N. alba* subsp. *alba* DSM 43377^T and *N. prasina* DSM 43845^T showed positive utilisation (Chen *et al*., 2010; Grund & Kroppenstedt, 1990; Yassin *et al*., 1997). Strain TPS2 was able to use D- mannitol, D- xylose and L- arabinose and *N. alba* subsp. *alba* DSM 43377^T could not (Grund & Kroppenstedt, 1990). Comparing with *N. prasina* DSM 43845^T, strain TPS2 could utilise D- xylose for growth but *N. prasina* DSM 43845^T was unable to utilise that (Yassin *et al*., 1997).

Presence of *meso*-DAP and iso-$C_{16:0}$ as a major fatty acid confirmed the taxonomic position of strain TPS2 within the family of *Nocardiopsaceae*. The novel strain also showed co-occurrence of $C_{18:0}$ 10- methyl and anteiso- $C_{17:0}$ as major fatty acids in the cell wall hydrolysates, which was also detected as the major cell wall fatty acids of *N. alba* subsp. *alba* DSM 43377^T, *N. terrae* YIM 90022^T and *N. prasina* DSM 43845^T. The

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predominant menaquinones in cell wall hydrolysate of strain TPS2 are belonging to the MK-10 series with minor amount of the MK-9 series, which is a typical characteristic of the *Nocardiopsis* species (Hozzein & Trujillo, 2012). Besides that, strain TPS2 also has phosphatidylinositol and phosphatidylglycerol as polar lipids, a characteristic that had been reported for *Nocardiopsis* spp. However, strain TPS2 showed the absence of phosphatidylmethylethanolamine and phosphatidylcholine, the polar lipids that had been reported for all three closest related matches. The full profiles of fatty acids, menaquinones and polar lipids of strain TPS2 and comparison with closest related matches are shown in Appendix E.

Comparison of genome details and genomic metrics was performed between strain TPS2 and the closely related species including *N. alba* subsp. *alba* DSM 43377^T, *N. prasina* DSM 43845^T and *N. listeri* NBRC 13360^T. Genome sequence of *N. terrae* DSM 43845^T is not available in GenBank, thus it is replaced with *N. listeri* NBRC 13360^T. Strain TPS2 shared 96.87% of 16S rRNA gene sequence with *N. listeri* NBRC 13360^T, ranked as the fifth closest related matches. Analysis of genome data and genomic metrics further confirm the taxonomic position of strain TPS2 as a novel *Nocardiopsis* species. The dDDH values calculated for strain TPS2 and the closely related species fall between 24.20% and 29.10%, which were obviously lower than the recommended cut-off values of 70%. As shown in table 4.21, both of the ANIb and AAI values calculated for the genomes of strain TPS2 and the closely related species were 81.44- 85.08% and 76.55- 85.08%. As highlighted in the recent literature that suggested using 94% and 98.41% as cut- off values for ANIb and AAI (Sangal *et al.*, 2016), respectively for potential novel species delineation, both of the ANIb and AAI values were well- below the recommended values. This suggested that strain TPS2 should be described as a novel species within the genus *Nocardiopsis*.

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5.3.2 Characterisation of *Streptomyces* sp. TPS3, TPS4, TPS114, TPS137, TPS143 and TPS183

Streptomyces is the type genus of the family *Streptomycetaceae* under the order *Actinomycetales*. It is the largest genus as compared to both genera: *Kitasatospora* and *Streptacidiphilus* within the family, created by Waksman and Henrinci in 1943 (Barka *et al.*, 2016). The genus *Streptomyces* is the most domain species in terrestrial soil and plays a role as decomposer of organic matters by recycling carbon trapped in inorganic debris, via production of diverse hydrolytic extracellular enzymes (Barka *et al.*, 2016). *Streptomyces* spp. is a natural chemistry factory that produces wide variety of bioactive secondary metabolites. *Streptomyces coelicolor* is well known as the role model system for bacterial antibiotics production. Genome sequencing of the actinobacteria revealed its potential to produce at least 15 distinct classes of specialized metabolites and novel enzymes, which are useful for synthetic biology approaches in production of fine and specialty chemicals (Challis, 2014).

Members of *Streptomyces* are aerobic and non-acid fast. They are a group of Gram-positive bacteria that produce extensive branching substrate and aerial mycelium. Aerial hyphae differentiated into spore chains upon cell maturation by production of cross-walls in the multinucleate aerial filaments. In terms of chemotaxonomy, *Streptomyces* spp. is characterized by the presence of LL- DAP isomer and glycine and absence of diagnostic sugar (cell wall chemotype I). The menaquinone and fatty acid profiles are diverse among the members. In general, fatty acids of straight chain, iso- and anteiso-branched were reported from cell wall components of *Streptomyces* spp., with carbon chain length of 14 to 18 atoms. Hydroxylated methyl esters were also observed to be present in certain *Streptomyces* spp. (Anderson & Wellington, 2001).

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5.3.2.1 Characterisation of *Streptomyces* sp. TPS3

Two strains namely *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T were selected as reference strains based on BLAST search on 16S rRNA gene sequences. *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T were isolated from terrestrial soil samples: grassland soil and sewage irrigation soil, respectively (Lin *et al.*, 2012, Lin *et al.*, 2013), whereas strain TPS3 was isolated from marine sediment sample. Due to the difference of habitat, comparison of growth of strain TPS3 with *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T was performed using two sets of media: one with 2.5% ASW and one without ASW. In addition, TPS3 is also closely related to *S. spongiae* Sp080513Sc-24^T by sharing 97.34% of the 16S rRNA gene similarity, in which the closest match was isolated from marine sponge *Haliclona* sp. (Khan *et al*., 2011). However, *S. spongiae* Sp080513Sc-24^T was not available and thus it is not being included in the parallel comparison study on strain characterisation.

Growth of strain TPS3 was affected by the presence of ASW in the culture media. The strain produced pale blue or pale purplish blue spores when it was cultured on ISP 2, ISP 4, ISP 5 and ISP 7 without supplementation of ASW. Formation of pale blue or pale purplish blue aerial mycelia was found absent when it was cultured on the media with supplementation of ASW, instead, the strain produced white aerial mycelia. On ISP 3 medium, the strain produced intense purplish blue aerial mycelium on medium without ASW. In contrast, the strain TPS3 produced less aerial mycelia that are very pale blue colour on ISP 3 with ASW.

Both reference strains were able to grow on culture media supplemented with ASW. Production of diffusible pigment by strain *S. ziwulingensis* F22^T was affected by the presence of ASW in ISP 2 and ISP 7 media. On both media supplemented with ASW, the strain produced more abundant of aerial mycelia as compared to the media without

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ASW. Colour of aerial mycelia and production of diffusible pigment were also found to be affected by the presence of ASW in the culture media for strain *S. shaanxiensis* CCNWHQ 0031^T. On ISP 2, strain *S. shaanxiensis* CCNWHQ 0031^T produced dark yellowish brown soluble pigment, which was absent when the growth was observed on ISP 2 without ASW. On ISP 4 and ISP 7 with ASW, strain *S. shaanxiensis* CCNWHQ 0031^T produced white aerial mycelium instead of the pale purplish blue aerial mycelium that was being recorded on ISP 2 medium without ASW. *S. spongiae* Sp080513Sc-24^T was also able to grow on media without supplementation of ASW by producing white aerial and substrate mycelia (Khan *et al.*, 2011).

Among the tested carbon sources, strain TPS3 differs from both reference strains in terms of utilisation of adonitol, D- arabinose, L- sorbose, glycine, L- lysine and L-methionine for growth. Strain TPS3 showed positive utilisation of D- arabinose, L-lysine and L- methionine but not adonitol, L- sorbose and glycine. *S. ziwulingensis* F22^T did not utilise all six carbon sources. *S. shaanxiensis* CCNWHQ 0031^T showed positive utilisation of adonitol, L- sorbose, glycine and L- lysine, and no utilisation of D-arabinose and L- methionine. Acid production from D- glucose, L- arabinose, D-fructose, D- galactose, D- lactose, D- maltose, D- mannitol, D- mannose, D- melibiose, D-ribose, sucrose, D- trehalose and xylose was also different among the three strains. Strain TPS3 utilised and produced acid from D- glucose, D- galactose, D- lactose, D-maltose, D- mannose, sucrose, D- trehalose and xylose. *S. ziwulingensis* F22^T utilised and produced acid from all the carbon sources mentioned for strain TPS3. In addition, *S. ziwulingensis* F22^T also produced acid from L- arabinose, D- fructose, D- mannitol, D-melibiose and D- ribose, of which strain TPS3 did not show acid production. *S. shaanxiensis* CCNWHQ 0031^T utilised and produced acid from L- arabinose and D-mannitol, and showed negative result for acid production from D- glucose, D- fructose, D- galactose, D- lactose, D- maltose, D- mannose, D- melibiose, D- ribose, sucrose, D-

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trehalose and xylose. On the other hand, both strain TPS3 and *S. spongiae* Sp080513SC-24^T were able to utilise glucose and maltose for growth and showed positive acid production from these carbon sources (Khan *et al.*, 2011).

Strain TPS3 differ from *S. shaanxiensis* CCNWHQ 0031^T in terms of growth tolerance to NaCl and pH. Strain TPS3 showed positive growth at pH 6- 11 and 0- 5% NaCl, however, strain *S. shaanxiensis* CCNWHQ 0031^T showed positive growth at pH 6- 9 and 0- 6% NaCl. There are no difference between strain TPS3 and *S. ziwulingensis* F22^T in terms of growth tolerance to pH, incubation temperature and NaCl. *S. spongiae* Sp080513SC-24^T were reported for positive growth at pH 5- 9 and 0- 5% NaCl (Khan *et al.*, 2011). Strain TPS3, *S. shaanxiensis* CCNWHQ 0031^T and *S. ziwulingensis* F22^T were growing optimally at 25- 37°C, pH 6- 8 and 0% NaCl. *S. spongiae* Sp080513SC-24^T was reported to show optimal growth at 25- 30°C at pH 6- 8. Aerial mycelia were absent when strain TPS3, *S. shaanxiensis* CCNWHQ 0031^T and *S. ziwulingensis* F22^T were allowed to grow on ISP 2 medium supplemented with 1% NaCl and above. Strain TPS3, *S. shaanxiensis* CCNWHQ 0031^T and *S. ziwulingensis* F22^T were able to produce amylase, proteinase, gelatinase, urease, catalase and decomposed adenine. Catalase positive was also reported for *S. spongiae* Sp080513SC-24^T (Khan *et al.*, 2011).

Presence of *LL*-DAP and hexa- and octahydrogenated menaquinones with nine isoprene units confirmed the taxonomic position of strain TPS3 as a species of *Streptomyces*. Besides that, the novel strain also showed a typical polar lipid profile of *Streptomyces* species that contains phosphatidylinositol, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. (Kämpfer, 2012a) The full profiles of fatty acids, menaquinones and polar lipids of strain TPS3 and comparison with closest related matches are shown in Appendix F.

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As genome sequences of *S. ziwulingensis* F22^T and *S. shaanxiensis* CCNWHQ 0031^T are not available, genome details and genomic metrics was compared to other closely related matches based on BLAST results on 16S rRNA gene sequences. These include *S. kanasensis* ZX01^T, *S. caeruleatus* NRRL B-24802^T and *S. griseoruber* NRRL B-1818^T. Strain TPS3 shared 96.47%, 95.83% and 95.55% of 16S rRNA gene sequence with *S. kanasensis* ZX01^T, *S. caeruleatus* NRRL B-24802^T and *S. griseoruber* NRRL B-1818^T, respectively. The dDDH values calculated for strain TPS3 and the closely related species fall between 23.20% and 26.90%, which were below the recommended cut-off values of 70%. As shown in table 4.29, both of the ANIb and AAI values calculated for the genomes of strain TPS3 and the closely related species were 77.42- 81.89% and 68.60- 76.39%., which were also below the cut- off values of 94% and 98.41% for ANIb and AAI (Sangal *et al.*, 2016), respectively for species separation. This suggested that strain TPS3 is a novel species of *Streptomyces*.

5.3.2.2 Characterisation of *Streptomyces* spp. TPS4 and TPS183

Based on the phylogenetic analysis of both strains TPS4 and TPS183 (Figure 4.28 and 4.32), they shared the same closely related matches: *S. carpaticus* NBRC 15390^T, *S. harbinensis* NEAU-Da3^T, *S. cheonanensis* VC-A46^T, and *S. xiamenensis* MCCC 1A01550^T, by sharing 96.69- 97.47% and 96.82- 97.80% of 16S rRNA gene sequence, respectively. Comparison of 16S rRNA genes of strains TPS4 and TPS183 were found to be 96.37% similar to each other by using ChunLab's online pairwise sequence alignment tool, suggested that both strains belong to different *Streptomyces* species

As colony morphology of *S. carpaticus* NBRC 15390^T was only recorded in the literature on ISP 3 and ISP 5, the comparison would be focusing on both ISP media. Strain TPS4 formed greyish brown substrate mycelia, olive grey aerial mycelia and dark olive diffusible pigment on ISP 3. Production of greyish olive substrate mycelium, olive

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gray aerial mycelia and dark greenish yellow diffusible pigment was recorded for strain TPS183. *S. carpaticus* NBRC 15390^T was reported to produce greyish gray-brown colour aerial mycelium and dark brown substrate mycelium and diffusible pigment, nearly black with reddish or olive shadow on ISP3 and ISP5 (Kämpfer, 2012a). By comparison, *S. harbinensis* NEAU-Da3^T produced dark greyish blue substrate mycelium, dark bluish gray aerial mycelium and dark yellowish green diffusible pigment on ISP 3 (Liu *et al.*, 2013a). Formation of grey aerial mycelium and olive substrate mycelium along with production of dark grey diffusible pigment was observed on ISP 3 for *S. cheonanensis* VC-A46^T (Kim *et al.*, 2006b). The reference strain *S. xiamenensis* MCCC 1A01550^T produced gray- green diffusible pigment, moderate yellow substrate mycelium and grey- white aerial mycelium with poor production of spores on ISP 3 (Xu *et al.*, 2009). Both TPS4 and TPS183 were observed to have good production of spores on ISP 3 with ASW.

When the strains were cultured on ISP 5 medium, strain TPS4 produced sparse aerial mycelia without production of diffusible pigment. Strain TPS183, on the other hand, produced abundant aerial mycelia and light olive diffusible pigment. Absent of growth was reported for *S. harbinensis* NEAU-Da3^T on ISP5 (Liu *et al.*, 2013a). *S. cheonanensis* VC-A46^T produced aerial and substrate mycelia and diffusible pigment of olive colour (Kim *et al.*, 2006b). *S. xiamenensis* MCCC 1A01550^T was reported to have poor production of spores on ISP 5 and produced light pink diffusible pigment (Xu *et al.*, 2009).

Strain TPS4 was observed to grow optimally at pH 8- 9 and 2- 3% NaCl, whereas strain TP183 grows optimally at pH 8- 10 and 2% NaCl. Strain TPS4 grew equally well at 15°C, 25°C, 28°C, 32°C and 37°C, whereas strain TPS183 grew optimally at 25°C, 28°C, 32°C and 37°C. Optimal growth condition was not tested for *S. carpaticus* NBRC

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15390^T and *S. cheonanensis* VC-A46^T according to literatures (Kämpfer, 2012a; Kim *et al.*, 2006b). *S. harbinensis* NEAU-Da3^T had optimum growth condition at pH 8 and 30°C and was able to grow in the presence of 0- 5% NaCl (Liu *et al.*, 2013a). *S. xiamenensis* MCCC 1A01550^T displayed optimum growth at 28°C and was able to grow in the presence of 0- 5% NaCl (Xu *et al.*, 2009). Tolerance of growth to pH was not available for strain *S. xiamenensis* MCCC 1A01550^T in the literature (Xu *et al.*, 2009).

Both strains TPS4 and TPS183 showed positive production of amylase by hydrolyse starch, and the same observation was obtained for *S. harbinensis* NEAU-Da3^T and *S. cheonanensis* VC-A46^T (Kämpfer, 2012a; Kim *et al.*, 2006b). Production of urease was negative for both TPS4 and TPS183, but *S. harbinensis* NEAU-Da3^T was reported to have positive production (Liu *et al.,* 2013a). Strains TPS4, TPS183 and *S. xiamenensis* MCCC 1A01550^T were able to produce gelatinase, but *S. harbinensis* NEAU-Da3^T was not able to produce gelatinase (Liu *et al.,* 2013a; Xu *et al.*, 2009).

Strain TPS4 and TPS183 showed a difference in utilisation of D- xylose, L- sorbose and L- lysine, in which TPS4 failed to utilise these carbon sources for growth and strain TPS183 showed positive growth in the presence of them. Strains TPS4 and TPS183 and *S. carpaticus* NBRC 15390^T, *S. cheonanensis* VC-A46^T and *S. xiamenensis* MCCC 1A01550^T were able to utilise mannitol for growth, but *S. harbinensis* NEAU-Da3^T was not able to utilise it (Kämpfer, 2012a; Kim *et al.*, 2006b; Liu *et al.*, 2013a; Xu *et al.*, 2009). All strains were able to utilise D- xylose, except for *S. xiamenensis* MCCC 1A01550^T (Kämpfer, 2012a; Kim *et al.*, 2006b; Liu *et al.*, 2013a; Xu *et al.*, 2009). Utilisation of sucrose was positive by strains TPS4, TPS183, *S. carpaticus* NBRC 15390^T, *S. cheonanensis* VC-A46^T and *S. xiamenensis* MCCC 1A01550^T, but *S.*

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harbinensis NEAU-Da3^T failed to utilise sucrose for growth (Kämpfer, 2012a; Kim *et al.*, 2006b; Liu *et al.*, 2013a; Xu *et al.*, 2009).

5.3.2.3 Characterisation of *Streptomyces* sp. TPS114

The strain TPS114 shared 97.88- 98.3% of 16S rRNA gene sequence similarity to *S. karpasiensis* K413^T, *S. nanshensis* SCSIO 01066^T and *S. glycovorans* YIM M 10366^T. Strain K413^T was isolated from the soil sample collected from Karpaz National Park, Magusa, Northern Cyprus (Veyisoglu *et al.*, 2014). *S. nanshensis* SCSIO 01066^T was recovered from a deep sea sediment sample at 2015 m depth from the sea area of the Nansha Islands in the South China Sea (Tian *et al.*, 2009c). *S. glycovorans* YIM M 10366^T was isolated from marine sediment samples collected from the Xisha Islands in the South China Sea at 778 m depth (Xu *et al.*, 2012).

