Marine-Derived Actinobacteria and Their Ability to Produce Antimicrobial Substances against Human Pathogens

Suthinee Sangkanu

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Microbiology

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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ เชื้อแอคติโนมัยสีทจากทะเลและความสามารถในการผลิตสารต้านจุลินทรีย์ก่อโรคในคน

ผู้เขียน นางสาวสุทธินี สังขานุ

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บทคัดย่อ

วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อแยกและคัดเลือกเชื้อแอคติโนมัยสีทจากทะเลที่มีความสามารถต้านเชื้อจุลินทรีย์ก่อโรคในคน โดยแยกได้ทั้งหมด 525 ไอโซเลท พบว่า แอคติโนมัยสีทที่คัดแยกได้ส่วนใหญ่จำแนกอยู่ในสกุล *Streptomyces* คัดเลือกตัวแทนเชื้อแอคติโนมัย - สีทจำนวน 274 ไอโซเลท มาทดสอบความสามารถในการต้านเชื้อจุลินทรีย์เบื้องต้นโดยวิธี cross streak และ hyphal inhibition พบว่า 57.7% สามารถยับยั้งเชื้อก่อโรคได้อย่างน้อย 1 สายพันธุ์ จากนั้นนำสารสกัดจำนวน 287 สาร จากเชื้อแอคติโนมัยสีท 104 ไอโซเลท ที่ออกฤทธิ์ยับยั้งและโตเร็วมาทดสอบฤทธิ์ยับยั้งจุลินทรีย์ที่ความเข้มข้น 200 ไมโครกรัมต่อมิลลิลิตร ด้วยวิธี colorimetric broth microdilution พบว่าสารสกัด 160 สาร จากเชื้อ 81 ไอโซเลท แสดงระดับการยับยั้งเชื้อที่แตกต่างกัน เมื่อนำสารสกัดดังกล่าวมาหาค่าความเข้มข้นต่ำสุดที่ยับยั้งเชื้อได้ (minimum inhibitory concentration, MIC) ความเข้มข้นต่ำสุดที่ฆ่าเชื้อแบคทีเรียได้ (minimum bactericidal concentration, MBC) และความเข้มข้นต่ำสุดที่ฆ่าเชื้อราได้ (minimum fungicidal concentration, MFC) พบว่าค่า MIC อยู่ในช่วง $\leq$0.03 ถึง 200 ไมโครกรัมต่อมิลลิลิตร ค่า MBC และ MFC อยู่ในช่วง 0.25 ถึง >200 และ 1 ถึง >200 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ในจำนวนนี้มีสารสกัด 39 สาร จากเชื้อแอคติโนมัยสีท 19 ไอโซเลท ที่แสดงฤทธิ์ต้าน

จุลินทรีย์ในระดับสูง (MIC $\leq$ 8 ไมโครกรัมต่อมิลลิลิตร) โดยมี 33 สารออกฤทธิ์ต้านเชื้อแบคทีเรียและ 6 สารต้านเชื้อรา สารสกัดจากส่วนเซลล์ด้วยเอทิลอะซีเตทจากเชื้อ AMA11 (AMA11CE) มีฤทธิ์ต้านแบคทีเรียกว้างที่สุดสามารถต้านได้ทั้งแบคทีเรียแกรมบวก (ค่า MIC ต่อเชื้อ *Staphylococcus aureus* และ methicillin-resistant *S. aureus*, MRSA เท่ากับ 0.5 ไมโครกรัมต่อมิลลิลิตร) และแบคทีเรียแกรมลบ (ค่า MIC ต่อเชื้อ *Acinetobacter baumannii* เท่ากับ 8 ถึง 64 ไมโครกรัมต่อมิลลิลิตร และต่อเชื้อ *Eschericiha coli* เท่ากับ 64 ไมโครกรัมต่อมิลลิลิตร) นอกจากนี้สารสกัด AMA11CE ยังสามารถยับยั้งการสร้างไบโอฟิล์มของเชื้อ *Staphylococcus epidermidis* และควอรัมเซนซิ่งของเชื้อ *Chromobacterium violaceum* เมื่อศึกษาองค์ประกอบของ AMA11CE ด้วยวิธี Gas chromatography-Mass spectrometry (GC-MS) พบว่าประกอบด้วยสารหลัก 2 สาร ได้แก่ 3-nitro-1,2-benzenedicarboxylic acid และ quinoxaline-2-carboxamide ที่อาจเกี่ยวข้องกับฤทธิ์ต้านแบคทีเรีย สารสกัดจำนวน 6 สาร สามารถต้านเชื้อราได้ในระดับสูง โดยสารสกัดจากส่วนเซลล์ด้วยเอทิลอะซีเตทจากเชื้อ *Streptomyces* sp. AMR71 (AMR71CE) ออกฤทธิ์ต้านเชื้อราได้ดีและกว้างที่สุด มีค่า MIC ต่อเชื้อ *Candida albicans Cryptococcus neoformans* และ *Talaromyces marneffei* เท่ากับ 4, 1 และ 4 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สารสกัดจากส่วนเซลล์ด้วยเฮกเซนจากเชื้อ *Streptomyces* sp. AMA50 (AMA50CH) ยับยั้ง *T. marneffei* ได้ดีที่สุด (MIC 0.5 ไมโครกรัมต่อมิลลิลิตร) และจากการทดลองในสิ่งมีชีวิตพบว่า AMA50CH สามารถป้องกัน *Caenorhabditis elegans* ที่ติดเชื้อ *T. marneffei* ให้รอดชีวิตได้ประมาณ 60 ถึง 70 เปอร์เซ็นต์เมื่อเทียบกับกลุ่มควบคุมที่ติดเชื้อ การศึกษาด้วย GC-MS พบว่า AMA50CH ประกอบด้วยสารหลัก 7 สาร ได้แก่ n-hexadecanoic acid, tetradecanoic acid, pentadecanoic acid, heptadecenoic acid, palmitoleic acid, hexadecanoic acid 2-hydroxyl-1-(hydroxymethyl) ethyl ester และ hexadecanoic acid methyl ester ซึ่งอาจเกี่ยวข้องกับฤทธิ์ต้านรา ผล

การศึกษาด้วยกล้องจุลทรรศน์อิเลกตรอนชนิดส่องกราด (scanning electron microscope, SEM) พบว่าสารสกัดที่มีฤทธิ์ต้านจุลินทรีย์ออกฤทธิ์ต่อผนังเซลล์และเซลล์เมมเบรน ในส่วนของการจัดจำแนกเชื้อแอคติโนมัยสีทจากทะเลจำนวน 63 ไอโซเลท โดยอาศัยการหาลำดับเบสของ 16S rDNA สามารถจัดจำแนกเชื้อทั้ง 63 ไอโซเลท อยู่ใน 6 orders และ 9 genera ได้แก่ *Streptomycetales* (*Streptomyces* และ Uncertain species), *Corynebacteriales* (*Gordonia*, *Nocardia* และ *Mycobacterium*), *Pseudonocardiales* (*Pseudonocardia*), *Streptosporangiales* (*Actinomadura*), *Jiangellales* (*Jiangella*) และ *Micromonosporales* (*Micromonospora*) นอกจากนี้ ยังจำแนกสายพันธุ์ AMA120^{T} เป็นเชื้อชนิดใหม่ในจีนัส *Gordonia* โดยศึกษาคุณสมบัติทาง ทางเคมี และทางสายสัมพันธ์เชิงวิวัฒนาการ ที่แตกต่างจากเชื้อต้นแบบที่ใกล้เคียง ดังนั้นจึงเสนอให้สายพันธุ์ AMA120^{T} เป็นเชื้อสายพันธุ์ใหม่ที่มีชื่อว่า *Gordonia sediminis* ผลการศึกษาในครั้งนี้ชี้ให้เห็นว่าสิ่งแวดล้อมทางทะเลในภาคใต้ของประเทศไทยเป็นแหล่งของเชื้อแอคติโนมัยสีทจากทะเลชนิดใหม่ และเชื้อที่มีความสามารถในการสร้างสารต้านจุลินทรีย์

คำสำคัญ: แอคติโนมัยสีทจากทะเล กิจกรรมการต้านจุลินทรีย์ องค์ประกอบหลักของสารสกัดที่ออกฤทธิ์ เชื้อสายพันธุ์ใหม่

Thesis Title	Marine-Derived Actinobacteria and Their Ability to Produce Antimicrobial Substances against Human Pathogens
Author	Ms. Suthinee Sangkanu
Major Program	Microbiology
Acedemic Year	2018

ABSTRACT

The aims of this study were to isolate marine-derived actinomycetes from sediments and marine organisms from the South of Thailand and screen for their antimicrobial activity against human pathogens. A total of 525 isolates were obtained with members of the genus *Streptomyces* as the dominant population. Among the 274 representative actinomycetes, 57.7% exhibited antimicrobial activity against at least one test strain by a cross streak and hyphal inhibition tests. Two hundred and eighty-seven extracts from the 104 fast-growing active isolates were preliminary tested at 200 µg/ml by colorimetric broth microdilution methods. Among them, 160 extracts from 81 isolates showed varying spectrum of activity and their minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC) were further determined. The MIC values of active extracts ranged from ≤0.03 to 200 µg/ml and the MBCs and MFCs from 0.25 to >200 µg/ml and 1 to >200 µg/ml, respectively. Thirty-nine extracts from 19 isolates exhibited strong inhibitory activity (MICs ≤8 µg/ml), 33 extracts against bacteria and six extracts against fungi. The cell ethyl acetate extract from AMA11 (AMA11CE) exhibited the broadest activity against both Gram-positive bacteria, *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) (MIC 0.5 µg/ml) and Gram-negative bacteria, *Acinetobacter baumannii* (MICs 8 to 64 µg/ml) and *Escherichia coli* (64 µg/ml). In addition, AMA11CE inhibited biofilm formation of *Staphylococcus epidermidis* and quorum sensing of *Chromobacterium violaceum*. Gas chromatography-Mass spectrometry (GC-MS) analysis revealed that AMA11CE contained two compounds namely 3-nitro-1,2-benzenedicarboxylic acid and

quinoxaline-2-carboxamide that may be associated with antibacterial activity. Six extracts displayed strong antifungal activity. The cell ethyl acetate extract from *Streptomyces* sp. AMR71 (AMR71CE) had the broadest antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Talaromyces marneffei* with MICs of 4, 1 and 4 µg/ml, respectively. The cell hexane extract from *Streptomyces* sp. AMA50 (AMA50CH) exhibited the strongest activity against *T. marneffei* (MIC 0.5 µg/ml). Furthermore, *in vivo* study showed that AMA50CH protected the survival of *T. marneffei* infected *Caenorhabditis elegans* 60 to 70% as compared to the infected control group. GC-MS analysis revealed that AMA50CH contained seven compounds comprising n-hexadecanoic acid, tetradecanoic acid, pentadecanoic acid, heptadecenoic acid, palmitoleic acid, hexadecanoic acid 2-hydroxyl-1-(hydroxymethyl) ethyl ester and hexadecanoic acidmethyl ester that may be responsible for the antifungal activity. Scanning electron microscopic studies revealed that these active extracts may act on cell wall and cell membrane of pathogens. Finally, 63 selected isolates of marine-derived actinomycetes were identified by 16S rDNA sequence analysis. They could be divided into six orders and eight genera comprising the *Streptomycetales* (*Streptomyces* and Uncertain species), *Corynebacteriales* (*Gordonia*, *Nocardia* and *Mycobacterium*), *Pseudonocardiales* (*Pseudonocardia*), *Streptosporangiales* (*Actinomadura*), *Jiangellales* (*Jiangella*) and *Micromonosporales* (*Micromonospora*). In addition, strain AMA120^{T} was classified as a novel species in the genus *Gordonia* based on a polyphasic study consisting of phenotypic, chemotaxonomic and phylogenetic properties. Therefore, the strain AMA120^{T} was proposed as the type strain of a novel species with the name *Gordonia sediminis* sp. nov. The results from this study revealed that marine environments in the South of Thailand would be a good source of novel and antimicrobial producing marine-derived actinomycetes.

Keywords: marine-derived actinomycete, antimicrobial activity, major compound of active extract, novel species

ACKNOWLEDGMENTS

I would like to express my sincere grateful thanks and sincere appreciation to my advisor, Professor Dr.Souwalak Phongpaichit, for her helpful suggestion, valuable guidance and comments throughout my study. Sincere appreciation is extended to Dr.Chanwit Suriyachadkun my co-advisor, for his valuable contribution and advice relating to this study.

I would like to thank Dr.Wipawadee Sianglum and Assistant Professor Dr.Nattawut Rungjindamai for their comments and advice relating to this study.

I wish to express my gratitude to the scholarship from the Thailand Graduate Institute of Science and Technology (TGIST) (grant number TGIST 01-57-048) for my Ph.D. study. The Graduate School, Prince of Songkla University is also acknowledged for partially support.

I would like to thank Prof. Dr.Vatcharin Rukachaisirikul for her suggestions and assistance in part of chemical extaction.

I would like to express my thanks to all beloved friends at PR504 Microbiology Laboratory, members of CH402 Chemical Laboratory and the BIOTEC Culture Collection, Thailand for their kind and helpful advice during my study.

Finally, I wish to thank my beloved family and all best friends for their supports and encouragements.

Suthinee Sangkanu

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CHAPTER 1

INTRODUCTION

1.1 Background and rationale

The development of resistance to multiple drugs is a main problem in the treatment of infectious diseases caused by pathogenic microorganisms. This multidrug resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these multidrug resistant pathogens (Farha and Brown, 2019). The filamentous Actinomycetes (phylum *Actinobacteria*) species are the largest producer group of bioactive microbial metabolites. Over 10,000 bioactive compounds, 7,600 produced by *Streptomyces* and 2,500 from rare actinomycetes (Bérdy, 2005; Azman *et al*., 2017; Giräo *et al*., 2019). They are responsible for the production of about half of the discovered bioactive secondary metabolites, especially antibiotics, anti-tumor, immunosuppressive agents and enzymes. However, the discovery rate of new compound from terrestrial actinomycetes has declined, whereas the rate of re-isolation of known compounds has increased (Lam, 2006). One of the efficient ways of discovering novel bioactive metabolites is through isolating new group of actinomycetes from various methods and unexplored habitats (Omura, 2003; Lam, 2006; Giräo *et al*., 2019).

The world's oceans cover over 70% of the earth's surface and include some of the most biodiverse ecosystem on the planet (Xie *et al*., 2018). In some marine ecosystems, such as deep sea floor and coral reefs, the biological diversity is higher than in the tropical rainforest (Haefner, 2003). Furthermore, the distributions of actinomycetes in the sea remain largely undescribed. The evidence that these bacteria play important ecological roles in the marine environment has remained undefined (Mincer *et al*., 2002). During the last decades, new members of actinomycetes and

novel metabolites recovered from marine environments such as deep-sea sediments and marine flora and fauna have been reported (Subramani and Sipkema, 2019). For example, forazoline A produced by *Actinomadura* sp. displayed anti-candida activity (Wyche *et al*., 2014). Glycerol 1-hydroxy-2,5-dimethyl benzoate from *Verrucosispora* sp. showed anti-MRSA activity (Huang *et al*., 2016). Branimycins B and C, new antibiotics in macrolide group displayed antibacterial activity against both Gram-positive and Gram-negative bacteria (Braña *et al*., 2017). Thus, the extensive exploration of marine environments to untap marine-derived actinomycete diversity may lead to the discovery of new species and potential novel antibiotics.

1.2 Review of the literature

1.2.1 Marine microbiology

The study of marine microbiology started in 1884s by the German Professor Bernard Fischer who reported the first cultivation of marine bacteria from deep sea waters and suggested their roles in this habitat (Azam, 2001). By the 1940s reports highlighted the importance of these microorganisms in different functions as the degradation of organic matter and nutrient recycling have been presented (Prieto-Devó, 2008). In the 1970s, marine microbial ecology was extensively studied because it exhibited the major respiration in the oceans. According to the bacterial size fraction, it is estimated that 10^{30} microbial cells presented in the oceans with more than 95% of the total respiration (Pedrós-Alió, 2006). Furthermore, Hobbies *et al*. (1977) confirmed the abundance of bacteria in high numbers (10^6/ml) in the ocean using the combination between epifluorescence microscopy techniques and nucleopore filters and found that small bacterial cells (0.5 µm or less in diameters) could be observed. Several years later, the first phylogeny-based studies of the diverse microbial life began from evaluation of small subunit rRNA oligonucleotide studies for microbial diversity and evolution (Lane *et al*., 1985; Pace *et al*., 1985; Stahl *et al*., 1985).

The world ocean is the largest ecosystem of the earth which covers 70% of the planet and provides good and inhabitable areas for the majority of the microbial population (Glöckner *et al*., 2012). The marine environment is an extreme habitat ranging from sunlit surface water to the deep ocean with pressures exceeding 100 MPa, salinity about 3.5%, and temperatures range from sea ice in the Polar Regions to high temperature at deep sea hydrothermal vents. Marine microorganisms are able to thrive in this environment, either surviving as free-living organisms (Kennedy *et al*., 2010) or association with other vertebrate and invertebrate organisms (Ward and Bora 2006). Marine microorganisms, the 'gatekeepers' of the earth system are the controllers of energy, organic matter cycles and major producers of the global primary productivity (Falkowski *et al*., 1998; Glöckner and Joint, 2010). They play important roles in marine food webs and cycles of carbon and energy (Glöckner *et al*., 2012). Sediment microorganisms degrade dead marine organisms and marine snow. The marine environments are covered by the three dominating microorganisms such as *Archaea*, *Bacteria* and *Eukarya*. Different environments create different microbes with adopting survival and growth strategies (Kennedy *et al*., 2010). For example, Morris *et al*. (2002) used rRNA sequence analysis to study the group of bacterial lineages in the Sargasso Sea and found that SAR 11 group (a lineage of bacteria that is extremely common in the ocean including *Alphaproteobacteria* with no known cultured representatives) was very abundant in the Sargasso Sea. SAR 11 has also been found in closely marine environment that has been sampled. In addition, the application of PCR showed that *Archaea* were originally in marine environments (Delong, 1992; Fuhrman *et al*., 1992) which was in agreement with the study of Venter *et al*. (2004) on the microbial diversity in the surface water of the Sargasso Sea. They used different phylogenetic biomarker genes to elucidate the bacterial community and the results showed nine major bacterial phyla (e.g. *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, *Bacteriodetes*, *Chloroflexi*, *Spirochaetes*, *Fusobacteria* and *Deinococcus-Thermus*) and two archaeal phyla (e.g. *Crenarchaeota* and *Euryarchaeota*). Later, Gontang *et al*. (2007) studied the diversity of marine bacteria from marine sediment in the Republic of Palau by culture dependent method and found that the dominant populations were Gram-positive bacteria consisting 22 families. By using 16S rRNA gene sequencing, 124 out of 189 representative isolates

(65.6%) belonged to the class *Actinobacteria* while the remaining 65 (34.4%) were members of the class *Bacilli*. Studies on diversity of marine-derived microorganisms from macroorganisms (e.g. algae, ascidians and sponges) are increasing due to their ability to produce diverse bioactive compounds including cytotoxic, antibacterial, antifungal, antiviral and anti-inflmmatory substances (Blunt *et al*., 2008; Osinga *et al*., 2001; Schirmer *et al*., 2005; Zhang *et al*., 2006). Menezes *et al*. (2010) investigated the diversity of bacteria and filamentous fungi isolated from marine algae (e.g. *Sargassum* sp.) and invertebrate samples (the sponges *Amphimedon viridis*, *Axinella corrugate*, *Dragmacidon reticulate*, *Geodia corticostylfera*, *Mycale laxissima* and *Mycale acgulosa*; the ascidians *Didemnum ligulum* and *Didemnum* sp.) from São Paulo State, Brazil, collected at depths between 5 and 10 m. Results of the identification derived from ARDRA (amplified ribosomal DNA restriction analysis) and sequencing analysis revealed that 256 filamentous fungal strains were obtained from invertebrate samples. The majority of these fungi belonged to phylum *Ascomycota* among 7 orders 18 genera followed by phyla *Zygomycota* and *Basidiomycota*. Some of them have been reported in the literatures as marine invertebrate-inhabiting fungi such as *Pestalotiopsis*, *Xylaria*, *Botryosphaeria* and *Cunninghamella*. A total of 181 bacterial isolates obtained from algae, sponge and ascidians were related to phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. However, bacterial diversity and community are varied by seasonal changes. The monitoring of bacterial community in several seasons (spring, summer, autum) of northern Antartic Peninsula revealed that *Alpha-proteobacteria* was higher in spring. Winter was dominant by *Thaumarchaeota* (*Nitrosopumilus*), *Euryarchaeota*, member of *Oceanospirillales*, SAR324. Summer and autumn were characterized by *Flavobacteria* (NS5 marine group and *Polaribacter*), *Alpha-proteobacteria* and *Gamma-proteobacteria (Oceanospirillales/Balneatrix* and *Cellcibrionales)* (Signori *et al*., 2018). Actinomycetes from sediment and water in Alexandria were also affected by seasonal variation. The highest munbers of actinomycetes were in autum and summer whereas they significantly decreased in spring and disappeared in winter (Ghanem *et al*., 2000).

1.2.2 Actinomycetes

Actinomycetes are defined as Gram-positive bacteria with high guanine-plus-cytosine (G+C) content (>55%) in their genomes, aerobic but some genera are anaerobic (e.g. *Actinomyces*), which show different morphologies from cocci, short rods, to branching hyphae. Most of them produce single or short chains of spores located either on the aerial mycelium or on both the aerial and substrate mycelium (Williams, 1989). They have no nuclear membrane and are sensitive to common antibacterial agents such as lysozyme (Sykes and Skinner, 1973).

Actinomycetes are commonly saprophytic and largely distributed in diverse natural environments such as soil, air, water and small amount of them live together with plants. Actinomycetes are common soil bacteria which degrade recalcitrant organic materials (Sonia *et al*., 2011). They have been identified as one of the dominant groups with diverse soil type and soil condition (e.g. geographical location, soil temperature, soil type, soil pH, organic matter content, agricultural activities, aeration, nutrient availability, moisture content and soil vegetation) which influenced the diversity of these microorganisms. Genus *Streptomyces* is the dominant group in soil. Other actinomycetes commonly found in soil are such as *Nocardia*, *Microbispora*, *Micromonospora*, *Actinomyces*, *Actinoplanes* and *Streptosporangium* (Arifuzzaman *et al*., 2010). *Planomonospora* and *Saccharothrix/Actinophytocola* (Chaouch *et al.*, 2018). It has been reported that the viable count numbers of actinomycetes is relatively high in dry, humic and calcareous soils reaching to 10^6 cfu/g of dry weight soil. In contrast, they are found in a small amount, 10^3 cfu/g of dry weight soil, in waterlogged, anaerobic soils and acidic soils (El-Tarabily and Sivasithamparam, 2006).

The air environment has been contaminated with actinomycete spores in many occupational facilities such as agriculture and waste composting facilities (Williams, 1989; Nielsen *et al*., 1997). Airborne spores of various actinomycetes species such as *Saccharopolyspora rectivirgula*, *Micropolyspora faeni*, *Thermoactinomyces vulgaris* and *Streptomyces albus* have been related to the incidence of allergic alveolitis and other health effect (Dales *et al*., 1991; Lacey and Dutkiewicz, 1994).

In the 19th century some actinomycetes were found closely associated with living plants, giving beneficial and/or cause of symptoms in their host plants such as genus *Frankia*, isolated from non-legume root nodules in 1886 and *Streptomyces scabies*, a first pathogenic actinomycetes of potato scab in 1890 (Hasegawa *et al*., 2006). Most endophytic actinomycetes are considered as producers of vast secondary metabolites with useful applications in human and agriculture. As reported by de Araújo *et al*. (2000), 43.4% of actinomycete genera *Microbispora*, *Streptomyces* and *Streptosporangium* isolated from leaves and root of maize, showed antimicrobial activity against *S. aureus, M. luteus, B. subtilis* and *C. albicans*. Mastsumoto and Takahashi (2017) isolated more than 1000 strains of endophytic actinomycetes from plant roots and rhizospheric soil and found that strains from rhizospheric soil are more capable of producing bioactive metabolites than those from the roots. Some *Actinomycetales* species are pathogenic to man or animals. The genus *Mycobacterium* contains many pathogenic species to human and animals and *M. tuberculosis* is the best known one (Sykes and Skinner, 1973). Genera *Gordonia, Nocardia, Rhodococcus, Tsukamurella* and some strains of *Streptomyces* are considered to be of medical importance which can be isolated from clinical samples (e.g. inflammatory exudate, sputum, transtracheal aspirate, lymph node biopsy, skin biopsy, corneal ulcer, blood and bronchial washes). Nocardiosis is the major infection caused by pathogenic aerobic actinomycetes (Poonwan *et al*., 2005) usually characterized by primary pulmonary lesions that may be subclinical or overt, chronic or, rarely and acute, later, the disease may spread to other organs or tissue such as skin or kidney (Boiron *et al*., 1992).

1.2.3 Marine actinomycetes

Marine actinomycetes are considered as potential sources of bioactive compounds, and several works have confirmed that these microbes are the richest sources of secondary metabolites. Thus, their diversity is important targets for the screening of novel metabolites and other using in industrial and biotechnology (Dhevagi *et al*., 2017). Biological diversity in some marine environments such as the deep sea floor and coral reefs is higher than in the tropical rainforest (Haefner, 2003).

As marine environmental conditions are extremely different from the terrestrial ones, it is assumed that marine actinomycetes have characteristics different from terrestrial actinomycetes and may produce different types of bioactive compounds (Xie *et al*., 2018). The ecological roles of marine actinomycetes are an antibiotic production, decomposition of recalcitrant organic materials such as chitin, a biopolymer that is particularly abundant in the sea (Jensen *et al*., 2005; Manivasagan *et al*., 2013). It has long been recognized that actinomycetes can be recovered from marine samples (Weyland, 1969). However, it is less clear whether the existence of indigenous populations of marine actinomycetes are that the terrestrial bacteria produce resistant spores that are known to be washed into sea, where they can remain available but dormant for many years (Fenical and Jensen, 2006). Therefore, the experts need to establish some criteria for defining what is or is not indigenous marine actinomycetes such as the ability to grow optimally at *in situ* salinity, pressure, temperature and nutrient concentrations. These criteria demonstrate that the organism is active *in situ* and the recognition of metabolic signatures of marine adapted organisms might be taken as prima facie evidence for indigenicity (Bull *et al*., 2005).

In 1984, the first marine actinomycete, *Rhodococcus marinonascens* was isolated from marine bottom sediment and able to growth in media containing sea water of 75 to 100% or an equivalent salt concentration with 20°C optimal temperature (Helmke and Weyland, 1984). Subsequently, Jensen *et al*. (1991) reported the first cultivation of an obligate marine actinomycetes, *Salinospora*, that isolated from marine sediment collected around the world including the Caribbean Sea, the Sea of Cortez, the Red Sea and the tropical Pacific Ocean off Guam. Following the discovery of the genus *Salinospora*, Jensen *et al*. (2005) began to culture actinomycetes from marine sediments using the same criteria with *Salinospora* as a guide. They could isolate the genus *Marinophilus* and added to the family *Streptomycetaceae*. In addition, culture-independent studies showed that the diversity of actinomycetes in marine sediment is different from terrestrial environment (Stach *et al*., 2003; Gontang *et al*., 2007). Sediment samples are commonly used for the isolation of marine-derived actinomycetes. The samples may be treated to remove fungi, Gram-negative bacteria and other unwanted microorganinsms. Pre-treatment samples enhance the chance to find novel species especially the rare actinomycetes.

Commonly, pre-treatment methods include the dilution and mixing with sterile natural seawater, drying the samples in laminar air flow, heating at high temperature or treating with chemicals such as phenol, sodium dodecyl sulfate, and benaethonium (Subramani and Sipkema, 2019).

1.2.4 Taxonomy of *Actinobacteria*

The *Actinobacteria*, a phylum of Gram-positive bacteria was proposed and described by Stackebrandt *et al*. (1997), including 6 classes namely *Actinobacteria*, *Acidimicrobila*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria* and *Thermoleophilia* (Gao and Gupta, 2012). The class *Actinobacteria* is further divided into 16 orders comprising *Actinopolysporales, Actinomycetales, Bifidobacteriales, Catenulisporales, Corynebacteriales, Frankiales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales*, and *Incertae sedis* (Puttasawamygowda *et al*., 2019). The *Actinomycetales* members are commonly referred to as actinomycetes (Goodfellow *et al.*, 2012; http://www.bacterio.net/). Morphology and chemotaxonomy were used to delineate the taxonomy of *Actinobacteria* at the genus levels. However, some organisms in this phylum have been reclassified on the basis of molecular analyses. For instance, the genus *Kitasatospora* was separated from the genus *Streptomyces* by genome sequencing (Barka *et al.*, 2016).

1.2.4.1 Morphological classification

Morphological characteristic is the tool for simple identification of actinomycetes using a brightfield microscope equipped with a long working distance lens. For certain features as the determination of the surfaces of spores, an electron microscope is essential (Williams and Davies, 1967). Morphological features include mycelium, conidia, sporangia and other structures (William, 1989).

Mycelium, some actinomycetes form either substrate or aerial mycelium where as some genera form both types of mycelium which may be a fragmenting substrate mycelium and limited aerial mycelium that can bear chains of arthrospores

(e.g. *Nocardia* species) or fragmentation into rod-shaped or coccoid elements (e.g. *Rhodococcus*). The mycelium may bear intercalary vesicles that do not contain spores (e.g. *Intrasporangium*) or contain many spores (e.g. *Frankia*) (Bell *et al*., 1998; William, 1989).

Conidia, actinomycetes can produce conidia in a variety of ways as described in the following. Single conidia presents in various genera of the actinomycetes such as *Micromonospora* strains produce non-motile single conidia on sporophores branched from substrate mycelium (Figure 1) (Bredholdt *et al*., 2007). *Thermomonospora* strains produce single conidia on branched and unbranched sporophores on aerial mycelium (Figure 1). Representatives of the other monosporic actinomycete genera, *Thermoactinomyces* strains form single conidia on short branches arising from the aerial and substrate mycelium (Waksman and Corke, 1953) and *Saccharomonospora* produces single conidia on aerial mycelium (Figure 1) (Runmao, 1987).

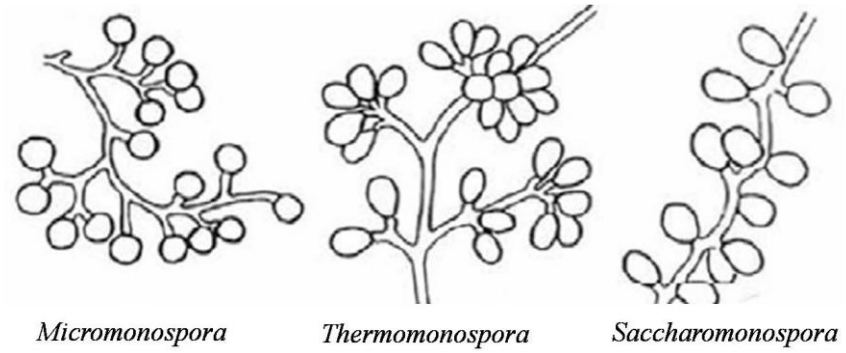


Figure 1 Single conidia types of actinomycetes (Vobis, 1997)

Pairs of conidia are the important characteristic of the genus *Microbispora* (Figure 2). This genus produces conidia in longitudinal pairs on the aerial mycelium but not usually forms on the substrate mycelium (Miyadoh *et al*., 1985; William, 1989). Short chain of conidia is usually considered up to 20 spores. The representative genera that formed short chain conidia as the genus *Microtetraspora*, *Nocardia*, *Pseudonocardia*, *Faenia*, *Streptoverticilium*. The genus *Microtetraspora*

develops a chain of four conidia on the short aerial mycelium (Thiemann *et al*., 1968; William, 1989). The genus *Faenia* produces short chain of conidia on unbranched lateral or terminal sporophores on both aerial and substrate mycelium. *Streptoverticillium* species form whorls and umbels short chains of conidia on their long aerial mycelium (Williams, 1989).

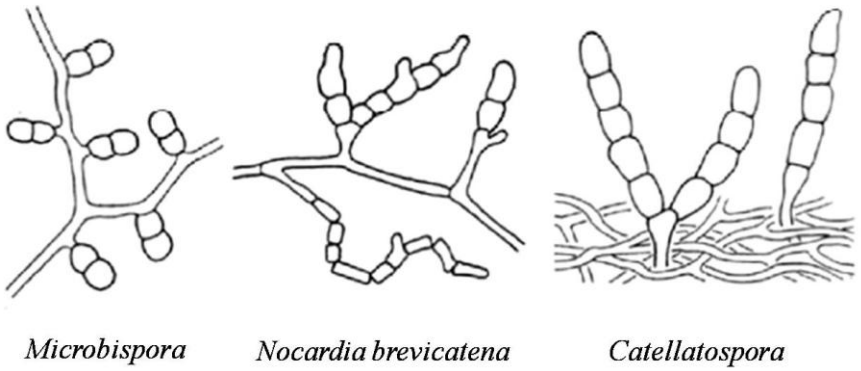


Figure 2 Pair and short chain of conidia types of actinomycetes (Vobis, 1997)

Long chains of conidia are formed by various genera such as the genus *Streptomyces* which produces three to many conidia on aerial mycelium and some species produce short chains of conidia on substrate mycelium (Figure 3). The genus *Actinopolyspora* produces 20 or more conidia on branched sporophores (Williams, 1989).

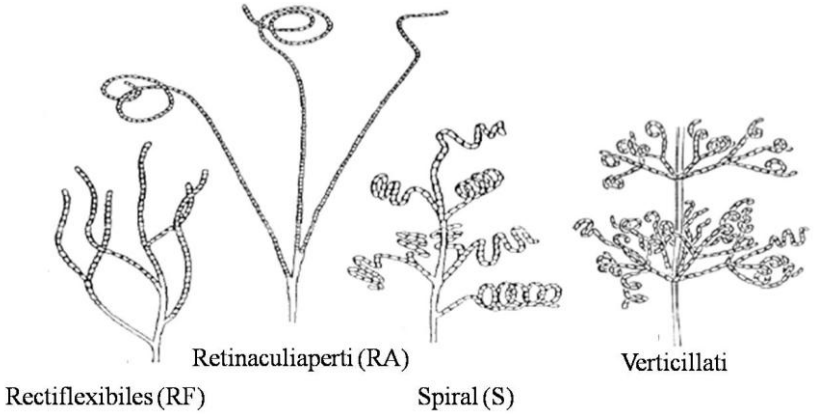


Figure 3 Long chain of conidia types of genus *Streptomyces* (Vobis, 1997)

Sporangia may be borne on well-developed aerial mycelium or on the surface of colonies with or without aerial mycelium such as *Actinoplanes* produces sporangia (spore vesicles) which containing motile spores on short sporangiophores at the tip of mycelium (Figure 4). Genus *Dactylosporangium* produce finger-shaped to claviform sporangia on short sporangiophores on substrate mycelium. Each sporangium contains three to four motile spores in rows (Figure 4). Genus *Ampullariella* produces different shape and size of sporangia in various species such as irregular, cylindrical, lobate, bottle shaped, flask shaped or digitate shaped. Their number ranges from 1-12 motile spores are arranged in row of sporangia. Genus *Planomonospora* produces cylindrical to clavate sporangia on aerial mycelium containing single motile spores (Figure 5). Genus *Streptosporangium* produces globose sporangia containing non-motile spores on aerial mycelium (Figure 5) (William, 1989).

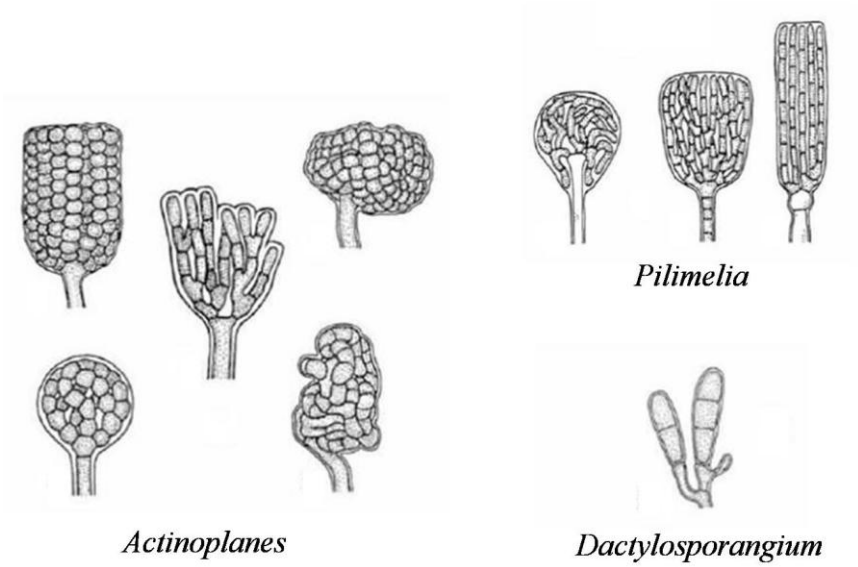


Figure 4 Sporangia types developed on substrate mycelium of actinomycetes (Vobis, 1997)

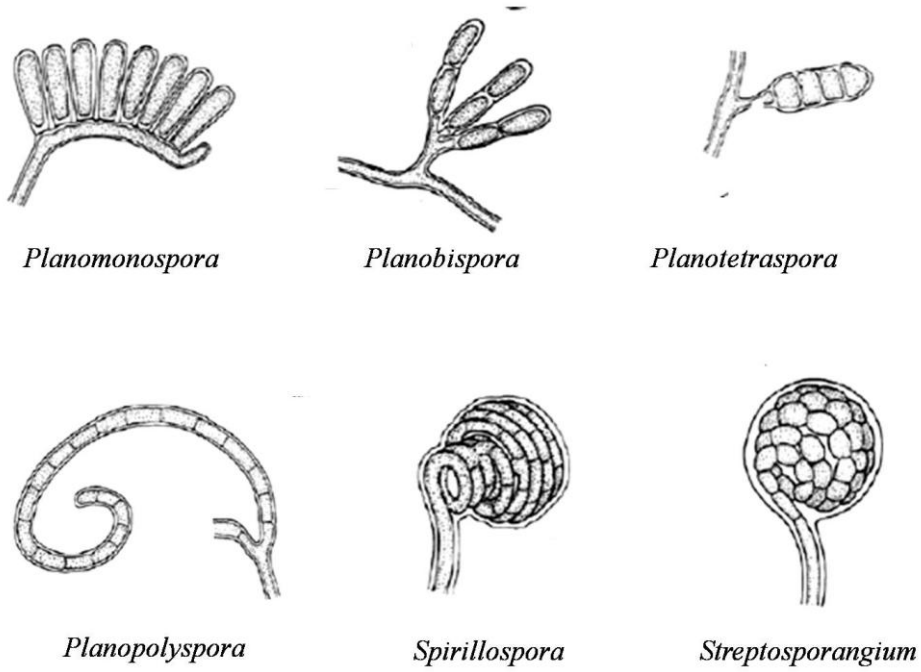


Figure 5 Sporangia types developed on aerial mycelium of actinomycetes (Vobis, 1997)

1.2.4.2 Chemotaxonomic classification

Cell chemical characteristic is considered to separate actinomycetes into broad groups. The most useful chemical compositions are such as diaminopimelic acid (DAP) isomers, whole cell sugars, polar lipids, mycolic acids and fatty acids (Stackebrandt, 1991).

The difference of DAP type is readily achieved by thin layer chromatography (TLC) or paper chromatography. DAP patterns can divide actinomycete cell wall into 9 chemotypes as shown in Table 1 (Lechevalier and Lechevalier, 1970).

Table 1 Cell wall chemotypes of the actinomycetes

Type	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX*** **
DAB							+		
Lysine					+	+	+		
Ornithine					+				
Aspartic acid						+	+	+	
Glycine	+	+			*	*	*	*	
meso-DAP		+**	+	+					+
LL-DAP	+								
Arabinose				+					
Galactose				+					

* Glycine is variably present in these groups

** Hydroxyl DAP may also be present

*** Many amino acids present.

DAP = 2,6 diaminopimelic acid

DAB = 2,4-diaminobutyric acid

Source: Lechevalier and Lechevalier, 1970

The sugar composition is an essential key on the classification as well as the DAP isomers. Paper chromatography can be used to separate and study the pattern of whole cell sugar. Actinomycetes containing *meso*-DAP can be devided into 5 types based on whole cell sugar pattern as shown in Table 2 (Lechevalier and Lechevalier, 1970).

Table 2 Whole cell sugar pattern of aerobic actinomycetes containing *meso*-DAP

Pattern	**Whole cell sugar**				
	Arabinose	**Fructose**	**Galactose**	**Madurose**	**Xylose**
A	+		+		
B				+	
C	No diagnostic sugars				
D	+				+
E		+			

Source: Lechevalier and Lechevalier, 1970

The 5 phospholipid patterns (Colwell and Grigorova, 1987) observed in the *Actinomycetales* are shown in Table 3 and these patterns are important tool to separate some genera such as *Amycolata* and *Amycolatopsis*.

Table 3 The patterns of phospholipid in the *Actinomycetales*

Type	**Marker phospholipids**				
	PG	**PC**	**PE**	**PME**	**GLUNU**
PI	*				
PII			+		
PIII	+	+		+	
PIV			+		+
PV	+				+

PG = phosphatidylglycerol PE = phosphatidylethanolamine

PC = phosphatidylcholine PME = phosphatidylmethylthanolamine

GULU = unknown lipid containing glucosamine * Variable

Source: Lechevalier and Lechevalier, 1970

Fatty acids of bacterial cells that occurred in membrane can be divided into two major groups. One is the straight-chain fatty acid group, which are synthesized from acetyl coenzyme A. The straight-chain includes palmitic, stearic, hexadecenoic, octadecenoic, cyclopropanic, 10-methylhexadecanoic and 2- or 3-hydroxyl fatty acids. The other one is the branched-chain fatty acid group, which includes iso-, anteiso- and ω-alicyclic fatty acids with or without a substitution (unsaturtion and hydroxylation) (Kaneda, 1991).

Mycolic acids (3-hydroxyl long fatty acids substituted at the 2-position with a moderately long aliphatic chain) are major components of cell envelope in genera *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Gordonia*, *Dietzia*, *Tsukamurella* and *Corynebacterium* (Nishiuchi *et al*., 1999). Mycolic acids are analyzed by thin layer chromatography (TLC) of ethanol : diethyl ether (1:1 v/v) extracts of these bacteria. The other procedure, as TLC of acid methanolysates of dry bacteria has been developed for detection the content of mycolic acid and other long chain constituents (Goodfellow *et al*., 1976).

Isoprenoid quinones are components of bacterial plasma membrane. The general structures of isoprenoid quinones can be divided into two groups such as naphthoquinones (phylloquinones and menaquinones) and benzoquinones (plastoquinones and ubiquinones) (Collins and Jones, 1981). In microorganisms, menaquinones and ubiquinones are commonly occurred and easily extracted from lyophilized cells with an appropriate organic solvent mixture (chloroform : methanol, 2:1, v/v) or of wet cells with acetone. These extracts are separated by TLC or column chromatography prior to HPLC technique (Hiraishi, 1999).

1.2.4.3 Molecular classification

The analysis of 16S rRNA gene (16S rDNA) sequence has been used to classify and identify the types and relationships of microorganisms worldwide (Hugenholtz *et al.*, 1998) because the primary structure of the 16S rDNA is highly conserved (Stackebrandt and Goebel, 1994). The rate of change of 16S rRNA gene is a sign of evolutionary distance and relatedness of organisms. The variable and conserved regions of 16S rRNA gene are covered with the gene sequence of about 1,550 bp. In addition, several housekeeping genes have been subjected for classification of the class *Actinobacteria* (Mohammadipanah and Dehhaghi, 2017). Previous study recommended that the suitable gene should fulfill the following conditions: (i) the genes must be ubiquitous with orthologous sequences in all cellular life as is the case of 16S rRNA gene; (ii) the genes must be present in single copy among genomes, without close paralogues that could confuse analysis; (iii) the individual genes must be long enough (900 bases) to contain sufficient information; (iv) the genes should not be prone to horizontal gene transfer (HGT) or recombination; (v) closely linked genes should also be avoided; (vi) the genes should contain at least two highly conserved regions to allow the design of appropriate amplification and sequencing primers, and (vii) the sequence must predict whole-genome relationships with acceptable precision (Adékambi *et al*., 2011).

The partial sequences of the *rpoB* gene (DNA directed RNA polymerase subunit beta) provided a correct tool for phylogenetic tree study of the genera *Mycobacterium* and *Streptomyces*. The *rpoB* gene has some advantages over 16S

rRNA gene. Firstly, *rpoB* is a single-copy gene, therefore, direct sequence analysis of this gene can be universally applied to streptomycete strains. The 16S rDNA is a multicopy gene and some streptomycete strains possess multiple gene copies with different sequences (Ueda *et al.*, 1999). In this case, direct sequencing is not possible because of the ambiguous results produced by the different sequences. Secondly, *rpoB* is a protein encoding gene. Therefore, the deduced amino acids, in addition to DNA sequences, can be used for the delineation of species within the genus *Streptomyces.* Thirdly, no gaps or additions were found in the multiple alignment of partial *rpoB* sequences, which means that all the sequence information can be considered for phylogenetic analyses without deleting sequence gaps. This cannot be done in the case of 16S rDNA (Kim *et al.*, 2004).

The *gyrB* gene (encoding for the B-subunit of DNA gyrase, a type II DNA topoisomerase) is a single-copy gene present in all bacteria and distribute universally among bacterial species. It cannot spread horizontally among different bacterial species, therefore it is a suitable alternative marker in bacterial classification. The *gyrB* gene sequences have been used for classification among members of the genera *Gordonia, Pseudomonas, Acinetobacter, Mycobacterium, Micromonospora, Pandorea* and the family *Microbacteriaceae* (Shen *et al.*, 2006).

1.2.5 Secondary metabolites from marine actinomycetes

Most antibiotics of microbial origin came from bacteria which are the majority producers of commercially important metabolites, both known and new drugs (Bull, 2004). Among these, bacteria belonging to the class *Actinobacteria* and more specially, the order *Actinomycetales* (commonly called actinomycetes) are one of the most economically and biotechnologically priceless prokaryotes. Genera *Streptomyces*, *Actinomyces*, *Arthrobacter*, *Corynebacterium*, *Frankia*, *Micrococcus* and *Micromonospora* are representatives of organism among actinomycetes, especially the genus *Streptomyces* which accounts approximately 80% of actinomycete natural products (Jensen *et al*., 2005; Solanki *et al*., 2008). A large number of actinomycetes have been isolated from terrestrial soil but presently the novel strains and metabolites from this habitat have decreased. This is a reason for the

switching over to new environments for discovering novel secondary metabolites and for combating resistant human pathogens (Dhevagi *et al*., 2017). The marine environment has been recently demonstrated as the best source of novel actinomycetes which comprise about 10% of all marine bacteria and the vast majority of secondary metabolites derived from these bacteria (Subramani and Aaldersberg, 2012). Marine actinomycetes show a wide variety of secondary metabolites such as antifungal, antitumor, antibacterial, immunosuppressive, insecticidal and enzyme inhibitor (Solanki *et al*., 2008). Mainly, the genus *Streptomyces* is a group of an enormous producer that remains unchallenged among other microbial groups. However, during this 6 years period, the list of the many different diverse structures with secondary metabolites from rare actinomycetes (non-streptomycetes) has been shown (Subramani and Aaldersberg, 2012). Table 4 shows some examples of new secondary metabolites from marine actinomycetes isolated from 2013 to 2019.

Table 4 Novel secondary metabolites produced by marine actinomycetes between 2013 to 2019

Compound	**Marine actinomycetes source**	**Biological activity**	**Reference**
Juvenimicin C	*Micromonospora* sp.	Chemopreventive	Carlson *et al*., 2013
Marfuraquinocins	*Streptomyces niveus* SCSIO 3406	Cytotoxic, antibacterial activity	Song *et al*., 2013
Kocurin	*Kocuria* sp.	Antibacterial	Palomo *et al*., 2013
Halomadurones A-D	*Actinomadura* sp.	Potent Nrf2-ARE activation	Wyche *et al*., 2013
Levantilide C	*Micromonospora* sp.	Antiproliferative activity	Peng *et al*., 2013
Nocapyrones H–J	*Nocardiopsis* sp.	Pro-inflammatory factor, stronger inhibitory effect on nitric oxide	Kim *et al*., 2013
Nocardiamide A and B	*Nocardiopsis* sp.	Antimicrobial activity	Wu *et al*., 2013a
Cyanogramide	*Actinoalloteichus cyanogriseus*	Multidrug-resistance (MDR) reversing activity	Lane *et al*., 2013
Desotamides B−D	*Streptomyces scopuliridis* SCSIO ZJ46	Antibacterial activity	Song *et al*., 2014
Actinosporins A and B	*Actinokineospora* sp.	Moderate activity against *Trypanosoma brucei*	Abdelmohsen *et al*., 2014

Table 4 (Cont.) Novel secondary metabolites produced by marine actinomycetes between 2013 to 2019

Compound	**Marine actinomycetes source**	**Biological activity**	**Reference**
Solwaric acids A and B	*Solwaraspora* sp.	Antibacterial activity against MDR pathogens	Ellis *et al*., 2014
Microbacterins A and B	*Microbacterium sediminis*	Potent cytotoxic activity	Liu *et al*., 2015
Micromonohalimane A and B	*Micromonospora* sp.	Modest antibacterial activity against MRSA, bacteriostatic	Zhang *et al*, 2016
Nocapyrones O–S	*Nocardiopsis* sp.	Cytotoxicity	Zhang *et al*., 2017
Saccharomonopyrones A–C	*Saccharomonospora* sp.	Weak antioxidant activity	Yim *et al*., 2017
Microsporanates A–F	*Micromonospora harpali*	Antibacterial activity	Gui *et al*., 2017
Tetrocarcin P	*Micromonospora harpali*	Antibacterial activity	Gui *et al*., 2017
Nocazines F and G	*Nocardiopsis* sp.	Excellent cytotoxicity	Sun *et al*., 2017
Kribellosides A-D	*Kribbella* sp.	Antifungal and RNA 5'-triphosphatase inhibitor	Igarashi *et al*., 2017

Table 4 (Cont.) Novel secondary metabolites produced by marine actinomycetes between 2013 to 2019

Compound	**Marine actinomycetes source**	**Biological activity**	**Reference**
Streptoglycerides A-D	*Streptomyces* sp.	Inhibitory effect on the production of nitric oxide in BV-2 microglia cells	Choi *et al*., 2018
Fridamycin H	*Actinokineospora spheciospongiae*	Anti-*Trypanosoma brucei*	Tawfke *et al.,* 2019
Akazamicin	*Nonomuraea* sp. AKA32	Cytotoxicity	Yang *et al*., 2019
Albumycin	*Streptomyces albus* J1074 l	Weak antibacterial activity	Huang *et al.,* 2019
Kenalactam E	*Nocardiopsis* CG3	Cytotoxicity	Messaoudi *et al*., 2019

1.3 Objectives

1. To isolate marine-derived actinobacteria from sediments and invertebrates.
2. To identify marine-derived actinobacteria by morphological characteristics and molecular method based on 16S rRNA sequencing
3. To screen for antimicrobial activity of marine-derived actinobacteria against various human pathogens.
4. To determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC) of extracts from the isolated marine-derived actinobacteria.
5. To determine possible antimicrobial effect of potential extracts using scanning electron microscopy (SEM).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Collection sites

Forty-five samples for marine-derived actinomycete isolation were collected from five provinces in the South of Thailand (Figure 6) including Nakhon Si Thammarat and Songkhla on the western shore of the Gulf of Thailand and Phuket, Trang and Satun on the western side of Malay Peninsula facing the Andaman Sea.

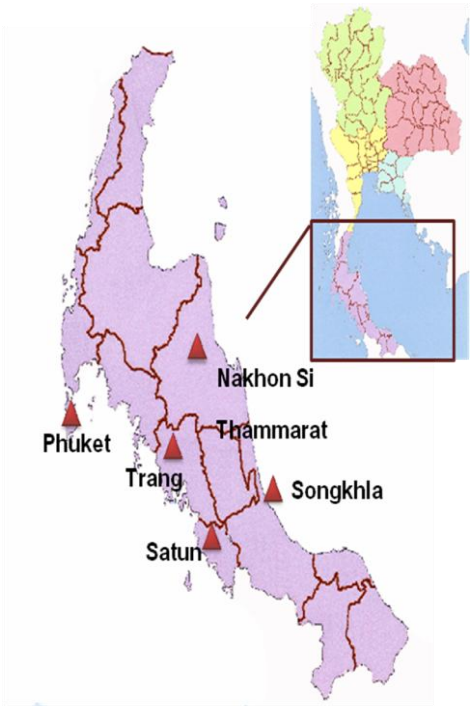


Figure 6 Map of Southern Thailand showing the five provinces for sample collection

2.1.2 Antimicrobial test microorganisms

Bacteria

- Gram-positive bacteria

Staphylococcus aureus ATCC25923

Staphylococcus epidermidis ATCC35984

Clinical isolate of methicillin-resistant *Staphylococcus aureus* SK1 obtained from the Pathology Department, Faculty of Medicine, Prince of Songkla University.

- Gram-negative bacteria

Chromobacterium violaceum DMST21761

Escherichia coli ATCC25922

Pseudomonas aeruginosa ATCC27853

Acinetobacter baumannii NPRC001, 002, 003, 004, 005, 007 obtained from the Natural Product Research Center of Excellence, Faculty of Science, Prince of Songkla University.

Fungi

- Yeast

Candida albicans ATCC90028

Cryptococcus neoformans ATCC90112

- Filamentous fungi

Clinical isolate of *Microsporum gypseum* SH-MU4 obtsained from The Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University.

Clinical isolate of *Talaromyces marneffei* PSU-SKH1 obtained from the Pathology Department, Faculty of Medicine, Prince of Songkla University. *T. marneffei* CI-2, CI-5 and CI-7 isolates were kindly provided by Prof. Dr. Nongnuch Vanittanakom, Faculty of Medicine, Chiang Mai University.

2.1.3 Chemicals

Normal saline solution (0.85% NaCl)	
Ethanol (commercial grade)	
Glycerol	(Fluka)
McFarland Standard	
M9 buffer	(Appendix)
Dimethyl sulfoxide (DMSO)	(Merck)
D-glucose	(BDH Prolabo)
Phosphate buffer saline (PBS) pH 7.0	
Resazurin (1.8%)	
Sodium dodecyl sulfate (0.03% SDS)	
Phenol (1.5% phenol)	
Crystal violet (0.1%)	
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)	

2.1.4 Media

Actinomycete isolation agar (AIA)	(Difco)
Brain heart infusion (BHI)	(Difco)
Chitin agar	(Appendix)
Granulated agar	(Difco)
Humic acid vitamin agar (HV)	(Appendix)
International *Streptomyces* Project (ISP) 2	(Appendix)
Luria Bertani (LB)	(Difco)
Malt extract	(Difco)
Modified soil extract agar (A9)	(Appendix)
Mueller-Hinton broth (MHB)	(Difco)
Nematode growth medium (NGM)	(Appendix)
Nutrient agar (NA)	(Difco)
Nutrient broth (NB)	(Difco)
Potato dextrose agar (PDA)	(Appendix)

RPMI-1640 medium	(Sigma)
Sabouraud dextrose agar (SDA)	(Difco)
Sabouraud dextrose broth (SDB)	(Difco)
Starch nitrate agar (SN)	(Appendix)
Starch-yeast extract-peptone-seawater agar (SYP-SW)	(Appendix)
Tryptic Soy Broth (TSB)	(Difco)
Yeast extract	(Difco)

2.1.5 Antibiotics

Amphotericin B	(Bristol-Mayer Squibb Co., USA)
Colistin	(Atlantic Laboratories Co., Ltd Thailand)
Gentamicin	(NIDA Pharma Inc., Thailand)
Kanamycin	(Sigma Chemical Co., USA)
Miconazole	(Sigma Chemical Co., USA)
Vancomycin	(Sigma Chemical Co.,USA)

2.1.6 Chemicals for DNA extraction

TE buffer	
Lysozyme	(Sigma)
Sodium dodecyl sulfate (10% SDS)	
Phenol/chloroform/isoamyl alcohol (25:24:1)	
3M sodium acetate pH 5.2	
Absolute ethanol	

2.1.7 Instruments

Stereozoom microscope	(Olympus SZ-PT)
Light microscope	(Olympus CX31)
Long working distance len (50×)	(Olympus)
Hemacytometer	

Microcentrifuge tube	(Eppendorf)
Pipette tips	(Axygen)
Automatic pipette	(Eppendorf)
Multi channel automatic pipette	(Eppendorf)
Hot plate stirrer	(Thermolyne)
Biosafety cabinet class II	
Autoclave	(Tomy)
Incubator	(Brandt)
Incubator shaker	
Hot air oven	(Binder)
Freezer -20°C	(Sanyo)
Freezer -80°C	(New Brunswick Scientific)
Electronic balance	(Satorius)
PCR thermal cycle	(Bio-Rad)
Centrifuge	
Gel electrophoresis machine	
Gel documentation	(Model Syngene Gene Genius)

2.2 Methods

2.2.1 Sample collection and isolation of marine-derived actinomycetes

Forty-five samples were collected from various marine environments in the South of Thailand (Table 5). Sediment samples from mangrove site in Songkhla province were collected at a depth of 10 cm. Sediment samples from lake and coastal areas were taken from different depths using a sediment corer. Sponge, nudibranch and sea anemone samples were collected by divers. Sea squirt, sea fan and algae samples were collected around coastal area and water surface. Marine organisms were identified by Mr. Wiani Pransuk from the Excellence Center for Biodiversity of Peninsular Thailand (CBIPT), Prince of Songkla University (appendix). The samples were taken to the laboratory in plastic bags containing natural seawater and kept at 4°C until processing. Wet sediment samples were dried at room temperature (RT) for 7 days and pretreated with chemical and physical methods as follows: 0.05% SDS + 5% yeast extract (Hayakawa and Nonomura, 1989), 1.5% phenol (Hayakawa *et al*., 1991), dry heat at 120°C 1 h (Bredholt *et al*., 2008; Abidin *et al*., 2016) and rehydration and centrifugation (Hayakawa, 2000). Algae and invertebrate samples were rinsed in sterile seawater and cut into small pieces, subsequently pretreated with lyophilization process. Water samples were passed through a sterilized Whatman No.1 and the filter paper was incubated on the isolation medium (Kinsey *et al*., 1999). Treated samples aseptically diluted with 60% sterile seawater. One hundred microliters of the dilution were spread on the surface of isolation media containing 60% seawater and supplemented with 20 mg/l nalidixic acid and 50 mg/l cycloheximide for inhibition of Gram-negative bacteria and fungi, respectively. Isolation media including actinomycete isolation agar (AIA), modified soil extract agar (A9) (Hamaki *et al*., 2005; Zhang and Zhang, 2011), humic acid vitamin agar (HV) (Zhang and Zhang, 2011) and starch nitrate agar (SN) (Ghanem *et al*., 2000) were used for sediment samples and water. Starch-yeast extract-peptone-seawater agar (SYP-SW) (Kennedy *et al*., 2009), chitin agar (Bredholt *et al*., 2008) and modified soil extract agar (A9) (Zhang and Zhang, 2011) were used for marine

organisms. The inoculated plates were incubated at 28°C for 4 weeks. Different colonial morphologies were picked and re-streaked until free of contaminants on A9 and International *Streptomyces* Project (ISP) No. 2 medium (with or without 60% seawater). The actinomycete isolates were maintained for long-term in 20% glycerol at -80°C.