Strain TPS114 produced sparse aerial mycelia on most of the IPS media, except for ISP 7, on which formation of abundant white aerial mycelium was observed. *S. karpasiensis* K413^T produced white aerial mycelia on ISP 3, 4, 5 and 7 (Veyisoglu *et al.*, 2014). White aerial mycelia were produced by *S. nanshensis* SCSIO 01066^T on ISP 2, 4 and 5 media (Tian *et al.*, 2009c). *S. glycovorans* YIM M 10366^T produced yellow-white aerial mycelia on ISP 2 (Xu *et al.*, 2012). Strain TPS114 and all reference strains did not produce diffusible pigment on all ISP media (Tian *et al.*, 2009c; Veyisoglu *et al.*, 2014; Xu *et al.*, 2012).

Optimum growth of strain TPS114 was observed at pH 7- 9 and 1- 4% of NaCl and good growth at 15°C, 25°C, 28°C, 32°C and 37°C. According to previous findings, *S. karpasiensis* K413^T produced optimum growth at pH 7.2 and 28°C of incubation temperature (Veyisoglu *et al.*, 2014). *S. nanshensis* SCSIO 01066^T grew optimally at pH7 in the presence of 0- 3% NaCl at 28°C. Optimum growth condition reported for *S. glycovorans* YIM M 10366^T was on media adjusted to pH 7 in the presence of 0- 3%

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NaCl, incubated at 28- 37°C. Strains TPS114, *S. karpasiensis* K413^T and *S. nanshensis* SCSIO 01066^T produced urease, but *S. glycovorans* YIM M 10366^T was not producing the enzyme (Tian *et al.*, 2009c; Veyisoglu *et al.*, 2014; Xu *et al.*, 2012). Production of amylase was positive in TPS114 and negative in YIM M 10366^T (Xu *et al.*, 2012). Production of gelatinase was positive for strain TPS114 and *S. glycovorans* YIM M 10366^T but *S. nanshensis* SCSIO 01066^T showed no production (Tian *et al.*, 2009c; Xu *et al.*, 2012). Hydrolysis of casein was positive for strain TPS114 and negative for *S. nanshensis* SCSIO 01066^T (Tian *et al.*, 2009c). However, both strain TPS114 and *S. nanshensis* SCSIO 01066^T were not able to hydrolyse adenine crystals (Tian *et al.*, 2009c). Catalase- positive and oxidase- negative were detected for strain TPS114 and reference strains including *S. karpasiensis* K413^T and *S. glycovorans* YIM M 10366^T (Veyisoglu *et al.*, 2014; Xu *et al.*, 2012). Catalase and oxidase production was not stated in the published paper for *S. nanshensis* SCSIO 01066^T (Tian *et al.*, 2009c).

Strain TPS114 and the reference strains were able to utilise D- mannitol for growth but not utilizing D- sorbitol. D- Galactose was utilised by strain TPS114, *S. karpasiensis* K413^T and *S. nanshensis* SCSIO 01066^T, whereas *S. glycovorans* YIM M 10366^T was not able to utilise it. Strain TPS114 and K413^T showed positive utilisation of D- lactose and the other two reference strains could not utilise it. Strains TPS114, *S. nanshensis* SCSIO 01066^T and *S. glycovorans* YIM M 10366^T could utilise maltose and sucrose for growth while strain K413^T could not.

5.3.2.4 Characterisation of *Streptomyces* spp. TPS137 and TPS143

Strains TPS137 and TPS143 were closely related to *S. sedi* YIM 65188^T, *S. zhaozhouensis* NEAU-LZS-5^T, *S. hainanensis* YIM 47672^T, *S. specialis* GW 41-1564^T and *S. mayteni* YIM 60475^T, with the percentage of similarity fall in the range of 96.95-97.65% for strain TPS137 and 96.88- 98.07% for strain TPS143, based on phylogenetic

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analyses of the almost full length 16S rRNA gene sequences. *S. sedi* YIM 65188^T, *S. zhaozhouensis* NEAU-LZS-5^T and *S. mayteni* YIM 60475^T are associated with plant tissues, including the root tissue of *Sedum* sp., the leaf of candelabra aloe and the roots of a traditional Chinese medicinal plant (*Maytenus austroyunnanensis*), respectively (Chen *et al.*, 2009; He *et al.*, 2014; Li *et al.*, 2009) . *S. hainanensis* YIM 47672^T and *S. specialis* GW 41-1564^T were isolated from soil samples (Jiang *et al.*, 2007; Kämpfer *et al.*, 2008).

Colony colour varied for strains TPS137 and TPS143 and the closest related matches on ISP 2. Strain TPS137 produced deep yellowish brown aerial mycelia and white coloured spores, along with production of light orange diffusible pigment. The strain TPS143 formed greyish greenish yellow aerial mycelia and light brown diffusible pigment. The reference strains, which associated with plant tissue, including *S. sedi* YIM 65188^T, *S. zhaozhouensis* NEAU-LZS-5^T and *S. mayteni* YIM 60475^T were reported to produce white aerial mycelia without diffusible pigment (Chen *et al.*, 2008; He *et al.,* 2014; Li *et al.*, 2009). Strains GW 41-1564^T and *S. hainanensis* YIM 47672^T from soil samples produced pinkish white aerial mycelia and diffusible pigment (Jiang *et al.*, 2007; Kämpfer, 2008). *S. specialis* GW 41-1564^T produced black diffusible pigment, whereas the *S. hainanensis* YIM 47672^T produced orange- yellow diffusible pigment (Jiang *et al.*, 2007; Kämpfer, 2008). Besides that, growth of strains TPS137, TPS143 and *S. hainanensis* YIM 47672^T were found different on ISP 3. Strain TPS137 produced light pink diffusible pigment on ISP 3 but formation of aerial mycelia was scarce. Strain TPS143 also displayed poor formation of aerial mycelia, which associate with production of dark orange yellow diffusible pigment. *S. hainanensis* YIM 47672^T produced pale pink aerial mycelium and orange- yellow diffusible pigment on ISP 3 (Jiang *et al.*, 2007).

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Both strains TPS137 and TPS143 were able to grow at a wide range of pH. As compared to the reference strains, both TPS strains showed positive growth at pH 7- 12, whereas the reference strains could tolerate up to a maximum of pH 10 only. Strain TPS137 could grow at pH 7- 12 in the presence of 0- 7% NaCl at 15°C, 25°C, 28°C, 32°C and 37°C with optimum growth occurred at pH 7- 8. Growth of strain TPS143 was positive at pH 6- 12, up to 5% of NaCl at 15°C, 25°C, 28°C, 32°C and 37°C. Strain TPS143 grew optimally at pH 7.5- 10 on medium containing 2- 3% NaCl. Strain YIM 65188^T showed positive growth at pH 7- 8, 15- 37°C and the strain could tolerate up to 5% NaCl (Li *et al.*, 2009). *S. zhaozhouensis* NEAU-LZS-5^T was able to grow at pH 6- 10 and 16- 35°C and tolerate up to 7% NaCl, with optimum growth being reported on medium with pH 7 that contained 1% NaCl at 28°C (He *et al.*, 2014). *S. hainanensis* YIM 47672^T grows at pH 6- 9 and tolerates up to 10% NaCl, with optimum growth at pH 7 on culture media without addition of NaCl (Jiang *et al.*, 2007). Growth tolerance of *S. specialis* GW 41-1564^T was not available in the literature (Kämpfer, 2008). *S. mayteni* YIM 60475^T was able to grow on medium with pH 6- 8 at 18- 32°C supplemented with 0- 3% NaCl with optimum growth at pH 7 and 28°C without NaCl (Chen *et al.*, 2008).

The strains TPS137 and TPS143 differ from each other based on utilisation of D-arabinose, D- melezitose, D- ribose, sucrose, L- sorbose, L- methionine and lysine. Strain TPS143 could utilise all these carbon sources for growth but strain TPS137 did not show growth on media that contained these carbon sources. Table 5.2 highlighted the different in carbon sources utilisation profiles of strains TPS137 and TPS143 as compared to the reference strains. In terms of enzyme production, strain TPS137 was able to produce urease while strain TPS143 was not able to produce the enzyme. Production of gelatinase was positive for strains TPS137, TPS143 and *S. zhaozhouensis* NEAU-LZS-5^T, but negative for *S. sedi* YIM 65188^T, YIM 47672^T and YIM 60475^T

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(Chen *et al.*, 2008; He *et al.,* 2014; Jiang *et al.*, 2007; Li *et al.*, 2009). In addition, both strains TPS137 and TPS143 and the reference strains including YIM 47672^T and YIM 60475^T were able to produce amylase (Chen *et al.*, 2008; Jiang *et al.*, 2007). Amylase production was negative for *S. sedi* YIM 65188^T and *S. zhaozhouensis* NEAU-LZS-5^T (He *et al.,* 2014; Li *et al.*, 2009). Hydrolysis tests results were not included in the previous study on description of strain GW 41-1564^T and thus not be able to compare with strains TPS137 and TPS143 (Kämpfer, 2008).

Table 5.2: Highlight of difference in carbon sources utilisation profiles of strain TPS137, TPS143 and the reference strains. Carbon utilisation profile of the reference strains were obtained from previously published papers (Jiang *et al.*, 2007; Chen *et al.*, 2008; Kämpfer, 2008; Li *et al.*, 2009; He *et al.,* 2014).

Carbon sources	**TPS137**	**TPS143**	**YIM 65188^T**	**NEAU-LZS-5^T**	**YIM 47672^T**	**GW 41-1564^T**	**YIM 60475^T**
D-Fructose	+	+	+	-	-	-	+
D-Galactose	+	+	-	+	-	-	+
D-Mannose	+	+	-	-	-	-	+
D-Sorbitol	-	-	-	-	-	+	-
D- Xylose	+	+	-	+	-	-	-
Inositol	+	+	-	-	-	+	-

5.3.3 Characterisation of *Blastococcus* spp. TPS166, TPS357, TPS418, TPS448 and TPS459

All five strains in the *Blastococcus* cluster, including TPS166, TPS357, TPS418, TPS448 and TPS459 were putatively identified as novel species based on 16S rRNA sequence analyses (Figure 4.19). Interestingly, the novel strains formed a distinct and stable lineage with high bootstrap value above 98% and was separated from the established *Blastococcus* species, which were isolated from terrestrial and beach environmental samples (Figure 4.19). Strain TPS166 was clearly separated from the other four strains and formed a distinct sub- cluster on its own. Pair- wise comparison of

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the 16S rRNA gene sequences of all five strains revealed that strains TPS418, TPS448 and TPS459 were closely related to each other, by sharing 98.61- 99.27% of gene similarity (Table 4.32). Strain TPS357 also shared a high similarity value of the 16S rRNA gene sequences to strain TPS448 (99.63%), however, strain TPS357 only shared 97.94- 98.39% of its 16S rRNA gene similarity to TPS418 and TPS459, of which the similarity percentage is much lower than the cut- off values 98.6% for species delineation. Therefore, DNA- DNA relatedness and genome comparison are required to clarify and confirm the taxonomic relationship between strains TPS357 and TPS418, TPS448 and TPS459.

At the time of writing, there are nine non- candidatus *Blastococcus* species that are validly named as *B. aggregatus*, *B. saxobsidens*, *B. colisei*, *B. jejuensis*, *B. endophyticus*, *B. capsensis*, *B. xanthinilyticus*, *B. atacamensis*, *B. litoris*, which were isolated from brackish water (Ahrens & Moll, 1970), monument stones (Urzì *et al.*, 2004; Hezbri *et al.*, 2017), beach sediment (Lee, 2006), medicinal plant leaves (Zhu *et al.*, 2013b), archaeological Roman pool (Hezbri *et al.*, 2016), hyperarid soil sample (Castro *et al.*, 2018), marble sample collected from monument (Hezbri *et al.*, 2018) and sea tidal flat sediment (Lee *et al.*, 2018), respectively. This study demonstrated isolation of members of the genus *Blastococcus* from a marine sediment sample. The *Blastococcus* genus is a member of the family *Geodermatophilaceae*, which contains members that are Gram- stain- positive, coccoidal bacteria that either occurs as single cells or in pairs that might be non- motile cocci or as motile rods and vibroid (Stackebrandt & Schumann, 2012b). The cells tend to form aggregates and might produce zoospores. The *Blastococcus* spp. contain *meso*- DAP as diagnostic diaminopimelic acid. Both unsaturated and iso- branched fatty acids and polar lipids including diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine,

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phosphatidylethanolamine and phosphatidylinositol as the predominant component present in the cell wall (Stackebrandt & Schumann, 2012b).

Five *Blastococcus* strains obtained from this study was closely related to *B. endophyticus* YIM 68236^T, *B. capsensis* BMG 804^T, *B. saxobsidens* BC448^T and *B. jejuensis* KST3-10^T based on phylogenetic analysis of the 16S rRNA gene (Figure 4.41 and table 4.11). They shared 97.4- 98.3% of 16S rRNA gene similarity to the reference strains. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that the five strains were clustered into two sub-clusters, with sub-cluster 1 contains TPS357, TPS418, TPS448 and TPS459 whereas the sub-cluster 2 contains TPS166, This result showed that the five novel strains were putatively identified as two distinct novel *Blastococcus* species.

B. endophyticus YIM 68236^T produced white colonies on TSA up to day 5 of culture and turns to pink and subsequently to black colonies (Zhu *et al.*, 2013b). *B. capsensis* BMG 804^T formed bright orange colonies (Hezbri *et al.*, 2016). Colonies of *B. saxobsidens* BC448^T are pink- or orange- pigmented (Urzì *et al.*, 2004). The colour of the colonies of strain KST3-10^T on ranged from cream to apricot depending on the incubation time (Lee *et al.*, 2006). Cell morphology of *B. endophyticus* YIM 68236^T, *B. capsensis* BMG 804^T and *B. saxobsidens* BC448^T were reported to be non- motile cocci that occurred singly or in tetrads and formed aggregates (Hezbri *et al.*, 2016; Urzì *et al.*, 2004; Zhu *et al.*, 2013b). The cells of *B. jejuensis* KST3-10^T were reported to be coccoid and occurred in pairs or as motile rods with flagella and showed bud formation (Lee *et al.*, 2006). The *Blastococcus* strains from this study was also found to occur as coccoid or as elongated rods that are highly aggregates, of which is in line with the observations reported for the reference strains in literatures.

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Comparison of growth on various culture media revealed that strain TPS166 produced dark reddish orange colour of colonies as compared to the other four strains, which produced brilliant orange colonies, on most of the tested culture media. Strains TPS166 and TPS357 were able to produce colonies on malt agar, of which the strains TPS418, TPS448 and TPS459 failed to produce colonies (Table 4.33). The reference strain *B. saxobsidens* BC448^T was also reported to produce sparse growth on LM medium and YCS- Glc agar and no growth was reported on YG- Glyc agar (Urzì *et al.*, 2004). *B. capsensis* BMG 804^T was reported to produce good growth on LM medium and *B. saxobsidens* BC448^T showed poor growth on the same medium (Hezbri *et al.*, 2016; Urzì *et al.*, 2004). By comparison, the *Blastococcus* strains obtained from this study were also showing good growth on LM medium, YCS- Glc agar and YG- Glyc agar.

All five putative novel strains were able to grow from pH 6 to 12, with optimum range fall in pH 7- 10 for strain TPS166, pH 6- 9 for strains TPS418 and TPS448, and pH 6- 10 for strain TPS459. The reference strains *B. endophyticus* YIM 68236^T, *B. capsensis* BMG 804^T, *B. saxobsidens* BC448^T and *B. jejuensis* KST3-10^T were reported to only tolerate up to pH 8- 10 with optimum growth at pH 6- 8 (Hezbri *et al.*, 2016; Lee *et al.*, 2006; Urzì *et al.*, 2004; Zhu *et al.*, 2013b). In terms of NaCl tolerance, all strains were able to tolerate up to 8% NaCl, with optimum growth in the presence of 0-4% NaCl. In contrast, the four reference strains are only capable of tolerating up to 3% NaCl (Hezbri *et al.*, 2016; Lee *et al.*, 2006; Urzì *et al.*, 2004; Zhu *et al.*, 2013b). The *Blastococcus* strains from this study was able to grow at 45°C and so *B. endophyticus* YIM 68236^T, however, *B. capsensis* BMG 804^T, *B. saxobsidens* BC448^T and *B. jejuensis* KST3-10^T were only able to tolerate up to 37°C (Hezbri *et al.*, 2016; Lee *et al.*, 2006; Urzì *et al.*, 2004; Zhu *et al.*, 2013b). Catalase- positive and oxidase negative were reported for all reference strains, except for *B. capsensis* BMG 804^T, which was

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reported as catalase- and oxidase- negative (Hezbri *et al.*, 2016; Lee *et al.*, 2006; Urzì *et al.*, 2004; Zhu *et al.*, 2013b). Comparison of carbon sources utilisation profile of the putative novel *Blastococcus* strains obtained from this study was not be able to be performed, as carbon sources utilisation profile by all the reference strains was tested on Biology GP2 but the putative novel strains obtained in this study was tested on ISP 9 (Hezbri *et al.*, 2016; Lee *et al.*, 2006; Urzì *et al.*, 2004; Zhu *et al.*, 2013b).

Production of hydrolytic enzymes and assimilation of carbon sources on API 20 NE was only performed for strain TPS166, TPS357 and TPS418 due to limited number of the API strips in the lab and limited funding to do purchasing. The test will be performed for strains TPS448 and TPS459 in the near future. The API 20 NE test was also not determined for *B. capsensis* BMG 804^T (Hezbri *et al.*, 2016) and thus comparison of API 20 NE test for strains TPS166, TPS357 and TPS418 will only be performed with the other three reference strains (Lee *et al.*, 2006; Urzì *et al.*, 2004; Zhu *et al.*, 2013b), as shown in Table 5.3.

Strain TPS166 and TPS459 had been selected for cell wall characterisation based on the phylogenetic analysis. As shown in the phylogenetic tree in figure 4.45, the novel strain TPS166 was selected as it forms a separate sub-cluster from the rest of the novel *Blastococcus* strains, which indicated that a significant phylogenetic difference. Since the four strains TPS357, TPS418, TPS448 and TPS459 are highly similar (more than 98.6% of similarity) in terms of 16S rRNA gene, this suggested that all four strains are belonging to the same species, but this need to be further confirmed by genomic analysis. As such, strain TPS459 was randomly selected as the representative for cell wall chemotaxonomy analysis due to limited funding. The chemotaxonomy analysis of cell walls of strains TPS357, TPS418 and TPS448 will be performed in the near future. Presences of *meso*- DAP as diagnostic diamino acid, MK-9(H_4) as predominant

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menaquinone and iso- $C_{16:0}$, $C_{18:1}\omega9c$ and iso- $C_{15:0}$ as major type of fatty acids showed that the novel strains belonging to the *Blastococcus* species, in addition to the presence of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylcholine as polar lipids (Stackebrandt & Schumann, 2012b). The full profiles of fatty acids, menaquinones and polar lipids of strain TPS166 and TPS459 and comparison with closest related matches are shown in Appendices H and I.

Table 5.3: Testing on enzyme production and assimilation of carbon sources using API 20 NE strips. 'nd' denoted no data is available and 'w' denoted weak positive result.

Reaction	**TPS166**	**TPS357**	**TPS418**	**YIM 68236^T**	**BC448^T**	**KST3-10^T**
Nitrate reduction	+	-	-	-	-	-
Production of:						
Arginine dihydrolase	+	+	+	-	+	-
Urease	+	+	+	-	-	-
β- glucosidase	+	+	+	+	-	-
Gelatinase	-	+	-	-	-	-
Assimilation of:						
Glucose	+	+	+	nd	-	-
Mannose	+	+	+	nd	-	-
Mannitol	+	+	+	nd	-	-
N- acetyl- glucosamine	+	+	+	nd	-	-
Maltose	+	+	+	nd	-	-
Potassium gluconate	+	+	+	nd	w	-
Capric acid	-	-	+	-	-	-
Adipic acid	+	+	+	-	-	-
Malate	-	+	+	nd	+	-
Trisodium citrate	-	-	+	-	-	-
Phenylacetic acid	+	+	+	-	-	-

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5.3.4 Characterisation of *Nonomuraea* sp. TPS358a

The genus *Nonomuraea* is a member of the family *Streptosporangiaceae*, houses aerobic and Gram- positive bacteria that produce extensively branched substrate and aerial mycelia, which further differentiate into straight, hooked or spiral spore chains (Zhang *et al.*, 1998). Cell wall characteristics of the genus *Nonomuraea* include the presence of *meso*- DAP and madurose as cell wall sugar, the predominant menaquinones that consists of MK-9(H_4), MK-9(H_2) and MK-9(H_0), the fatty acid profiles that are dominated by 10- methyl- 17- and iso- 16- branched fatty acids and occurrence of diphosphatidylglycerol, phosphatidylethanolamine and hydroxylated phosphatidylethanolamine as predominant cell wall phospholipids (Zhang *et al.*, 1998). The *Nonomuraea* spp. were widely associated with soil (Saricaoglu *et al.*, 2018), rhizosphere (Zhang *et al.*, 2014), plant tissues and roots (Li *et al.*, 2017; Niemhom *et al.*, 2017), cave (Fang *et al.*, 2017), costal (Xi *et al.*, 2011) and mangrove sediments (Suksaard *et al.*, 2016).

The putative novel strain TPS358a formed a distinct cluster from the closest related match *Nonomuraea salmonea* DSM 43678^T and as a separate sub- cluster from the second closest related matches *Nonomuraea maheshkhaliensis* 16-5-14^T, which clustered with *Nonomuraea fuscirosea* NEAU dht8^T and *Nonomuraea kuesteri* NRRL B-24325^T (Figure 4.48). The novel strain TPS358a shared 97.68- 98.33% of 16S rRNA gene similarity to these reference strains. *N. maheshkhaliensis* 16-5-14^T was isolated from mangrove rhizosphere mud while *N. fuscirosea* NEAU dht8^T from rhizosphere soil of rehmannia (Ara *et al.*, 2007; Zhang *et al.*, 2014). *N. kuesteri* NRRL B-24325^T was isolated from soil (Kämpfer *et al.*, 2005).

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Strain TPS358a produced white aerial mycelia on ISP 2 and white to brilliant yellow aerial mycelium on ISP 3. *N. salmonea* DSM 43678^T produced cream coloured to pinkish coloured aerial mycelia on ISP 2 and ISP 3 (Kämpfer, 2012b). *N. maheshkhaliensis* 16-5-14^T was not producing aerial mycelium on ISP 2 but produced white aerial mycelium on ISP 3 (Ara *et al.*, 2007). *N. fuscirosea* NEAU dht8^T produced white aerial mycelium on both ISP 2 and ISP 3 (Zhang *et al.*, 2014). *N. kuesteri* NRRL B-24325^T only produced traces of aerial mycelium on ISP 3 (Kämpfer *et al.*, 2005). Strain TPS358a was not producing diffusible pigment on ISP 2 and ISP 3. Production of diffusible pigment was also not reported for *N. salmonea* DSM 43678^T, *N.maheshkhaliensis* 16-5-14^T, *N. fuscirosea* NEAU dht8^T and *N. kuesteri* NRRL B-24325^T on these media (Ara *et al.*, 2007; Kämpfer *et al.*, 2005; Kämpfer, 2012b; Zhang *et al.*, 2014). Strain TPS358a was observed to produce long and branching substrate and aerial mycelia without fragmentation that further differentiate into long spore chains, which is in line with the observation reported in literatures on typical characteristic of mycelia of *Nonomuraea* species.

Strain TPS358a grew optimally at pH 7- 9 and 0- 3% NaCl at 15°C, 25°C, 28°C and 32°C. When strain TPS358a could tolerate pH 6- 12, *N. maheshkhaliensis* 16-5-14^T and *N. fuscirosea* NEAU dht8^T showed positive growth at pH 5- 9 and pH 6- 9, respectively (Ara *et al.*, 2007; Zhang *et al.*, 2014). When strain TPS358a and *N. fuscirosea* NEAU dht8^T were not able to grow at 45°C, *N. maheshkhaliensis* 16-5-14^T showed positive growth. Strain TPS358a was able to tolerate NaCl up to 4%, by comparison, *N. maheshkhaliensis* 16-5-14^T and *N. fuscirosea* NEAU dht8^T were able to tolerate up to 3% and 2% NaCl, respectively (Ara *et al.*, 2007; Zhang *et al.*, 2014). Both *N. salmonea* DSM 43678^T and *N. kuesteri* NRRL B-24325^T were not reported for their ability to tolerate various growth temperatures, pH of culture media and NaCl concentrations in the previous reports (Kämpfer *et al.*, 2005; Kämpfer, 2012b).

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Production of gelatinase and urease were positive for strains TPS358a and *N. salmonea* DSM 43678^T but negative for strains *N. fuscirosea* NEAU dht8^T and *N. kuesteri* NRRL B-24325^T (Kämpfer *et al.*, 2005; Kämpfer, 2012b; Zhang *et al.*, 2014). Hydrolysis of starch and by amylase and proteinase were also reported positive for strain TPS358a and *Nonomuraea salmonea* DSM 43678^T, while *N. fuscirosea* NEAU dht8^T and *N. kuesteri* NRRL B-24325^T showed negative hydrolysis of starch and casein (Kämpfer *et al.*, 2005; Kämpfer, 2012b; Zhang *et al.*, 2014). Hydrolysis activity of *N. maheshkhaliensis* 16-5-14^T was not reported in the respective paper (Ara *et al.*, 2007).

Strain TPS358a showed positive utilisation of D- fructose, D- mannose, D- xylose and inositol, in contrast, *N. salmonea* DSM 43678^T was not utilizing all of these in the report (Kämpfer, 2012b). However, both TPS358a and *N. salmonea* DSM 43678^T were able to utilise sucrose for growth (Kämpfer, 2012b). Both strains TPS358a and *N. maheshkhaliensis* 16-5-14^T were able to utilise D- fructose, D- mannitol, D- mannose, D- xylose, inositol and sucrose (Ara *et al.*, 2007). When comparing to *N. fuscirosea* NEAU dht8^T, both TPS358a and the reference strain were able to D- galactose, D- glucose, D- maltose, D- mannitol, D- mannose, lactose and sucrose, however, both strains were not capable of utilizing sorbitol and glycine (Zhang *et al.*, 2014). Strain TPS358a was able to utilise D- fructose, D- ribose, D- xylose, L- glutamine and inositol, *N. fuscirosea* NEAU dht8^T showed negative utilisation of these carbon sources (Zhang *et al.*, 2014). Both TPS358a and *N. kuesteri* NRRL B-24325^T could utilise adonitol, D- fructose, D- galactose, D- glucose, D- mannitol, D- mannose, D- melibiose, D- trehalose, D- xylose, lactose and sucrose (Kämpfer *et al.*, 2005). Negative utilisation of sorbitol and glycine was also observed for the pair TPS358a and *N. kuesteri* NRRL B-24325^T (Kämpfer *et al.*, 2005). Strains TPS358a were able to utilise D- maltose, D- ribose and inositol, but *N. kuesteri* NRRL B-24325^T was not able to utilise those (Kämpfer *et al.*, 2005).

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The presence of *meso*- diaminopimelic in the whole cell hydrolysates of strain TPS358a, MK-9(H_4) and MK-9(H_2) as major menaquinones, $C_{17:0}$ 10- methyl and C_{16} iso -branched fatty acids indicated the phylogenetic relationship of the novel strain with *Nonomuraea* species (Kämpfer, 2012b). Species belonging to the genus of *Nonomuraea* was reported to contain diphosphatidylglycerol, phosphatidylethanolamine, hydroxylated phosphatidylethanolamine, and ninhydrin and sugar positive phospholipids as predominant phospholipids (Kämpfer, 2012b). However, strain TPS358A was lacking of these polar lipids, instead, it contained phosphatidylinositol that was also detected in the cell wall of the closest related matches including *N. salmonea* DSM 43678^T, *N. kuesteri* NRRL B-24325^T and *N. fuscirosea* NEAU dht8^T (Kämpfer *et al.*, 2005; Kämpfer, 2012b; Zhang *et al.*, 2014). The full profiles of fatty acids, menaquinones and polar lipids of strain TPS358a and comparison with closest related matches are shown in Appendices J and K.

5.3.5 Characterisation of *Glycomyces* sp. TPM287

The genus *Glycomyces* belongs to the *Glycomycetaceae* family. The *Glycomyces* species are Gram- stain and catalase- positive bacteria that produce branching substrate mycelia. Aerial mycelia may be produced depends on the culture media (Labeda, 2012c). Formation of oval, spherical or rod- like spores could be produced on the substrate mycelia and square- ended conidia could be produced on aerial mycelia in some species (Labeda, 2012c). Presence of *meso*-DAP without diagnostic whole cell sugar pattern is one of the main characteristics of *Glycomyces* spp. (Labeda, 2012c; Li *et al.*, 2018). The predominant polar lipid is diphosphatidylglycerol (Li *et al.*, 2018). Various combinations of iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$_{C16:0}$ and anteiso-$C_{17:0}$ make up the fatty acid profiles of *Glycomyces* species (Li *et al.*, 2018). The predominant menaquinones could be a combination of MK-10 and MK-11, MK-10, MK-9 or MK-12 (Li *et al.*, 2018). The *Glycomyces* species had been reported from soil (Labeda &

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Kroppenstedt, 2004; Mu *et al.*, 2018), marine sediment (Mohammadipanah *et al.*, 2018), lake (Wang *et al.*, 2018b), hypersaline habitat (Guan *et al.*, 2011; Lv *et al.*, 2015) and plant tissue (Gu *et al.*, 2007).

Strain TPM287 was clustered with *Glycomyces phytohabitans* KLBMP 1483^T, which was separated from the single member cluster containing only *Glycomyces tarimensis* TRM 45387^T and another cluster consists of *Glycomyces arizonensis* DSM 44726^T and *Glycomyces tenuis* DSM 44171^T (Figure 4.50). The novel strain shared 96.12- 98.34% of 16S rRNA gene similarity to the reference strains. *G. phytohabitans* KLBMP 1483^T was isolated from the stem of the coastal plant *Dendranthema indicum* (Linn.) Des Moul (Xing *et al.*, 2014). *G. tarimensis* TRM 45387^T was obtained from a saline- alkali soil (Lv *et al.*, 2015). *G. arizonensis* DSM 44726^T was isolated from soil (Labeda & Kroppenstedt, 2004). *G. tenuis* DSM 44171^T was isolated as an associate with the soil-originated *Streptomyces galilaeus* INA 5888 (Evtushenko *et al.*, 1991).

The strain TPM287 produced long and straight substrate mycelia and aerial mycelium was not observed. This is largely due to examination of its micromorphology was done using culture growing on ISP 2 medium, which did not encourage formation of aerial mycelia. *G. phytohabitans* KLBMP 1483^T produced branching substrate mycelia and aerial mycelia from which chains of square-ended conidia were produced (Xing *et al.*, 2014). The media including ISP 3, ISP 4, NA and Czapek's agar were reported to support aerial mycelia formation for strain *G. phytohabitans* KLBMP 1483^T (Xing *et al.*, 2014). The aerial mycelium of *G. tarimensis* TRM 45387^T was straight or flexuous with few branches with development of oval or rod-like sporangia the ends of aerial mycelia on ISP 4 (Lv *et al.*, 2015). Aerial mycelia were also reported absent for *G. arizonensis* DSM 44726^T and DSM 44171^T (Evtushenko *et al.*, 1991; Labeda &

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Kroppenstedt, 2004). Substrate mycelia of *G. tenuis* DSM 44171^T were very thin and long and densely branched (Evtushenko *et al.*, 1991).

Both strains TPM287 and *G. tarimensis* TRM 45387^T were not producing aerial mycelia and diffusible pigment on ISP 2, however, they produced light yellow and brown substrate mycelia, respectively (Lv *et al.*, 2015). Strain TPM287 and the reference strains *G. phytohabitans* KLBMP 1483^T and *G. tarimensis* TRM 45387^T produced white aerial mycelium on ISP 3 (Lv *et al.*, 2015; Xing *et al.*, 2014). Strain TPM287 did not produce aerial mycelium on ISP 4 but *G. phytohabitans* KLBMP 1483^T and *G. tarimensis* TRM 45387^T did produce white aerial mycelia (Lv *et al.*, 2015; Xing *et al.*, 2014). Production of diffusible pigment was not observed for strains TPS287 and *G. tarimensis* TRM 45387^T on ISP 3 and ISP 4, whereas black diffusible pigment was produced by *G. phytohabitans* KLBMP 1483^T (Lv *et al.*, 2015; Xing *et al.*, 2014). Strains TPM287 and TRM 45387^T produced white aerial mycelium on ISP 5, however, absent of aerial mycelia was reported for *G. phytohabitans* KLBMP 1483^T (Lv *et al.*, 2015; Xing *et al.*, 2014). *G. tarimensis* TRM 45387^T was also reported to produce white aerial mycelia on ISP 6 while strain TPM287 did not show production of aerial mycelia (Lv *et al.*, 2015). Strain DSM 44726^T produced white to yellowish white, waxy, plicate growth on most media, also without aerial mycelia (Labeda & Kroppenstedt, 2004). Faint pinkish–yellowish to yellow soluble pigment was produced by strain DSM 44726^T on some media, of which the media were not specified in the published article (Labeda & Kroppenstedt, 2004). *G. tenuis* DSM 44171^T formed white or sometimes cream or slightly pink substrate mycelia on ISP media with absent of aerial mycelia (Evtushenko *et al.*, 1991).

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Strain TPM287 showed optimum growth at pH 7- 11 and 2- 3% NaCl and grew well at 25°C, 28°C, 32°C, 37°C and 45°C. It can tolerate up to pH 12 and 4% NaCl. Interestingly, strain TPM287 required at least 1% NaCl and pH 7 for growth to occur. By comparison, *G. phytohabitans* KLBMP 1483^T and *G. tarimensis* TRM 45387^T can grow at pH 6 and tolerate up to pH 11 and pH 9, respectively (Lv *et al.*, 2015; Xing *et al.*, 2014). Growth of the reference strains occurred at 10- 37°C (*G. phytohabitans* KLBMP 1483^T), 18- 42°C (*G. tarimensis* TRM 45387^T), 20- 37°C (*G. arizonensis* DSM 44726^T) and 15- 37°C (*G. tenuis* DSM 44171^T) (Evtushenko *et al.*, 1991; Labeda & Kroppenstedt, 2004; Lv *et al.*, 2015; Xing *et al.*, 2014). In terms of tolerance to NaCl, strain TPM287 tolerated a narrower range of NaCl concentration as compared to the reference strains, which can tolerate up to 5% (*G. arizonensis* DSM 44726^T and *G. tenuis* DSM 44171^T), 7% (*G. phytohabitans* KLBMP 1483^T) and 8% NaCl (*G. tarimensis* TRM 45387^T) (Evtushenko *et al.*, 1991; Labeda & Kroppenstedt, 2004; Lv *et al.*, 2015; Xing *et al.*, 2014).

Production of proteinase was positive for strain TPS287 and most of the reference strains, except for *G. tarimensis* TRM 45387^T that was reported negative for hydrolysis of casein (Evtushenko *et al.*, 1991; Labeda & Kroppenstedt, 2004; Lv *et al.*, 2015; Xing *et al.*, 2014). Production of gelatinase was positive for strain TPM287 and all of the reference strains were reported negative for liquefaction of gelatine (Evtushenko *et al.*, 1991; Labeda & Kroppenstedt, 2004; Lv *et al.*, 2015; Xing *et al.*, 2014). Amylase production was positive for strain TPS287 and all the reference strains, except for *G. arizonensis* DSM 44726^T that did not hydrolyse starch (Evtushenko *et al.*, 1991; Labeda & Kroppenstedt, 2004; Lv *et al.*, 2015; Xing *et al.*, 2014). Production of urease was negative for strain TPS287 and the reference strains including *G. tarimensis* TRM 45387^T and *G. arizonensis* DSM 44726^T (Labeda & Kroppenstedt, 2004; Lv *et al.*, 2015). *G. phytohabitans* KLBMP 1483^T and DSM 44171^T were reported positive for

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hydrolysis of urea (Evtushenko *et al.*, 1991; Xing *et al.*, 2014). Strain TPM287 showed positive catalase reaction, which is in line with the characteristics of *Glycomyces* species. Comparison of carbon utilisation profile of strain TPM287 with the reference strains including *G. phytohabitans* KLBMP 1483^T, *G. tarimensis* TRM 45387^T and *G. tenuis* DSM 44171^T was showed in the Table 5.4 (Evtushenko *et al.*, 1991; Lv *et al.*, 2015; Xing *et al.*, 2014). The strain DSM 44726^T was not tested for utilisation of the same carbon sources as strain TPM287 and other reference strains in the previously published paper (Labeda & Kroppenstedt, 2004), hence, comparison was not available at the time of writing this thesis.