Table 5 Locations and total number of samples collected for marine-derived actinomycete isolation

Location	**Number of sample**								
	Sediment	**Water**	**Sponge**	**Nudibranch**	**Sea anemone**	**Sea squirt**	**Sea fan**	**Algae**	**Total**
Mangrove forest, Songkhla province	5	5	-	-	-	-	-	-	10
Songkhla Lake, Songkhla province	6	-	-	-	-	-	-	-	6
Songkhla Coast, Songkhla province	-	-	2	-	-	-	-	-	2
Koh Bulon-Lae, Satun province	3	-	3	1	-	-	-	-	7
Pakmeng Beach, Trang province	-	-	1	-	-	-	-	-	1
Si Kao Bay, Trang province	3	-	-	-	3	-	-	1	7
Phuket Coastal Fisheries Research and Development Center, Phuket province	-	-	-	-	-	5	1	3	9
Khao Phlai Dam, Nakhon Si Thammarat province	-	-	-	-	-	-	-	3	3
Total	17	5	6	1	3	5	1	7	45

2.2.2 Antimicrobial screening of marine-derived actinomycetes by a cross streak technique and hyphal inhibition test

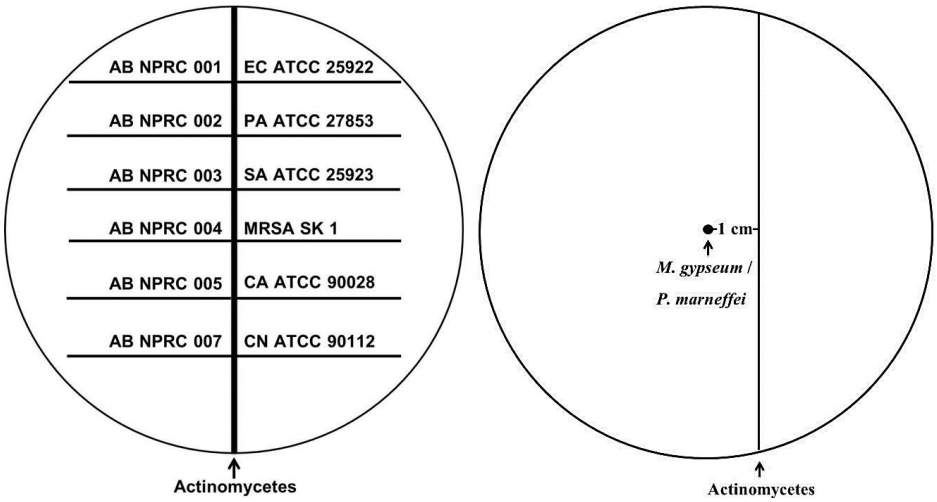
Antibacterial and anti-yeast screening was performed by a cross streak technique as previously described (Williston *et al*., 1947). Each actinomycete was streaked on the center of ISP 2 agar (60% seawater) and incubated at RT for 1 week or until sporulation occurred. After that, one loop of each tested strain was streaked on the agar perpendicular to the edge of the actinomycete streak as shown in Figure 7a. Plates were incubated at 35°C for 18-24 h for bacteria and *C. albicans*. *C. neoformans* was incubated RT for 24-48 h. Antimicrobial activity was recorded by measuring the inhibition zones of the tested strain. Control plate was maintained without inoculating actinomycetes.

Screening test against filamentous fungi was performed by hyphal inhibition test. Each actinomycete was streaked onto one half of the ISP 2 agar (60% seawater) and incubated at RT for 1 week or until sporulation occurred. Then, a mycelial plug of an actively growing fungus was placed about 1 cm from the edge of actinomycete streak as shown in Figure 7b and incubated at RT for 1 week. Then, radii of the tested fungal colony and control were measured and calculated using the formula by Gamliel *et al*. (1989).

$$\% \text{ inhibition} = 100 - [(R^2 / r^2) \times 100]$$

R = average radii of test fungal colony
r = average radii of control fugal colony

The actinomycete strains exhibiting inhibitory zone over 15 mm by a cross streak technique or over 80% inhibition by hyphal inhibition were selected for further study.



a b

Figure 7 Diagram of antimicrobial screening of marine-derived actinomycetes by a across streak technique (a) and fungal inhibition test (b).

AB = *Acinetobacter baumannii* NPRC001-005,007 SA = *Staphylococcus aureus* ATCC25923
MRSA = methicillin-resistant *Staphylococcus aureus* SK1 EC = *Escherichia coli* ATCC25922
PA = *Pseudomonas aeruginosa* ATCC27653 CA = *Candida albicans* ATCC90028
CN = *Cryptococcus neoformans* ATCC90112

2.2.3 Actinomycete fermentation and extraction

Seed culture of actinomycetes was cultivated in 100 ml of ISP 2 broth on the rotary shaker at 200 rpm, 28°C for 1 week. Then 1 ml of seed culture was added to 100 ml ISP 2 broth and incubated at RT for 6 weeks. The extraction procedure is as shown in Figure 8. The culture filtrate was extracted three times with an equal volume of ethyl acetate (EtOAc). Then, the ethyl acetate extract was recovered and concentrated to obtain broth ethyl aceate extract (BE). The cells of actinomycetes were soaked in 200 ml of methanol (MeOH) for 2 days. The MeOH layer was evaporated and 50 ml of water was added. The solution was mixed with 100 ml of hexane using a separatory funnel and allowed the mixture to separate. The aqueous layer was extracted with EtOAc as for the culture filtrate to give cell ethyl acetate extract (CE). The hexane layer was evaporated to obtain cell hexane extract (CH).

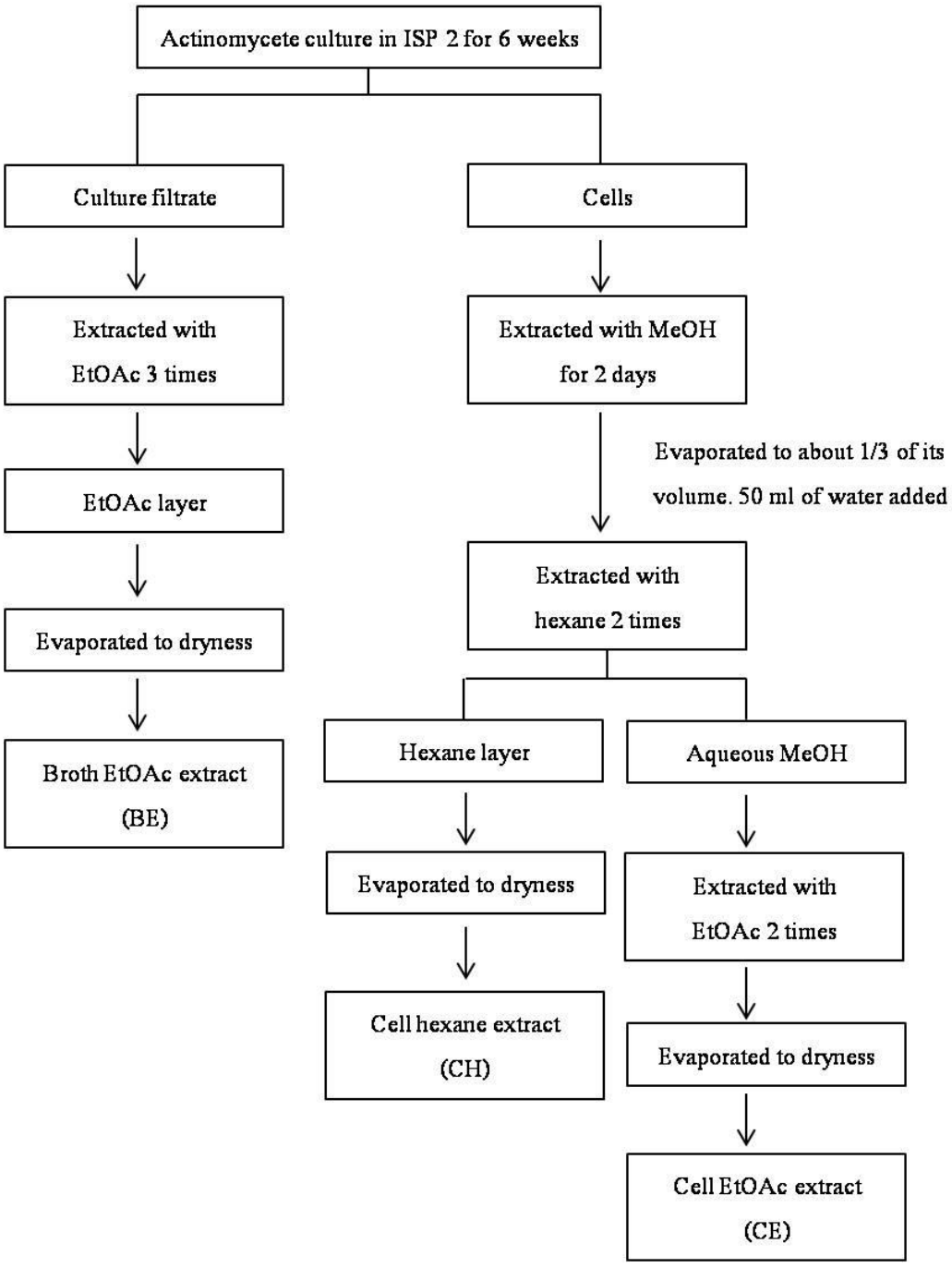


Figure 8 Extraction of antimicrobial metabolites from marine-derived actinomycetes

2.2.4 Antimicrobial activity testing of marine-derived actinomycete extract

The extract was primarily screened for antimicrobial activity at a concentration 200 µg/ml by colorimetric broth microdilution methods according to a modification of Clinical and Laboratory Standards Institute (CLSI 2008a, b; CLSI 2012; Liu *et al*., 2007; Sarker *et al*., 2007).

2.2.4.1 Extract preparation

Each extract was dissolved in dimethyl sulphoxide (DMSO) to prepare a stock solution (10 mg/ml) and stored at -20°C.

2.2.4.2 Inoculum preparation

Tested bacterial strains such as *S. aureus* ATCC25923, MRSA SK1, *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *A. baumannii* NPRC001-005, 007 were cultured on nutrient agar (NA) and incubated at 35°C for 18-24 h. *C. albicans* ATCC90028 and *C. neoformans* ATCC90112 were streaked on Sabouraud dextrose agar (SDA) and incubated at 35°C for 18-24 h and at RT for 48 h, respectively. The suspensions of the tested strains were prepared by picking up 4-5 single colonies of bacterium or yeast and transferred to nutrient broth (NB) and RPMI-1640, respectively. These were incubated on a rotary shaker (150 rpm) at 35°C for 3-5 h. After that, bacterial and yeast suspensions were adjusted to equal the turbidity of 0.5 and 2.0 McFarland standard (MF) with sterile normal saline solution (NSS), respectively and further diluted 1:200 using MHB and 1:20 using RPMI-1640 to obtain approximately 10^6 CFU/ml.

M. gypseum MU-SH4 and *T. marneffei* PSU-SKH1 were maintained on SDA at 25°C for 7 days. Conidia were harvested by scaping the surface of fungal colony gently with sterile glass beads and then suspended with sterile NSS. Finally, the conidial suspension was transferred to a new sterile tube and further adjusted to a concentration of 4×10^3 - 5×10^4 conidia/ml using a hemacytometer.

2.2.4.3 Antimicrobial activity screening of extract

The extract solution (10 mg/ml) was diluted to obtain a concentration of 400 µg/ml with MHB and RPMI-1640 medium for bacteria and yeast, respectively. Then, 50 µl of extract samples were transferred to each well in triplicate and 50 µl of testing inoculum suspension (10^6 CFU/ml) was added to give a final extract concentration of 200 µg/ml. Plates were incubated at 35°C, 15 h for bacteria, 35°C, 18 h for *C. albicans* ATCC90028 and RT 24 h for *C. neoformans* ATCC90112. The inhibitory endpoint was checked by adding 30 µl of resazurin solution (18 µg/well). Plates were re-incubated at the same temperatures for 3-5 h, 6 h and 24 h for bacteria, *C. albicans*, and *C. neoformans*, respectively.

Testing of antifungal activity against *M. gypseum* MU-SH4 and *T. marneffei* PSU-SKH1 was performed in the same manner as against yeasts but 4×10^3 - 5×10^3 conidia/ml was used as inoculum and test plates were incubated at 25°C for 7 days. The inhibition was observed under a stereo zoom microscope for fungal growth.

Standard antibiotics as shown in Table 6 were used as positive controls and DMSO was used as solvent control. A growth control containing no antibiotic (negative control) and a sterile control without inoculum were also included.

Table 6 Standard drugs used in the antimicrobial screening test

Antibiotic	**Final concentration (µg/ml)**	**Test microorganism**
Vancomycin	10	*S. aureus* and MRSA
Gentamicin	10	*E. coli* and *P. aeruginosa*
Colistin	30	*A. baumannii*
Amphotericin B	10	*C. albicans*, *C. neoformans* and *T. marneffei*
Miconazole	32	*M. gypseum*

The results of antimicrobial activity were read according to a change of resazurin color from blue to pink (resorufin) which indicates the growth of microorganisms (negative result). On the other hand, the color of resazurin remained

blue indicates inhibition of growth (positive result). For filamentous fungi, the inhibition was read by observation of visible fungal growth under a stereo zoom microscope.

The extracts showing antimicrobial activity at a screening concentration of 200 µg/ml were further determined for their minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC).

2.2.4.4 Determination of minimum inhibitory concentration (MIC)

The MICs of active extracts were determined by a colorimetric broth microdilution method according to a modification of CLSI M7-A9 (CLSI, 2012), CLSI M27-A3 (CLSI, 2008a) and CLSI M38-A2 (CLSI, 2008b) against bacteria, yeasts and filamentous fungi (*M. gypseum* and *T. marneffei*), respectively.

The determination of MIC was performed in the same manner as the screening test but 10 concentrations of serially 2-fold dilution of each extract from 0.25-128 µg/ml were tested in triplicate. After adding resazurin and incubation under suitable conditions, the MIC endpoint as the lowest concentration of each extract at which there was no color change (blue color) was recorded. In case of no inhibition at a concentration of 128 µg/ml, the MIC value was recorded as 200 µg/ml.

Drug controls (Table 7), growth control, solvent control and sterility control were also tested.

Table 7 Standard drugs used in the MIC determination

Antibiotic	**Final concentration range (µg/ml)**	**Test microorganism**
Vancomycin	0.0625-32	*S. aureus* and MRSA
Gentamicin	0.0625-32	*E.coli* and *P. aeruginosa*
Colistin	0.0625-32	*A.baumannii*
Amphotericin B	0.03125-16	*C. albicans*, *C. neoformans* and *T. marneffei*
Miconazole	0.0625-32	*M. gypseum*

2.2.4.5 Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

MBCs and MFCs were determined by subculturing 10 µl from each well with an extract concentration higher than the MIC and equal to the MIC on NA plates for bacteria and SDA plates for yeasts and filamentous fungi. Incubation temperatures were the same as those used for the MIC determinations. Incubation times were 16-18 h, 18-24 h, 48 h and 7 d for bacteria, *C. albicans*, *C. neoformans* and filamentous fungi, respectively. The MBC and MFC endpoints were defined as the lowest concentration of crude extract that killed >99.9% of the initial inoculum population where no visible growth was observed on the media plates.

2.2.5 Effect of active extract on target cells detected by a scanning electron microscopy (SEM)

Tested bacteria and yeasts were streaked onto MHA and SDA, respectively. Culture conditions were 35°C, 24 h for bacteria and *C. albicans* and RT, 48 h for *C. neoformans*. Three to five single colonies of them were subcultured into MHB and SDB and incubated at the same conditions previously described. Cells were collected by centrifugation at 5,000 rpm, 5 min and resuspended in MHB and SDB (10 ml).

Finally, the cells were treated with the active extracts at 4×MIC concentration and incubated at 35°C, 24 h for bacteria and *C. albicans* and RT, 48 h for *C. neoformans*.

Filamentous fungi were cultivated on PDA for 7 days. Then, the mycelial plugs (1 cm^2) of actively growing fungi were soaked in SDB containing with the test extract at 4×MIC concentration and incubated at 25°C for 1-4 days. Mycelilal plugs treated with 1% DMSO were used as control. Treated samples were washed with phosphate buffer saline solution (PBS) 3 times, fixed in 2.5% glutaradehyde in PBS for 1-2 h and washed with PBS 3 times. They were then fixed in 1% osmium tetroxide for 1-2 h, washed with sterile distilled water 3 times and dehydrated with alcohol series (50%, 70%, 80%, 90% and 100% of ethanol) before processing for SEM at the Scientific Equipment Center, Prince of Songkla University.

2.2.6 Anti-quorum sensing potential of marine-derived actinomycete extracts by a disk diffusion method

A modified method from Chenia (2013) was used in this experiment. *C. violaceum* DMST21761 was grown overnight on Luria Bertani (LB) plate and the turbidity of cell suspensions were adjusted to a turbidity of 0.5 McFarland standard. *C. violaceum* inoculum was inoculated onto LB plates by streaking swabs, followed by the application of the disks containing extract (100 µg/disk). Plates were then incubated for 24 h at 30°C. QS inhibition was detected by measuring a ring of colorless but viable cells around the disks. DMSO was used as a negative control and cinnamaldehyde (64 µg/disk) as a posive control.

2.2.7 Bacteriolytic activity

MRSA SK1 was grown in Nutreint broth (NB) medium at 35°C for 24 h and then suspended in NSS (10^8 CFU/ml). Ethyl acetate extract from cells of AMA11 (AMA11CE) at final concentrations equivalent to MIC, 2×MIC, 4×MIC and 8×MIC were added to bacterial suspension and incubated at 35°C. The OD_{620} was measurd at 0, 2, 4, 6, 8, 10, 12 and 24 h. The diltuions of extracts alone in NSS were used as blanks, and 1% DMSO was used as negative control. Bacterial cell lysis was indicated

by decrease in the absorbance at OD_{620}. All assays were carried out in triplicate. The results were expressed as a ratio of the OD at each time interval versus the OD at 0 min (in percent) (Carson *et al*., 2002).

2.2.8 Anti-biofilm assay

S. epidermidis ATCC35984 was grown in Tryptic Soy broth (TSB) supplemented with 0.5% glucose at 35°C for 24 h, then diluted to 10^6 CFU/ml. A 100 µl of bacterial suspension was added to each well of a 96-well microtiter plate containing 80 µl of TSB and 20 µl extract or vancomycin at different concentrations. Plates were incubated at 35°C for 24 h in a static condition. After incubation, the plates were washed twice with PBS, air-dried at RT, and stained with 200 µl of 0.1% crystal violet solutions for 30 min. Crystal violet was discarded and the plates were washed with water and air-dried. Absorbed crystal violet stain from the biofilms was eluted with 200 µl DMSO and OD_{570} was measured using a microtiter plate reader (Karaolis *et al.,* 2005). The results were based on data obtained in triplicate. The percentage inhibition of biofilm was defined as (mean OD_{570} of control well – mean OD_{570} of the treated well/mean OD_{570} of control well) × 100 (Santhakumari *et al.,* 2016).

2.2.9 Effect of actinomycete extracts on the established biofilms

S. epidermidis ATCC35984 was cultured in NB medium at 35°C for 24 h. Two hundred microlitres of overnight culture (10^6 CFU/ml) was transferred to a 96-well microtitre plate. After 24 h of incubation at 35°C, the medium was gently removed and the wells were washed twice with PBS. TSB (180 µl) and 20 µl of the extracts and vancomycin at different concentrations were added. The plates were incubated for 24 h at 35°C. The medium was removed and filled with 200 µl PBS supplemented with 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml). The plates were incubated at 35°C for 2 h and MTT solution was removed. DMSO (200 µl) was added into the well to dissolve the insoluble purple formazan and the OD_{570} was measured. The percentage viability of

biofilm was calculated as follows: % viability = (mean OD_{570} of the treated well/mean OD_{570} of control well) × 100 (Kuźma *et al*., 2007).

2.2.10 Toxicity test in *C. elegans* model (modification of Huang *et al*., 2014)

C. elegans was grown at 20°C on a nematode growth medium (NGM) with *E. coli* DSM498. Synchronized nematodes in the L4 stage were washed off the plate with sterile distilled water. About 250 of L4 stage nematodes were put into 1 ml liquid medium assay consisting of 80% M9 buffer, 20% BHI, 45 µg/ml kanamycin containing extracts, amphotericin B and 1% DMSO. The nematodes were incubated at 25°C. Dead nematodes were counted at 24 h intervals for 7 days. Nematodes were considered dead if they were unresponsive to touch. Wells containing *C. elegans* and medium served as a control.

2.2.11 *T. marneffei* virulence test in *C. elegans* (Huang *et al*., 2014)

About 250 worms were transferred to 24-wells plate containing 10^5 conidia/ml of various strains (PSU-SKH1, CI-2, CI-5, CI-7) of *T. marneffei* in liquid medium assay. Plates were incubated at 25°C and nematode survival was scored at 24 h intervals for 7 days. Nematodes were considered dead if they were unresponsive to touch. Wells containing *C. elegans* and medium served as a control.

2.2.12 *C. elegans* survival assay (Huang *et al*., 2014)

The L4 stage nematodes were infected with 10^5 conidia/ml of *T. marneffei* CI-2 for 24 h in a liquid medium assay consisting of 80% M9 buffer, 20% BHI, 45 µg/ml kanamycin supplemented with either extracts or amphotericin B at various concentrations (MIC, 2×MIC, 4×MIC and 8×MIC). Plates were incubated at 25°C and dead nematodes were examined at 24 h intervals for 7 days. Wells containing *C. elegans* and medium served as the control.

2.2.13 Partial purification of the AMA11CE extract

The AMA11CE extract was subjected to silica gel column chromatography (20×2 cm, Silica gel 100, Merck) using a gradient solvent system of methanol and water. Fractions obtained were evaporated to dryness by a rotary evaporator and screened for antibacterial activity against Gram-positive (MRSA SK1) and Gram-negative (*C. violaceum* DMST21761) bacteria. The chemical constituents of the active fractions with good antibacterial activity were analyzed using the gas-chromatography-mass spectrometry (GC-MS).

2.2.14 Gas chromatography-Mass spectrometry (GC-MS) analysis

GC-MS analysis of the most active extracts (AMA11CE, AMA11CE fractions and AMA50CH) was performed using a Agilent Technologies 7890 B (GC) equipped with 5977A Mass Selective Detector (MS). VF-WAXms capillary column of dimensions 30 m×250×0.25 µm was used with helium gas as the carrier at a flow rate of 1 ml/min. The column temperature was programmed initially at 60°C, which was increased to 160°C at 10°C/min and further increased to 250°C at 2.5°C/min, held time for 15 min. The mass spectrometer was operated in the electron ionization mode at 70eV with a source temperature of 230°C, with continuous scanning from 35 – 500 m/z. The chemical constituents were identified by comparing their mass spectral data with those from the Wiley library.

2.2.15 Identification of actinomycetes

2.2.15.1 Morphological characteristics

Morphological observations were conducted by criteria according to the guide described in Bergey's manual of systematic bacteriology volume 4 (Willims, 1989). The actinomycetes were grown on modified soil extract agar (Hamaki *et al.*, 2005: Zhang and Zhang, 2011). Plates were incubated at 28°C for 7-14 days and determined

for the characteristics of the spore by direct microscopic examination with long working distance lens (50×).

2.2.15.2 Molecular identification and phylogenetic analysis

The potential isolates were identified based on 16S rDNA sequence analysis.

2.2.15.2.1 DNA extraction (modification of Marmur, 1961)

Selected actinomycetes were cultured in ISP 2 broth in a rotary shaker at 200 rpm, 28°C for 3-4 days. Cells were collected by centrifugation at 13500 rpm for 3-5 min, washed with TE buffer twice, and then suspended in TE buffer. Small amount of lysozyme was added into the microcentrifuge tube and incubated at 37°C for 3 h. 10% SDS was added and the tube was further incubated at 55°C for 1 h. The phenol extraction of DNA was performed by adding phenol/chloroform/isoamyl alcohol (25:24:1), mixed well and frozen at -20°C overnight, then centrifuged at 13000 rpm for 20 min. The upper layer was collected and transferred into a new microcentrifuge tube. One tenth volume of 3M sodium acetate pH 5.2 was added to clear solution. DNA was precipitated by adding 2 volumes of cold absolute ethanol. Subsequently, DNA was harvested by pooling with a small glass rod and dried at RT. Finally, the dried DNA was dissolved in TE buffer and stored at -20°C.

2.2.15.2.2 PCR, sequencing and phylogenetic tree analysis (modification of Trcek and Teuber, 2002)

The PCR amplification of 16S rRNA gene was performed in a total volume of 100 µl of reaction consisting of 15 ng of DNA template, 2.5 units of Taq DNA polymerase, 10 µl of 10×Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM of dNTP mixture, 2.0 µmoles of forward primer 20F (5'-AGTTTGATCCTGGCTCAG-3', positions 9–27) and reverse primer 1500R (5'-GTTACCTTGTTACGACTT-3', positions 1509–1492). The final volume was adjusted with dH_2O. The PCR amplification was carried out with DNA Thermal Cycler, which was programmed as follows:

Initial denaturation	95 ˚C	3	min	
Denaturation	95 ˚C	1	min	30 cycles
Annealing	50 ˚C	1	min	
Extension	72 ˚C	2	min	
Final extension	72 ˚C	3	min	

The PCR products were visualized by running 3-5 µl of the reaction mixture on a 0.8% agarose gel. The PCR product was purified with a Geneaid PCR DNA fragments extraction kit (Geneaid, Taiwan) before sending to Macrogen Inc. Korea for sequencing using 27F, 518F, 800R and 1492R primers.

The sequences obtained from Macrogen were assembled using BioEdit Sequence Alignment Editor (version 7.2.5). A BLAST comparison of the 16S rRNA gene sequence was made and the similarities with the type strains were calculated via EzBio-Cloud's database (Yoon *et al*., 2017). Multiple alignments were performed with the program CLUSTAL W (Thompson *et al*., 1994) of the software BioEdit Sequence Alignment Editor (version 7.2.5) (Hall, 1999).

The phylogenetic tree was constructed using the neighbor-joining (Saitou and Nei, 1987) methods in the MEGA 6 program (version 6.06) (Tamura *et al*., 2013). The neighbour-joining algorithm was calculated with the Kimura two-parameter model. Bootstrap analysis was used to evaluate the topology of each tree based on 1000 replications (Felsenstein, 1985).

2.2.16 Description of the novel species

2.2.16.1 Phenotypic characteristics

Strain AMA120^{T} was culture on ISP no. 2 medium at 28°C for 7 days. Cell morphology was determined using scanning electron microscope (SEM). Pigmentation was assessed on various media such as ISP medium nos. 2, 3, 4, 5, 7 and TSA (Shirling and Gottlieb, 1966). Colony colors were determined after incubation at 28°C for 7 and 14 days using the ISCC-NBS color chart (Kelly, 1964). Growth was determined at 5, 10, 15, 20, 28, 37 and 45°C for 7 and 14 days. The

initial pH range for growth was observed on ISP medium no. 2 broth at pH 4.0 to 14.0 (intervals of 1.0 pH using biological buffers: phosphate, citrate, bicarbonate-carbonate buffer) at 28°C for 7 and 14 days. The NaCl tolerance range for growth was tested at concentrations between 1 and 10% w/v at 1% intervals at 28°C for 7 and 14 days. Sole carbon source utilization was tested on ISP medium no. 9 as a basal medium supplemented with a final concentration of 1% of the carbon sources. Acid production from carbohydrates was studied in a Tryptone yeast extract medium containing 0.5% (w/v) carbon sources (Blackall *et al*., 1989). Hydrolysis of hypoxanthine, chitin, casein, skim milk, gelatin and nitrate was investigated as described by Gordon *et al* (1974). Catalase activity was determined by bubble production in 3% (v/v) H_2O_2 (Greenwood and Pickett, 1979). Enzyme hydrolysis patterns against various substrates were determined using API-ZYM (BioMerieux).

2.2.16.2 Genotypic and phylogenetic analyses

The 16S rRNA gene sequences of strain AMA120^T were determined as previously described in 2.2.15.2. For genome sequencing, the DNA of AMA120^T was extracted from cell pellets using Maxwell 16 DNA Purification Kits (Promega) according to the manufacturer's instruction. The genome was sequenced using the Illumina platform at Macrogen, Inc., South Korea. Libraries of genomic DNA were prepared using a TruSeq Nano DNA kit (Illumina) and pooled libraries were subjected to multiplexed paired-end sequencing. Sequencing read assembly was performed using SPAdes version 3.10.1 (Serrano *et al*., 2018). The nucleotide sequence of *gyrB* (1,200 bp) of AMA120^T was obtained from whole genome sequence and the sequence was BLAST with the type strains from the GenBank/EMBL/DDBJ database (Drzyzga *et al*., 2009). The average nucleotide identity (ANI) of whole genome of AMA120^T with closely related type strains was calculated using the JSpeciesWS web server (Richter *et al*., 2016).

The phylogenetic tree was constructed using the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Felsenstein, 1983) and maximum-likelihood (Felsenstein, 1981) methods in the MEGA 6 program (version 6.06) (Tamura *et al*., 2013). The neighbour-joining algorithm was calculated with the Kimura two-

parameter model. The maximum-likelihood was calculated according ti the Tamura-Nei model. Bootstrap analysis was used to evaluate the topology of each tree based on 1000 replications (Felsenstein, 1985).

DNA-DNA hybridization was performed by microdilution wells using a photobiotin-labelling method (Ezaki *et al*., 1989) and determined using a colorimetric method (Verlander, 1992). Relatedness values (%) were calculated according to the method of Ezaki et al (Ezaki *et al*., 1989) with three replications. The DNA G+C content of AMA120^T was determined from the whole genomic DNA sequence.

2.2.16.3 Chemotaxonomic characteristics

Freeze-dried cells were used for chemotaxonomic analyses.

2.2.16.3.1 Diaminopimelic acid (DAP) and whole-cell sugars analysis (Staneck and Roberts, 1974).

Approximately 3 mg of freeze-dried cells was placed into a small ampoule with 1 ml of 6 N HCl, and then kept at 100°C in an oven for 18 h. After cooling, the hydrolysate was filtered by Whatman no. 1 paper. The filtrate was evaporated to dryness in a boiling water bath, redissolved in 1 ml of distilled water, and taken to dryness again. This residue was dissolved in 0.3 ml of distilled water, and 2 µl was applied at the base line of the TLC sheet. TLC was performed with the solvent system methanol : distilled water : 6 N HCl : pyridine (80:26:4:10, vol/vol) for 3.5 h. After TLC was air dried, spots were visualized by spraying with 0.2% ninhydrin in acetone and heating at 100°C for 3 min. As a DAP standard, 1 µl of 0.01 M DL-DAP (Sigma Chemical Co.) containing both *meso*- and *L*-DAP isomers, was used as control. For whole-cell sugar analysis, TLC was performed with the solvent system of n-butanol-distilled waterpyridine-toluene (10: 6: 6:1, v/v) (4) for 3 hr. After the sheet was dried in a draft, spots were visualized by spraying with acid aniline phthalate and heating at 100°C for 4 min. As the standard sugars, 1 µl of solution containing galactose, glucose, mannose, arabinose, xylose, and ribose each at 1% concentration was spotted on TLC together.

2.2.16.3.2 Menaquinone analysis (Collins *et al*., 1977)

Equal volumes (2 ml) of aqueous methanolic NaCl (10 ml 0.3%, w/v, aqueous NaCl added to 100 ml methanol) and petroleum ether (b.p. 60-80 "C) were added to 100 mg biomass in polytetrafluoroethylene-lined capped tubes and mixed on a tube rotator for 15 min. The tubes were centrifuged at low speed for 5 min, the upper layer was removed and the extraction repeated with 1 ml petroleum ether. The extracts were evaporated under nitrogen gas at about 37°C to provide crude isoprenoid quinones. Crude isoprenoid quinone samples were dissolved in petroleum ether, applied as 0.5 cm bands to 10×10 cm pieces of plastic-backed silica gel sheet, and developed with petroleum ether/acetone (96:4, v/v) in the first direction and petroleum ether/toluene (50:50, v/v) in the second direction. Separated menaquinones were detected with short-wave UV light (254 nm) and menaquinone bands cut out and extracted by shaking with diethyl ether (1 ml) for 15 min. The extraction was repeated with a further 1 ml diethyl ether. The combined extracts was passed through a Pasteur pipette plugged with cotton wool prewashed with diethyl ether and the filtrate was evaporated to dryness using nitrogen gas (around 37°C) and stored in the dark at - 20°C. Purified menaquinones were analysed using reverse-phase HPLC [Cosmosil $5C_{18}$ column (4.6×150 mm); NacalaiTesque] with a mixture of methanol and 2-propanol (2:1, v/v) as elution solvent.

In addition, polar lipids were extracted and identified by two-dimensional TLC as described by Minnikin *et al* (1984). Fatty acid analysis followed the RTSBA6 method of the Microbial Identification System (MIDI, Sherlock Version 6.2B) (Sasser, 2001). The occurrence of mycolic acid was investigated by TLC according to the method of Minnikin *et al* (1975).

CHAPTER 3

RESULTS

3.1 Isolation of marine-derived actinomycetes

A total of 525 isolates of marine-derived actinomycetes were obtained from sediments and marine organisms. No isolate was recovered from water samples. The highest number of total isolates was from lake sediments (ALA, 183 isolates from 6 samples), followed by marine organisms (AMR, 160 isolates from 23 samples), mangrove sediments (AMA, 156 isolates from 5 samples) and coastal sediments (ASE, 26 isolates from 6 samples). Morphological characters such as color of mycelium, production of diffusible pigment and spore morphology were used for family classification. Results are shown in Table 8. *Streptomycetaceae* (256 isolates) was the predominant family found in this study, followed by *Micromonosporaceae* (178 isolates), *Pseudonocardiaceae* (45 isolates), *Gordoniaceae* (12 isolates), *Nocardiaceae* (11 isolates), *Thermomonosporaceae* (7 isolates), *Jiangellaceae* (2 isolates), *Streptosporangiaceae* (1 isolate) and unidentified actinomycetes (13 isolates).

Table 8 Families of marine-derived actinomycetes obtained from various sample types

Family	No. of actinomycetes				Total (%)
	AMA	ALA	ASE	AMR	
Gordoniaceae	7	0	0	5	12 (2.3)
Jiangellaceae	0	0	2	0	2 (0.4)
Micromonosporaceae	33	94	2	49	178 (33.9)
Nocardiaceae	10	0	0	1	11 (2.1)
Pseudonocardiaceae	1	37	7	0	45 (8.6)
Streptomycetaceae	99	52	8	97	256 (48.8)
Streptosporangiaceae	1	0	0	0	1 (0.2)
Thermomonosporaceae	5	0	2	0	7 (1.3)
Unidentified actinomycetes	0	0	5	8	13 (2.5)
Total	156	183	26	160	525

AMA = actinomycetes from mangrove sediment ALA = actinomycetes from Songkhla lake

ASE = actinomycetes from coastal sediment AMR = actinomycetes from marine organisms

3.2 Antimicrobial screening by a cross streak and hyphal inhibition tests

Among 525 isolated actinomycetes, 274 isolates were selected as morphological representatives for antimicrobial activity testing against the standard ATCC and clinical strains including *S. aureus* ATCC25923, MRSA SK1, *A. baumannii* NPRC001-005, 007, *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *C. albicans* ATCC90028, *C. neoformans* ATCC90112, *M. gypseum* SH-MU4 and *T. marneffei* PSU-SKH1 by a cross streak technique and hyphal inhibition methods (Figure 9). The results showed that 158 isolates (57.7%) possessed inhibitory activity against at least one test strain. The representatives from all families except the

Jiangellaceae and *Nocardiaceae* exhibited antimicrobial activity (Table 9). They were highly active against *M. gypseum* (65.2%), *T. marneffei* (42.4%), MRSA (40.5%), *S. aureus* (31.1%), *C. neoformans* (25.9%), *C. albicans* (19.0%), *E. coli* (5.1%), *A. baumannii* (3.8-5.7%) and *P. aeruginosa* (1.3%) (Figure 10).

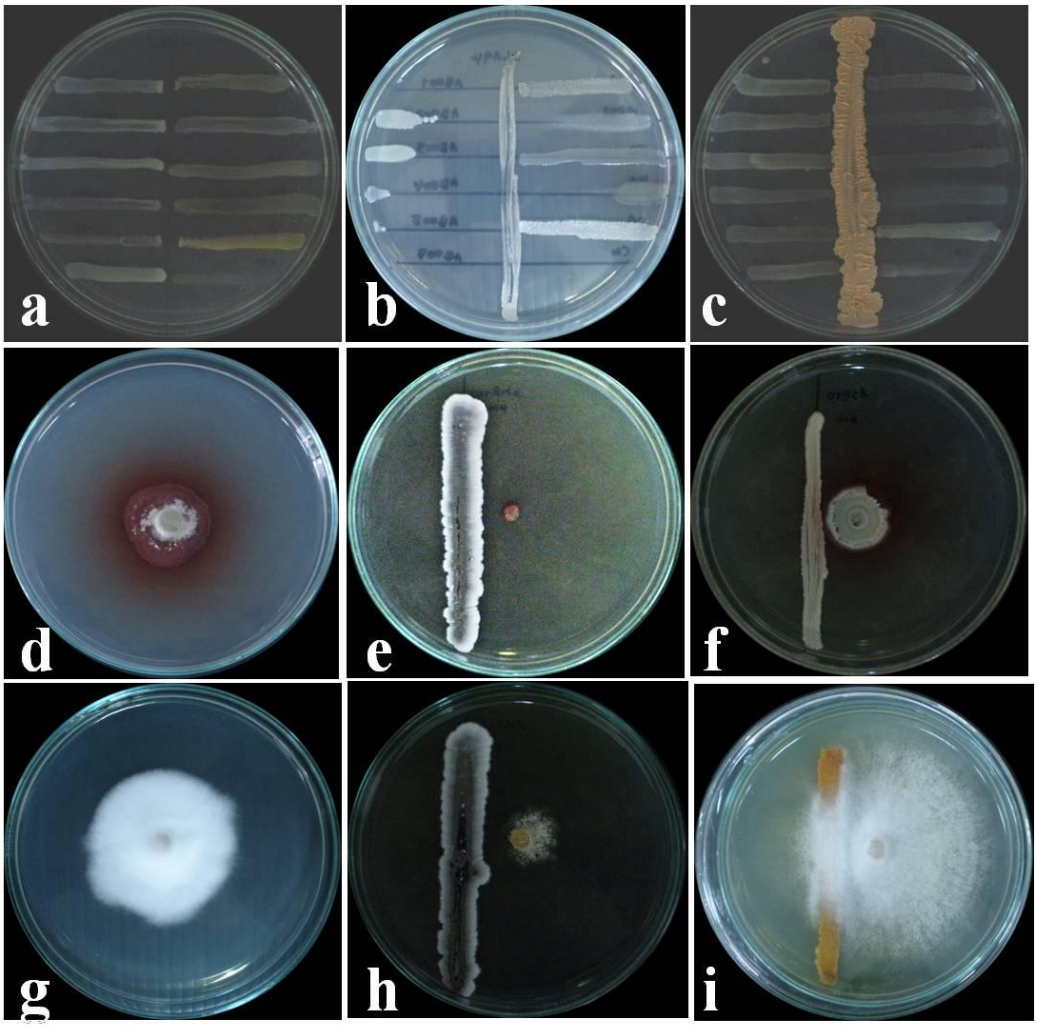


Figure 9 Primary antimicrobial screening by a cross streak method and hyphal inhibition tests. Plates showing antibacterial and anti-yeast activities (a-c), anti-*T. marneffei* (d-f) and anti-*M. gypseum* (g-i). All rows consist of growth controls (a, d, g), positive (b, e, h) and negative results (c, f, i) for each test.

Table 9 Antimicrobial activity of marine-derived actinomycetes from various families

Family	Total isolates tested	Total active isolates (%)
Gordoniaceae	6	1 (16.7)
Jiangellaceae	1	0 (0)
Micromonosporaceae	62	14 (22.6)
Nocardiaceae	6	0 (0)
Pseudonocardiaceae	22	10 (45.5)
Streptomycetaceae	166	125 (75.3)
Streptosporangiaceae	1	1 (100)
Thermomonosporaceae	5	4 (80)
Unidentified group	5	3 (60)
Total	274	158 (57.7)

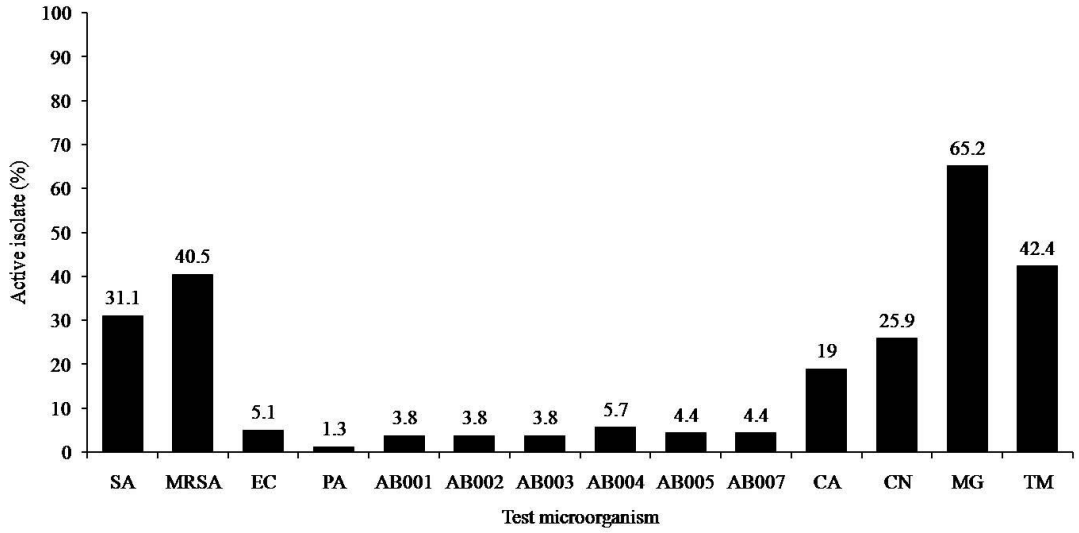


Figure 10 Antimicrobial activities of marine-derived actinomycetes tested by a cross streak and hyphal inhibition tests

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1

EC = *Escherichia coli* ATCC25922 PA = *Pseudomonas aeruginosa* ATCC27853

AB = *Acinetobacter baumannii* NPRC001-005, 007 CA = *Candida albicans* ATCC90028

CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH1

TM = *Talaromyces marneffei* PSU-SKH1

3.3 Preliminary antimicrobial activity of marine-derived actinomycete extracts at the concentration of 200 µg/ml

The promising and fast-growing active isolates exhibiting inhibition zones over 15 mm from the screening test were further tested for their antimicrobial secondary metabolite production under submerged fermentations. Two hundred and eighty-seven extracts from 104 isolates were screened for their antimicrobial activities against 9 pathogenic microorganisms including *S. aureus* ACTCC25923, MRSA SK1, *A. baumannii* NPRC004, *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *C. albicans* ATCC90028, *C. neoformans* ATCC90112, *M. gypseum* SH-MU4 and *T. marneffei* PSU-SKH1.

Out of 287 extracts, 160 (55.7%) from 81 isolates showed antimicrobial activity inhibiting at least one of the test pathogens. Among the active extracts, 78.8, 69.4, 19.4 and 1.9% displayed inhibitory activity against *S. aureus*, MRSA, *A. baumannii*, *E. coli*, respectively. None of the extracts was active against *P. aeruginosa*. For antifungal activity, 13.8, 13.8, 8.8 and 2.5% were active against *C. neoformans*, *T. marneffei*, *C. albicans* and *M. gypseum* (Figure 11).

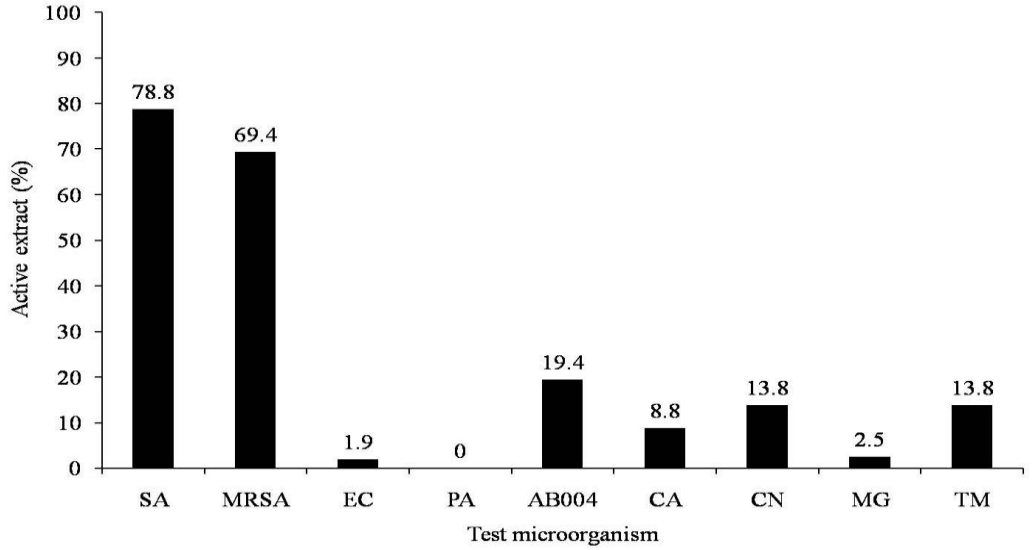


Figure 11 Antimicrobial activities of marine-derived actinomycete extracts against individual test microorganism

SA = *Staphylococcus aureus* ATCC25923
MRSA = methicillin-resistant *S. aureus* SK1
EC = *Escherichia coli* ATCC25922
PA = *Pseudomonas aeruginosa* ATCC27853
AB = *Acinetobacter baumannii* NPRC004
CA = *Candida albicans* ATCC90028
CN = *Cryptococcus neoformans* ATCC90112
MG = *Microsporum gypseum* MU-SH1
TM = *Talaromyces marneffei* PSU-SKH1

According to the types of extracts, the results revealed that the most active extracts was the ethyl acetate extract from cells (CE, n=59), followed by the hexane extract from cells (CH, n=51) and the ethyl acetate extract from culture broth (BE, n=50) (Figure 12). The BE and CE extracts showed antimicrobial activity against 1 to 6 tested strains, whereas CH inhibited 1 to 5 tested strains (Figure 13). Table 10 shows antimicrobial activity of each extract against individual test strain. All types of extracts showed a broad range activity against bacteria, yeasts and filamentous fungi with the highest number of extracts against Gram-positive bacteria. *S. aureus* was found to be most susceptible to the CE and CH (n >40), whereas MRSA was most susceptible to the CE (n = 40). Among Gram-negative bacteria, *A. baumanii* was the most susceptible strain that was inhibited by the CH. Only two BE and one CE inhibited *E. coli*. For yeasts and filamentous fungi, CH could most inhibit *T. marneffei*, *C. neoformans*, *C. albicans* and *M. gypseum*.

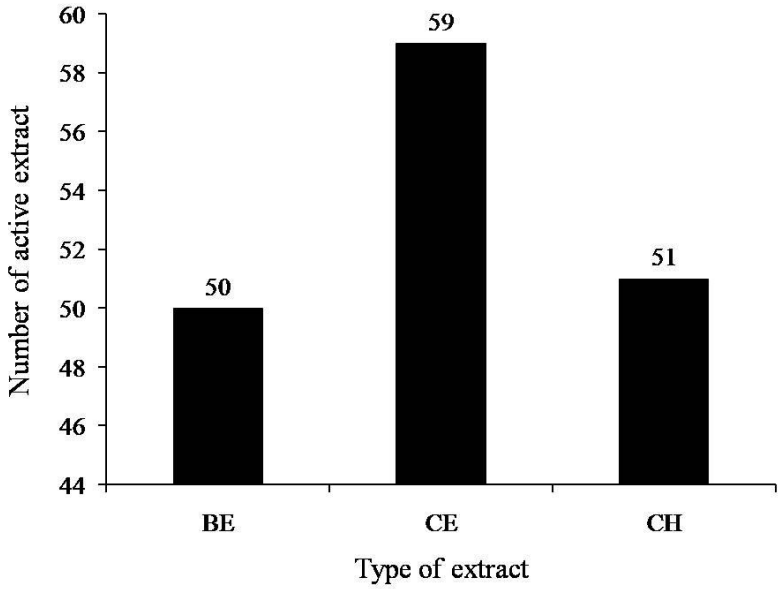


Figure 12 Number of each type of active extracts from marine-derived actinomycetes

BE = ethyl acetate extract from culture broth CE = ethyl acetate extract from cells

CH = hexane extract from cells

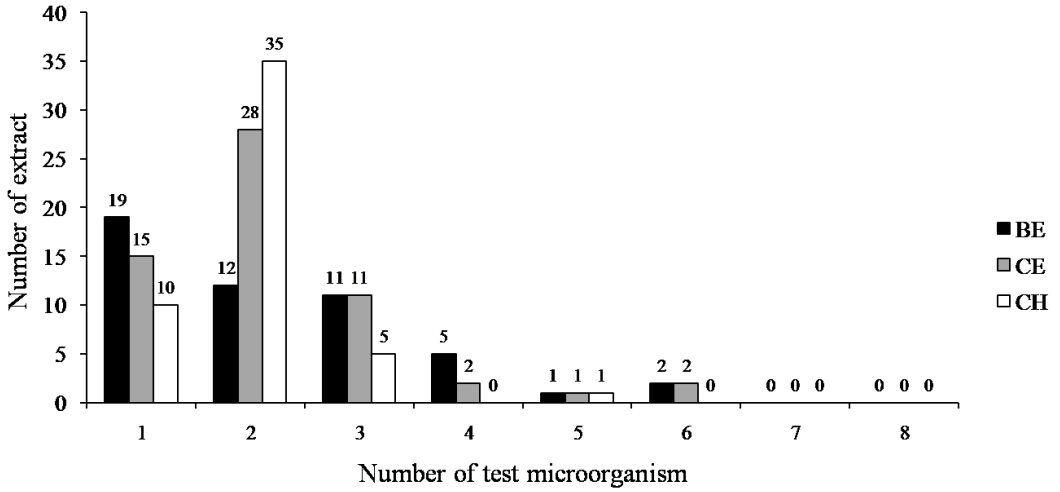


Figure 13 Number of test microorganisms inhibited by each type of extracts

BE = ethyl acetate extract from culture broth CE = ethyl acetate extract from cells

CH = hexane extract from cells

Table 10 Antimicrobial activity of each type of extracts against individual test microorganisms

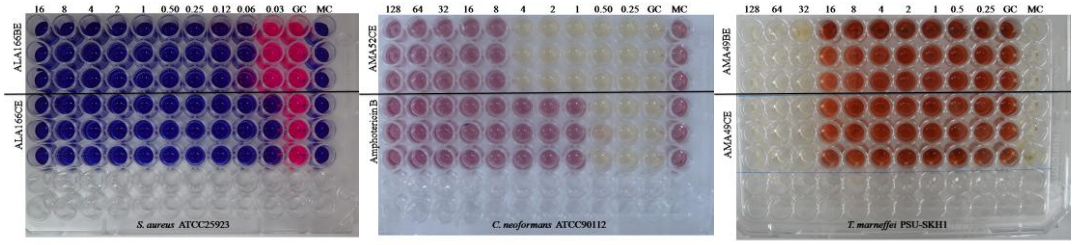
Type of extract (no. of active extract)	Test microorganism							
	SA	**MRSA**	**EC**	**AB004**	**CA**	**CN**	**MG**	**TM**
BE (n=50)	32	34	2	19	6	8	2	9
CE (n=59)	49	40	1	9	6	10	2	8
CH (n=51)	45	37	0	31	14	22	4	22

BE = ethyl acetate extract from culture broth
CE = ethyl acetate extract from cells
CH = hexane extract from cells
SA = *Staphylococcus aureus* ATCC25923
MRSA = methicillin-resistant *S. aureus* SK1
EC = *Escherichia coli* ATCC25922
AB = *Acinetobacter baumannii* NPRC004
CA = *Candida albicans* ATCC90028
CN = *Cryptococcus neoformans* ATCC90112
MG = *Microsporum gypseum* MU-SH4
TM = *Talaromyces marneffei* PSU-SKH1

3.4 Determination of the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal (MFC) concentration

One hundred and sixty extracts that showed inhibitory activities at 200 µg/ml were further determined for their MICs by the colorimetric broth dilution methods using resazurin as growth indicator, except for *T. marneffei* which had its own red pigment (Figure 14). The MIC values of extracts ranged from ≤0.03 to 200 µg/ml (Table 11). The MIC range of extracts (≤0.03-200 µg/ml) was recorded against *S. aureus* ATCC25923 and MRSA SK1, followed by *T. marneffei* PSU-SKH1 (0.5-200 µg/ml), *C. neoformans* ATCC90112 (1-200 µg/ml), *C. albicans* ATCC90028 (4-200 µg/ml), *A. baumannii* (8-200 µg/ml), and *E. coli* ATCC25922 and *M. gypseum* SH-MU4 (64-200 µg/ml).

The extracts exhibited MBCs and MFCs at 0.25->200 µg/ml and 1->200 µg/ml, respectively (Table 11). The lowest MBC/MFC value was shown against MRSA SK1 (0.25 µg/ml), followed by *S. aureus* ATCC25923 (0.5 µg/ml), *C. neoformans* ATCC90112 (1 µg/ml), *C. albicans* ATCC90028 and *T. marneffei* PSU-SKH1 (4 µg/ml), *E. coli* ATCC25922 (128 µg/ml) and *A. baumannii* NPRC001-NPRC 005, NPRC007 and *M. gypseum* SH-MU4 (>200 µg/ml).



a b c

Figure 14 Determination of MICs of actinomycete extracts by colorimetric microdilution methods, *S. aureus* ATCC25923 inhibited by extracts (blue color of wells indicated inhibition of growth (a), *C. neoformans* ATCC90112 inhibited by extracts and amphotericin B (purple color of wells indicated inhibition of growth) (b) and *T. marneffei* PSU-SKH1 inhibited by extracts (colorless of wells indicated no growth)(c)

Table 11 MIC, MBC and MFC ranges of extracts from marine-derived actinomycetes against each test strain

Test	Test microorganism												
	Bacteria									Yeast		Filamentous fungi	
	SA	MRSA	EC	AB001	AB002	AB003	AB004	AB005	AB007	CA	CN	TM	MG
Extracts MIC (µg/ml)	≤0.03 - 200	≤0.03 -200	64 - 200	8 - 200	64 - 200	32 - 200	32 - 200	64 - 200	64 - 200	4 - 200	1 - 200	0.5 - 200	64 - 200
MBC or MFC (µg/ml)	0.5 - >200	0.25->200	128 - >200	>200	>200	>200	>200	>200	>200	4 ->200	1 ->200	4 - >200	>200
Vancomycin (MIC/MBC) (µg/ml)	1/2	2/2											
Gentamicin (MIC/MBC) (µg/ml)			0.5/0.5										
Colistin (MIC/MBC) (µg/ml)				16/64	16/64	16/64	16/64	32/64	16/64				
Amphotericin B (MIC/MFC) (µg/ml)										0.5/0.5	0.5/0.5	1/2	
Miconazole (MIC/MFC) (µg/ml)													2/2

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 EC = *Escherichia coli* ATCC25922

AB = *Acinetobacter baumannii* NPR 001-005, 007 CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112

MG = *Microsporum gypseum* MU-SH4 TM = *Talaromyces marneffei* PSU-SKH1

In this study, the MIC values could be divided into 3 levels as strong activity (≤0.03-8 µg/ml), moderate activity (16-64 µg/ml) and weak activity (128-200 µg/ml). Most marine-derived actinomycete extracts exhibited weak activity, followed by moderate activity and strong activity.

Thirty-nine extracts from 19 isolates exhibited strong inhibitory activity (MIC ≤8 µg/ml). The highest number of extract showing strong activity was against *S. aureus* ATCC25923 (31 extracts), followed by MRSA SK1 (30 extracts), *T. marneffei* PSU-SKH1 (5 extracts), *C. neoformans* ATCC90112 (2 extracts), *A. baumannii* NPRC001 and *C. albicans* ATCC90028 (1 extract each) (Figure 15 and Table 12).