Table 5.4: Carbon utilisation profiles of strain TPS287 and the reference strains: *G. phytohabitans* KLBMP 1483^T, *G. tarimensis* TRM 45387^T, *G. tenuis* DSM 44171^T. 'nd' denoted that the data was not reported for the reference strain in the previously published paper.

Carbon source	TPM287	KLBMP 1483^T	TRM 45387^T	DSM 44171^T
Adonitol	-	nd	nd	+
D- arabinose	+	+	nd	+
D- fructose	-	+	nd	+
D- galactose	+	+	+	+
D- glucose	+	+	+	nd
D- ribose	+	+	nd	nd
D- xylose	+	+	nd	+
Inositol	-	nd	+	+
D- lactose	+	+	+	+
D- maltose	+	+	+	+
D- mannitol	+	nd	nd	+
D- mannose	+	nd	+	nd
D- sorbitol	-	nd	-	+
L- sorbose	-	nd	nd	+
Sucrose	+	nd	+	+
D- trehalose	+	+	+	nd
L- glutamine	+	nd	+	nd
Glycine	-	-	-	nd
L- lysine	-	-	-	nd

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5.3.6 Characterisation of *Agromyces* sp. TPS92

The genus *Agromyces* is classified under the family *Microbacteriaceae*, containing Gram- stain positive, aerobic to microaerophilic bacteria that produced branched substrate mycelia, short branching filaments or irregular rods that fragmented into diphtheroid, rod- like to coccoid elements. They produced whitish or yellowish round colonies, which were either with convex, smooth or rough surface that often penetrate into agar media. Cell wall of *Agromyces* spp. was characterized by the presence of 2, 4-diaminobutyric acid that was mainly composed of the L- isomer. The predominant menaquinones components are MK-12, MK-13 and MK-11. The fatty acids $C_{15:0}$ anteiso, $C_{17:0}$ anteiso and $C_{16:0}$ iso are mostly present. The *Agromyces* species had been reported from various environments including terrestrial and mangrove soil (Corretto *et al.*, 2016; Huang *et al.*, 2016; Wang *et al.*, 2018c), cave (Jurado *et al.*, 2005a), plants (Dorofeeva *et al.*, 2003; Rivas *et al.*, 2004), fermented seafood (Park *et al.*, 2010), marine sediment (Hamada *et al.*, 2014) and wall of tomb (Jurado *et al.*, 2005b).

The strain TPS92 was clustered with *Agromyces luteolus* IFO 16235^T and *Agromyces brachium* IFO 16238^T, which was separated from *Agromyces aurantiacus* YIM 21741^T and *Agromyces binzhouensis* OAct353^T (Figure 4.52). It shared 97.57- 98.09% of 16S rRNA gene similarity to the reference strains. *A. luteolus* IFO 16235^T and *A. brachium* IFO 16238^T were both isolated from rhizosphere soils of mangroves in the estuary of the Shiira River, Iriomote Island (Takeuchi & Hatano, 2001). *A. aurantiacus* YIM 21741^T and *A. binzhouensis* OAct353^T were isolated from soil (Chen *et al.*, 2016; Li *et al.*, 2003).

Straight to slightly curved rods was observed for strain TPS92. By comparison, *A. luteolus* IFO 16235^T and *A. brachium* IFO 16238^T were irregular rods that form filaments (Takeuchi & Hatano, 2001). *A. aurantiacus* YIM 21741^T and *A. binzhouensis*

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OAct353^T produced branching hyphae that break up into diphtheroid and rod- like or irregular fragments (Chen *et al.*, 2016; Li *et al.*, 2003). Aerial mycelium was not produced by strain TPS92, *A. aurantiacus* YIM 21741^T and *A. binzhouensis* OAct353^T on the ISP media (Chen *et al.*, 2016; Li *et al.*, 2003). However, *A. aurantiacus* YIM 21741^T was reported to form pink- white substrate mycelium and light yellow brown aerial mycelium on ISP 5 along with production of deep yellow brown soluble pigment (Li *et al.*, 2003). Strain TPS92 was not producing any diffusible pigment on all ISP media, whereas *A. aurantiacus* YIM 21741^T produced orange- yellow or pink- grey diffusible pigment on the ISP media (Li *et al.*, 2003). Colony morphology and diffusible pigment production were not reported for *A. luteolus* IFO 16235^T and *A. brachium* IFO 16238^T (Takeuchi & Hatano, 2001).

Strain TPS92 grew optimally at 25°C, 28°C, 32°C and 37°C in the presence of 1- 2% NaCl at pH 7- 12. Strain TPS92 tolerated up to 6% NaCl and showed growth at 15°C. *A. luteolus* IFO 16235^T and *A. brachium* IFO 16238^T grew optimally at 20- 30°C and good growth was observed at 5% NaCl and no growth at 10°C and 37°C in the presence of 8% NaCl (Takeuchi & Hatano, 2001). Strain OAct353^T grew at pH 5- 8 and 12- 37°C with NaCl concentration up to 10% (Chen *et al.*, 2016). Tolerance of *A. aurantiacus* YIM 21741^T to various physiological growth conditions was not reported (Li *et al.*, 2003). Catalase production was positive for strain TPS92, *A. luteolus* IFO 16235^T and *A. brachium* IFO 16238^T and negative for *A. aurantiacus* YIM 21741^T and *A. binzhouensis* OAct353^T (Chen *et al.*, 2016; Li *et al.*, 2003; Takeuchi & Hatano, 2001). Both TPS92 and OAct353^T were negative for oxidase production and no data on oxidase production was reported for *A. luteolus* IFO 16235^T and *A. brachium* IFO 16238^T (Chen *et al.*, 2016; Li *et al.*, 2003; Takeuchi & Hatano, 2001).

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Carbon utilisation profile was determined on ISP 9 for strain TPS92, whereas the profiles for all reference strains were determined using API 50 CHE strip (Chen *et al.*, 2016; Li *et al.*, 2003; Takeuchi & Hatano, 2001). As the API strip and the reference strains were not available in this study, comparison of carbon utilisation profiles will be performed in the future. Production of gelatinase by strain TPS92, *A. aurantiacus* YIM 21741^T and *A. binzhouensis* OAct353^T was negative, but positive results was reported for *A. luteolus* IFO 16235^T and *A. brachium* IFO 16238^T (Chen *et al.*, 2016; Li *et al.*, 2003; Takeuchi & Hatano, 2001). Hydrolysis of starch via amylase production was positive for strains TPS92, *A. luteolus* IFO 16235^T, *A. brachium* IFO 16238^T and *A. aurantiacus* YIM 21741^T, but negative for *A. binzhouensis* OAct353^T (Chen *et al.*, 2016; Li *et al.*, 2003; Takeuchi & Hatano, 2001). Production of urease was negative for strain TPS92 and all reference strains (Chen *et al.*, 2016; Li *et al.*, 2003; Takeuchi & Hatano, 2001).

Chemotaxonomy analysis of the cell wall hydrolysates of strain TPS92 revealed the presence of MK-11 and MK-12 as the major menaquinones and anteiso- $C_{15:0}$, anteiso- $C_{17:0}$ and iso- $C_{16:0}$ as major types of fatty acids, indicated that the novel strain was belonging to the genus *Agromcyes* (Akimov & Evtushenko, 2012). Moreover, the strain TPS92 also contained diphosphatidylglycerol and phosphatidylglycerol as the cell wall polar lipids. Both polar lipids diphosphatidylglycerol and phosphatidylglycerol have been reported to be the principal polar lipids for *Agromyces* species. The full profiles of fatty acids, menaquinones and polar lipids of the novel strain TPS92 and comparison with closest related matches is shown in Appendix L.

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5.3.7 Characterisation of *Mycobacterium* sp. TPM181

Mycobacterium is the type genus of the family *Mycobacteriaceae*, consists of aerobic to microaerophilic bacteria that are acid- alcohol- fast (Magee & Ward, 2012). Cells are slightly curved, appeared as straight rods or filamentous, which become fragmented into rods or coccoid elements. Cell wall was characterized by the presence of *meso*-DAP, arabinose, galactose and mycolic acids. The predominant polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phospatidylinositol mannosides. Fatty acid profiles are primarily made up of straight-chain saturated, unsaturated and 10-methyloctadecanoic (tuberculostearic) fatty acids. The MK-9(H_2) isoprenolog is the predominant menaquinones among the *Mycobacterium* species. White- to cream- coloured and yellow- or orange- pigmented colonies had been reported for *Mycobacterium* species, in which the pigment production could be stimulated by exposure to light. Mycobacteria are commonly known as human and animal pathogens; however, they are also ubiquitous in the natural environment, such as the soil (Gcebe *et al.*, 2017; Hormisch *et al.*, 2004), marine organisms (Padgitt & Moshier, 1987; Rhodes *et al.*, 2005), river water (Pickup *et al.*, 2005) and estuarine sediments (Khan *et al.*, 2002).

In general, mycobacteria were classified based on growth rate, pigment producing characteristics on culture media and pathogenic potential of a species (Eisenstadt & Hall, 1995). Occurrence of growth in less than seven days is considered as fast grower, else, as the slow grower. Light stimulation on carotenoid pigment production classifies the mycobacterial species as photochromogens, scotochromogens or non-chromogens (Eisenstadt & Hall, 1995). Photochromogens produce non- pigmented colonies in the dark and pigmented colonies following exposure to light and reincubation. Scotochromogens produce deep yellow to orange pigmented colonies without affected by exposure to light. Non- chromogens produced non- pigmented colonies, both in the

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dark and upon exposure to light, or produce pale yellow, buff or tan pigment colonies that are not intensified by exposure to light. Grouping of mycobacteria based on pathogenic potential separate the species into two major groups: *M. tuberculosis* complex and non- tuberculous mycobacteria (Sinha *et al.*, 2016). *M. tuberculosis, M. africanum, M. canettii, M. bovis, M. microti, M. orygis, M. caprae, M. pinnipedii, M. suricattae* and *M. mungi* constituted the *M. tuberculosis* complex group that causes tuberculosis, leprosy and Hansen's disease (Sinha *et al.*, 2016). The non- tuberculous mycobacteria comprised of other *Mycobacterium* spp. that cause chronic pulmonary infections, lymphadenitis, disseminated disease and soft tissue and skeletal infections, in particularly affecting patients with immune compromise such as genetic or acquired defects of the interferon-gamma/ interleukin-12 pathway or with disorders of mucociliary clearance (Jarzembowski & Young, 2008; Szymanski *et al.*, 2015).

Strain TPM181 was identified as putative novel *Mycobacterium* species that formed a single cluster with *M. houstonense* ATCC 49403^T, which was separated from its closest related matches including *M. peregrinum* ATCC 14467^T and *M. longobardum* DSM 45394^T. The novel strain shared 98.12- 98.34% of 16S rRNA gene similarity to the reference strains. Although strain TPM181 is an environmental isolate, *M. houstonense* ATCC 49403^T, *M. peregrinum* ATCC 14467^T and *M. longobardum* DSM 45394^T were recovered from face wound (Schinsky *et al.*, 2004), bronchial aspiration from a Mexican child (Kusunoki & Ezaki, 1992) and clinical specimens associated with tuberculosis, COPD, pneumonia, bronchitis, broncho-pneumonitis, lung cancer, suspected tuberculosis in systemic lupus erythematosus patient (Tortoli *et al.*, 2013), respectively.

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Strain TPM181 was observed as acid- fast rods, filaments and aerial mycelia were not produced, which is similar to the descriptions of micromorphology for *M. peregrinum* ATCC 14467^T and *M. longobardum* DSM 45394^T. *M. houstonense* ATCC 49403^T was described as acid- fast pleomorphic rods with co-occurrence of long filamentous forms. On solid medium, strain TPM181 starts to form visible orange coloured colonies with smooth surface followed by four to five days of incubation in the dark at 28°C and developed into its full morphology after seven days of incubation on ISP 2. Hence, strain TPM181 can be considered as fast growing scotochromogens. *M. houstonense* ATCC 49403^T produced white to slightly beige colonies on heart infusion agar with 5% rabbit blood at 35°C for 2 days (Schinsky *et al.*, 2004). Colonies of *M. houstonense* ATCC 49403^T were mucoid, convex, round and entire- edged without production of aerial mycelia (Schinsky *et al.*, 2004). *M. peregrinum* ATCC 14467^T formed colonies that were intermediate between smooth and rough and white to slightly yellowish but non-photochromogenic on Ogawa egg medium with 5% NaCl after seven days at 28°C and 37°C (Kusunoki & Ezaki, 1992). *M. longobardum* DSM 45394^T produced rough and non- pigmented colonies on Middlebrook 7H11 medium at 25-37°C for 7- 14 days (Tortoli *et al.*, 2013).

Although phylogenetic data and comparison of colony morphological features support the description of strain TPM181 as a novel species, data available in this thesis at the point of writing is not sufficient for the formal species description. According to the minimum standards for describing new species of the genus *Mycobacterium*, the putative novel strain is needed to be compared in parallel with all three reference strains using phenotypic assays (Table 5.5), antimicrobial susceptibility profiling, acid- fastness and mycolic acid profiling, DNA- DNA hybridization profiling and recently, sequence-based analysis of four mycobacterial housekeeping genes namely the 16S rRNA, *hsp*65, *rpo*B and *sod*A genes was also used for comparison study and species description

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(Gcebe *et al.*, 2017; Levy-Frebault & Portaels, 1992). In addition, GC content should also be determined for the novel strain.

Table 5.5: Criteria for description of novel species belong to the genus *Mycobacterium* using phenotypic assays (Gcebe *et al.*, 2017; Levy-Frebault & Portaels, 1992).

Criteria	Details
Morphological criteria including shape of the cells, organization of cells and ranges of length and width of bacilli or filaments, colony morphology and pigmentation on solid media	Löwenstein- Jensen medium, Ogawa egg yolk medium and Middlebrook 7H10 or 7H11 medium
Growth at various incubation temperatures	25°C, 30°C, 33°C, 37°C, 42°C and 45°C
Resistance to chemicals	Isoniazid (1 and 10 µg/ ml), thiophene-2-carboxylic hydrazide (2 µg/ ml), hydroxylamine (500 µg/ ml), *p*- nitrobenzoic acid (500 µg/ ml), thiacetazone (10 µg/ ml), oleate (250 µg/ ml), NaCl (5%), picrate (0.2%)
Production of enzymes	Catalase, nitrate reductase, acid phosphatase, arylsulfatase, pyrazinamidase, α- esterase, urease
Utilisation of carbon sources	D- mannitol, inositol, L- rhamnose, L- arabinose
Others	Niacin production, hydrolysis of Tween 80 and esculin

Strain TPM181 produced optimal growth at pH 6- 10 in the presence of 1- 8% NaCl. Good growth was also observed for strain TPM181 at 37°C but growth was absent at 45°C. *M. houstonense* ATCC 49403^T was reported to grow in the presence of 5% NaCl at 28°C, 35°C and 42°C (Schinsky *et al.*, 2004). *M. peregrinum* ATCC 14467^T was reported to grow at 25- 37°C in the presence of 5% NaCl but not at 42°C (Kusunoki & Ezaki, 1992). Growth of strain DSM 45394^T was reported at 25- 37°C and growth was not observed at 45°C (Tortoli *et al.*, 2013). Growth of *M. longobardum* DSM 45394^T at 5% NaCl was not reported in the published paper. Catalase production was positive for strain TPM181, and the same result was reported for all three reference strains as well (Kusunoki & Ezaki, 1992; Schinsky *et al.*, 2004; Tortoli *et al.*, 2013). Production of urease is positive for strain TPM181, *M. houstonense* ATCC 49403^T and *M. peregrinum*

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ATCC 14467^T (Kusunoki & Ezaki, 1992; Schinsky *et al.*, 2004). Negative result on production of urease was reported for *M. longobardum* DSM 45394^T (Tortoli *et al.*, 2013). Carbon utilisation profile of strain TPM181 was compared to *M. houstonense* ATCC 49403^T (Schinsky *et al.*, 2004) and *M. peregrinum* ATCC 14467^T (Kusunoki & Ezaki, 1992) as shown in Table 5.6. Utilisation of carbon source by *M. longobardum* DSM 45394^T was not available in the published paper (Tortoli *et al.*, 2013).

Table 5.6: Carbon utilisation profiles of strain TPS287 and the reference strains: *M. houstonense* ATCC 49403^T, *M. peregrinum* ATCC 14467^T. 'ND' denoted that the data was not reported for the reference strain in the previously published paper.

Carbon source	**TPM181**	**ATCC 49403^T**	**ATCC 14467^T**
Adonitol	+	-	-
D- fructose	+	+	+
D- galactose	+	-	-
D- glucose	+	+	+
D- lactose	+	-	ND
D- mannitol	+	+	+
D- sorbitol	+	+	-
D- trehalose	+	+	+
D- xylose	+	-	-
Inositol	+	-	+
L- arabinose	+	-	-
Maltose	+	-	ND
Sucrose	+	-	-

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5.4 Draft genome sequences of selected novel strains TPS16, TPS81, TPS83, TPS2 and TPS3

5.4.1 Presence of marine adaptation gene and resistance to heavy metals

Genome comparison between the obligate marine actinobacteria, the *Salinispora* species, with the terrestrial actinobacterial species had led to identification of marine adaptation genes, which involved in the electron transport and those encode various transporters (Penn & Jensen, 2012). Inability of *Salinispora* to grow in media with low osmotic strength was correlated to the loss of *Msc*L gene encoding a mechanosensitive channel. The *Salinispora* species was able to grow on media without supplementation of sea salt was restored by genetic complementation of the lost gene (Bucarey *et al.*, 2012).

The marine adaptation gene pool from *Salinispora tropica* and *S. arenicola* include *Pst*, *Liv*, *Mrp* and *Msc*L genes, the partial 2 *Nuo* (NADH- ubiquinone oxidoreductase chain) genes that encode a partial NDH-1 complex (*nuo*N, *nuo*M, *nuo*L, *nuo*K, *nuo*J, *nuo*H and *nuo*A), as well as the genes encode for Na^+/ Ca^{2+} exchanger, Na^+/ Ca^{2+} antiporter and Na^+/ Bile symporter (Penn & Jensen, 2012). In addition, the partial 2 *Nuo* genes had also been identified from *Streptomyces* spp. associated with marine sponges, together with the genes encoding putative *Trk* potassium uptake proteins *Trk*A and *Trk*H, which were also relevant to marine adaptation (Ian *et al.*, 2014). The presence of nuo genes in the genomes of *Streptomyces* spp. was responsible for growth and faster differentiation of *Streptomyces* spp. on solid media with sea water than media without seawater (Ian *et al.*, 2014). Interestingly, the presence of marine adaptation genes including partial 2 *Nuo* genes and genes encoding putative *Trk* potassium uptake proteins were also annotated from the genomes of strains TPS16, TPS81, TPS83, TPS2 and TPS3. This result suggests that the novel actinobacterial strains isolated from Tioman marine sediment also displayed marine adaptation to a certain extent, as shown by their ability to gow in the presence of high concentration of salt.

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NADH- ubiquinone oxidoreductase (complex I) catalysed NADH- and ubiquinone-1-dependent oxygen turnover to hydrogen peroxide, which occurs at the first stage of electron transfer in the respiratory chain, meanwhile, also serves as the site for production of superoxide (Johnson *et al.*, 2003; Peng *et al.*, 2018). On the other hand, the *Trk* proteins are involved in potassium ions uptake system, which helps to maintain intracellular potassium ion concentration at 200- 600 mM (Binepal *et al.*, 2016). Accumulation of potassium ions enabled prokaryotic cells to survive dehydration and membrane damage, to regulate Na^+/ H^+- dependent cell energetic and pH homeostasis and to minimize interference with the structures and the functions of intracellular proteins. Previous report showed that absent of *Trk* system led to failure of *E. coli* TK2420 to grow in hypersaline conditions and culture medium with low pH (Binepal *et al.*, 2016).

Presence of high concentration of heavy metals in various natural habitat including water and sediments were notably caused by anthropogenic pollution from industries, agriculture and urbanization (Alvarez *et al.*, 2017). Presence of highly concentrated heavy metals in the environment, in particularly of iron, zinc, manganese, copper, cobalt, nickel, vanadium and molybdenum, causes oxidative stress damage to the fundamental macromolecules. The marine microorganisms, therefore, develop various mechanisms through molecular adaptation to survive the heavy metal stress. This is evidenced by the presence of heavy metal resistance genes in the genomes of the five novel actinobacterial strains TPS16, TPS81, TPS83, TPS2 and TPS3. Previous study had reported the ability of a marine- derived *Streptomyces* sp. to tolerate the highly toxic tellurite for the first time, in addition to a diverse set of heavy metals that include copper, cobalt, mercury, chromate and nickel (Undabarrena *et al.*, 2017). Interestingly, the novel strain TPS3 also showed the presence of tellurite resistance gene.

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5.4.2 Potential of novel actinobacterial species to produce secondary metabolites based on prediction of BGCs

Concentration of heavy metals and exposure to oxidative stress had been reported to stimulate and regulate biosynthesis of secondary metabolites by actinobacteria. Prediction of BGCs from the genomes of *Nocardiopsaceae* strains TPS16, TPS81 and TPS83, *Nocardiopsis* sp. TPS2 and *Streptomyces* sp. TPS3 disclosed the potential of these novel actinobacterial strains to produce a wide variety of natural products. Compounds listed in the Tables 4.41, 4.42 and 4.43, as predicted from the genome with antiSMASH, were searched against literature for their biological activities, as shown in the following tables (Tables 5.7). Notably, most compounds predicted from those genomes exhibit antibacterial, antifungal, antiviral, anti-parasitic and cytotoxic activities. Mode of action of the compounds predicted from these actinobacterial genomes was listed in Appendix H.

The same predicted secondary metabolites as strain TPS16 had been determined for strains TPS81 and TPS83, however, also predicted from their genome on the potential to produce auricin, coelimycin, simocyclinone, microbisporicin, planosporicin, desotamide, friulimicin, laspartomycin, mannopeptimycin and marfomycins. The three strains, TPS16, TPS81 and TPS83, are hypothesized to have the same predicted secondary metabolites profile, as they are isolated as clone derivatives. However, strain TPS16 was observed to have higher number of biosynthetic gene clusters than the other two strains. The difference of the number of biosynthetic gene clusters could be caused by occurrence of gene fragmentation at the region of the gene encoding for the BGCs. By improving the quality of genome assembly may resolve the difference of metabolite profile between these three strains.

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The compounds predicted from all sequenced genomes include desferrioxamine B, ectoine, friulimicin, griseoviridin/ viridogrisein, laspartomycin, marfomycins, pristinamycin, scabichelin, simocyclinone and skyllamycin (Table 5.7). Strain TPS2 was predicted to have potential to produce carotenoid, desotamide, elaiophylin, enduracidin, isorenieratene, lasalocid, sioxanthin and SW-163, which were also produced by strains TPS16, TPS81 or TPS83 (Table 5.7). Frankiamicin, kanamycin, lankamycin, phosphonoglycans and pradimicin were predicted from the genomes of the *Nocardiopsaceae* strains TPS16, TPS81, TPS83 and *Streptomyces* sp. strain TPS3 (Table 5.7). On the other hand, the *Nocardiopsis* sp. strain TPS2 and strain TPS3 were predicted to have BGCs encoded for 2- methylisoborneol, 9- methylstreptimidone, cycloheximide/ actiphenol, dorrigocin/ migrastatin, etnangien, griselimycin, lactimidomycin, leucanicidin, lipopeptide 8D1-1/ 8D1-2, midecamycin, sorangicin and telomycin (Table 5.7).

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Table 5.7: Biological activities of predicted secondary metabolites (compounds) from the genomes of novel actinobacterial strains belong to the *Nocardiopsaceae* family: TPS16, TPS81 and TPS83. Abbreviation of activity: 'AB' as antibacterial, 'AF' as antifungal, 'AT' as cytotoxic, 'AP' as anti- parasitic and 'AV' as antiviral. 'ND' denoted as not determined.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16, TPS81, TPS83	Ectoine	Ectoine	Osmotic stress protector	Improve protein folding and protect the whole cells against osmotic stress and low growth temperature.	Han *et al*., 2018; Kuhlmann *et al.*, 2011
	Siderophore	Desferrioxamine B	Iron chelator	Is activated under low- iron conditions and repressed under iron- rich conditions, regulated by a complex between the repressor protein Fur (ferric uptake regulation) associate with ferrous iron as the corepressor.	Gunter *et al.*, 1993
	T1- PKS	R1128	AT	Non-steroidal estrogen- receptor antagonist that inhibits the colony formation of MCF-7 cells in the absence of estradiol.	Hori *et al.*, 1993
	T1- PKS	Neocarzilin	Anti- leukemic	A polyenones that consists of a bicyclodiynene chromophore.	Nozoe *et al.*, 1992
	T1- PKS	Pristinamycin	AB	A streptogramin- type antibiotic containing pristinamycin I (PI) and pristinamycin II (PII). PI is a branched cyclic hexadepsipeptide of the B group of streptogramins. PII is a polyunsaturated cyclo-peptidic macrolactone belonging to the A group of streptogramins.	Mast *et al.*, 2011
	T1- PKS	Tiacumicin B	AB	A glycosylated macrolide antibiotic features an 18-membered macrolactone core with four chiral centers and a high degree of unsaturation.	Glaus & Altmann, 2015
	T1- PKS	Isofuranonaphthoquinone	AB, AT, AP, iron chelator and antioxidant	Featuring a tricyclic naphthol (2,3- c) furan (or pyrrole)- 4,9-dione ring scaffold, with varying substitutions at rings A, B or C.	Bezabih *et al*., 2001; Guo *et al.*, 2017; Motohashi *et al.*, 2016; Zhang *et al.*, 2009
	T1- PKS	Griseoviridin/ viridogrisein	AB	A streptogramin antibiotic. Both molecules exhibited synergistic antibacterial activity.	Haste *et al.*, 2010

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16, TPS81, TPS83	Fatty acid-butyrolactone-other KS	Colabomycin	AB, AT, anti-inflammatory	A manumycin- type metabolites, consists of 2-amino-5,6-epoxy-4-hydroxycyclohex-2-en-1-one (mC_7N unit) as the core structure.	Grote *et al.*, 1988; Petříčková *et al.*, 2014
	Other	Paromomycin	AB, AP	An oligosaccharide aminoglycoside antibiotic.	Davidson *et al.*, 2009
	Lassopeptide	SSV-2083	ND	A peptide natural product.	Kersten *et al.*, 2012
TPS16, TPS81	Fatty acid-butyrolactone-other KS	Skyllamycin	AB, AT	A cyclic peptide that inhibits the binding of platelet- derived growth factor (PDGF) BB to its receptor PDGF beta. PDGF-signaling plays a role in the development of cancer, fibrosis or arteriosclerosis.	Schubert *et al.*, 2014; Toki *et al.*, 2001
	NRPS	Lobosamide	AP	A polyene 26 membered macrolactam.	Schulze *et al.*, 2015a
	NRPS	Methylpendolmycin	AT	An indolactam alkaloid with an N- methylisoleucine moiety incorporated in the nine- membered indolactam ring. It inhibits the PDBu (phorbol ester) binding to protein kinase C.	Huang *et al.*, 2011; Sun *et al.*, 1991
	NRPS	Salinilactam	ND	A polyene macrolactam consists of two isolated polyene fragments, a 1,2,3,5-tetrahydroxy alkane moiety, three methyl groups and an amide functionality.	Udwary *et al.*, 2007
	Putative	A54145	AB	A complex of acidic lipopeptide antibiotics.	Counter *et al.*, 1990; Fukuda *et al.*, 1990
	Terpene	Carotenoid	Antioxidant	Contain electron- rich conjugated system, acts by quenching singlet oxygen and scavenging radicals to terminate the chain reactions.	Tian *et al.*, 2014
	Terpene	Isorenieratene	Antioxidant	An aryl carotenoid that is a radical scavenger and singlet oxygen quencher.	Kohl *et al.*, 1983; Wagener *et al.*, 2012

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16, TPS81	Terpene	Sioxanthin	Antioxidant	A glycosylated carotenoid.	Richter *et al.*, 2015
TPS81, TPS83	NRPS	Desotamide	AB	A cyclohexapeptide.	Song *et al.*, 2014
	NRPS	Friulimicin	AB	A cyclic lipopeptide antibiotic.	Aretz *et al.*, 2000; Schneider *et al.*, 2009
	NRPS	Laspartomycin	AB	An acidic lipopetide antibiotic.	Borders *et al.*, 2007
	NRPS	Mannopeptimycin	AB	A cyclic glycopeptide antibiotic.	Singh *et al.*, 2003
	NRPS	Marfomycins	AB	A cycloheptadepsipeptide antibiotic.	Zhou *et al.*, 2014
TPS16	Fatty acid	Chlorizidine A	AT	An alkaloid that contains a chlorinated 2,3-dihydropyrrolizine ring attached to a chlorinated 5*H*- pyrrolo [2,1-*a*]isoindol-5-one.	Alvarez-mico *et al.*, 2013
	Fatty- acid- NRPS	Calicheamicin	AB, AT	The core structure has a conjugated 10- membered enediyne unit and a cyclohexenone ring associate with a carbamate moiety and an exocyclic olefinic appendage ending with a methyltrisulfide chain.	Maiese *et al.*, 1989; Nicolaou *et al.*, 2012
	Fatty- acid- NRPS	Elaiophylin	AB, AT, AP, Immunosuppresive activity	A 16- membered macrodiolide abtibiotic with C2 symmetry that inhibits activation of B cells by lipopolysaccharide and cell proliferation. Is an autophagy inhibitor.	Haydock *et al.*, 2004; Takahashi *et al.*, 1967; Wang *et al.*, 2017b; Zhao *et al.*, 2015
	Fatty- acid- NRPS	Lankamycin	AB	A neutral macrolide with a 14- membered ring aglycone and lankolide, also contains sugar moieties 4-*O*-acetyl-arcanose and chalcose at C3 and C5, respectively.	Martin *et al.*, 1976

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16	Fatty- acid- NRPS	AT2433	AB, AT	An indolocarbazole antibiotic.	Golik *et al*., 1989; Matson *et al.*, 1989
	Fatty- acid- NRPS	Lipomycin	AB	An acylic polyene antibiotic.	Bihlmaier *et al.*, 2006
	Fatty- acid- NRPS	Pradimicin	AB, AF, AV	A nonpeptidic benzonaphtacenequinone antibiotic that contains the moieties including D- xylose, 4,6-dideoxy-4-methylamino-D-galactose, D- alanine and a substituted 5,6-dihydrobenzo-α-naphtacenequinone	Balzarini *et al.*, 2007; Oki *et al.*, 1990; Tomita *et al.*, 1990
	Fatty- acid- NRPS	Versipelostatin	GRP78/Bip molecular chaperone down regulator	A macrocyclic compound consists of an alpha- acyltetronic acid and sugar moieties. It helps to reduce stress responose induced by GRP78 that involve in the resistant mechanism against chemotherapy and hypoxic stress in solid tumour.	Park *et al.*, 2002
	NRPS	Lyngbyatoxin	Tumour promoter and skin irritants	An indole alkaloid containing nine membered lactam rings. Acts as a protein kinase C activator.	Cardellina *et al.*, 1979; Fujiki *et al.*, 1981
	NRPS	Micromonolactam	No activity	A polyene macarcylic lactam.	Skellam *et al.*, 2013
	NRPS	Teleocidin B	AP, also a tumour promoter	An indole alkaloid, with an indolactam and a monoterpenoid moiety fused with C-6 and C-7 of the indole. A protein kinase C activator.	Awaka *et al*., 2014; Takashima & Sakai, 1960
	NRPS	WS9326	AP	A congener of depsipeptide that inhibit expression of asparaginyl- tRNA synthetase genes.	Yu *et al.*, 2012
	Putative	Aclacinomycin	AB, AT	An anthracycline glycoside antibiotic.	Oki *et al.*, 1975; Oki, 1979
	Putative	Arimetamycin	AT	Feature a 20 carbon (type II) anthracycline core with 2 distinct glycosylation.	Kang & Brady, 2013
	Putative	Avermectin	Insecticidal	An antibiotic class of macrocyclic lactones.	Lasota & Dybas, 1991

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16	Putative	Bottromycin A2	AB	A microtermolide that contains a 12- membered cyclic skeleton with an amidine moiety that is formed through condensation of cyclic tetrapeptide and linear tripeptide units.	Hou *et al.*, 2012; Shimamura *et al.*, 2009
	Putative	C- 1027/ Lidamycin	AB, AT	An acidic protein containing nine- membered cyclic enediynes.	Hu *et al.*, 1988; Jiang *et al.*, 1995; Shao & Zhen, 2008
	Putative	Chartreusin	AB, AT	A glycosidic antibiotic that contains a disaccharide unit composed of D- fucose and D- digitalose.	Leach *et al.*, 1953; Portugal, 2003
	Putative	Chloroeremomycin	AB	A glycopeptide antibiotic that contains a central core heptapeptide.	Prowse *et al.*, 1995; Rodriguez *et al.*, 1998
	Putative	Cinerubin B	AT	An anthracycline antibiotic that inhibit hypoxia- inducible-factor- 1- alpha activation.	David *et al.*, 2005; Matsuzawa & Oki, 1981; Yamazaki *et al.*, 2006
	Putative	Coelichelin	AB	An alkaloid containing a functionalized 1, 5-oxathiocane.	Gomez-Escribano *et al.*, 2012
	Putative	Congocidine and distamycin	AV	A member of pyrrolamide family of NRPS that contain pyrrole-2-carboxamide moieties in the core structure and inhibit viral DNA replication, by reversibly binding to at least four consecutive A–T minor-groove pairs.	Al-Mestarihi *et al.*, 2015
	Putative	Cosmomycin	AB, AT	An anthracycline antibiotic that acts as an inducer of cell differentiation, also a DNA intercalator that interacts with minor groove of DNA.	Furlan *et al.*, 2004; Morioka *et al.*, 1985
	Putative	Echosides	AB, AT	Belonging to para- terphenyl natural products that exhibit strong topoisomerase I and modest topoisomerase II (alpha) inhibitory activity.	Deng *et al.*, 2014; Zhu *et al.*, 2014

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16	Putative	D- cycloserine	AB, NMDA agonist	An NMDA agonist that acts on the glycine-binding site of NMDA receptor. It disrupts bacterial cell wall formation by inhibiting the incorporation of DL-alanine-1-^{14}C into bacterial cell wall and protein fractions.	Neuhaus & Lynch, 1964; Schade & Paulus, 2016
	Putative	Euduracidin	AB	A basic peptide antibiotic containing chlorine that inhibits the transglycosylation step of peptidoglycan biosynthesis.	Fang *et al.*, 2006; Higashide *et al.*, 1968
	Putative	Fluostatin	AB, AT	Contain fluorenone chromophore that bears two phenolic hydroxyl groups and attached with aliphatic ring. Are dipeptidyl peptidases inhibitors.	Akiyama *et al.*, 1998; Chaudhary *et al.*, 2013
	Putative	Frankiamicin	AB	A pentagular polyketide.	Ogasawara *et al.*, 2015
	Putative	Glycopeptidolipid	No activity	Involve in sliding motility and biofilm formation.	Schorey & Sweet, 2008
	Putative	Gobichelin	Iron chelator	Peptide natural product, which is also a siderophore.	Chen *et al.*, 2013
	Putative	Herboxidiene	AT, herbicide	A phytotoxic polyketide that inhibits seeds germination, activation of low- density lipid receptor, increase G1 and G2 cell phase population.	Isaac *et al.*, 1992; Murray & Forsyth, 2008
	Putative	Hedamycin	AT, AP	An aromatic polyketide, consists of a planar 4*H*-anthra[1,2-b] pyran polycyclic aglycone with a bisepoxide side chain, two aminosugars attached via C- glycoside linkages. It inhibits the cell cylce progression and induces substantial S- phase arrest.	Das & Khosla, 2009; Tu *et al.*, 2004; Ui *et al.*, 2007
	Putative	Himastatin	AB, AT	A unique dimeric cyclohexadepsipeptide joined through a biphenyl linkage between two oxidized tryptophan units.	Lam *et al.*, 1990; Mamber *et al.*, 1994
	Putative	Kanamycin	AB	An aminoglycoside antibiotic with the neamine core, which is composed of a six membered aminocylitol glycosidically linked to a glucosaminopyranose. Promote misreading and inhibit the translocation of the tRNA-mRNA complex.	Gourevitch *et al.*, 1958; Salian *et al.*, 2012