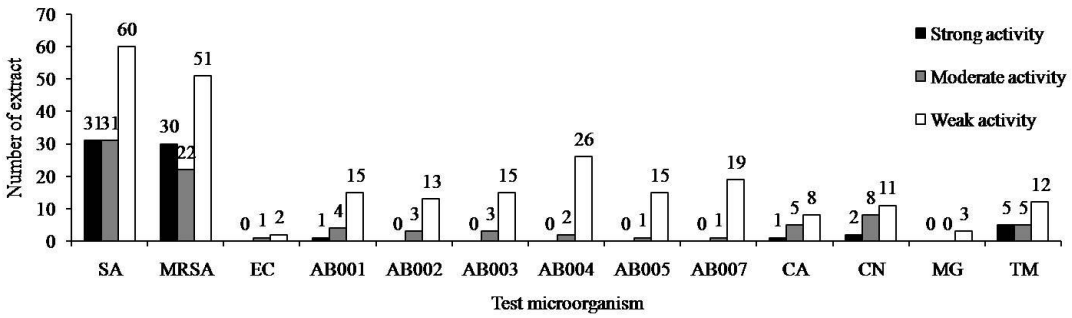


Figure 15 Number of active extracts against each test microorganism

SA = *Staphylococcus aureus* ATCC 25923 MRSA = methicillin-resistant *S. aureus* SK 1

EC = *Escherichia coli* ATCC 25922 AB = *Acinetobacter baumannii* NPRC 001-005, 007

CA = *Candida albicans* ATCC 90028 CN = *Cryptococcus neoformans* ATCC 90112

MG = *Microsporum gypseum* MU-SH 1 TM = *Talaromyces marneffei* PSU-SKH 1

Strong activity, MIC ≤ 8 µg/ml; moderate activty, MIC 16-64 µg/ml; weak activity, 128-200 µg/ml.

Antibacterial activity

Among 160 active extracts, 146 (91.3%) showed antibacterial activity inhibiting at least one test pathogen with MIC and MBC values of ≤0.03-200 and 0.25->200 µg/ml, respectively. Thirty-three extracts from 19 isolates showed strong inhibitory activity against either *S. aureus* or MRSA or both (MIC ≤8 µg/ml) and this was comparable to the antibacterial drug vancomycin (*S. aureus*: MIC/MBC = 1/2 µg/ml, MRSA: MIC/MBC = 2/2 µg/ml). Only one extract (AMA11CE) displayed strong inhibitory activity against a Gram-negative bacterium, *A. baumannii* NPRC001

(MIC 8 µg/ml) whereas the control drug colistin had MIC value of 16 µg/ml (Table 12). Moreover, AMA11CE exhibited the broadest activity against both Gram-positive bacteria, *S aureus* and MRSA (MIC 0.5 µg/ml) and Gram-negative bacteria, *A. baumannii* (MICs 8-64 µg/ml) and *E. coli*, (64 µg/ml).

Antifungal activity

Thirty four out of 160 active extracts (21.23%) showed antifungal activity with MIC/MFC values ranged from 0.5-200/1->200 µg/ml. Six extracts from three isolates (AMA50BE, AMA50CE, AMA50CH, AMA52CE, AMR71CE and AMR71CH) displayed strong activity (MIC ≤8 µg/ml) (Table 12). Among them, only AMR71CE inhibited *C. albicans* with MIC/MBC value of 4/4 µg/ml. Two extracts, AMA52CE and AMR71CE presented strong activity against *C. neoformans* with MIC/MFC values of 8/200 and 1/1 µg/ml, respectively. Five extracts exhibited strong activity against *T. marneffei* comprising AMA50BE (MIC/MFC 8/>200 µg/ml), AMA50CE (MIC/MFC 4/>200 µg/ml), AMA50CH (MIC/MFC 0.5/64 µg/ml), AMR71CE and CH; (MIC/MFC 4/4 µg/ml). AMA71CE had the broadest and antifungal activity against *C. albicans, C. neoformans* and *T. marneffei*.

Table 12 MIC, MBC and MFC values of marine-derived actinomycete extracts having strong antimicrobial activity (MIC ≤8 µg/ml)

Actinomycete code	Extract	MIC/MBC or MFC (µg/ml)												
		SA	**MRSA**	**EC**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
AMA11	BE	**2**/>200	**2**/64		64/>200	200/>200	200/>200	200/>200	200/>200	200/>200				
	CE	**0.5**/128	**0.5**/64	64/128	**8**/>200	64/>200	32/>200	32/>200	64/>200	64/>200				
AMA12	BE	**2**/>200	**1**/>200	200/>200	32/>200	200/>200	128/>200	128/>200	200/>200	200/>200				
	CE	**4**/>200	**4**/32		64/>200			200/>200						
AMA21	CE	**4**/>200	**4**/>200		32/>200	200/>200	200/>200	200/>200	200/>200	200/>200				
AMA44	BE	**8**/>200	**4**/16											
	CE	**4**/>200	**8**/64											
AMA50	BE													**8**/>200
	CE													**4**/>200
	CH	200/>200												**0.5**/64
AMA52	CE											**8**/200		
AMA74	BE	**4**/>200	**8**/>200											
	CE	**4**/>200	**8**/>200											
	CH	**4**/>200	16/>200											
ASE22	BE	**2**/>200	**4**/>200											
	CE	**4**/>200	16/>200											
	CH	**1**/>200	**4**/>200											
ALA166	BE	**0.06**/64	**0.06**/0.5		200/>200	200/>200	200/>200	200/>200	200/>200	200/>200				
	CE	**≤0.03**/64	**≤0.03**/0.25		200/>200	200/>200	200/>200	200/>200	200/>200	200/>200	128/>200	32/64		
	CH	**0.5**/>200	**1**/16		200/>200	200/>200	200/>200	200/>200	200/>200	200/>200				

Table 12 (Cont.) MIC, MBC and MFC values of marine-derived actinomycete extracts having strong antimicrobial activity (MIC ≤8 µg/ml)

Actinomycete code	Extract	MIC/MBC or MFC (µg/ml)												
		SA	**MRSA**	**EC**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
ALA167	BE	**0.25**/>200	**0.13**/>200											
	CE	**0.25**/>200	**0.06**/>200									128/128		
ALA175	CE	**4**/>200	**8**/128											
AMR9	BE	**2**/>200	**1**/>200		200/>200	200/>200	200/>200	200/>200	200/>200	200/>200	200/>200			
AMR67	BE	**0.5**/>200	**2**/>200									128/200		200/200
	CE	**0.06**/0.5	**0.5**/4								16/32	16/200	128/>200	128/128
	CH	**≤0.03**/>200	**≤0.03**/32											
AMR69	BE	**≤0.03**/200	**≤0.03**/0.5		200/>200	64/>200	64/>200	128/>200	200/>200	128/>200		64/200		200/>200
	CE	**0.13**/200	**0.06**/2		200/>200	200/>200	200/>200	128/>200	200/>200	200/>200				
	CH	**≤0.03**/>200	**≤0.03**/0.5		128/>200	64/>200	64/>200	32/>200	128/>200	128/>200		64/200		200/>200
AMR71	CE										**4**/4	**1**/1		**4**/4
	CH											32/64		**4**/4
AMR77	BE	**8**/>200	16/>200		200/>200	200/>200	200/>200	200/>200	200/>200	200/>200				
	CE	**2**/>200	**4**/>200		200/>200	200/>200	200/>200	200/>200	200/>200	200/>200				
AMR82	BE	16/64	**4**/8	200/>200	200/>200	200/>200	200/>200	200/>200	200/>200	200/>200	32/64	128/200		
AMR92	BE	16/>200	**8**/>200		200/>200	200/>200	200/>200	200/>200	200/>200	200/>200				
AMR107	BE	**1**/>200	**4**/>200								32/64			200/200
	CE	**0.5**/>200	**2**/128											
	CH	**0.25**/>200	**2**/4											

Table 12 (Cont.) MIC, MBC and MFC values of marine-derived actinomycete extracts having strong antimicrobial activity (MIC ≤8 µg/ml)

Actinomycete code	Extract	MIC/MBC or MFC (µg/ml)												
		SA	**MRSA**	**EC**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
Number of strong active extract		31	30		1						1	2		5
Vancomycin		1/2	1/2											
Gentamicin				0.5/0.5										
Colistin					16/64	16/64	16/64	16/64	32/64	16/64				
Amphotericin B											0.5/0.5	0.5/0.5		1/2
Miconazole													2/2	

BE = ethyl acetate extract from culture broth CE = ethyl acetate extract from cells CH = hexane extract from cells SA = *Staphylococcus aureus* ATCC25923

MRSA = methicillin-resistant *S. aureus* SK1 EC = *Escherichia coli* ATCC25922 AB = *Acinetobacter baumannii* NPRC001-005,007

CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 MG = *Microsporum gypseum* MU-SH4

TM = *Talaromyces marneffei* PSU-SKH1 AMA = actinomycetes from mangrove sediment ALA = actinomycetes from Songkhla lake

ASE = actinomycetes from coastal sediment AMR = actinomycetes from marine organisms

3.5 Antimicrobial effect of the active extracts on their targeted cells

The effect of the most active extracts (MIC ≤ 0.03 µg/ml) against MRSA was investigated by SEM (Figure 16). MRSA SK1 cells treated with marine-derived actinomycete extracts were compared with 1% DMSO and vancomycin. DMSO treated cells had smooth and spherical morphology (Figure 16a). In contrast, bacterial cells exposed to 4×MIC (4 µg/ml) of vancomycin, 2 µg/ml of AMA11CE and 0.12 µg/ml of ALA166CE, AMR67CH, AMR69BE and AMR69CH showed drastic morphological alterations such as pores on the cell surface, cytoplasm protusion, cell surface collapse and broken cells (Figures 16b-g).

Figure 17 shows the scanning electron micrographs of *C. albicans* ATCC90028 and *C. neoformans* ATCC90112. The normal cells are presented in Figures 17a and d. Cells treated with amphotericin B (Figures 17b and e) were completely deformed and collapsed as well as cells treated with AMR71CE (Figures 17c and f).

In addition, *T. marneffei* PSU-SKH1 were also treated with AMR71CE. The morphological changes such as pores, surface wrinkle and flattened mycelia were observed in the sample treated with amphotericin B and AMR71CE as compared with the control healthy mycelia (Figures 18e-f).

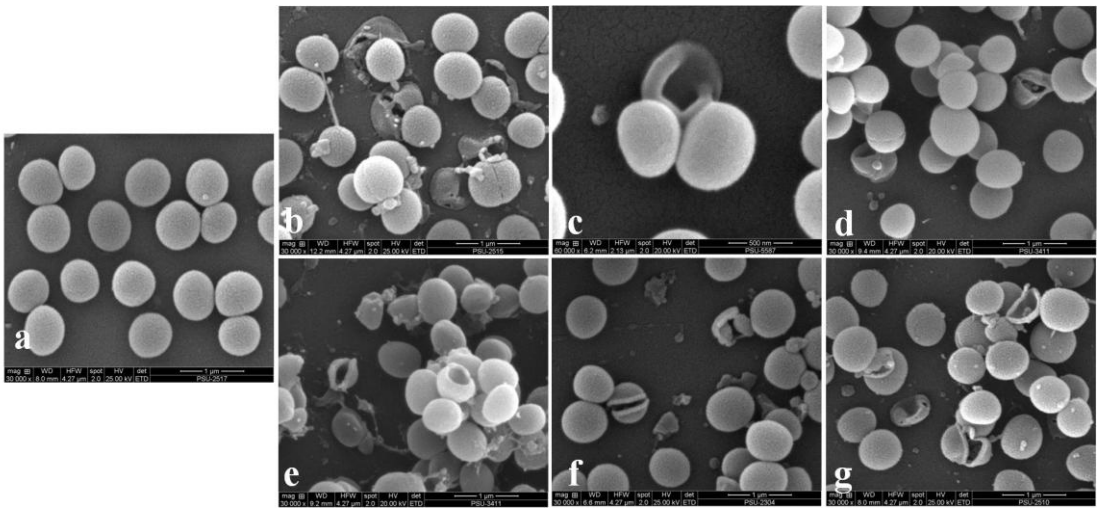


Figure 16 Scanning electron micrographs of methicillin-resistant *Staphylococcus aureus* SK1 after treated with 1% DMSO (a), 4 µg/ml (4×MIC) vancomycin (b), 2 µg/ml (4×MIC) AMA11CE (c: this figure was published in Sangkanu *et al*., 2017), 0.12 µg/ml (4×MIC) ALA166CE (d), AMR67CH (e), AMR69BE (f) and AMR69CH (g) at 25^{o}C for 24 h.

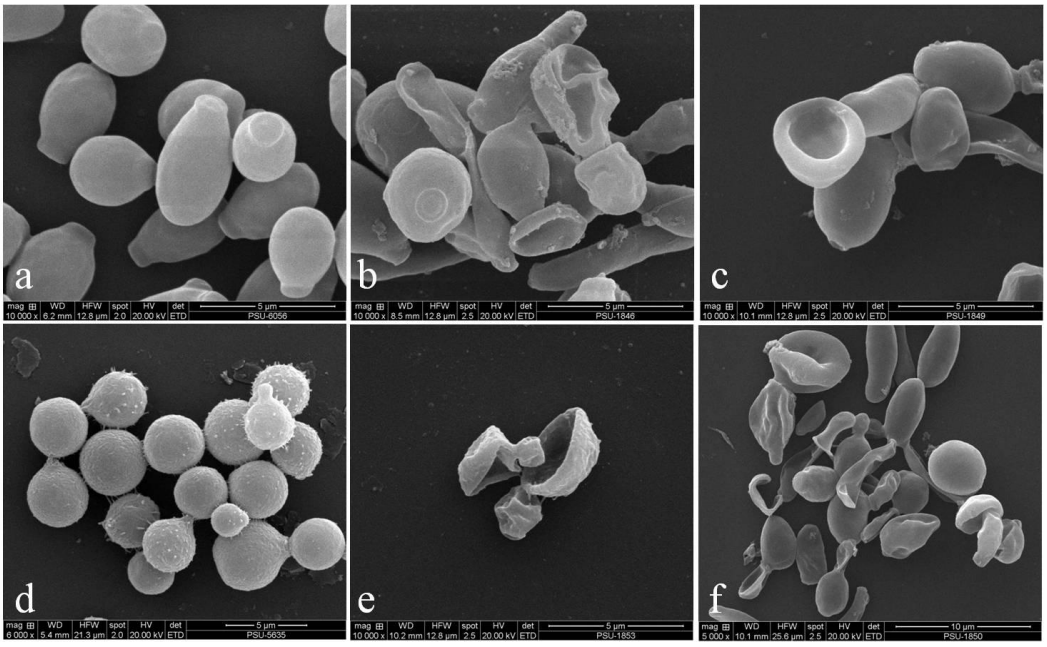


Figure 17 Scanning electron micrographs of *C. albicans* ATCC90028 (a-c) and *C. neoformans* ATCC90112 (d-f). Normal control cells after treated with 1% DMSO (a and d), cells treated with 4 µg/ml (4×MIC) of amphotericin B (b and e) and cells treated with 16 and 4 µg/ml (4×MIC) of AMR71CE (c and f), respectively.

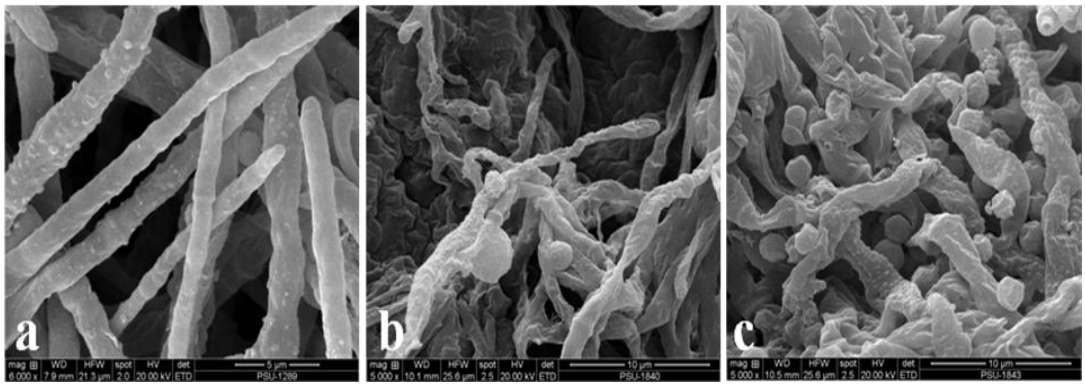
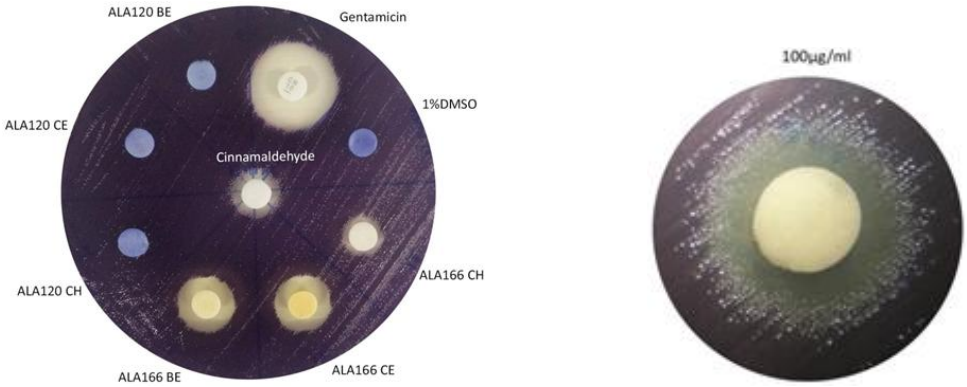


Figure 18 Scanning electron micrographs of *T. marneffei* PSU-SKH1 after treated with 1% DMSO (a), 4 µg/ml (4×MIC) of amphotericin B (b) and 16 µg/ml (4×MIC) of AMR71CE (c).

3.6 Anti-quorum sensing potential of crude marine-derived actinomycete extracts by a disk diffusion method

C. violaceum produces the purple pigment, violacein, as a result of QS. Loss of purple pigment in *C. violaceum* around the impregnated disks was indicative of QS inhibition by the extracts from marine-derived actinomycetes (Figure 19 and Table 13). Antimicrobial activity is indicated by the inner clear zone. Only nine out of 287 extracts (3.1%) presented the results of quorum sensing inhibition at 100 µg/disk while eleven extracts inhibited the growth of *C. violaceum*. Two extracts showed both antibacterial and anti-quorum sensing activities similar to cinnamaldehyde (64 µg/disk), a positive control of QS inhibitor. Gentamicin (10 µg/disk) was used as antimicrobial control drug and only growth inhibition zone was observed. Neither growth inhibition zone nor violacein production inhibition zone was observed around the 1%DMSO solvent control.



a b

Figure 19 Anti-quorum sensing assays of marine-derived actinomycete extracts against *C. violaceum* DMST21761 by a disk diffusion method. Test plate (a) and enlargment of colorless violacein production inhibition zone by AMR82BE (b).

Table 13 Zones of quorum sensing inhibition and antimicrobial activity (mm) of marine-derived actinomycete extracts at 100 µg/disk by *Chrobacterium violaceum* disk assay

Extract	Inhibition zone (mm)	
	Inhibition of violacein production	**Inhibition of growth**
AMA11BE	7.70	-
AMA11CE	2.25	8.35
AMA12BE	4.00	6.95
AMA12CE	10.25	-
AMA21CE	9.60	-
AMA22BE	8.60	-
AMA54BE	8.00	-
ALA65BE	1.75	-
ALA166BE	-	12.25
ALA166CE	-	11.75
ALA166CH	-	7.75
ALA167BE	-	11.25
ALA167CE	-	9.50
ALA174BE	-	7.00
AMR69BE	-	15.25
AMR69CE	-	13.00
AMR69CH	-	14.25
AMR82BE	2.25	-
DMSO (1%)	-	-
Cinnamaldehyde (64 µg/disk)	1.21	9.00
Gentamicin (10 ug/disk)	-	15.08

AMA = actinomycetes from mangrove sediment ALA = actinomycetes from Songkhla lake
ASE = actinomycetes from coastal sediment AMR = actinomycetes from marine organisms
BE = ethyl acetate extract from culture broth CE = ethyl acetate extract from cells
CH = hexane extract from cells

3.7 Bacteriolytic activity

Ethyl acetate extract from cells of AMA11 (AMA11CE) was tested for its bacteriolytic activity on MRSA. The results are shown in Figure 20. At all concentrations tested (MIC to 8×MICs), AMA11CE showed no bacteriolytic effect on MRSA within 24 h (% relative absorbance at OD_{620} range from 79 to 82%) as well as 1% DMSO, the control solvent.

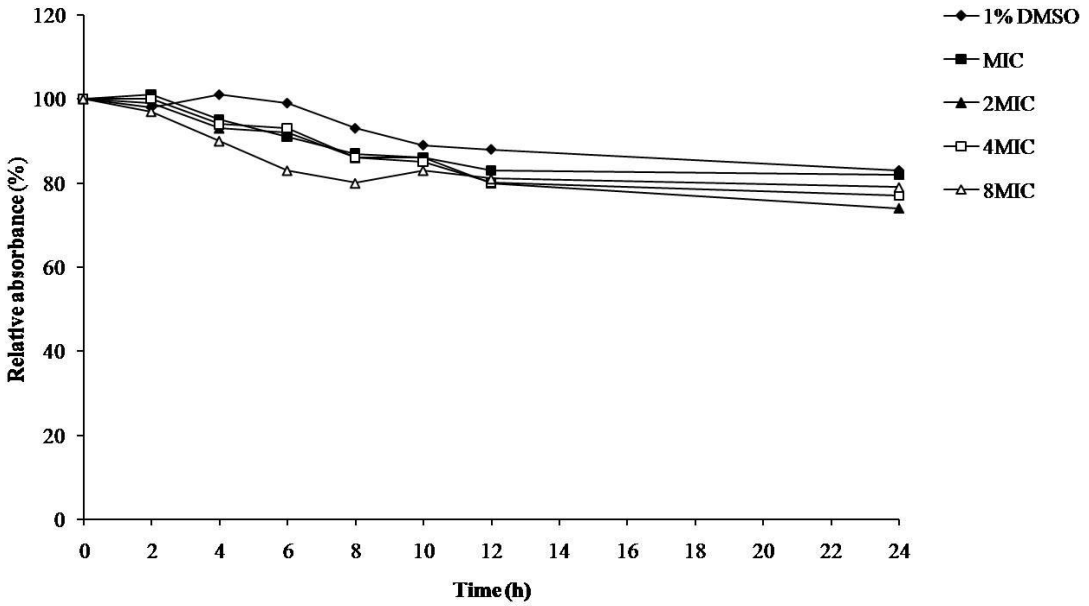


Figure 20 Bacteriolytic activity of ethyl acetate extract from cells of AMA11 against methicillin-resistant *Staphylococcus aureus* SK1. The results at MIC, 2×MIC, 4×MIC, 8×MIC, and 1% DMSO were expressed in percent as the ratio of OD_{620} at each time interval versus OD_{620} at 0 min (% relative absorbance). (This result was published in Sangkanu *et al.*, 2017).

3.8 Anti-biofilm activity

The inhibitory effect of five actinomycete extracts on biofilm formation of *S. epidermidis* ATCC35984 was determined at sub-MIC concentrations (0.125, 0.25 and 0.5 times its MIC). Actinomycete extracts were effective in reducing the biofilm formation while vancomycin had no effect at all tested concentrations as shown in Figure 21. The AMA11BE, AMA11CE and AMA12BE at 0.5×MIC clearly inhibited biofilm formation over 50% and these concentrations did not affect the growth of *S. epidermidis*.

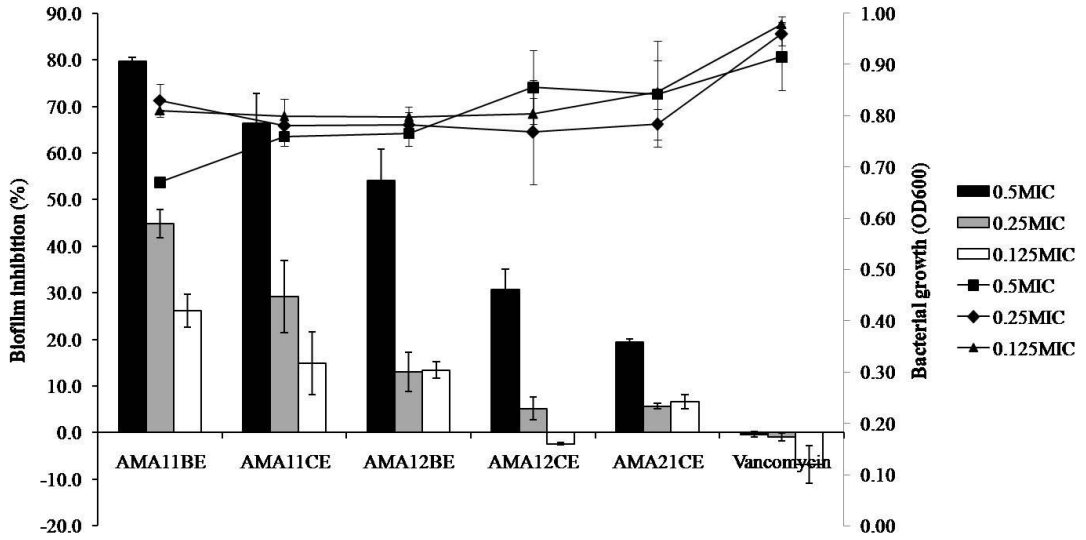


Figure 21 Effects of actinomycete extracts and vancomycin on biofilm formation of *Staphylococcus epidermidis* ATCC35984. The growth of bacterial cell present in biofilm (connected dot, right axis) was compared with the biofilm inhibition (bar, left axis). The data represent the mean ± standard deviation (SD) of triplicate experiments. (This result was published in Sangkanu *et al.*, 2017).

3.9 Effect of actinomycete extracts on established biofilms

Five extrtacts exhibiting anti-biofilm formation were further tested against the 24-h established biofilm. After 24 h of incubation of the preformed biofilm and extracts, 49.5%, 59.6%, 65.8% and 71.5% survival of *S. epidermidis* in biofilms were recorded at 8×MIC of AMA11BE, AMA12BE, AMA12CE and AMA11CE, respectively while 90% viability was obtained from AMA21CE and vancomycin (Figure 22).

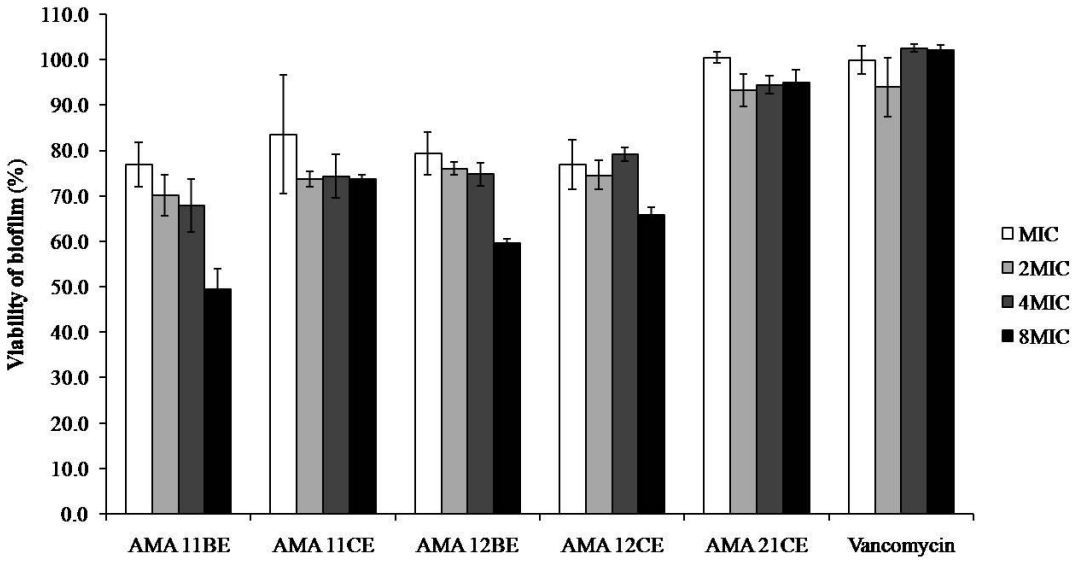


Figure 22 Effects of actinomycete extracts and vancomycin on *Staphylococcus epidermidis* ATCC35984 in biofilms viability for 24 h. The data represent the mean±standard deviation (SD) of triplicate experiments. (This result was published in Sangkanu *et al.*, 2017).

3.10 *C. elegans* virulence model for *T. marneffei*

Hexane extract from cells of AMA50 (AMA50CH) was the most potential extract that inhibited *T. marneffei* PSU-SKH1 with the lowest MIC value of 0.5

µg/ml. Therefore, its *in vivo* toxicity and its protective effect in *T. marneffei* infection were further studied using *C. elegans* model.

3.10.1 Toxicity test

Various concentrations of AMA50CH (MIC to 8×MIC) were injected into *C. elegans* and its viability was observed daily for 7 days. Results are shown in Figure 23. At MIC concentration, AMA50CH did not show toxicity against *C. elegans* as well as 1% DMSO (untreated group). However, at 2 to 8×MIC, the nematode survial was decreased to 60-70% at 7 days post injection. No toxicity was seen in all concentrations of amphotericin B up to 8×MIC (data not shown).