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16	Putative	Kedarcidin	AB, AT	An enediyne containing chromophore embedded in a highly acidic single chain polypeptide. Also known as a chromoprotein. Antitumour activity is given by DNA damaging chromophore and a protease like apoprotein. It cleaves duplex DNA site specifically in a single stranded manner.	Lam *et al.*, 1991; Zein *et al.*, 1993
	Putative	Kosinostatin	AB, AT, anti- yeast	A quinocycline antibiotic that induce cell apoptosis.	Rambabu *et al.*, 2015
	Putative	Lasalocid	AB	A polyether antibiotic.	Westley *et al.*, 1974
	Putative	Lomaiviticin	AB	Aromatic polyketides of the angucycline family that contains a diazotetrahydrobenzo[b]fluorene scaffold with dimeric diazobenzofluorene glycoside, which inhibit cell proliferation by inducing double strand breaks in DNA.	Janso *et al.*, 2014; Herzon, 2017
	Putative	Nocathiacin	AB	A subclass of thiazolyl peptides belonging to the nosiheptide-class of tricyclic peptide antibiotic. Binds to the 23S rRNA of the 50S ribosomal unit and prevents normal conformational transition that occurs from 23S-L11 interaction and inhibits translation of genes involve in the elongation step of bacterial protein synthesis.	Pucci *et al.*, 2004; Singh *et al.*, 2017
	Putative	Nogalamycin	AT	An anthracycline antibiotic that inhibits DNA- directed synthesis by binding to adenine or thymine of the DNA primer.	Bhuyan & Reusser, 1970
	Putative	Paromomycin	AB, AP	An aminoglycoside antibiotic that binds to the 30S ribosomal subunit causes impairment of bacterial protein synthesis. It inhibits the metabolism of parasite and mitochondrial inhibition.	Davidson *et al.*, 2009; Kanyok *et al.*, 1994; Thompson *et al.*, 1959
	Putative	Pentalenolactone	AB, AT, AV	A sesquiterpenoid antibiotic that inhibits nucleic acid synthesis in bacterial cells and acts as a specific inhibitor of glyceraldehyde-3-phopshate dehydrogenase in the glycolytic pathway.	Cane *et al.*, 1990; Hartmann *et al.*, 1978; Takahashi *et al.*, 1983

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16	Putative	Phenalinolactones	AB	A terpenoglycoside antibiotic that contains a diterpenoid tricycle, a 2, 3, 6- trideoxysugar, a pyrrole-carboxylic acid and an oxidized unsaturated γ-lactone.	Gebhardt *et al.*, 2011
	Putative	Ristocetin (Ristomycin A)	AB	A glycopeptide antibiotic that contains a central core heptapeptide and inhibits bacterial cell wall peptidoglycan synthesis.	Barña & Williams, 1984
	Putative	Salinomycin	AB, AP, AT	A polyether ionophore antibiotic that inhibits the Wnt/ β-catenin signalling pathway and leads to inhibitin of proliferation, induction of apoptosis and reduction of mobility of cancer cell lines.	Antoszczak *et al.*, 2014; He *et al.*, 2013; Miyazaki *et al.*, 1974
	Putative	Scabichelin	Iron chelator	A tris-hydroxamate siderophore.	Kodani *et al.*, 2013
	Putative	Sporolide	AV	A polycyclic macrolide that inhibit the HIV-1 reverse transcriptase.	Dineshkumar *et al.*, 2014
	Putative	Staurosporine	AT, muscle relaxing agent, inhibit platelet aggregation, induce neuron outgrowth	An alkaloid that blocks cell cycle by inhibiting protein kinase C and cyclin- dependent kinase, inducing apoptosis via inhibition of expression of the cell cycle proteins at the G2/M checkpoint.	Antonsson & Persson, 2009; Omura *et al.*, 1994; Tamaoki *et al.*, 1986
	Putative	SW- 163	AF, Immunosuppressive	An antibiotic with large ring ester structure.	Takahashi *et al.*, 2001
	Putative	Teicoplanin	AB	A lipoglycopeptide antibiotic that binds to the terminal amino acyl-D-alanyl-D-alanine and thus interferes with peptidoglycan biosynthesis including glycan polymerization and cross linking.	Jung *et al.*, 2009; Parenti, 1986
	Putative	Vancomycin	AB	A glycopeptide antibiotic contains a central core heptapeptide that binds to the terminal amino acyl-D-alanyl-D-alanine and inhibits the peptidoglycan cell wall synthesis.	Nagarajan, 1991

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Table 5.7, continued.

Strains	Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
TPS16	Putative	SF2575	AB, AT	A tetracycline polyketide antibiotic that exhibits antitumour activity by targeting the DNA topoisomerases I and II.	Pickens *et al.*, 2009
	Saccharide	Phosphonoglycans	No activity	Phosphonate containing exopolysaccharides that involve in ion buffering capacity, ion transport, provide enzyme resistance, inter- and intra- species communication.	Hilderbrand, 2018; Yu *et al.*, 2014
TPS81	Fatty acid-butyrolactone-other KS	Auricin	AB	An angucycline polyketide antibiotic that conjugate to an amino deoxyhexose D- forosamine.	Rehakova *et al.*, 2013
	Fatty acid-butyrolactone-other KS	Coelimycin	No activity	A polyketide alkaloid that contains a functionalized 1, 5-oxathiocane.	Gomez-Escribano *et al.*, 2012
	Fatty acid-butyrolactone-other KS	Simocyclinone	AB, Cytostatic activity	An angucycline antibiotic consists of an angucyclic polyketide core, a deoxyhexose (D-olivose), a tetraene side chain and a halogenated aminocoumarin.	Schimana *et al.*, 2000; Trefzer *et al.*, 2002
	NRPS	Microbisporicin	AB	A lantibiotic that contains two post- translational modifications in A1 and A2 with bis-hydroxylated proline and 5-chloro-trypthopan and mono- proline, respectively.	Castiglione *et al.*, 2008
	Terpene	Planosporicin	AB	Belonging to the actagardine- mersacidin subgroup of type B lantibiotics that selectively blocks peptidoglycan biosynthesis and causes accumulation of uridine triphosphate - linked peptidoglycan precursors in growing bacterial cells. Also serves as an extracellular signalling molecule to elicit precocious production of it.	Castiglione *et al.*, 2007; Sherwood & Bibb, 2013
	Terpene	Sioxanthin	Antioxidant activity	A C_{40}-carotenoid, glycosylated on one end of the molecule and containing an aryl moiety on the opposite end.	Richter *et al.*, 2015

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Table 5.8: Biological activities of predicted secondary metabolites (compounds) from the genomes of the *Nocardiopsis* sp. strain TPS2. Abbreviation of activity: 'AB' as antibacterial, 'AF' as antifungal, 'AT' as cytotoxic, 'AP' as anti- parasitic and 'AV' as antiviral.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
Thiopeptide-*trans*- AT PKS	9- methylstreptimidone	AT, AF, AV	A glutarimide antibiotic, featuring a glutarimide moiety and a terminal diene moiety, which introduce interferon- inducing activity in viral infected cells and induce apoptosis.	Otani *et al.*, 1989; Saito *et al.*, 1976; Takeiri *et al.*, 2012
	Cycloheximide/ actiphenol	AF	Both belonging to glutarimide- containing polyketide family of natural products. Actiphenol has a phenol in place of cyclohexane moiety. Both inhibit eukaryotic translation.	Schneider- Poetsch *et al*., 2010; Whiffen *et al.*, 1946; Yin *et al.*, 2014
	Dorrigocin/ migrastatin	AT (migrastatin), AF (dorrigocin)	Migrastatin and dorrigocin are shunt metabolites of iso-migrastatin. Migrastatin is a 14 membered lactone (14 membered macrolide) with a glutarimide side chain. Dorrigocin A and B are glutarimide containing polyketides.	Hochlowski *et al.*, 1994; Ju *et al.*, 2005; Karwowski *et al.*, 1994
	Etnangien	AB, AV	A macrolactone antibiotic that inhibits nucleic acids polymerase.	Irschik *et al.*, 2007
	Lactimidomycin	AT, AF	A 12- membered unsaturated macrolide antibiotic with glutarimide side chain. As inhibitor of tumour cell migration.	Ju *et al.*, 2009; Sugawara *et al.*, 1992
	Sorangicin	AB	A macrolide polyether antibiotic that specifically inhibits eubacterial RNA polymerase by binding to the β- subunit pocket of RNA polymerase.	Campbell *et al.*, 2005; Irschik *et al*., 1986; Smith *et al.*, 2009
trans- AT PKS	Bongkrekic acid	No activity	A heat stable, highly saturated tricarboxylic fatty acid that is a mitochondrial toxin	Anwar *et al.*, 2007
Terpene	2- methylisoborneol	No activity	A saturated cyclic tertiary alcohol that causes earthy or musty taste and odour in natural waters	Giglio *et al.*, 2011; Izaguirre *et al.*, 1982
NRPS	Cremimycin	AB, AT	A 19- membered macrolactam antibiotic with a 2,6-dideoxy-3-*O*-methylallose (cymarose) and a bicyclic 19-membered macrolactam ring.	Amagai *et al.*, 2011; Igarashi *et al.*, 1998

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Table 5.8, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
T1 PKS	Filipin	AF	A 28- membered ring pentane macrolide that are devoid of sugar and constitutes the archetype of non-glycosylated polyenes. It interacts with the fungal membrane ergoserol and cholesterol that leads to alteration of membrane structure.	Bergy & Eble, 1968; Payero *et al.*, 2015
	Leucanicidin	Insecticidal	A plecomacrolide antibiotic with a 16- membered macrolactone containing two conjugated diene units connected with a six- membered hemiacetal side chain through a C_3 spacer.	Isogai *et al.*, 1984; Wu *et al.*, 2016
	Midecamycin	AB	A 16- membered macrolide antibiotic. It inhibits staphylococcal enteroxin B- induced mRNA expression of Th2 cytokines interleukin- 4 and -5 in human peripheral blood monunuclear cells from atopic dermatitis patients. Also, it suppresses lipoteichoic acid- induced or peptidoglycan-induced interleukin- 5 mRNA expressions in the patients, leads to synthesis of cytokines in blood mononuclear cells, indicating that it controls Th2 cytokine production.	Matsui & Nishikawa, 2004; Tsuruoka *et al.*, 1971
	Spinosad	Insecticidal	A tetracyclic polyketide aglycone attached to a neutral saccharide substituent (2, 3, 4-tri-*O*-methyla- L-rhamnosyl) on the C-9 hydroxyl group and an aminosugar moiety (β-D-forosaminyl) on the C-17 hydroxyl group. This tetracyclic ring system contains *cis*-anti-*trans*-5, 6, 5-tricyclic moiety that is fused to a 12-membered lactone.	Kirst, 2010
	Streptolydigin	AB, AT	Belonging to tetramic acids family. It is comprised of an epoxide- containing bicyclic ketal connected by a polyene spacer to a glycosylated acyl tetramic acid. A bacterial RNA polymerase inhibitor that prevents the nucelotide triphosphate insertion step and interferes with RNA chain elongation process and inhibits DNA transcription. Also, it inhibits expression of terminal deoxynucleotidyl transferase in leukocytes from leukemic patients.	Olano *et al.*, 2009; Pronin & Kozmin, 2010; Siddhikol *et al.*, 1969
	Tirandamycin	AB, AP	It is a tetramic acid featured by a pyrrolidine- 2, 4- dione ring system and an itriguing bicyclic ketal moiety. It targets bacterial RNA polymerases and blocks the transcriptional elongation process. Also, it targets the asparaginyl- tRNA synthetase and kills the parasites.	Carlson *et al.*, 2009; Espinosa *et al.*, 2012; Rateb *et al.*, 2014; Zhang *et al.*, 2016b
NRPS	Incednine	AT	A 24- membered macrolactam glycoside with two unusual aminosugars that inhibits the anti-apoptotic functions of the molecular target Bcl-2/Bcl-xL in oncology.	Futamura *et al.*, 2008; Takaishi *et al.*, 2008

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Table 5.8, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
NRPS	Feglymycin	AB, AV	A linear tridecapeptide with nine non-proteinogenic amino acids that contains a central stretch of amino acids with alternating D/L configurations at the sterogenic α- carbon and forms a β-helical dimeric structure. It targets the peptidoglycan biosynthesis pathway and inhibits cytoplasmic enzymes MurA/ MurC (UDP-N-acetylglucosamine enolpyruvyl transferase/ UDP-N-acetylmuramyl-L-alanine ligase). It also inhibits HIV cell- to- cell transfer between HIV infected T cells and uninfected $CD4^+$ T cells and the DC- SIGN- mediated viral transfer to $CD4^+$ T cells. It is also a GP120/CD4 binding inhibitor.	Férir *et al.*, 2012; Gonsior *et al.*, 2015; Hänchen *et al.*, 2013
	Griselimycin	AB	Cyclic depsidecapeptide contains three (2S, 4R)-4-methyl-prolines that blocks oxidative degradation and increases metabolic stability in animal models. Active against *Mycobacterium tuberculosis* by inhibiting the DNA polymerase sliding clamp DnaN.	Kling *et al*., 2015; Lukat *et al.*, 2017
	Jawsamycin	AF	An antibiotic containing 5'-amino-5'-deoxy-5,6-duhydrouridine and a highly unusual polycyclopropanated fatty acids. It acts as an inhibitor of the cholesterol ester transfer protein.	Hiratsuka *et al.*, 2014; Yoshida *et al.*, 1990
	Lipopeptide 8D1-1/ 8D1-2	AB	Both 8D1-1 and 8D1-2 are linear lipopeptides with an undecapeptide. 8D1-2 is an epoxy-ring-opened derivative of 8D1-1.	Xu *et al.*, 2016
	Telomycin	AB	A lipopetide antibiotic with a cyclic depsipeptide that contains 11 amino acids including five non-proteinogenic amino acids, a nonapeptide lactone ring that is formed between the Thr hydroxyl group and the C- terminal caboxyl group.	Fu *et al.*, 2015
	Thiocoraline	AB, AT	A twofold- symmetric bicyclic non-ribosomal octathiodepsipeptide that contains two moieties of the chromophore 3-hydroxy-quinaldic acid. It inhibits DNA and RNA synthesis by targeting DNA polymerase, RNA polymerase and thymidylate synthase due to the DNA bisintercalative properties and DNA polymerase α inhibitory activity.	Erba *et al.*, 1999; Lombó *et al.*, 2006; Romero *et al.*, 1997
NRPS	Triostin A	AB, AT	A quinoxaline antibiotic with a disulfide- bridged, bicyclic, depsipeptide scaffold that pre-organizes two quinoxaline intercalating units. It recognises C-G sequence through interaction with the DNA bases by π- stacking with the DNA bases and the hydrogen bonding between the peptide scaffold and double stranded DNA. It also inhibits DNA binding of hypoxia-inducible factor-1 (HIF-1) and HIF-1α protein accumulation.	Cornish *et al.*, 1983; Hattori *et al.*, 2016; Ray & Diederichsen, 2009

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Table 5.9: Biological activities of predicted secondary metabolites (compounds) from the genomes of the *Streptomyces* sp. strain TPS3. Abbreviation of activity: 'AB' as antibacterial, 'AF' as antifungal, 'AT' as cytotoxic, 'AP' as anti- parasitic and 'AV' as antiviral.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
trans- AT PKS	Pactamycin	AB, AT, AP	An aminocyclopentitol antibiotic that inhibits protein translation and RNA interactions in eukaryotic, bacterial and archaea cells, by promoting structural changes in the 30S ribosomal subunit and prevents tRNA from binding. Also, it affects the elongation process of prokaryotic ribosomes.	Broderson *et al.*, 2000; Hanessian *et al.*, 2013
	Viguiepinol	AF	A diterpene with an *ent*- primarane skeleton.	Bi & Yu, 2016; Kawasaki *et al.*, 2006
Butyrolactone	Alnumycins	AB, AT	Aromatic polyketides that contain a 4'- hydroxy- 5'-hydroxymethyl- 2', 7'- dioxane moiety and is attached to an isochromanequinone aglycone via a C_8-C_1' bond.	Bieber *et al.,* 1998; Oja *et al.*, 2012
	Aranciamycins	AB, AT	Anthracycline antibiotics with amino acids being absent on the sugar moiety. It is an inhibitor of collagenase, an enzyme that is present in high level during progression of tumor metathesis and arthritis.	Khalil *et al.*, 2015
	Erdacin	Antioxidant	A pentacyclic polyketide with a pentacyclic skeleton.	King *et al.*, 2010
	Pyralomicin	AB	An antibiotic with an aminocarbasugar unit as the glycone that is attached to a polyketide- peptide derived core structure. They have a benzopyranopyrrole chromophore that is linked to cyclohexene ring or a tetrahydropyran ring through a nitrogen atom.	Flatt *et al.*, 2013; Kinoshita *et al.*, 1995
	Steffimycin	AB, AT	An anthracycline antibiotic that inhibits DNA- directed RNA synthesis by binding to the bacterial double stranded DNA template.	Bergy & Reusser, 1967; Brodasky & Reusser, 1974; Reusser, 1967
T1 PKS- Butyrolactone- NRPS	Abyssomicin	AB	A tetronic polyketide antibiotic that contains an oxo- bridge from the tetronate that forms a bicyclic system, which covalently binds to the aminodesoxychorismate synthase and inhibits *p*- aminobenzoate formation and folate biosynthesis in bacterial cells.	Gottardi *et al.*, 2011