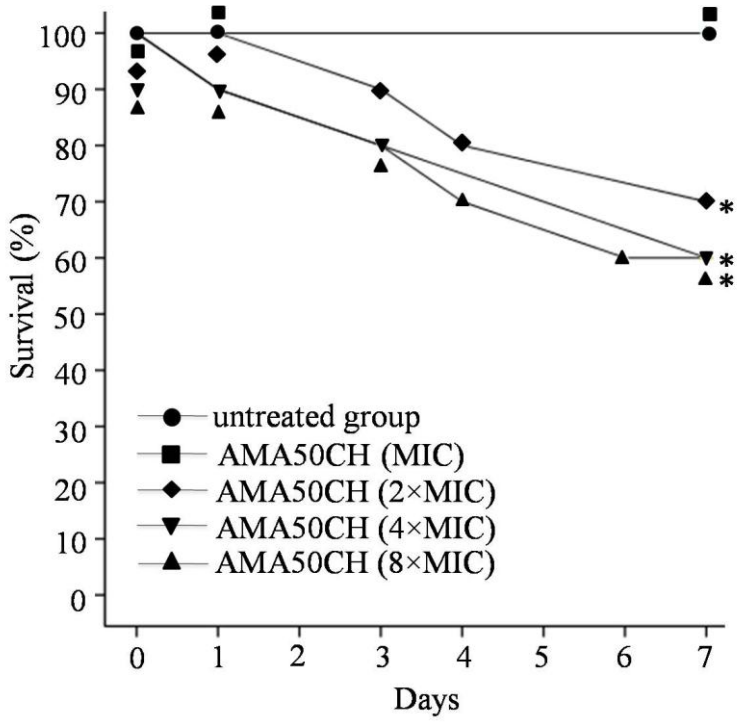


Figure 23 Survival curves of *Caenorhabditis elegans* in pathogens-free medium after treated with various concentrations of hexane extract from cells of AMA50 (AMA50CH). *Significantly difference compared with the control (p<0.05)

3.10.2 *C. elegans* virulence model

Four strains of *T. marneffei* were determined for their virulence in *C. elegans*. Results are shown in Figure 24. At 10^5 spores/ml, the CI-2 strain was the most virulent strain. As shown in Figure 24a, the nematodes infected with CI-2 began to die at day 2 after infection, and were completely killed at day 4 after infection whereas 50% of the nematodes infected with CI-5, CI-7 and PSU-SKH1 still survived at day 4. In addition, *C. elegans* infected with CI-2 were fully covered with hyphae that had penetrated through the nematodes. In CI-7 infected worm, red pigment was found throughout the entire body of the dead nematode. No red pigment was seen in *C. elegans* infected with PSU-SKH1 and CI-5 (Figure 24b).

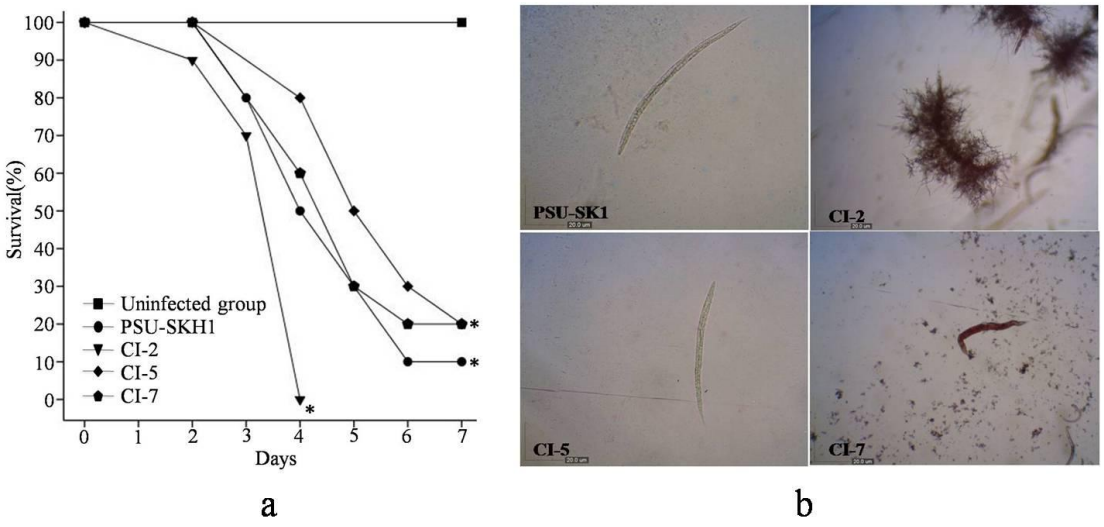


Figure 24 Virulence assessment of four strains of *Talaromyces marneffei* in *Caenorhabditis elegans* infection model. Survival curves of nematodes after infections with various strains of *T. marneffei* in liquid medium assay (a). Significant differences were found between the tested groups and the uninfected control (*p<0.05). Light microscope images of *C. elegans* at day 4 after infected with *T. marneffei* strains PSU-SKH1, CI-2, CI-5 and CI-7 (b). No obvious red pigment or hyphae was seen in the *C. elegans* intestine after infected with PSU-SKH1 and CI-5. Hyphal protrusion from the nematodes after infected with CI-2 and red pigment production in the nematode intestine after infected with CI-7 were clearly seen.

3.10.3 Protective effect of actinomycete extract in *T. marneffei*-infected nematodes

T. marneffei CI-2 was used to study the protective effect of actinomycete extract in menatodes since it was the most virulent strain. AMA50CH inhibited *T. marneffei* CI-2 with MIC value of 1 µg/ml while MIC of amphotericin B was 0.5 µg/ml (data not shown). The protective effects of AMA50CH and amphotericin B at MIC to 8×MIC were studied. Results are shown in Figure 25. Percent survival of *C. elegans* in the treatment groups with AMA50CH at all tested concentrations (60-70% at day 7) were significantly higher than that in the infection control group (0% at day 5) (Figure 25a). Amphotericin B at MIC concentration could only increase *C. elegans* survival to 30% after 7 days of treatment. However, amphotericin B at 2, 4 and 8×MIC prolonged *C. elegans* survival to 70-80% (Figure 25b).

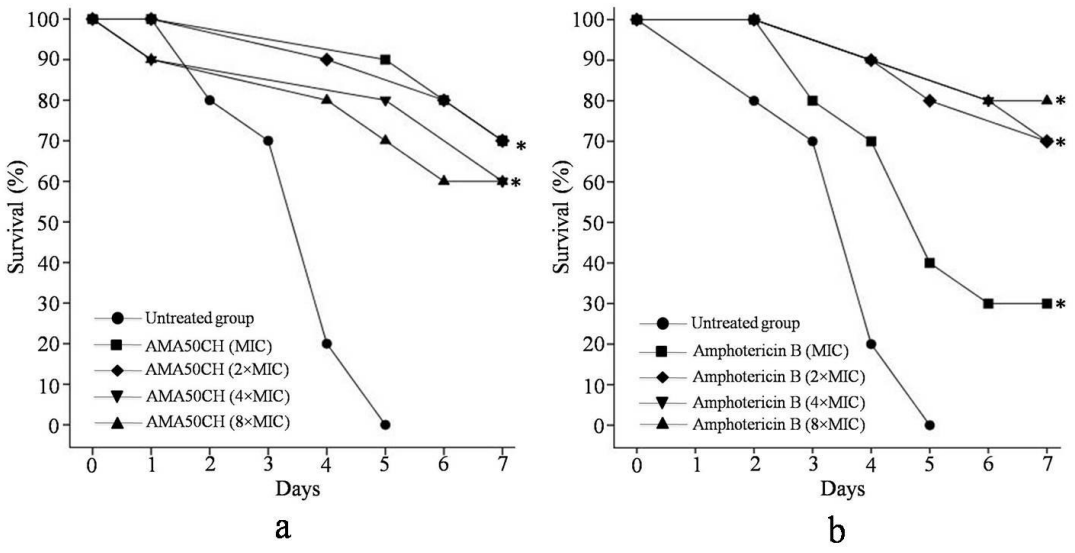


Figure 25 Survival curves of *Caenorhabditis elegans* infected with *Talaromyces marneffei* CI-2 for 24 h before treated with various concentrations of hexane extract of cells from AMA50 (AMA50CH) (a) and amphotericin B (b). Significant differences were compared with the untreated control group (*p<0.05).

3.11 Partial purification and identification of active extract

3.11.1 AMA11CE

AMA11CE that showed a broad spectrum of activity against all the tested bacterial strains was selected for partial purification. The large scale fermentation of AMA11 was cultured and extracted. A new lot of AMA11CE was confirmed to have antibacterial activity with MIC/MBC values of 2/16 and 128/128 µg/ml against MRSA and *C. violaceum*, respectively. AMA11CE was subjected to silica gel column chromatography to obtain nine fractions (appendix). Fraction 7 was found to possess the highest inhibitory activity against MRSA and *C. violaceum* with MIC values of 0.25 and 4 µg/ml, repectively. Fractions 5 and 6 showed moderate activity towards MRSA (MIC 64 and 32 µg/ml), whereas fractions 1-4 and 9 were inactive (Table 14).

Fractions 5, 6 and 7 were analyzed by GC-MS. Results are shown in Table 15. Fraction 5 contained hexadecanoic acid (56.56%) as a major component and pentadecanoic acid (5.89%) as a minor constituent. Fractions 6 and 7 consisted of 3-nitro-1,2-benzenedicarboxylic acid (14.31% and 28.86%, respectively) and hexadecanoic acid (10.86% and 8.65%) as the major compounds. The minor components were pentadecanoic acid (2.09% and 1.61%) and quinoxaline-2-carboximide (0.74% and 5.84%). Antimicrobial activity of standard hexadecanoic acid and pentadecanoic acid were also tested against MRSA and *C. violaceum*. They showed no activity at 200 µg/ml (Table 14).

Table 14 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of standard compounds, AMA11CE extract and its fractions

Fraction/ standard compound	**MIC/MBC values (µg/ml)**	
	MRSA	**CV**
Hexadecanoic acid	-	-
Pentadecanoic acid	-	-
Extract	2/16	128/128
Fraction 1	-	-
Fraction 2	-	-
Fraction 3	-	-
Fraction 4	-	-
Fraction 5	64/128	-
Fraction 6	32/128	-
Fraction 7	0.25/16	4/8
Fraction 8	128/>128	-
Fraction 9	-	-
Vancomycin	0.5/1	ND
Gentamicin	ND	1/1

ND = not detected MRSA = methicillin-resistant *Staphylococcus aureus* SK1

CV = *Chromobacterium violaceum* DMST21761

(This result was published in Sangkanu *et al*., 2017)

Table 15 GC-MS analysis of the active fractions from the AMA11CE. The highest percentages of peak areas are printed in bold

Compound name	**Peak area (%)**		
	Fraction 5	**Fraction 6**	**Fraction 7**
Pentadecanoic acid	5.89	2.09	1.61
Hexadecanoic acid	**56.56**	10.86	8.65
3-Nitro-1,2-benzenedicarboxylic acid	-	**14.31**	**28.86**
Quinoxaline-2-carboxamine	-	0.74	4.84

(This result was published in Sangkanu *et al*., 2017)

3.11.2 AMA50CH

The chemical composition of the active extract AMA50CH was also investigated. Seven compounds were identified as its major components comprising n-hexadecanoic acid (26.1%), tetradecanoic acid (13.4%), pentadecanoic acid (9.0%), heptadecenoic acid (2.5%), palmitoleic acid (2.4%), hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl) ethyl ester (1.6%) and hexadecanoic acid, methyl ester (1.5%) (Table 16).

Table 16 Major components of the hexane extract from cells of AMA50 (AMA50CH) identified by gas chromatography-mass spectrometry (GC-MS) analysis

No.	RT (min)	Name of the compound	Mol. formula	Peak area (%)
1.	10.2686	Tetradecanoic acid	$C_{14}H_{28}O_2$	13.4
2.	13.5314	Pentadecanoic acid	$C_{15}H_{30}O_2$	9.0
3.	15.1217	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	1.5
4.	15.9807	Palmitoleic acid	$C_{16}H_{30}O_2$	2.4
5.	16.9187	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	26.1
6.	20.6115	Heptadecenoic acid	$C_{17}H_{32}O_2$	2.5
7.	37.1906	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	1.6

RT, retention time

3.12 Identification of marine-derived actinomycetes

Sixty three isolates of active marine-derived actinomycetes were selected for phylogenetic analysis based on their morphologies and antimicrobial activities. Their complete 16S rRNA gene sequences were obtained. EzBioCloud nucleotide BLAST searches revealed that all isolates were Gram-positive bacteria and shared 97.06-100% similarity with the most closest members of the class *Actinobacteria*. They consisted of 6 clusters comprising orders *Streptomycetales* (*Streptomyces* 35 isolates and Uncertain species 2 isolates), *Corynebacteriales* (*Gordonia* 4 isolates, *Nocardia* 3 isolates and *Mycobacterium* 1 isolate), *Pseudonocardiales* (*Pseudonocardia* 4 isolates), *Streptosporangiales* (*Actinomadura* 4 isolates), *Jiangellales* (*Jiangella* 1 isolate) and *Micromonosporales* (*Micromonospora* 9 isolates), encompassing 9 genera of the class *Actinobacteria* (Table 17). Based on phylogenetic result (data not shown) and morphological characteristics, 16 isolates were confirmly identified as

known species and 47 isolates as unknown species (Table 17 and Table 22 in appendix).

Nineteen active isolates of marine-derived actinomycetes were classified into the genera *Streptomyces* (16 isolates), Uncertain species (2 isolates) and *Actinomadura* (1 isolate). The generated tree of active isolates presented in Figure 26a-c. Their morphological characteristics, antimicrobial activities, GenBank accession numbers and BCC/TBRC accession numbers are described in Table 18

Figure 26a comprised nine active isolates. Strain AMR92 formed a phyletic line with *S. griseorubens* NBRC 12780^T. They produced white-gray or gray color spores borne in chains forming spiral (S) or open loop (RA). Strain AMR77 produced S or RA chains on white-gray spore mass that showed the same morphology of closely related type strain *S. spongiicola* HNM0071^T. Strains AMR71, AMA50, AMA52 and AMA74 produced the matured brown color spores formed in S chains while the type strains *S. misionensis* DSM 40306^T and *S. phaeoluteichromatogenes* NRRL 5799^T produced gray spore borne in chains forming S and flexuous (RF), respectively. Strain AMA44 produced white color spore mass in RA and S chains that differentiated from the type strain, *S. aurantiogriseus* NBRC 12842^T produced yellow color spore mass in S chains. Strain AMR9 produced green aerial spore mass that differentiated from type strains, *S. naganishii* NBRC 12892^T and *S. spiralis* NBRC 14215^T. All of these strains formed S chains. Strain AMA21 produced gray color spore mass with RA chains and it formed a distinct lineage among the most closely type strains *S. gramineus* JR-43^T, *S. griseoluteus* NBRC 13375^T, *S. recifensis* NBRC 12813^T and *S. seoulensis* NRRL B-24310^T.

Figure 26b comprised 9 active isolates. Strains ALA166 and ALA167 formed RA chains on white color spore while *S. bungoensis* DSM41781^T produced gray color spore borne in chains forming S. Type strains *S. antibioticus* NBRC12838^T and AMR69 produced gray color spore borne in S chains. Strains AMA11 and AMA12 were grouped with the type strain of the *Kitasatospora psammotica* NBRC13971^T. All strains produced gray or green color spore mass borne in S chains. Strain ALA175 and type strains produced S chains on white-gray or gray color spore mass. Strain AMR82 and closely related strain produced white or gray color spore mass borne in flexuous (RF) chains. Gray color spore mass with S chains was found in strain

AMR107 and *S. malaysienis* NBRC 16446^{T}. Strain AMR67 grouped with *S. javensis* NBRC 100777T, *S. violaceusniger* NBRC 13459^{T} and *S. yogyakartensis* NBRC 100779^{T}. All type strains produced yellow aerial spore mass and formed S chains while strain AMR67 formed RF chains on gray spore.

Figure 26c showed a neighbor-joining tree of strain ASE22 that belonged to the genus *Actinomadura*. Strain ASE22 and closely related type strain *A. geliboluensis* A8036^{T} produced white color spore mass with hook spore chains.

Table 17 BLAST results of 63 isolates performed on the EzBioCloud nucleotide BLAST searches.

Actinomycete	Hit taxon name	Hit strain name	% Similarity	Diff/Total nt	Note
AMA53	*Actinomadura maheshkhaliensis*	13-12-50 ^{T}	99.86	2/1421	Known species
AMA125	*Actinomadura maheshkhaliensis*	13-12-50 ^{T}	99.93	1/1421	Known species
ASE12	*Actinomadura geliboluensis*	A 8036 ^{T}	100.00	0/1440	Known species
ASE22	*Actinomadura geliboluensis*	A 8036 ^{T}	100.00	0/1440	Known species
AMA47	*Gordonia alkanivorans*	NBRC 16433 ^{T}	98.61	20/1440	Unknown species
AMA120	*Gordonia rhizosphera*	NBRC 16068 ^{T}	98.96	15/1440	Unknown species
AMR3	*Gordonia sputi*	NBRC 100414 ^{T}	99.79	3/1405	Unknown species
AMR98	*Gordonia rhizosphera*	NBRC 16068 ^{T}	99.86	2/1442	Known species
AMA11	*Kitasatospora psammotica*	NBRC 13971 ^{T}	99.49	7/1386	Unknown species
AMA12	*Kitasatospora psammotica*	NBRC 13971 ^{T}	99.30	10/1437	Unknown species
AMA2	*Micromonospora auratinigra*	DSM 44815 ^{T}	99.72	4/1436	Known species
AMA8	*Micromonospora maritima*	D10-9-5 ^{T}	99.70	4/1351	Unknown species
AMA141	*Micromonospora zhanjiangensis*	2902at01 ^{T}	99.72	4/1433	Unknown species
AMR21	*Micromonospora chalcea*	DSM 43026 ^{T}	99.72	4/1435	Unknown species
AMR101	*Micromonospora tulbaghiae*	DSM 45142 ^{T}	99.44	8/1437	Unknown species
AMR165	*Micromonospora tulbaghiae*	DSM 45142 ^{T}	99.37	9/1434	Unknown species

Table 17 (Cont.) BLAST results of 63 isolates performed on the EzBioCloud nucleotide BLAST searches.

Actinomycete	Hit taxon name	Hit strain name	% Similarity	Diff/Total nt	Note
ASE10	*Jiangella alba*	DSM 45237 ^{T}	99.92	1/1281	Known species
AMA11	*Kitasatospora psammotica*	NBRC 13971^{T}	99.49	7/1386	Unknown species
AMA12	*Kitasatospora psammotica*	NBRC 13971 ^{T}	99.30	10/1437	Unknown species
AMA2	*Micromonospora auratinigra*	DSM 44815^{T}	99.72	4/1436	Known species
AMA8	*Micromonospora maritima*	D10-9-5^{T}	99.70	4/1351	Unknown species
AMA141	*Micromonospora zhanjiangensis*	2902at01 ^{T}	99.72	4/1433	Unknown species
AMR21	*Micromonospora chalcea*	DSM 43026 ^{T}	99.72	4/1435	Unknown species
AMR101	*Micromonospora tulbaghiae*	DSM 45142 ^{T}	99.44	8/1437	Unknown species
AMR165	*Micromonospora tulbaghiae*	DSM 45142 ^{T}	99.37	9/1434	Unknown species
ALA1	*Micromonospora chalcea*	DSM 43026 ^{T}	98.54	21/1434	Unknown species
ALA14	*Micromonospora endophytica*	DCWR9-8-2 ^{T}	99.65	5/1413	Known species
ALA147	*Micromonospora inositola*	DSM 43819 ^{T}	99.22	11/1413	Unknown species
AMR130	*Mycobacterium florentinum*	DSM 44852 ^{T}	98.09	26/1364	Unknown species
AMA36	*Nocardia nova*	NBRC 15556 ^{T}	99.86	2/1439	Known species
AMA60	*Nocardia nova*	NBRC 15556 ^{T}	99.10	13/1439	Unknown species

Table 17 (Cont.) BLAST results of 63 isolates performed on the EzBioCloud nucleotide BLAST searches.

Actinomycete	Hit taxon name	Hit strain name	% Similarity	Diff/Total nt	Note
AMR47	*Nocardia amamiensis*	TT 00-78 ^{T}	99.65	5/1441	Unknown species
AMA3	*Pseudonocardia oroxyli*	D10^{T}	98.80	17/1413	Unknown species
ALA88	*Pseudonocardia antitumoralis*	SCSIO 01299 ^{T}	99.86	2/1394	Known species
ALA168	*Pseudonocardia carboxydivorans*	Y8 ^{T}	99.93	1/1416	Known species
ASE19	*Pseudonocardia nantongensis*	KLBMP 1282 ^{T}	97.06	42/1431	Unknown species
AMA13	*Streptomyces phaeoluteichromatogenes*	NRRL 5799 ^{T}	99.58	6/1420	Unknown species
AMA21	*Streptomyces violaceorubidus*	LMG 20319 ^{T}	98.61	20/1440	Unknown species
AMA22	*Streptomyces coelicoflavus*	NBRC 15399 ^{T}	100.00	0/1450	Known species
AMA26	*Streptomyces laurentii*	ATCC 31255 ^{T}	99.24	11/1440	Unknown species
AMA30	*Streptomyces phaeoluteichromatogenes*	NRRL 5799 ^{T}	99.58	6/1427	Unknown species
AMA44	*Streptomyces levis*	NBRC 15423 ^{T}	99.09	13/1425	Unknown species
AMA48	*Streptomyces catenulae*	NRRL B-2342 ^{T}	99.59	6/1448	Unknown species
AMA49	*Streptomyces phaeoluteichromatogenes*	NRRL 5799 ^{T}	99.49	7/1385	Unknown species
AMA50	*Streptomyces phaeoluteichromatogenes*	NRRL 5799 ^{T}	98.70	18/1385	Unknown species
AMA52	*Streptomyces phaeoluteichromatogenes*	NRRL 5799 ^{T}	99.51	7/1428	Unknown species
AMA54	*Streptomyces hyaluromycini*	NBRC 110483 ^{T}	98.62	15/1085	Unknown species

Table 17 (Cont.) BLAST results of 63 isolates performed on the EzBioCloud nucleotide BLAST searches.

Actinomycete	Hit taxon name	Hit strain name	% Similarity	Diff/Total nt	Note
AMA59	*Streptomyces coacervatus*	AS-0823 ^{T}	99.36	9/1412	Unknown species
AMA64	*Streptomyces glomeratus*	LMG 19903 ^{T}	99.10	13/1444	Unknown species
AMA66	*Streptomyces coacervatus*	AS-0823 ^{T}	99.17	12/1439	Unknown species
AMA74	*Streptomyces misionensis*	DSM 40306 ^{T}	99.33	8/1199	Unknown species
AMR1	*Streptomyces olivaceus*	NRRL B-3009 ^{T}	99.93	1/1448	Known species
AMR2	*Streptomyces wuyuanensis*	CGMCC 4.7042 ^{T}	99.79	3/1438	Unknown species
AMR6	*Streptomyces wuyuanensis*	CGMCC 4.7042 ^{T}	99.57	6/1397	Unknown species
AMR8	*Streptomyces pseudovenezuelae*	DSM 40212 ^{T}	99.38	9/1448	Unknown species
AMR9	*Streptomyces coeruleofuscus*	NBRC 12757 ^{T}	99.45	8/1443	Unknown species
AMR67	*Streptomyces yogyakartensis*	NBRC 100779 ^{T}	99.93	1/1448	Unknown species
AMR69	*Streptomyces antibioticus*	NBRC 12838 ^{T}	99.75	3/1194	Unknown species
AMR82	*Streptomyces pluripotens*	MUSC 135 ^{T}	99.86	2/1440	Known species
AMR92	*Streptomyces griseorubens*	NBRC 12780 ^{T}	99.65	5/1445	Known species
AMR71	*Streptomyces phaeoluteichromatogenes*	NRRL 5799 ^{T}	99.44	8/1428	Unknown species
AMR77	*Streptomyces spongiicola*	HNM0071 ^{T}	99.49	7/1369	Unknown species
AMR107	*Streptomyces malaysiensis*	NBRC 16446 ^{T}	100.00	0/1441	Known species

Table 17 (Cont.) BLAST results of 63 isolates performed on the EzBioCloud nucleotide BLAST searches.

Actinomycete	Hit taxon name	Hit strain name	% Similarity	Diff/Total nt	Note
ALA166	*Streptomyces bungoensis*	DSM 41781 ^{T}	99.58	6/1442	Unknown species
ALA167	*Streptomyces bungoensis*	DSM 41781 ^{T}	99.76	3/1237	Unknown species
ALA175	*Streptomyces thermoviolaceus subsp. thermoviolaceus*	DSM 40443 ^{T}	98.96	15/1440	Unknown species
ALA181	*Streptomyces glomeratus*	LMG 19903 ^{T}	98.26	25/1433	Unknown species
ASE1	*Streptomyces spongiicola*	HNM 0071 ^{T}	99.49	7/1369	Unknown species
ASE2	*Streptomyces spongiicola*	HNM 0071 ^{T}	99.27	10/1369	Unknown species
ASE4	*Streptomyces prasinopilosus*	NRRL B-2711 ^{T}	98.00	29/1448	Unknown species
ASE5	*Streptomyces specialis*	GW41-1564 ^{T}	98.55	21/1452	Unknown species

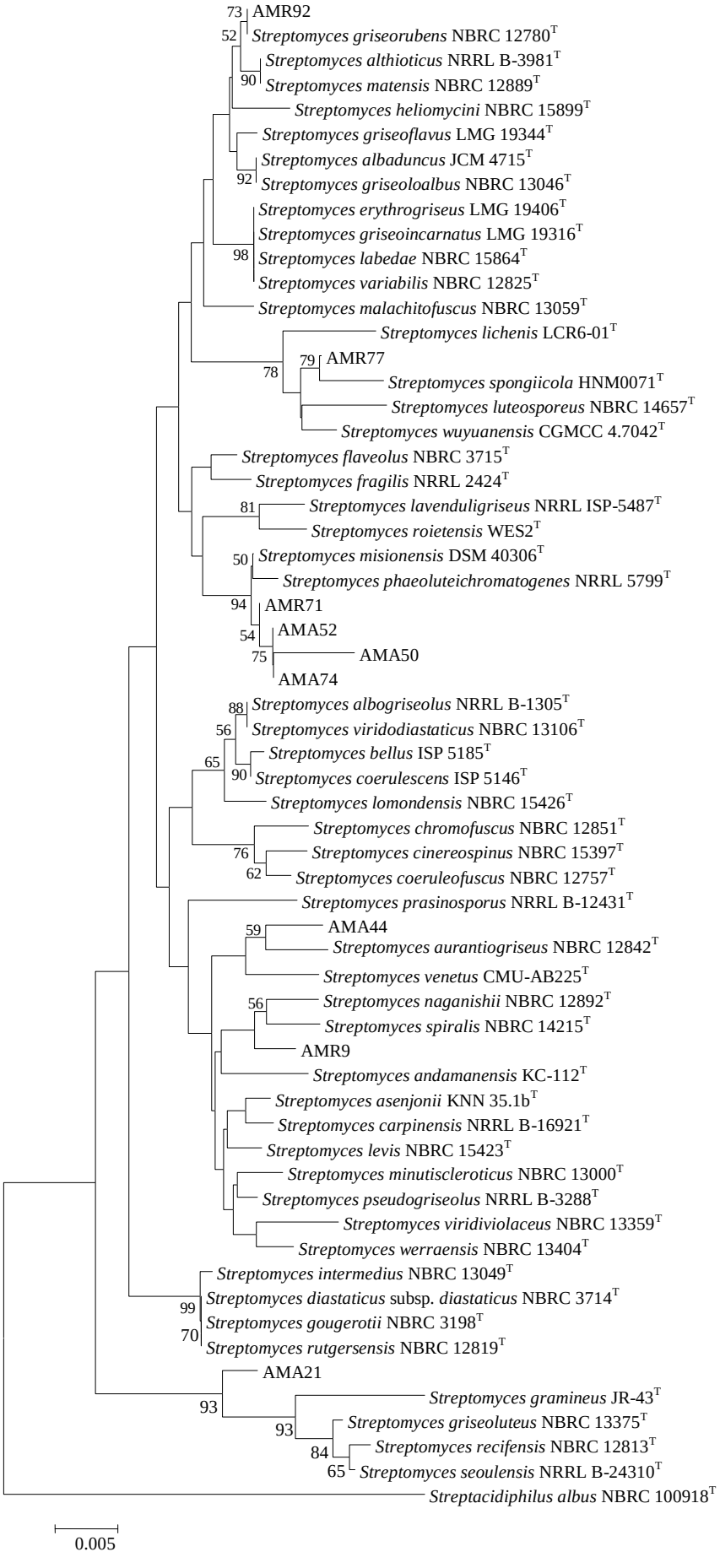

73
AMR92
52
Streptomyces griseorubens NBRC 12780T
Streptomyces althioticus NRRL B-3981T
90
Streptomyces matensis NBRC 12889T
Streptomyces heliomycini NBRC 15899T
Streptomyces griseoflavus LMG 19344T
Streptomyces albaduncus JCM 4715T
92
Streptomyces griseoloalbus NBRC 13046T
Streptomyces erythrogriseus LMG 19406T
Streptomyces griseoincarnatus LMG 19316T
98
Streptomyces labedae NBRC 15864T
Streptomyces variabilis NBRC 12825T
Streptomyces malachitofuscus NBRC 13059T
Streptomyces lichenis LCR6-01T
79
AMR77
78
Streptomyces spongiicola HNM0071T
Streptomyces luteosporeus NBRC 14657T
Streptomyces wuyuanensis CGMCC 4.7042T
Streptomyces flaveolus NBRC 3715T
Streptomyces fragilis NRRL 2424T
81
Streptomyces lavenduligriseus NRRL ISP-5487T
Streptomyces roietensis WES2T
50
Streptomyces misionensis DSM 40306T
Streptomyces phaeoluteichromatogenes NRRL 5799T
94
AMR71
54
AMA52
75
AMA50
AMA74
88
Streptomyces albogriseolus NRRL B-1305T
56
Streptomyces viridodiastaticus NBRC 13106T
Streptomyces bellus ISP 5185T
65
90
Streptomyces coerulescens ISP 5146T
Streptomyces lomondensis NBRC 15426T
Streptomyces chromofuscus NBRC 12851T
76
Streptomyces cinereospinus NBRC 15397T
62
Streptomyces coeruleofuscus NBRC 12757T
Streptomyces prasinosporus NRRL B-12431T
59
AMA44
Streptomyces aurantiogriseus NBRC 12842T
Streptomyces venetus CMU-AB225T
56
Streptomyces naganishii NBRC 12892T
Streptomyces spiralis NBRC 14215T
AMR9
Streptomyces andamanensis KC-112T
Streptomyces asenjonii KNN 35.1bT
Streptomyces carpinensis NRRL B-16921T
Streptomyces levis NBRC 15423T
Streptomyces minutiscleroticus NBRC 13000T
Streptomyces pseudogriseolus NRRL B-3288T
Streptomyces viridiviolaceus NBRC 13359T
Streptomyces werraensis NBRC 13404T
Streptomyces intermedius NBRC 13049T
Streptomyces diastaticus subsp. diastaticus NBRC 3714T
99
Streptomyces gougerotii NBRC 3198T
70
Streptomyces rutgersensis NBRC 12819T
AMA21
Streptomyces gramineus JR-43T
93
93
Streptomyces griseoluteus NBRC 13375T
84
Streptomyces recifensis NBRC 12813T
65
Streptomyces seoulensis NRRL B-24310T
Streptacidiphilus albus NBRC 100918T
0.005

Figure 26a Phylogenetic relationship of active isolates of marine-derived actinomycetes and related taxa obtained from EzBioCloud's database, based on the 16S rDNA analysis. The tree created by neighbor-joining method with 1000 bootstrap re-sampling: value lower than 50% are not shown. Scale bar represents the number of changes per base position.