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Table 5.9, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
T1 PKS-Butyrolactone-NRPS	Chlorothricin	AB	A spirotetronate antibiotic with an aglycone that contains tetronic acid conjugated with a *trans*- decalin system by a carboxylic ester. It is a cholesterol biosynthesis inhibitor and an inhibitor of bacterial pyruvate carboxylase.	Jia *et al.*, 2006; Kawashima *et al.*, 1991; Schindler & Zäuhner, 1973
	Kijanimicin	AB, AP	A spirotetronate antibiotic consists of a pentacyclic core with four L-digitoxose units and a nitro sugar (D-kijanose).	Zhang *et al.*, 2007b
	Lobophorins	AB, AT, anti-inflammatory	They are spirotetronate antibiotics that could induce unfolded protein response (UPR)-associated gene expression, inhibit growth of oral squamous carcinoma cells and lead to cell death in murine fibroblasts.	Cruz *et al.*, 2015; Jiang *et al.*, 1999; Niu *et al.*, 2011; Wei *et al*., 2011
	Maklamicin	AB, AF, AT	A spirotetronate antibiotic contains a trans-decalin unit and a tetronic acid moiety spiro-linked with a cyclohexene ring.	Igarashi *et al.*, 2011
	Quartromicin	AV	A spirotetronate antibiotic with the spirotetronate units being connected by enone linkers.	He *et al.*, 2012; Tsunakawa *et al.*, 1992
	RK- 682	AT	A tetronic acid with a saturated fatty acid at C-3 and a 5- hydroxymethyl moiety. It inhibits G1/S transition and dephosphorylation activity.	Hamaguchi *et al.*, 1995
	Tetronomycin	AB	A polycyclic ionophore polyether with acyl-ylidenetetronic acid moiety.	Keller-Juslén *et al.*, 1982
T2 PKS-Other- PKS	A-74528	AT, AV	A polycyclic polyketide that inhibits 2', 5'- specific phosphodiesterase and enhance synthesis of 2', 5'- oligoadenylates and interferons activity, which leads to degradation of viral and cellular RNAs and inhibition of protein synthsis in virus- infected cells.	Fujita *et al.*, 2005
	FD- 594	AB, AT	A naphthoxanthene antibiotic that contains a pyrano[4',3':6,7]naphtho[1,2-b]xanthene polyketide with a trisaccharide of 2,6-dideoxysugars.	Kudo *et al.*, 2011; Qiao *et al.*, 1998
	Fredericamycin	AB, AF, AT	Contains an entirely spiro ring system by inhibiting protein synthesis in tumour cells.	Misra & Pandey, 1982; Warnick-Pickle *et al.*, 1981

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Table 5.9, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
T2 PKS- Other- PKS	Granaticin	AB, AT	A benzoisochromanequinones polyketide antibiotic that inhibits RNA synthesis and rRNA maturation.	Elson *et al.*, 1988; Heinstein, 1982
	Griseorhodin	AB, AT, AV	A subset of the rubromycins that contains dense oxygen functionality that is located on the spiroketal core. It inhibits eukaryotic translation elongation and viral reverse transcriptase.	Atkinson & Brimble, 2015
	Hexaricins	Antioxidant	Pradimicin- like polyketides with pentangular polyphenols that acts free radical scavengers.	Gao *et al.*, 2018; Tian *et al.*, 2016
	Lysolipin	AB, cytostatic	Aromatic polyketide that has high affinity for lipids. It interacts with the C55- lipid carrier bactoprenol, which is involved in bacterial cell wall biosynthesis.	Lopez *et al.*, 2010
	Medermycin	AB, AT	An aromatic C- glycoside containing benzoisochromanequinones antibiotic.	Ichinose *et al.*, 2003; Takano *et al.*, 1976; Tanaka *et al*., 1985
	Rubromycin	AB, AT, AF, AP	A family of antibiotics that consists of a naphthazarin and isocoumarin framework linked through a 5, 6- bisbenzannulated spiroketal. Each individual compound can be distinguished by differing oxidation states at C-3', C-3, C-4 and the functionality at C-7. It also inhibits eukaryotic translation elongation and viral reverse transcriptase.	Atkinson & Brimble, 2015
	TLN-05220	AB, AT	An echinosporamicin-type antibiotic.	Banskota *et al.*, 2009
	Xantholipin	AB, AF, AT	A xanthone- derived antibiotic with a hexacyclic angular fused skeleton and contains a xanthone scaffold, a methylene dioxybridge and a β- lactam structure. As an inhibitor of HSP47 heat- shock protein gene expression and inhibits collagen production as induced by TGF-beta.	Terui *et al.*, 2003; Wu *et al.*, 2017
T2 PKS- T1 PKS	Arixanthomycin	AB, AT	Pentangular polyphenols that belonging to a subfamily of aromatic polyketides that use C24 to C30 polyacetate precursors.	Kang & Brady, 2014

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Table 5.9, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
T2 PKS- T1 PKS	Curamycin	AB	Chlorine containing glycosidic antibiotic.	Galmarini & Deulofeu, 1961
	Landomycins	AB, AT	Aromatic polycyclic angucycline polyketides that are featured by the presence of benz(a)anthracene tetracyclic framework with an angularly condensed ring. They inhibit tumour colony formation and interfere with DNA synthesis.	Ostash *et al.*, 2009
	Polyketomycin	AB, AT	A tetracyclic quinone glycoside.	Daum *et al.*, 2009; Momose *et al.*, 1998
T1 PKS	Aculeximycin	AB, AF	A 30- membered macrolide with a trisaccharide (aculexitriose), D- mannose and L- vancosamine that targets mitochondrial respiration system as a potent uncoupler of oxidative phosphorylation.	Ikemoto *et al.*, 1983; Miyoshi *et al.*, 1996
	Angolamycin	AB	A 16-membered epoxyenone macrolide antibiotic.	Kadar-Pauncz *et al.*, 1992; Sakamoto *et al.*, 1984
	Bafilomycins	AB, AF, AT, AP	A family of polyene macrolides containing 16- member lactone ring that acts as V-ATPase and autophagy inhibitor and promoting apoptosis.	Van Schalkwyk *et al.*, 2010; Werner *et al.*, 1984; Yoshimori *et al.*, 1991; Yuan *et al.*, 2015; Zhang *et al.*, 2011
	Candicidin/ FR-008	AF	An aromatic macrolide heptanes containing the amino sugar mycosamine and *p*- aminoacetophenone, connected through a glucoside linkage and aldol linkage, respectively.	Kligman & Lewis, 1953; Lechevalier *et al.*, 1953
	ECO-02301	AF	A linear polyene polyketide.	McAlpine *et al.*, 2005
	Indanomycin	AB	A carboxylic acid ionophore that is closely related to polyether antibiotics.	Liu *et al.*, 1979; Westley *et al.*, 1979

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Table 5.9, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
T1 PKS	JBIR-100	AT	A 16- membered tetraene macrolide that acts as V-ATPase and autophagy inhibitor and promoting apoptosis.	Ueda *et al.*, 2010
	Macbecin	AB, AT, AP	A benzenoid ansamycins antibiotic that acts an inhibitor of Hsp90, which is an overexpressed protein in tumour cells.	Martin *et al.*, 2008; Muroi *et al.*, 1980; Tanida *et al.*, 1980
	Meridamycin	Immunoregulant and neuroprotectant	A 27- membered macrolide. It is an immunoregulant that inhibits the immunosuppressive activity of FK506 and rapamycin and this makes it useful as a neuroprotectant in various neurodegenerative disorders.	Liu *et al.*, 2016; Salituro *et al.*, 1995
	ML-449	AB, AF	A 20 membered macrolactam antibiotic.	Jørgensen *et al.*, 2010
	Monensin	AB, AT, AV	A polyether ionophore antibiotic that promotes apoptosis, reduces DNA synthesis during viral replication and induces reduction of early viral antigens and impairs functions of nutrients and vacuolar organelles of the parasite and lead to cell death	Łowicki & Huczyński, 2013; Yoon *et al.*, 2013
	Naphthomycins	AB, AF, AT	Naphthalenoid ansamycins antibiotics that contain 29- membered ansamacrolactams. The compounds inhibit synthesis of fatty acid with selective toxicity against prokaryotes and production of enzymes involved in nucleic acid biosynthesis.	Keller-Schierlein *et al.*, 1983; Okabe *et al.*, 1985; Wu *et al.*, 2011b
	Nemadectin	AP	A polyether ionophorous antibiotic with a 16- membered macrocyclic lactone. Although it has not been tested for anti-tumour activity, it could inhibit P-glycoprotein efflux and lead to accumulation of rhodamine 123 in adriamycin resistant- breast cancer cell lines (MCF-7/adr).	Doscher *et al.*, 1989; Yang *et al.*, 2013
	Nigericin	AB, AF, AP, herbicide	A polyether antibiotic that activates caspase- 1 and generation, maturation and release of IL-1β in lipopolysaccharide- primed monocytes and macrophages, followed by induction of cell death.	Gumila *et al.*, 1997; Heisey & Putnam, 1986; Hentze *et al.*, 2003; Shoji *et al.*, 1968
	Nocardiopsin	Immunosuppresant	A macrolide polyketide that shows binding of FK-506 to FKBP 12.	Raju *et al.*, 2010

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Table 5.9, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
T1 PKS	Phoslactomycin B	AF, AT	A polyketide- derived antibiotic with a fully reduced cyclohexyl ring that selectively inhibits PP2A protein and helps to regulate apoptosis and activation of natural killer cells and cytotoxic T- lymphocytes.	Fotso *et al.*, 2013; Ozasa *et al.*, 1989; Palaniappan *et al*., 2003
	Pimaricins	AF, AP	Macrolide polyketides of polyene class that contain 26- membered macrolactone ring with four conjugated double bonds as the chromophore. They target ergosterol in fungal membranes with low toxicity and are widely used in food industries to control fungal contamination.	Aparicio *et al.*, 2016; Medina *et al.*, 2007
	Pladienolide	AT	A 12- membered macrolide that inhibits hypoxia- induced- factor dependent transcription pathway responsible for angiogenesis, causes mRNA splicing impairment and induces apoptosis.	(Mizui *et al.*, 2004; Sakai *et al.*, 2004; Sato *et al.*, 2014)
	PM100117/ PM100118	AF, AT	Polyhydroxyl macrolide lactones contains a macrocyclic lactone, three deoxysugars and a 1,4- naphthoquinone chromophore. They are antibiotics that alter membrane integrity and cause cell premeabilization that give rise to necrotic cell death.	Pérez *et al.*, 2016
	Stambomycin	AB, AT	A family of 51- membered glycosylated macrolides.	Laureti *et al.*, 2011
	Streptazone E	No anti-tumour activity	A piperidine alkaloid containing an intriguing cyclopenta(b)pyridine scaffold.	Liu *et al.*, 2013b; Ohno *et al.*, 2015
	Thuggacin	AB	Thiazole- containing macrolides that inhibits oxygen consumption and reduced nicotinamide adenine dinucleotide oxidation at the cytoplasmic membranes.	Steinmetz *et al.*, 2007
NRPS	Albachelin	Iron chelator	A linear peptide siderophore containing amino acids including serine, cyclic N-hydroxyornithine, N-a-acethyl-N-d-hydroxy-N-d-formylornithine and N-amethyl-N-d-hydroxyornithine.	Kodani *et al.*, 2015
	Lactonamycin	AB, AT	A heterocyclic quinone antibiotic.	Matsumoto *et al.*, 1999

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Table 5.9, continued.

Type of BGC	Compound	Biological activities	Compound description/ mode of action	References
NRPS	Mithramycin	AT	An aureolic acid group of antibiotic that binds to chromosomal DNA and core histones present in chromatin, which in turn causes alteration of post- translational modification of histone H3 and disrupts DNA- binding transcription factors.	(Banerjee *et al.*, 2014)
	Saframycin A	AT	A tetrahydroisoquinoline antibiotic that contains a bisquinone core with an α-amino nitrile moiety. It targets the minor groove of DNA and cuases single strand breaks when it is reduced with NADPH or sodium borohydride.	Li *et al.*, 2008b; Lown *et al.*, 1982
Terpene	Hopene	No activity	Pentacyclic triterpenoid that acts as bacterial membrane integrity stabilizer.	Dobritsa *et al.*, 2001; Siedenburg & Jendrossek, 2011
	Informatipeptin	No activity	A lantipeptide class of compound.	Mohimani *et al.*, 2014
	Rishirilide B	α- 2- macroglobulin inhibitor	A tricyclic compound with a partial- aromatic backbone and a C- 4 isopentyl side chain, which is a blood coagulation inhibitor and is widely used in prevention and treatment of fibrinolytic accentuated thrombosis.	Iwaki *et al.*, 1984; Yan *et al.*, 2012
Other	Borrelidin	AB, AT, AV, AP	An 18- membered nitrile- containing macrolide that inhibits threonyl- rRNA synthetase and mediate G1 cell cycle arrest and induce apoptosis.	Dickinson *et al.*, 1965; Habibi *et al.*, 2012; Kim *et al.*, 2017; Liu *et al.*, 2012; Sugawara *et al.*, 2013

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5.5 Antibacterial activity of actinobacteria by agar plug diffusion assay

In this study, five production media were employed for assessment of antibacterial activity of actinobacterial strains against Gram- positive and Gram- negative pathogens by agar plug diffusion assay including PM3, soybean meal glucose, micromonospora medium, ATCC medium 241 and starch yeast peptone media. Actinobacterial strains isolated from marine sediment in the Trondheim Fjord, Norway that were cultured on the oatmeal- based PM3 medium were able to inhibit growth of the Gram- positive *Micrococcus luteus* and the yeast, *Candida albicans* (Bredholt *et al.*, 2008). The *Streptomyces* spp. and *Micromonospora* spp. isolated from sea hare, sea anemone and sea plants, which were growing on the soybean meal medium was reported to produce antibacterial, antifungal and antitumor activities (Zheng *et al.*, 2000). On the other hand, *Micromonospora* sp. M39 that was cultivated on micromonospora medium produced antifungal activity against rice blast pathogen *Pyricularia oryzae* MPO 293 (Ismet *et al.*, 2004). Starch yeast peptone medium was reported to support production of cyclomarazines A and B with antibacterial activity against methicillin- resistant *S. aureus* and vancomycin- resistant *Enterococcus faecium*, as well as cyclomarin D with cytotoxic activity from *Salinispora arenicola* isolated from marine sediments in Palau (Schultz *et al.*, 2008). The ATCC medium 241 was previously reported to support production of antibiotic against the Gram- positive *Enterococcus faecalis* and the Gram- negative *E. coli* by a near-shore marine intertidal environment-associated *Streptomyces* sp. USC-633 (English *et al.*, 2017).

The actinobacterial strains showed variation of inhibitory activities towards different production media. This observation agrees to the research finding by English *et al.* (2017) that also reported variation of antibiotic production by marine *Streptomyces* sp. in response to nutritional variation. The *Streptomyces* sp. USC-633 displayed larger colony mass formation and better production of antibacterial activity on starch and

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sugar media combinations, as compared to media that contain casein and chitin (English *et al.*, 2017). Expression of antibiotic producing genes leading to the production of antibiotics in cells is modulated by a complex sensing ability that functions as part of the cellular machinery that interprets availability of nutrients and initiates metabolic pathways for production of various secondary metabolites, by inducing expression of biosynthetic gene clusters under different triggering conditions that involve multiple growth parameters (Challis & Hopwood, 2003; English *et al.*, 2017).

5.5.1 Antibacterial activity of *Salinispora*- like actinobacterial strains

The *Salinispora*- like strains produced antibacterial activity on five production media, with the highest number of strains on starch yeast peptone (n= 27), followed by PM3 (n= 24), micromonospora medium (n= 22), ATCC medium 241 (n= 14) and soybean meal medium (n= 8). Although most of the *Salinispora* strains obtained in this study were shown to inhibit growth of the Gram- positive pathogens *S. aureus* ATCC 29213 or *B. subtilis* ATCC 23857 as shown in agar plug diffusion assay, two strains TPS104 and TPS178 were able to inhibit the growth of Gram- positive *B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213 and also the Gram- negative *P. aeruginosa* ATCC 27853.

Previous studies had reported discoveries of secondary metabolites with antibacterial activity from *Salinispora arenicola* and *Salinispora pacifica*. Lomaiviticins A and B are antibiotics isolated from *S. pacifica* that possess dimeric diazobenzofluorene glycoside structures, which were able to inhibit the growth of the Gram- positive *S. aureus* and *Enterococcus faecium* (He *et al.*, 2001). *S. arenicola* was known to be an alternative source of rifamycins antibiotics by producing rifamycin B and rifamycin SV targets staphylococci and *Mycobacterium tuberculosis*, which had also been used to treat biliary tract infection sustained by less sensitive Gram- negative microorganisms or

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mixed bacterial flora (Di Stefano *et al.*, 2011; Kim *et al.*, 2006a). Cyclomarazines A and B are diketopiperazine dipeptides isolated from *S. arenicola* CNS-205 that exhibited antibacterial activity against Gram- positive methicillin- resistant *S. aureus* and vancomycin- resistant *E. faecium* (Schultz *et al.*, 2008). Salinisporamycin is another rifamycin antibiotic produced by *S. arenicola* that actively against *S. aureus* IFO 12732 and *B. subtilis* IFO 3134, but did not inhibit growth of *E. coli* IFO 3301 and *P. aeruginosa* IFO 3446 (Matsuda *et al.*, 2009). Saliniquinone A was also isolated from S. arenicola but exhibit a weak activity against methicilin resistant *S. aureus* (Murphy *et al.*, 2010). In addition, the compound arenimycin was also identified from *S. arenicola* that actively inhibit growth of methicillin- resistant *S. aureus*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, vancomycin- resistant *E. faecium* and *Mycobacterium bacille*.

Although *S. arenicola* was reported to be a rich source of antibiotic, previous reports showed that the compounds targeted only Gram- positive pathogens. In this study, two *Salinispora* strains isolated from marine sediment were able to inhibit growth of Gram-negative *P. aeruginosa*. Further studies should focus on purification and identification of bioactive compounds that target the Gram- negative pathogen. Identification of biosynthetic gene clusters that are responsible for the bioactive compounds from these *Salinispora* strains is also worth a detailed study.

5.5.2 Antibacterial activity of non- *Salinispora*- like actinobacterial strains

Actinobacterial strains with antibacterial activity from these studies include the putatively novel and known members of the genera *Streptomyces*, *Saccharopolyspora* and *Salinispora* and the novel members of *Nocardiopsaceae* family. Antibacterial activity of *Salinispora* strains was discussed in section 5.2. The hgiest number of non-*Salinispora*- like strains produced activity on ATCC medium 241 (n= 12), and

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subsequently on micromonospora medium (n= 11), starch yeast peptone (n= 10), soybean meal (n= 8) and PM3 (n= 5).

The *Saccharopolyspora* strain TPS37 that shared high slimilarity to *S. hirsuta* subspecies *hirsuta* was able to inhibit the growth of Gram- positive *B. subtilis* and *S. aureus* as determined by agar plug diffusion assay (Figure 4.19). Previous literature had reported production of sporacins A and B by *S. hirsuta* subspecies *kobensis* that are active against Gram- positive *S. aureus* and *B. subtilis* as well as the Gram- negative *E. coli*, *Proteus vulgaris*, *P. aeruginosa*, *Serratia* sp. and *Mycobacterium smegmatis* (Deushi *et al.*, 1979).