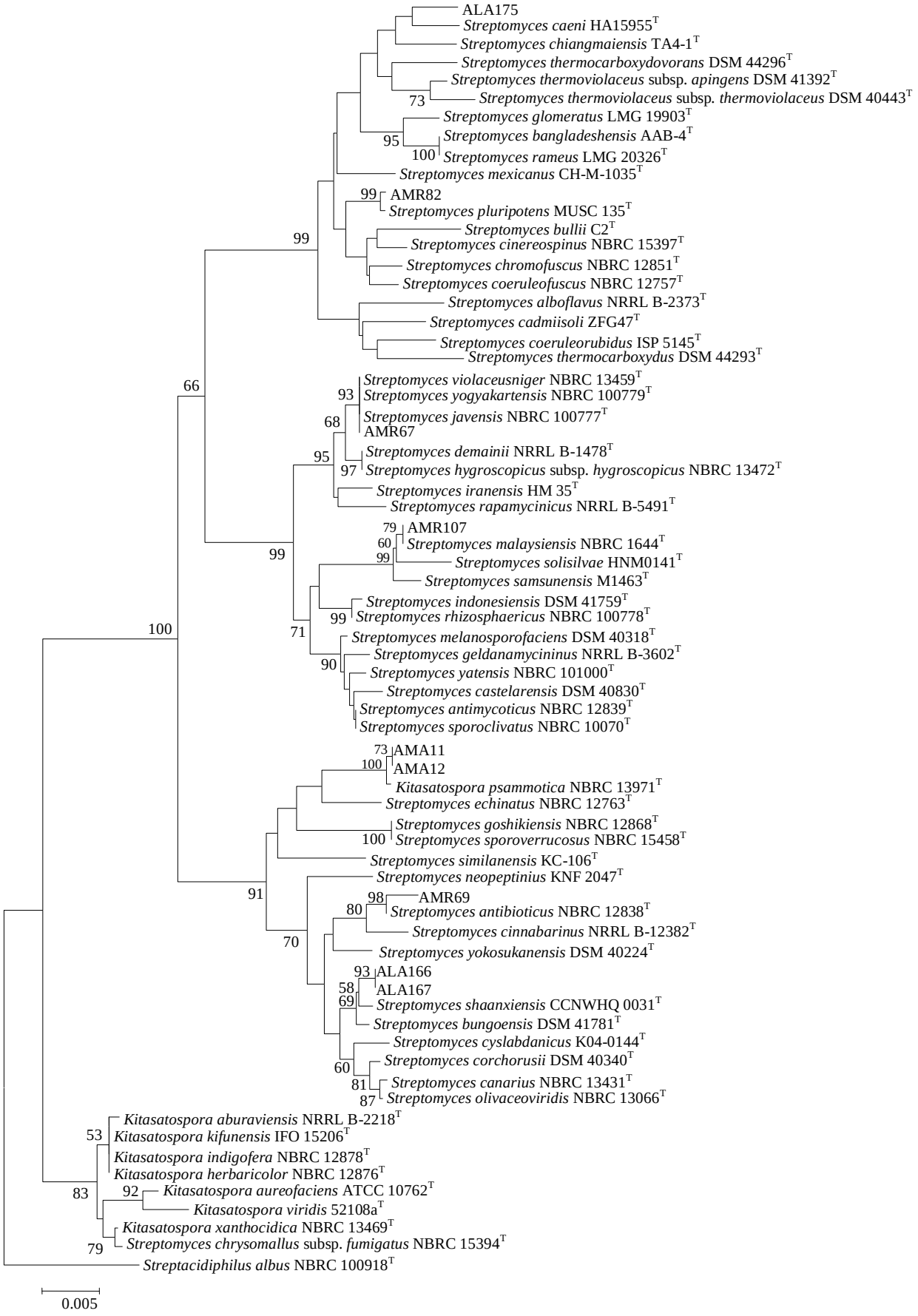

ALA175
Streptomyces caeni HA15955T
Streptomyces chiangmaiensis TA4-1T
Streptomyces thermocarboxydovorans DSM 44296T
Streptomyces thermoviolaceus subsp. apingens DSM 41392T
Streptomyces thermoviolaceus subsp. thermoviolaceus DSM 40443T
Streptomyces glomeratus LMG 19903T
Streptomyces bangladeshensis AAB-4T
Streptomyces rameus LMG 20326T
Streptomyces mexicanus CH-M-1035T
AMR82
Streptomyces pluripotens MUSC 135T
Streptomyces bullii C2T
Streptomyces cinereospinus NBRC 15397T
Streptomyces chromofuscus NBRC 12851T
Streptomyces coeruleofuscus NBRC 12757T
Streptomyces alboflavus NRRL B-2373T
Streptomyces cadmiisoli ZFG47T
Streptomyces coeruleorubidus ISP 5145T
Streptomyces thermocarboxydus DSM 44293T
Streptomyces violaceusniger NBRC 13459T
Streptomyces yogyakartensis NBRC 100779T
Streptomyces javensis NBRC 100777T
AMR67
Streptomyces demainii NRRL B-1478T
Streptomyces hygroscopicus subsp. hygroscopicus NBRC 13472T
Streptomyces iranensis HM 35T
Streptomyces rapamycinicus NRRL B-5491T
AMR107
Streptomyces malaysiensis NBRC 1644T
Streptomyces solisilvae HNM0141T
Streptomyces samsunensis M1463T
Streptomyces indonesiensis DSM 41759T
Streptomyces rhizosphaericus NBRC 100778T
Streptomyces melanosporofaciens DSM 40318T
Streptomyces geldanamycininus NRRL B-3602T
Streptomyces yatensis NBRC 101000T
Streptomyces castelarensis DSM 40830T
Streptomyces antimycoticus NBRC 12839T
Streptomyces sporoclivatus NBRC 10070T
AMA11
AMA12
Kitasatospora psammotica NBRC 13971T
Streptomyces echinatus NBRC 12763T
Streptomyces goshikiensis NBRC 12868T
Streptomyces sporoverrucosus NBRC 15458T
Streptomyces similanensis KC-106T
Streptomyces neopeptinius KNF 2047T
AMR69
Streptomyces antibioticus NBRC 12838T
Streptomyces cinnabarinus NRRL B-12382T
Streptomyces yokosukanensis DSM 40224T
ALA166
ALA167
Streptomyces shaanxiensis CCNWHQ 0031T
Streptomyces bungoensis DSM 41781T
Streptomyces cyslabdanicus K04-0144T
Streptomyces corchorusii DSM 40340T
Streptomyces canarius NBRC 13431T
Streptomyces olivaceoviridis NBRC 13066T
Kitasatospora aburaviensis NRRL B-2218T
Kitasatospora kifunensis IFO 15206T
Kitasatospora indigofera NBRC 12878T
Kitasatospora herbaricolor NBRC 12876T
Kitasatospora aureofaciens ATCC 10762T
Kitasatospora viridis 52108aT
Kitasatospora xanthocidica NBRC 13469T
Streptomyces chrysomallus subsp. fumigatus NBRC 15394T
Streptacidiphilus albus NBRC 100918T
0.005

Figure 26b Phylogenetic relationship of active isolates of marine-derived actinomycetes and related taxa obtained from EzBioCloud's database, based on the 16S rDNA analysis. The tree created by neighbor-joining method with 1000 bootstrap re-sampling: value lower than 50% are not shown. Scale bar represents the number of changes per base position.

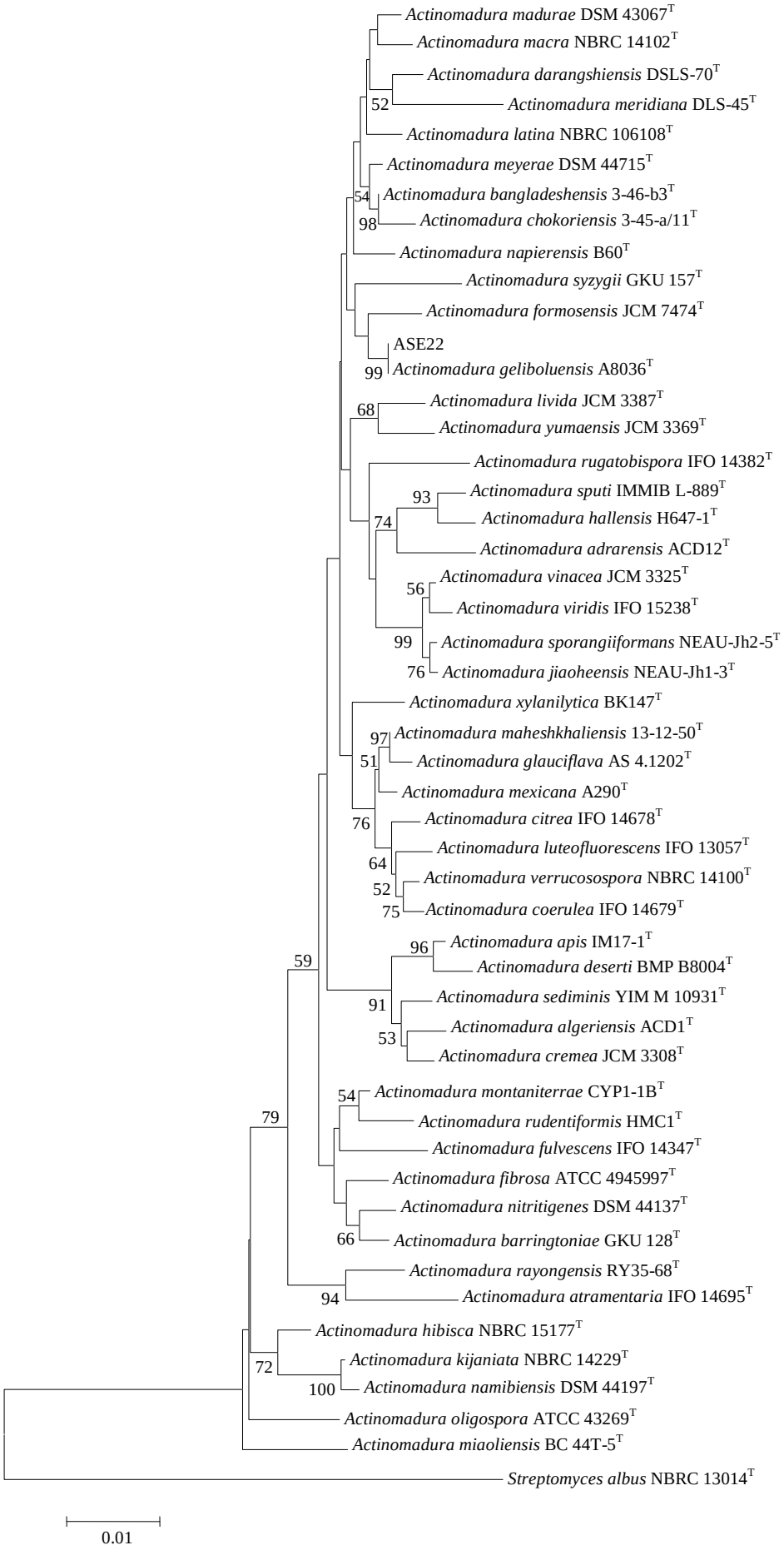

Actinomadura madurae DSM 43067^T
Actinomadura macra NBRC 14102^T
Actinomadura darangshiensis DSLS-70^T
52
Actinomadura meridiana DLS-45^T
Actinomadura latina NBRC 106108^T
Actinomadura meyerae DSM 44715^T
54
Actinomadura bangladeshensis 3-46-b3^T
98
Actinomadura chokoriensis 3-45-a/11^T
Actinomadura napierensis B60^T
Actinomadura syzygii GKU 157^T
Actinomadura formosensis JCM 7474^T
ASE22
99
Actinomadura geliboluensis A8036^T
68
Actinomadura livida JCM 3387^T
Actinomadura yumaensis JCM 3369^T
Actinomadura rugatobispora IFO 14382^T
93
Actinomadura sputi IMMIB L-889^T
74
Actinomadura hallensis H647-1^T
Actinomadura adrarensis ACD12^T
Actinomadura vinacea JCM 3325^T
56
Actinomadura viridis IFO 15238^T
99
Actinomadura sporangiiformans NEAU-Jh2-5^T
76
Actinomadura jiaoheensis NEAU-Jh1-3^T
Actinomadura xylanilytica BK147^T
97
Actinomadura maheshkhaliensis 13-12-50^T
51
Actinomadura glauciflava AS 4.1202^T
Actinomadura mexicana A290^T
76
Actinomadura citrea IFO 14678^T
64
Actinomadura luteofluorescens IFO 13057^T
52
Actinomadura verrucosospora NBRC 14100^T
75
Actinomadura coerulea IFO 14679^T
96
Actinomadura apis IM17-1^T
59
Actinomadura deserti BMP B8004^T
91
Actinomadura sediminis YIM M 10931^T
53
Actinomadura algeriensis ACD1^T
Actinomadura cremea JCM 3308^T
54
Actinomadura montaniterrae CYP1-1B^T
79
Actinomadura rudentiformis HMC1^T
Actinomadura fulvescens IFO 14347^T
Actinomadura fibrosa ATCC 4945997^T
Actinomadura nitritigenes DSM 44137^T
66
Actinomadura barringtoniae GKU 128^T
Actinomadura rayongensis RY35-68^T
94
Actinomadura atramentaria IFO 14695^T
Actinomadura hibisca NBRC 15177^T
72
Actinomadura kijaniata NBRC 14229^T
100
Actinomadura namibiensis DSM 44197^T
Actinomadura oligospora ATCC 43269^T
Actinomadura miaoliensis BC 44T-5^T
Streptomyces albus NBRC 13014^T
0.01

Figure 26c Phylogenetic relationship of active isolates of marine-derived actinomycetes and related taxa obtained from EzBioCloud's database, based on the 16S rDNA analysis. The tree created by neighbor-joining method with 1000 bootstrap re-sampling: value lower than 50% are not shown. Scale bar represents the number of changes per base position.

Table 18 Identification of the most active marine-derived actinomycetes with their antimicrobial activity.

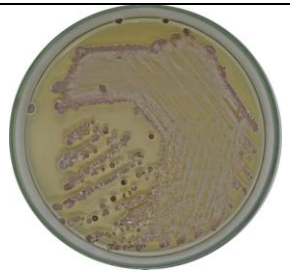
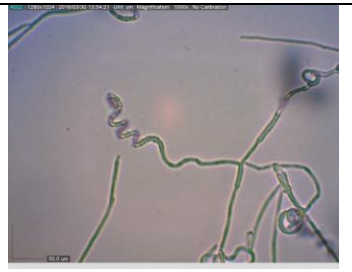
Atinomycete Code	**Species**	**GenBank accession no.**	**BCC/TBRC accession no.**	**Colony on ISP2 medium**	**Spore type (100X)**	**Strong activity against**
AMA11 AMA12	Uncertain species	KP893292 KX129882	BCC77513 TBRC5802			SA, MRSA SA, MRSA
AMA21	*Streptomyces* sp.	KX129883	TBRC5803			SA, MRSA
AMA44	*Streptomyces* sp.	KX129884	TBRC5804			SA, MRSA

Table 18 (Cont.) Identification of the most active marine-derived actinomycetes with their antimicrobial activity.

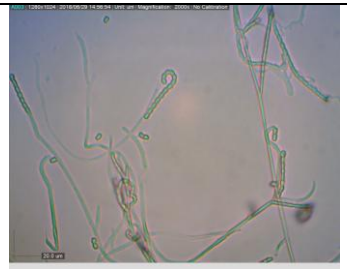
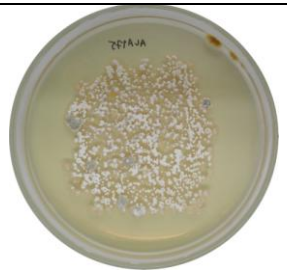
Atinomycete Code	**Species**	**GenBank accession no.**	**BCC/TBRC accession no.**	**Colony on ISP2 medium**	**Spore type (100X)**	**Strong activity against**
AMA50	*Streptomyces*	KX129899	BCC77515			TM
AMA52	sp.	KX129885	TBRC5805			CN
AMA74		KX129886	TBRC5805			SA, MRSA
AMR71		KX129893	TBRC5815			CA, CN, TM
ALA166	*Streptomyces*	KX129888	TBRC5810			SA, MRSA
ALA167	sp	KX129889	TBRC5811			SA, MRSA
ALA175	*Streptomyces*	KX129890	TBRC5812			SA, MRSA
	sp					

Table 18 (Cont.) Identification of the most active marine-derived actinomycetes with their antimicrobial activity.

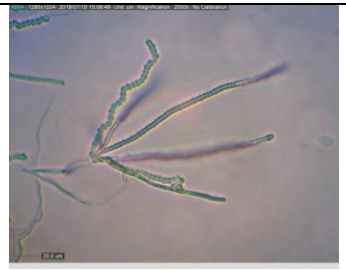
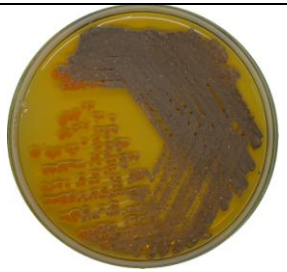
Atinomycete Code	**Species**	**GenBank accession no.**	**BCC/TBRC accession no.**	**Colony on ISP2 medium**	**Spore type (100X)**	**Strong activity against**
AMR9	*Streptomyces* sp.	KX129891	TBRC5813			SA, MRSA
AMR67	*Streptomyces* sp.	KX129892	TBRC5814			SA, MRSA
AMR69	*Streptomyces* sp.	KX129900	BCC77516			SA, MRSA

Table 18 (Cont.) Identification of the most active marine-derived actinomycetes with their antimicrobial activity.

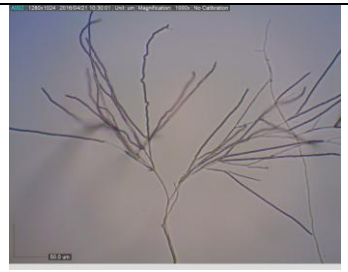
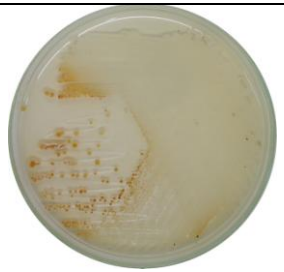
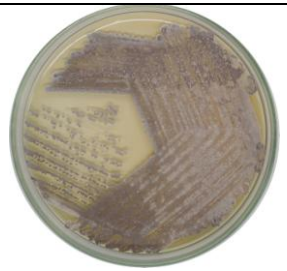
Atinomycete Code	**Species**	**GenBank accession no.**	**BCC/TBRC accession no.**	**Colony on ISP2 medium**	**Spore type (100X)**	**Strong activity against**
AMR77	*Streptomyces* sp.	KX129894	TBRC5816			SA, MRSA
AMR82	*S. pluripotens*	KX129895	TBRC5817			MRSA
AMR92	*S. griseorubens*	KX129896	TBRC5818			SA, MRSA

Table 18 (Cont.) Identification of the most active marine-derived actinomycetes with their antimicrobial activity.

Atinomycete Code	Species	GenBank accession no.	BCC/TBRC accession no.	Colony on ISP2 medium	Spore type (100X)	Strong activity against
AMR107	*S. malaysiensis*	KX129897	TBRC5819			SA, MRSA
ASE22	*A. geliboluensis*	KX129887	TBRC5808			SA, MRSA

SA = *Staphylococcus aureus* ATCC25923 MRSA = methicillin-resistant *S. aureus* SK1 AB = *Acinetobacter baumannii* NPRC001

CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112 TM = *Talaromyces marneffei* PSU-SKH1

AMA = actinomycetes from mangrove sediment ALA = actinomycetes from Songkhla lake ASE = actinomycetes from coastal sediment

AMR = actinomycetes from marine organisms

3.13 Description of *Gordonia sediminis* sp. nov.

Of these 63 isolates, 47 may represent novel species based on phylogenetic tree analysis and their morphological characteristics. In this study, AMA120^T was chosen for polyphasic study consisting of phenotypic, chemotaxonomic and phylogenetic studies. From 16S rRNA gene sequence analysis, strain AMA120^T was classified to the genus *Gordonia* and it was closely related to *Gordonia rhizosphera* NBRC16068^T (98.9%), *Gordonia polyisoprenivorans* NBRC16320^T (98.1%) and *Gordonia bronchialis* NBRC16047^T (98.1%). Phylogenetic trees constructed using NJ (Figure 27), ML and MP methods showed that AMA120^T formed a distinct clade with *G. rhizosphera* NBRC16068^T. A phylogenetic tree reconstructed from a fragment of the *gyrB* gene in Figure 28 showed that AMA120^T formed a distinct phyletic line with *G. rhizosphera* NBRC16068^T (95.4%). DNA-DNA relatedness values between AMA120^T and *G. rhizosphera* NBRC16068^T, *G. polyisoprenivorans* NBRC16320^T and *G. bronchialis* NBRC16047^T, its closest neighbours in 16S rRNA gene sequence similarity, were 51-55%, 3-12% and 8-10%, respectively. The draft genome of AMA120^T consisted of 205 contigs with 7.3 Mbp exhibiting a DNA G+C content 66.6 mol%. The genome was annotated using NCBI's Prokaryotic Genome Annotation Pipeline (PGAP) and contains 6,516 predicted protein-coding genes. The ANI of strain AMA120^T with *G. rhizosphera* NBRC16068^T was 92.4%, 75.0% with *G. bronchialis* NBRC16047^T and 74.5% with *G. polyisoprenivorans* NBRC16320^T. This ANI analysis indicated that strain AMA120^T belongs to a disticnt but closely related species in the genus *Gordonia*.

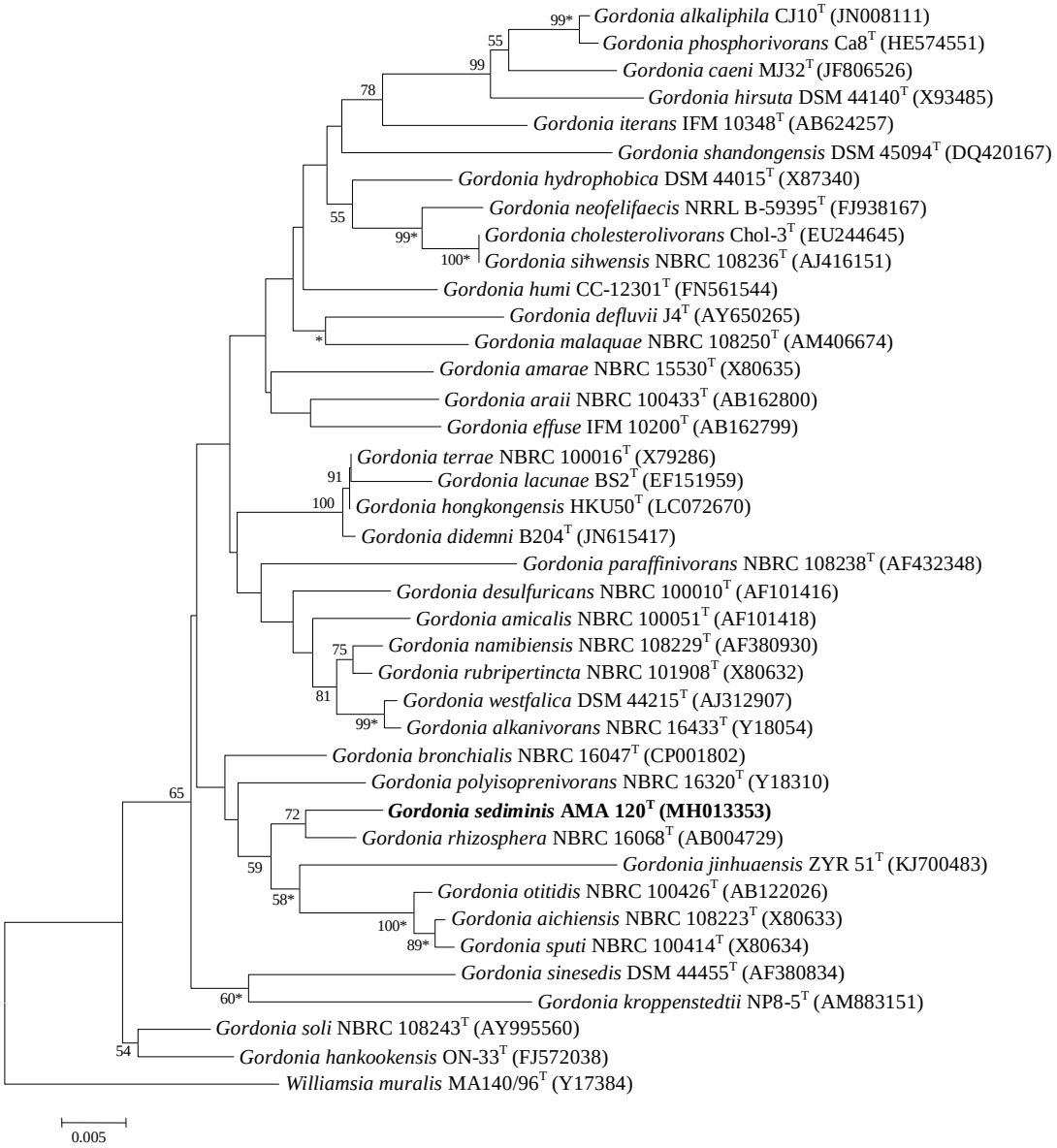


Figure 27 Phylogenetic tree based on 16S rRNA gene sequences created using the neighbour-joining method in MEGA version 6.06, showing the phylogenetic positions of strain AMA 120^T and type strains within the genus *Gordonia*. Numbers at branching points refer to percentages of bootstrap values from 1000 replications (only values greater than 50% are indicated). Asterisks represent clades that were also recovered using the maximun-likelihood and maximum-parsimony methods. Bar, 0.005 substitutions per nucleotide position. (This figure was published in Sangkanu *et al.*, 2019).