The *Streptomyces* strains obtained in this study were found to be active against Gram- positive or both Gram- positive and Gram- negative pathogens, the latter as showed by strains TPS6, TPS10, TPS12 and TPS17, which also inhibited the growth of Gram- negative *P. aeruginosa* and *E. coli*. *Streptomyces* is the richest known source of antibiotics. Antibiotic production is strongly correlated to stress response, in which the secondary metabolism was shown to be influenced by interaction of the regulatory proteins including the *TetR*-like protein specified by Sco3201, the *Abr*C cluster (Sco4596–8) encoding a response regulator AbrC3 and histidine protein kinases *Abr*C1 and *Abr*C2 with the small- molecule ligands associated with primary metabolisms (Rico *et al.*, 2014; Rodríguez *et al.*, 2015; Xu *et al.*, 2014).

Production of secondary metabolites will be focused on novel actinobacteria of the *Nocardiopsaceae* family, as represented by the strains TPS16, TPS81 and TPS83, which inhibited the growth of Gram-negative *E. coli* ATCC 47076 and Gram-positives *B. subtilis* and *S. aureus* ATCC 29213 in the preliminary screening using agar plug diffusion assay. Out of the five production media, the novel strains inhibited two Gram-positives and one Gram- negative on soybean meal glucose and micromonospora media,

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with larger inhibition zones were observed on soybean meal medium than on micromonospora medium. By contrast, only Gram- positive pathogens were inhibited on oatmeal- based PM3 and glucose- based medium ATCC medium 241 and starch yeast peptone medium. This finding suggested that variation of growth parameters such as alteration of media composition could unlock cryptic secondary metabolic pathways (Bode *et al.*, 2002; English *et al.*, 2017). Systematic alteration of cultivation parameters such as media formulation is one of the ways to reveal nature's chemical diversity based on "one strain- many compound (OSMAC) approach (Bode *et al.*, 2002).

Although all three strains shared 100% similarity of their 16S rRNA gene sequences, similar antibacterial activity profiles were obtained for three strains when cultured on different production media as shown in Table 4.15. Hierarchical clustering of the active fractions obtained from the crude extract of strain TPS83 with BioMap profiles revealed that all the fractions formed a separate and distinct cluster although closely related to fosfomycin. The antibiotic fosfomycin is only one of the few antibiotics that still remained active against broad spectrum targets including the multi-drug resistant and extensively-drug resistant pathogens (Borisova *et al.*, 2014; Falagas *et al.*, 2016). It is a bactericidal compound that interferes with the formation of UDP N-acetylmuramic acid, the peptidoglycan precursor, which is involved in the first cytoplasmic step of bacterial cell wall synthesis (Borisova *et al.*, 2014).

5.6 Identification of methylpendolmycin and nocapyrone R from crude extract of strain TPS83

Assessment of the six SPE fractionated crude extracts for antibacterial activity disclosed the growth inhibitory activity of fraction TPS83_D against the largest number of pathogens, including Gram-negative *P. alcalifaciens* ATCC 14990^{T}, as compared to other fractions. Two compounds had been purified and identified from the TPS83_D

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fraction, including nocapyrone R and methylpendolmycin. Identification of the compound was performed by interpreting the HRDARTMS and ^{1}H and ^{13}C NMR spectroscopic data and compared with those reported in literature (Huang *et al.*, 2011; Kim *et al.*, 2014b; Sun *et al.*, 1991).

Nocapyrone R was previously purified from a *Nocardiopsis* strain isolated from marine sediment as colourless amorphous (Kim *et al.*, 2014b). The compound was tested for induction of adinopectin and showed negative result, on the other hand, nocapyrones B, H and L were found to be positive (Kim *et al.*, 2014b). Nocapyrone B and nocapyrone A were also tested for antibacterial activity against *B. subtilis*, *S. epidermidis*, *E. coli* and *P. aeruginosa* and showed negative results. Cytotoxic activity against NIH-3T3, HepG2, and HT29 cell lines and enzyme inhibitory activity against phosphodiesterase 4, protein tyrosine phosphatase 1B, acetylcholinesterase, reverse transcriptase and glycogen synthase kinase 3β were also reported as negative for both nocapyrone A and B (Schneemann *et al.*, 2010). In this study, nocapyrone R was tested against a panel of Gram- positive and gram- negative pathogens, however, no antibacterial activity was observed for the compound.

Methylpendolmycin was first reported from a *Nocardiopsis* strain in 1991, in which it was isolated as amorphous solid, and later from *Marinactinospora thermotolerans* SCSIO 00652^{T} (Huang *et al.*, 2011; Sun *et al.*, 1991). Both *Nocardiopsis* sp. and *Marinactinospora* sp. are belonging to the *Nocardiopsaceae* family and strain TPS83 is taxonomically closely related to *M. thermotolerans* SCSIO 00652^{T}. Methylpendolmycin is a non- cytotoxic compound that acts as a protein kinase C- PDBu binding inhibitor (Table 5.6). Huang *et al.*, (2011) reported no significant cytotoxic activity against nine tumour cell lines including MCF-7 (human breast adenocarcinoma cell line), SW1990 (human pancreatic duct adenocarcinoma cell line), SMMC-7721 (human

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hepatocarcinoma cell line), NCI-H460 (human lung cancer cell line), A549 (human non-small cell lung cancer cell lines), HeLa (human epitheloid cervix carcinoma cell line), DU145 (human prostate carcinoma cell line), MDA-MB-231 (human breast cancer adenocarcinoma cell line). Antibacterial activity of methylpendolmycin was not tested and reported in the previous literature. A summary of biological activities of methylpendolmycin that had been reported in the previous studies is given as Table 5.10. The methylpendolmycin compound is present in a mixture within the active fraction, as shown by the NMR spectroscopy (Figure 4.66). Purification using reverse phase HPLC and chiral column on normal phase HPLC had been carried out and the compounds are still remained inseparable. Further purification using different chromatography methods would need to be carried out in the future.

Production of methylpendolmycin by strain TPS83 was also supported by the prediction of the compound from the genome sequences of its sister strains TPS16 and TPS81 using antiSMASH. The methylpendolmycin/ pendolmycin BGC is present at 5607 – 20933 bp of the scaffold 79 (accession number: QEIO01000080) for strain TPS16 and at 5493 – 20878 bp of the scaffold 44 (accession number: QEIN01000045) for strain TPS81 based on antiSMASH analysis and pairwise comparison of the gene sequence to methylpendolmycin/ pendolmycin BGC gene sequence of the reference strain *Marinactinospora thermotolerans* SCSIO00652^{T}. The methylpendolmycin BGC is not predicted from the genome of strain TPS83 and it could be due to occurrence of gene fragmentation in the genome during sequencing near the methylpendolmycin BGC or failure of genome assembly software to perform a paired- end mating for the gene fragments of methylpendolmycin BGC. This can be improvised by performing genome reassembly using other software or by performing a PCR that target the biosynthetic gene cluster using a specifically designed primer. A primer walking through the genome to scan for the BGC can be performed for primer design to detect the

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methylpendolmycin biosynthetic gene clusters. Nevertheless, this study showed that the soybean meal medium supported production of methylpendolmycin by inducing gene expression of methylpendolmycin/ pendolmycin BGC from strain TPS83 and the presence of methylpendolmycin/ pendolmycin BGC in the genome was supported by antiSMASH analysis of the sister strains TPS16 and TPS81.

Notably, the strain TPS83 along with its sister strains TPS16 and TPS81 are potential producers of a number of antibacterial compounds with broad spectrum activity against both Gram- positive and Gram- negative pathogens (Tables 4.41 and 5.7). Genome prediction of secondary metabolites production highlights potential of all three strains to produce paromomycin: a compound that was reported previously to be active against Gram- positive *Staphylococcus* spp. and *Mycobacterium* spp. and Gram- negative *Escherichia* spp., *Klebsiella* spp., *Proteus* spp. and *Shigella* spp. (Coffey *et al.*, 1959). Kanamycin, kosinostatin and calicheamicin are also antibacterial compounds with broad spectrum activity that had been predicted from the genome of strain TPS16, which theroretically should also be present in the genomes of both TPS81 and TPS83 (Furumai *et al.*, 2002; Gourevitch *et al.*, 1958; Maiese *et al.*, 1989).

In addition, compounds with antibacterial activity that target only the Gram- positive were also predicted from the genomes of strains TPS16, TPS81 and TPS83. These include isofuranonapthoquinone, tiacumicin B, pristinamycin, griseoviridin/ virdogrisein, colabomycin, mannopeptimycin, desotamide, marfomycins, laspartomycin, friulimicin, D- cycloserine, lasalocid, bottromycin A2, eudaracidin, salinomycin, nocathiacin, SF2575, kedarcidin, lidamycin, himastatin, phenalinolactone, teicoplanin, frankiamicin, vancomycin, ristocetin, lomaiviticin, chloroeremomycin, echosides, aclacinomycin, cosmomycin, chartreusin, lankamycin, elaiophylin, AT2433,

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pradimicin, lipomycin, desotamide, skyllamycin, simocyclinone, auricin, microbisporicin and planosporicin (Table 5.7, Appendix H).

Table 5.10: A summary of biological activity being tested for methylpendolmycin in this study and in the previous studies.

Producing strain	Biological activity	Reference
Nocardiopsis sp.	Inhibited phorbol dibutyrate receptor binding to protein kinase C (IC_{50} = 13.5 ng/ml)	Sun *et al.,* (1991)
M. thermotolerans SCSIO00652^T	The compound was not significantly cytotoxic against various tumor cell lines.	Huang *et al.,* (2011)
TPS83	Displayed antibacterial activity against Gram-positive *B. subtilis* 168, *E. faecium* ATCC 6569, *L. ivanovii* BAA-139, MRSA, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 14990.	This study

5.7 Future considerations and future work

A number of novel actinobacterial species had been identified in this study based on similarity of 16S rRNA gene and phylogenetic taxonomy. However, among the 19 novel actinobacterial strains, only description data for strains TPS16, TPS81 and TPS83 are sufficient to claim the novelty of these actinobacteria. The novel actinobacterial strains need to be characterized and compared in parallel with the closely related matches for the morphological, physiological and biochemical properties, the carbon utilisation and fatty acids profiling. In addition, profilings of the cell wall composition of the novel actinobacterial strains including cell wall sugar, polar lipids and fatty acids needs to be performed to confirm the placement of the strains as novel actinobacterial species. The *Mycobacterium* sp. strain TPM181 would need to be characterized for their morphology, production of extracellular hydrolytic enzymes, resistance to various chemicals, niacin production and hydrolysis of Tween 80 and esculin as outlined in Table 5.5 in a biosafety level 2 cabinet with biosafety level 3 work practices, of which our laboratory is lacking of.

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This study is mainly focusing on characterisation of novel actinobacterial species and production of antibacterial compounds from strain TPS83 only. Besides that, compound that is active against Gram- negative *P. alcalifaciens* is yet to be identified. Prediction of the genome sequences of strain TPS83 together with TPS16 and TPS81 revealed their potential as producer of a vast number of, antiviral, antitumour and antibacterial compounds with broad spectrum activity. Hence, it is worthwhile to study the full chemistry profiles of all three strains. The main challenge in natural product discovery from actinobacteria is the extremely low yield of novel bioactive secondary metabolites, which are always produced as the minor compounds. It often requires large volume fermentation in order to obtain enough materials for NMR spectrum analysis purpose to elucidate the complete chemical structure.

Although strains TPS2 and TPS3 were not active against any of the tested pathogens in agar plug diffusion assay, these strains were shown to be highly potential in producing antimicrobial, antiviral and antitumor compounds as predicted from their genome sequences. Growing the strains on other production media and heterologous expression of the cryptic biosynthetic gene clusters could be performed to induce expression and production of bioactive compounds from strains TPS2 and TPS3. The novel *Streptomyes* sp. strain TPS114, TPS137 and TPS143 were also tested positive inhibitory activity against the Gram- positive tested pathogens. This makes them interesting for chemistry profiling study. Genome analyses are in the progress.

Future study on comprehensive analysis of the intra-species diversity observed in the BOX-PCR results include sequencing of the 16S-23S rRNA ITS sequence of the isolates and compared the sequences with those deposited in GenBank database (Goo *et al.*, 2014). As the genus *Salinispora* is well- known to be a rich source of structurally diverse natural products and biosynthetic gene clusters associated with secondary

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metabolites were reported to be species-specific, secondary metabolites were suggested to be used to represent ecotype-defining traits for *S. tropica* and *S. arenicola* (Jensen *et al.*, 2007). Comparative genomics is another important approach that could delineate the *Salinispora* strains, of which could not be confidently resolved based on 16S rRNA gene phylogeny (Millán-Aguinãga *et al.*, 2017). In addition, as this study focuses more on the non- *Salinispora*- like actinobacterial strains, antibacterial screening of the *Salinispora*- like actinobacterial strains is still remained incomplete. *Salinispora* species are proven to be prolific producers of clinically important secondary metabolites in particularly of those with anti-tumour activity. Future study could be targeting the antibacterial profile of this *Salinispora*- like actinobacterial group.

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CHAPTER 6: CONCLUSIONS

Culture dependent techniques are important in recovering bioactive actinobacteria from marine environmental samples. This study demonstrated successful selective isolation of diverse actinobacterial strains from marine sediments using mannitol-based and humic acid vitamin media with skim milk/ HEPES or UV pre-treatments. A total of 183 actinobacterial strains were putatively identified to belong to twenty distinct genera including *Salinispora* (n= 84), *Streptomyces* (n= 60), *Micromonospora* (n= 8), *Blastococcus* (n= 5), *Mycobacterium* (n= 3), *Nocardia* (n= 3), *Rhodococcus* (n= 3), *Glycomyces* (n= 1), *Gordonia* (n= 2), *Jishengella* (n= 2), *Actinomadura* (n= 1), *Agromyces* (n= 1), *Nocardiopsis* (n= 1), *Nonomuraea* (n= 1), *Plantactinospora* (n= 1), *Pseudonocardia* (n= 1), *Saccharomonospora* (n= 1), *Saccharopolyspora* (n= 1), *Streptosporangium* (n= 1) and a novel genus of the *Nocardiopsaceae* family (n= 3). In addition, four actinobacterial strains isolated from corals *Porites* sp. and *Montipora* sp. were identified to be *Micrococcus* spp. and *Kocuria* spp.

Nineteen novel actinobacterial species isolated from two marine sediment samples have been identified and characterised using polyphasic taxonomy. Three strains (TPS16, TPS81 and TPS83) were fully characterised and has been described as *Marinitenerispora sediminis* gen. nov., sp. nov., within the *Nocardiopsaceae* family. In addition, the remaining 16 strains are found to be novel species of the genera *Streptomyces* (n= 6), *Blastococcus* (n= 5), *Agromyces* (n= 1), *Glycomyces* (n= 1), *Mycobacterium* (n= 1), *Nocardiopsis* (n= 1) and *Nonomuraea* (n= 1). The novel *Blastococcus* strains were putatively identified as two distinct species. This study is also the first to report novel *Blastococcus* strains isolated from tropical marine sediment. Description of these other novel strains is in progress.

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Among the actinobacterial strains screened for antibacterial activity using agar plug diffusion assay, 44 strains out of 84 *Salinispora*- like strains were observed to inhibit two Gram- positive pathogens (*B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213), including two strains (TPS104 and TPS178) that also inhibited growth of a Gram-negative pathogen (*P. aeruginosa* ATCC 27853). Furthermore, 20 actinobacterial strains from 80 non- *Salinispora*- like strains were found to inhibit two Gram- positive pathogens (*B. subtilis* ATCC 23857 and *S. aureus* ATCC 29213) and/ or a Gram-negative pathogen (*E. coli* ATCC 47076). Five different culture media were used for this primary screening. The *Salinispora*- like strains produced antibacterial activity on starch yeast peptone (n= 27), PM3 (n= 24), micromonospora medium (n= 22), ATCC medium 241 (n= 14) and soybean meal medium (n= 8). In contrast, the non-*Salinispora*- like strains produced activity on ATCC medium 241 (n= 12), micromonospora medium (n= 11), starch yeast peptone (n= 10), soybean meal (n= 8) and PM3 (n= 5).

The novel strain TPS83, which was identified as a novel species of a novel genus within the *Nocardiopsaceae* family based on polyphasic and whole genome sequence data, was found to produce indolactam alkaloid methylpendolmycin and α- pyrone nocapyrone R. Methylpendolmycin showed positive antibacterial activity against Gram-positive pathogens. The strain TPS83 could be a novel source for production of nocapyrone R and methylpendolmycin. This is also the first study that reported antibacterial activity from methylpendolmycin type of compounds.

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Whole genome analysis of *Marinitenerispora sediminis* TPS16, TPS81 and TPS83, *Streptomyces* sp. TPS3 and *Nocardiopsis* sp. TPS2 revealed the presence of marine adaptation genes and genes that code for type I- and type II- polyketide synthases as well as non- ribosomal peptide synthases. These biosynthetic genes are mainly associated with production of bioactive compounds with antibacterial, antifungal, antiviral and cytotoxic activities, suggesting the potential of the novel strains in biotechnology.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS:

1. **Ng, Z. Y.**, & Tan, G. Y. A. (2018). Selective isolation and characterisation of novel members of the family *Nocardiopsaceae* and other actinobacteria from a marine sediment of Tioman Island. *Antonie Van Leeuwenhoek*, *111*(5), 727-742.

2. **Ng, Z. Y**., Fang, B. Z., Li, W. J., & Tan, G. Y. A. (2019). *Marinitenerispora sediminis* gen. nov., sp. nov, a member of the family *Nocardiopsaceae* isolated from a marine sediment. *International Journal of Systematic and Evolutionary Microbiology* (Accepted for publication on 23rd June 2019).

ORAL PRESENTATION IN CONFERENCE:

1. **Ng, Z. Y**., & Tan, G. Y. A. Diversity of *Salinispora* spp. and marine actinobacteria isolated from Tioman Island. Presented in Asian Congress in Biotechnology 2015 (ACB 2015) at Istana Hotel, Kuala Lumpur, Malaysia on 15-19th November 2015.

2. Tan, G. Y. A., & **Ng, Z. Y**. Bioactive compounds and taxonomy of a novel member of the family *Nocardiopsaceae* isolated from a marine sediment. Presented in the 18th International Symposium on the Biology of Actinomycetes (ISBA18) on 23-27th May 2017 at International Convention Centre, Jeju Island, Korea.

POSTER PRESENTATION IN CONFERENCE:

1. **Ng, Z. Y.**, & Tan, G. Y. A. Draft genome sequence of a novel marine actinobacterium. Presented in the Microbiology Postgraduate Seminar 2018 (MSMPS2018) on 9th October 2018 at Dewan Annexe, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia.

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