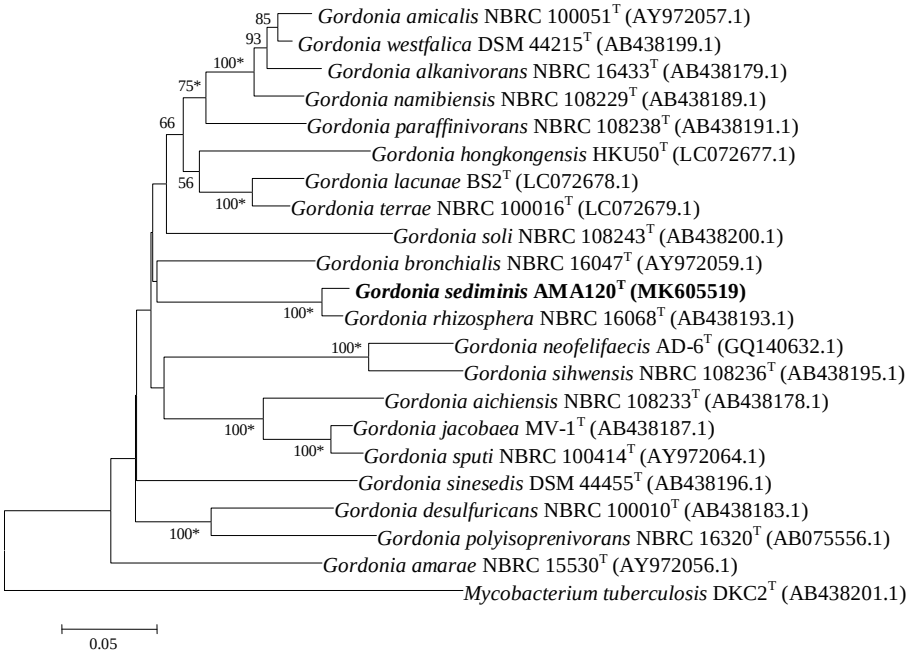


Figure 28 Phylogenetic tree analysis of *Gordonia* strains derived from *gyrB* gene nucleotide sequences. The tree was created using the neighbour-joining method. The numbers on the tree represent bootstrap values for the branch points. Bootstrap values greater than 50% significance are indicated. Asterisks represent clades that were also recovered using the maximun-likelihood and maximum-parsimony methods. Bar, 0.05 substitutions per nucleotide position. (This figure was published in Sangkanu *et al.*, 2019).

Cell wall of strain AMA120^T was also determined. It contained *meso*-diaminopimelic acid and arabinose and galactose as characteristic of whole-cell sugar (wall chemotype IV). Mycolic acid was found to be present. MK-9(H_2) was detected as the major menaquinone. The polar lipid consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and two unidentified phospholipids. The major fatty acids were $C_{16:0}$, $C_{18:1}\omega 9c$ and summed feature 3 ($C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$).

Cells of strain AMA120^T were Gram-positive with rod-shaped (Figure 29). It grew well on ISP medium nos. 2, 3 and TSA, showing brilliant orange; moderately on ISP medium no. 7, showing vivid reddish orange; but weakly on ISP medium nos.4

and 5, showing vivid orange. No diffusible pigment was observed in all of the media tested. Growth occurred at 15, 20 and 28°C (optimum 28°C) but no growth was observed at 5, 10, 37 and 45°C. Strain AMA120^{T} showed a broad pH range for growth (pH 4.0 to 13.0, optimum 6.0 to 9.0) and a wide tolerance to NaCl (1 to 8% w/v, optimum 1 to 5%). Catalase activity was positive but hydrolysis of hypoxanthine, chitin, and casein were negative. Biochemical and other physiological properties of strain AMA120^{T} are summarized in this description and are compared with the closely related type strains in Table 19

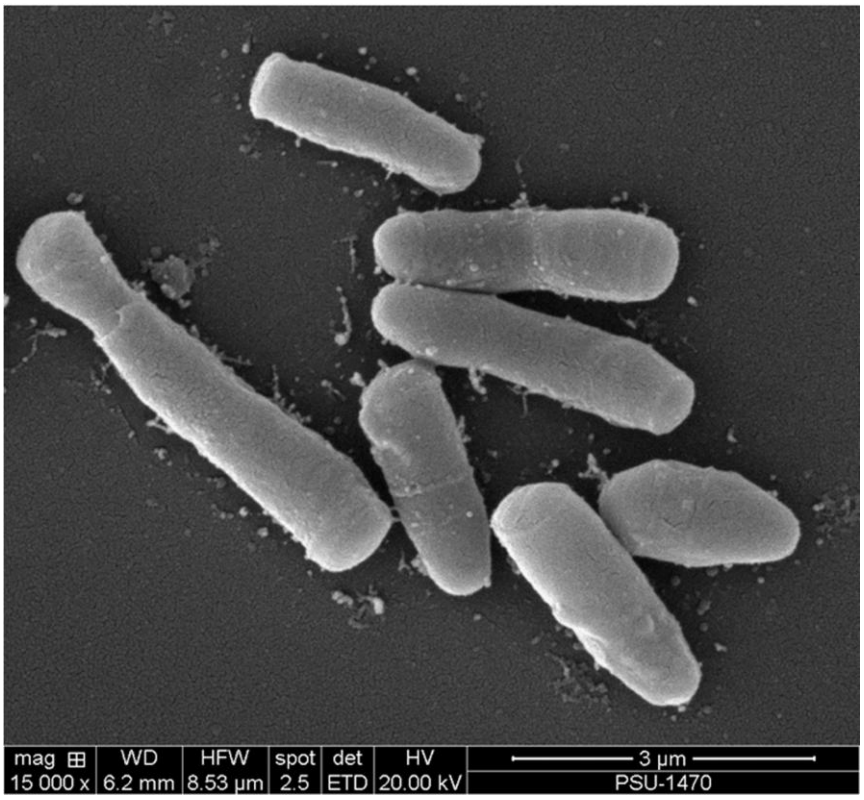


Figure 29 Scanning electron micrograph of strain AMA120^{T} grown on ISP medium no. 2 at 28°C for 7 days. Bar, 3 µm. (This figure was published in Sangkanu *et al.*, 2019).

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Table 19 Differential physiological characteristics between strain AMA120^T and related type strains of the genus *Gordonia*.

Characteristic	**1**	**2**	**3**	**4**
Colony on ISP medium no. 2	Brilliant orange	Brilliant orange	Brilliant orange yellow	Vivid orange
Growth at temperature 15°C	+	-	+	+
Growth at pH 4.0	+	w	+	w
Growth at pH 13.0	w	w	-	-
Growth in presence NaCl 8% (w/v)	w	-	-	-
Utilization of:				
D-trehalose	+	-	+	+
Xylitol	-	+	-	-
Melibiose	w	+	-	-
Mannose	-	+	+	w
L-rhamnose	+	+	+	-
D-galactose	-	-	+	+
Acid production from:				
Maltose	+	w	-	-
Mannitol	+	-	-	-
Fructose	+	-	-	-
Rhamnose	+	w	-	-
Sucrose	+	-	-	-
Activities of:				
Alkaline phosphatase	-	-	+	+
Esterase (C4)	-	-	+	+
Esterase lipase (C8)	-	-	+	+
Lipase (C14)	-	-	w	+
Cysteine arylamidase	+	+	-	+
Trypsin	+	+	-	w
α-Chymotrypsin	+	+	-	w
α-Galactosidase	+	-	-	-
β-Glucosidase	+	+	-	w

Strain: 1, *G. sediminis* AMA 120^T ; 2, *G. rhizosphera* NBRC 16068^T ; 3, *G. polyisoprenivorans* NBRC 16320^T ; 4, *G. bronchialis* NBRC 16047^T . +, Positive; -, Negative; w, weak positive. All data were obtained in this study. (This table was published in Sangkanu *et al*., 2019).

The differences of phenotypic properties (Table 19 and Figure 30) and the differential genotypic properties of strain AMA120^T are sufficient to classify AMA120^T as a novel taxonomic unit in the genus *Gordonia*. It is appropriated to propose AMA120^T as the type strain of a novel species with the name *Gordonia sediminis* sp. nov.

The type strain is AMA120^T (=TBRC 7109^T =NBRC 113236^T). The sequence of the *Gordonia sediminis* AMA120^T has been deposited in the GenBank database under the accession number MH013353 for the 16S rRNA gene, MK605519 for *gyrB* gene and RZUM00000000 for the whole genome.

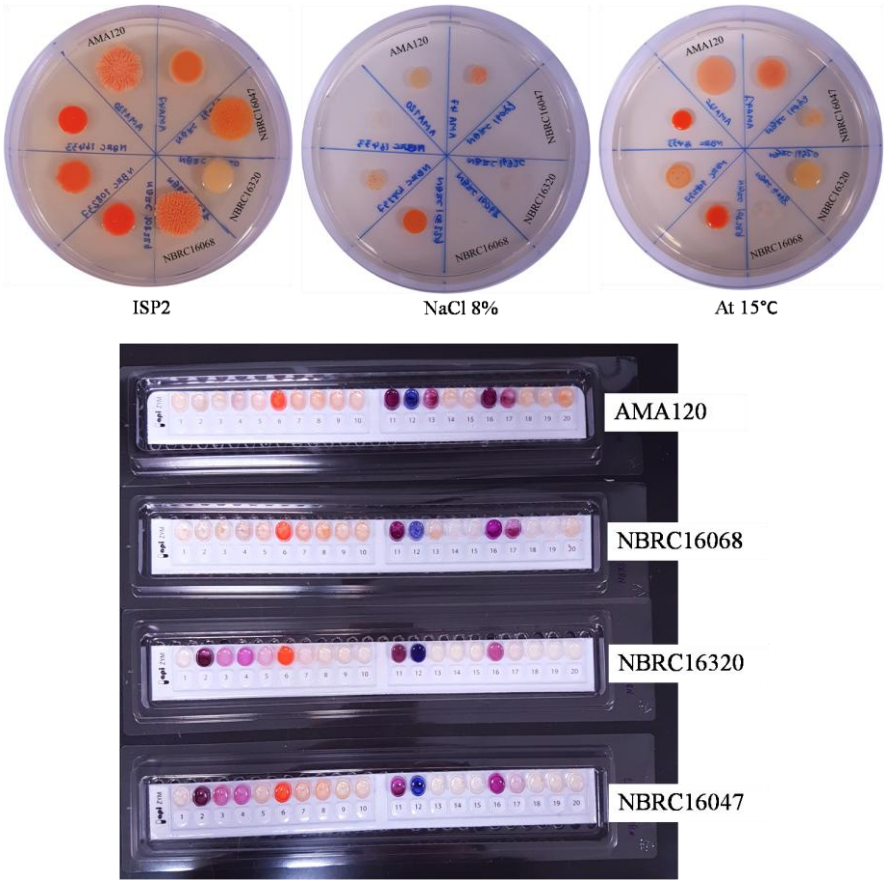

Figure 30 Some difference of phenotypic properties of AMA120^T and closely related type strains

CHAPTER 4

DISCUSSION

4.1 Isolation of marine-derived actinomycetes

Actinomycete is a Gram-positive bacterium with high mol% G+C content. They are filamentous or non-filamentous which some genera produce spores (Mohammadipanah and Wink, 2016). The actinomycete is a group of bacteria that exists in various environments and has originated from soil. Many of them are well known as a potential producer of bioactive secondary metabolites including antibiotics, antitumor agents, immunosuppressive agents and enzymes (Chaudhary *et al*., 2013). Recently, the discovery rate of new compounds from terrestrial actinomycetes has decreased, while the rate of re-isolation of known compounds has increased (Lam, 2006). Therefore, it is necessary to isolate new groups of actinomycetes from diverse habitats for the searching of novel bioactive compounds (Lam, 2006; Takahashi and Omura, 2003). The world ocean is the largest ecosystem of the earth which covers 70% of the planet and provides good and inhabitable areas for the majority of the microbial population (Das *et al*., 2006). Marine-derived *Actinobacteria* were found abundant in marine sediments and they are still an important source of novel compounds (Parera-Valadez *et al*., 2019). Although sediments are considered as sources for isolation of actinomycetes but several potential isolates have been obtained from marine organisms (Ramesh and Mathivanan, 2009). The present study isolated actinomycetes from marine samples consisting of sediments, water and marine organisms and determined their antimicrobial activities. Five hundred and twenty five isolates distributed among eight families. The highest number was obtained from sediments followed by marine organisms but no actinomycete was isolated from water. The number of actinomycetes from sediment or organism samples was higher than in water because these samples may be plentiful with a source of nutrients (Ghanem *et al*. 2000).

Family *Streptomycetaceae* and *Micromonosporaceae* were the dominant families in this study, followed by *Pseudonocardiaceae*, *Gordoniaceae*, *Nocardiaceae*, *Thermomonosporaceae*, *Jiangellaceae* and *Streptosporangiaceae* (Table 8). These results were in accordance with several previous studies. Maldonado *et al*. (2005) reported that *Micromonospora*, *Rhodococcus* and *Streptomyces* species were the dominant actinomycetes in marine environments. Likewise, Zhang *et al*. (2013a) also reported *Streptomyces* and *Micromonospora* as the dominant genera of culturable marine actinomycetes. However, cultivation dependent studies of sediments from New Brunswick, Canada revealed the presence of *Micromonospora*, *Nocardia*, *Nocardiopsis*, and *Pseudonocardia*, in addition to the genus *Streptomyces* (Duncan *et al*., 2014). All isolates in this study were not strictly marine actinomycetes because they can grow on media without seawater. These marine-derived actinomycetes may be transported from the land via rains and storms. Terrestrial actinomycetes produce resistant spores resulted in the survival in marine environments. Therefore, it has been normally claimed that marine-derived actinomycetes are merely the terrestrial origin (Dharmaraj, 2010). Our results indicated that the bacterial community of marine environments in Southern Thailand especially sediments and marine organisms is rich in actinomycetes group.

4.2 Antimicrobial activity of marine-derived actinomycetes

4.2.1 Primary and secondary screening

Since more than 500 isolates of marine-derived actinomycetes were isolated, representatives of each morphogroup were selected for further studies. The cross-streak method was used for the screening of antimicrobial activities against bacteria, yeasts and hyphal inhibition method against filamentous fungi. These two methods are widely used for this purpose because they are easy to perform and relatively rapid. The growth inhibition zones or hyphal inhibition zones were clearly seen. However, these methods detected only soluble antimicrobial metabolites that could diffuse into

the medium. In this study, 57.7% of the selected isolates showed inhibitory activity and the majority of them suppressed the growth of filamentous fungi, *M. gypseum* (65.2%) and *T. marneffei* (42.4%). Further analysis of the secondary metabolites, organic extracts from cultures of 104 fast-growing potential isolates were studied. The antimicrobial activities of the three types of extracts including the CE, CH and BE were in a similar range (59, 51 and 50%, respectively). The majority of extracts was best active against the Gram-positive bacteria that was different from the results of cross streak and hyphal inhibition. Ehtyl acetate is a medium polarity solvent that can extract compounds of intermediate polarity, while hexane is used to solubilize greatly lipophilic compounds (Seidel, 2006). The cross streak and hyphal inhibition methods detected mianly the activity of soluble or polar metabolites, whereas the extracts contained intermediate and less polar compounds. Another drawback of the cross streak method was the difficulty in obtaining quantitative data (Velho-Pereira and Kamat, 2011).

It has already been reported by many investigators that ethyl acetate extracts from marine actinomycetes showed a good antimicrobial activity. The ethyl acetate extract of *Nocardiopsis* sp. VITSVK5 isolated from marine sediment exhibited antifungl activity against *Aspergillus fumigatus*, *A. flavus* and *A. niger* at concentration of 1 mg/ml (Vimal *et al*., 2009). Antimycins antibiotics extracted by ethyl acetate from culture broth of *Streptomyces* sp. VY46 showed anticandidal activity (Charousová *et al*., 2016). The first antimycins have been detected in fermentation broth of *Streptomyces* sp. Ni-80 associated with unidentified sponge. These antibiotics exhibited inhibitory activity against *C. albicans* (Imamura *et al*., 1993). Ethyl acetate extracts from endophytic actinomycetes isolated from mangrove plant *Avicennia marina* showed antibacterial activity against muti-drug resistant uropathogens such as *E. coli*, *P. aeruginosa*, *K. pneumonia*e and *Enterobacter* sp. at 100 µg/ml (Ramachandran *et al*., 2018). However, secondary metabolite production depended on strains and culture conditions. Some strains showed the highest ability to produce extracellular metabolites, while others showed good ability to produce intracellular metabolites (Retnowati *et al*., 2018). In this study, 11 out of 14 active isolates produced both intracellular (CE and CH) and extracellular (BE) strongly active metabolites against *S. aureus* and MRSA.

4.2.2 Determination of MIC and MBC or MFC values

4.2.2.1 Antibacterial activity

In this study, most of the marine-derived actinomycete extracts showed weak activity (MIC values 128-200 µg/ml) against human pathogens. Thirty-one extracts from 19 isolates displayed strong antibacterial activity against *S. aureus* and 30 extracts against MRSA (MIC values <0.03-8 µg/ml). Only one extract, AMA11CE showed strong activity against Gram-negative bacteria, *A. baumanii* (MIC 8 µg/ml) (Table 12). Several studies have reported antibacterial activity of actinomycetes. Arenimycin, a new antibiotic from *Salinispora arenicola* had a potent antibacterial activity against five strains of MRSA with MIC values ranging from 0.05 to 1.03 µg/ml (Asolkar *et al*., 2010). Wu *et al* (2013b) isolated napypadiomycins from marine-derived *Streptomyces* sp. SCSIO 10428 that inhibited Gram-positive bacteria, *Staphylococcus* and *Bacillus* strains with MIC values of 0.25 to 32 µg/ml. Whereas Gram-negative bacteria, *E. coli* was less susceptible to these compounds (MIC >128 µg/ml). Our extracts form AMA11 (CE), ALA166 (BE, CE, CH), ALA167 (BE,CE), AMR67 (CE, CH) and AMA69 (BE, CE, CH) exhibited potential activity against both *S. aureus* ATCC25923 and MRSA with MIC less than or equal to the MIC of vancomycin standard drug (1 µg/ml). The active compounds from these extracts will be determined in the future.

In this study, only three extracts showed moderate (AMA11CE, MIC 64 µg/ml) and weak activity (AMA12BE and AMR82BE, MIC 200 µg/ml) against *E. coli*. AMA11CE also showed strong to moderate activity against six strains *A. baumannii* (MIC 8-64 µg/ml). The first anti-*A. baumannii* activity of actinomycetes was reported in 2010 by Lee *et al*. Their soil *Streptomyces* sp. KH29 produced a cyclo (L-tryptophanyl-L-tryptophanyl) that could inhibit 42 strains of *A. baumannii* with MIC ranges from 12.5 to 25 µg/ml but eight strains were not susceptible to this compound (MIC >100 µg/ml). John and Ramasamy (2014) found active fraction from *Streptomyces* sp. PM49 that exhibited antibacterial activity against *A. baumannii* and *P. aeruginosa*. Gram-negative bacteria are more resistant to many antibiotics than the Gram-positive ones because of the presence of lipopolysaccharide (LPS) in outer

membrane of Gram-negative bacteria. LPS is acted as barriers that prevent Gram-negative bacteria from the passive diffusion of hydrophobic solutes such as antibiotics or detergents into their cells (Zhang *et al*., 2013b). Therefore, the discovery of new compounds effective against Gram-negative bacteria is a challenge.

Compound having a narrow ratio of MIC/MBC or MIC/MFC less than or equal to 4 is considered to have bactericidal or fungicidal effect (Mathurin *et al.*, 2012). Among the strong active extracts (MIC ≤8 µg/ml) in this study, three extracts, AMA44BE (MIC/MBC 4/16 µg/ml), AMR82BE (4/8 µg/ml) and AMR107CH (2/4 µg/ml) exhibited bactericidal activity against MRSA with MIC/MBC ratios of 4, 2 and 2, respectively. However, these MIC values were higher than that of vancomycin (MIC/MBC 1/2 µg/ml). Vancomycin is the main antimicrobial drug for the treatment of MRSA infection. According to the CLSI interpretative standard for vancomycin, MIC ≤2 µg/ml is considered as susceptible (Philips *et al*., 2016).

The possible modes of action of the most active extracts having the lowest MIC (≤0.03 µg/ml) comprising ALA166CE, AMR67CH, AMR69BE and AMR69CH were studied using the SEM at 0.12 µg/ml (4 times their MICs). All SEM images of the treated cells revealed many abnomalities of the broken cells in the same manner as the vancomycin treatment. Vancomycin, a glycopeptides antibiotic acts on cell wall systhesis of Gram-positive bacteria (Kemung *et al.*, 2018). It inhibits the late-stage peptidoglycan biosynthesis by forming the complex with the D-Ala-D-Ala residues. This complex blocks the formation of peptoglycan cross-link step. In addition, this mechanism leads to cytoplasmic accumulation of UDP-linked MurNAc-pentapeptide precursor relating of an effect on a membrane-associated step (Allen and Nicas, 2003). Some cytoplasmic protusions were also observed in the treated cells indicating the interference with cell membranes. Therefore, the possible modes of action of our extracts from marine-derived actinomycetes may act on cell wall and cell membrane. As AMA11CE showed a broad spectrum activity against all bacteria tested except *P. aeruginosa*, the possible mechanisms of action of AMA11CE were also determined including the SEM study and bacteriolytic activity. Because of an insufficient amount obtained, only AMA11CE treated MRSA was studied by the SEM. The SEM

micrograph of MRSA treated with 4×MIC (2 µg/ml) of AMA11CE for 24 h exhibited irregular cell morphology. The appearance of small pores on the cell wall and a slight depression due to cell lysis were observed in some cells. However, this extract did not show bacteriolytic activity within 24 h. It is clearly shown that AMA11CE exhibited bacteriostatic activity as its MBC against MRSA SK1 was 128 times higher than the MIC. A few damaged cells seen by SEM were therefore correlated to the bacteriolytic test results which might have a delayed effect on the release of cell materials. Most of the previously reported antimicrobial compounds from several organisms exhibited their antibacterial activity in the same manner (Anam *et al*., 2010; Cardozo *et al*., 2013; Zhang *et al.,* 2013c).

Pathogenic bacteria trend to live in a community as biofilm. Biofilms are commonly associated many chronic infections. It is difficult for drugs to get through the biofilms and kill bacteria. Therefore, biofilms are more resistant (>10-100 folds) to antibiotics than the planktonic cells. Anti-biofilm agents are needed to prevent biofilm formation and infection. Anti-biofilm agent is therefore, a new strategy to combat resistant bacteria (Bueno, 2014; Khan *et al*., 2015; Taylor *et al*., 2014). In this study, the BE and CE of AMA11, AMA12 and CE of AMA21 were tested for anti-biofilm activity. The AMA11BE, AMA11CE and AMA12BE at 0.5×MIC showed good activity in preventing biofilm formation of *S. epidermidis*, while vancomycin had no activity. Moreover, the AMA11BE, AMA12BE and AMA12CE extracts were more effective to reduce the viability of biofilm grown cells than vancomycin. The glycocalyx of biofilm may act as a penetration barrier, resulting in the delayed diffusion of antibiotic to the bacterial cell inside biofilm (Darouiche *et al*.,1994). The results from this study indicated that both BE and CE extracts of AMA11 and AMA12 would give a considerable advantage against biofilm-forming organisms.

4.2.2.2 Antifungal activity

Six extracts from three marine-derived actinomycetes (AMA50, AMA52 and AMA71) possessed strong antifungal activity against *C. albicans*, *C. neoformans* and *T. marneffei* with MIC values ranging from 0.5 to 8 µg/ml. In particular, the

AMA50CH had the lowest MIC (0.5 µg/ml) against *T. marneffei* comparable to amphotericin B standard drug (MIC 1 µg/ml). Kumar and Kannabiran (2010) reported that ethyl acetate extract of *Streptomyces* VITSVK5 from marine sediment in India showed strong antifungal activity against *A. fumigatus* ATCC6645 and *A. niger* ATCC6404 with MIC values ranging from 0.25 to 0.5 µg/ml. In addition, this extract inhibited 11 clinical isolates of multridrug-resistant *Aspergillus* strains with low MIC values (0.125-4 µg/ml) when compared to standard antifungal drugs amphotericin B (MIC values 0.125-16 µg/ml), itraconazole (MIC values 2-16 µg/ml), fluconazole (MIC values 0.5-8 µg/ml) and ketoconazole (MIC values 2-16 µg/ml). Polyketide metabolite from marine *Streptomyces* sp. AP-123 inhibited *C. abicans* and *A. niger* with MIC values 12.5 and 25 µg/ml, respectively (Arasu *et al*., 2013). Marine-derived *Nocardia* sp. ALAA2000 produced four active compounds against bacteria and fungi. Among them, the novel antibiotic, ayamycin possessed strong antifungal activity against *Rhodotorula acuta*, *Pichia angusta*, *C. albicans*, *C. neoformans*, *A. niger* and *Botrytis fabae* with MIC ranging from 0.1 to 0.5 µg/ml (El-Gendy *et al.,* 2008). Previous studies have reported antifungal activity from marine actinomycetes against various fungi, but no studies on *T. marneffei*. Thus, strains AMA50 and AMR71 in this study may be the putative for futher antifungal study against *T. marneffei*, an important dimorphic fungus. *T. marnefei* is a cause of severe infections in immunocompromised patients in Southeast Asia (Yu *et al*., 2018).

AMR71CE exhibited a broad spectrum and strong activity against *C. albicans, C. neoformans* and *T. marneffei* with low MIC/MFC values of 4/4, 1/1 and 4/4 µg/ml, respectively indicating fungicidal activity. All fungal cells treated with AMR71CE were deformed and perforated as well as the amphotericin B treated cells. This might be confirmed the possible mode of action of this extract on cell membrane as compared to the action of amphotericin B. Amphotericin B is a member of the macrolide polyene antibiotics produced by soil actinomycete, *Streptomyces nodosus* (Hartsel nd Bolard, 1996). This drug acts by binding to ergosterol in the cell membrane of most fungi. After binding with ergosterol, it induces the formation of pores leading to loss of protons and monovalent cations which promoted the depletion of intracellular ions and cell death. In addtion, amphotericin B also induces oxidative

stress in the cells by producing free redicals. Finally, amphotericin B has a stimulatory effect of immune response in host (Mesa-Arango *et al*., 2012). Antifungal metabolites such as 2,5-bis(1,1-dimethylethyl) phenol and benzeneacetic acid were detected in extract of *Streptomyces* sp. PWS52. They inhibited *C. albicans* at a concentration of 50 µg/ml and SEM photographs of the treated cells showed cell shrinkage (Das *et al*., 2018).

Hexane extract from cells of AMA50 (AMA50CH) exhibited strong antifungal activity against *T. marneffei*. Therefore, this study also investigated the potential of AMA50CH *in vivo*. *C. elegans* is a self-fertilizing hermaphrodite with a rapid generation time. It is a useful and simple model that can be infected by several of human pathogens, including *Pseudomonas aeruginosa* (Tan *et al*., 1999) *Salmonella* enterica (Sem and Rhen, 2012) *Enterococcus faecalis* (Sim and Hibberd, 2016) *Staphylococcus aureus* (Sifri *et al*., 2003) *C. neoformans* (Mylonakis *et al*.,2002) and *C. albicans* (Breger *et al*., 2007). The evaluation of antifungal agents against *T. marneffei* in *C. elegans* model has been reported by Huang *et al*. (2014). They found that the antifungal agents such as amphotericin B, terbinafine, fluconazole and voriconazole successfully prolonged the survival of *C. elegans* infected by *T. marneffei*. In this study, *T. marneffei* CI-2 was used to infect *C. elegans* and found that the nematodes died in day 4. However, the survival percentage of pre-infection nematodes increased after treatment with AMA50CH and amphotericin B. Among the treatment groups, 1 µg/ml of AMA50CH showed the best protective effect and prolonged the survival of infected nematodes upto 60 to 70% after 7 days of treatment but the protective effect of this extract was decreased at higher concentrations. This may be the influence from the cytotoxic effect of AMA50CH at high concentrations. Previous study by Park *et al*. (2002) reported the toxic effect of metabolites from actinomycetes toward nematodes.

4.2.3 Anti-quorum sensing activity

C. violaceum is a Gram-negative bacterium that can grow on common laboratory media. Its colonies are violet color due to the production of violacein pigment (Kothari *et al*., 2017). *C. violaceum* is distributed in soil and water exposure

in tropical regions. Human infections caused by *C. violaceum* are rarely but fatal (Lin *et al*., 2016). Quorum-sensing system in *C. violaceum* is the production of violacein pigment that associated with the LuxI/LuxR homologues CviI/CviR (Stauff and Bassler, 2011). It is commonly used as an assay system to screen for quorum sensing inhibitor (Chenia, 2013). Furthermore, QS also has a major impact on biofilm formation (Wang *et al*., 2017). In this study, nine extracts from seven isolates exhibited quorum sensing inhibitory activity by the disk screening test with *C. violaceum*. The positve results showed the violacein inhibition zones of 1.75-10.25 mm at a concentration of 100 µg/disk. A similar result has been reported by Miao *et al*. (2017) who studied with actinomycetes isolated from seawater. They found that five strains possessed anti-quorum sensing activity with violacein inhibiton zones from 7.2-11 mm at a concentration of 250 µg/disk. They also identified secondary metabolite, actinomycin D from *S. parvulus* HY026O that showed anti-QS activity. Actinomycin C inhibited the violacein production of *C. violaceum* by 65% at 12.5 µg/ml and prodigosin of *Serratia proteamaculans* at 25 µg/disk. Fu *et al*. (2003) reported three new α-pyrones namely nocapyrones H, I and M from marine-derived *Nocardia dassonvillei* subsp. *dassonvillei* XG-8-1 that exhibited inhibitory activity on QS-controlled gene expression in *C. violaceum* and *P. aeruginosa* at a concentration 100 µg/ml.

Six out of nine anti-QS positive extracts comprising AMA11BE, AMA11CE, AMA12BE, AMA12CE, AMA21CE and AMR82BE had strong antibacterial activity (Table 12). The other three extracts, AMA22BE, AMA54BE and ALA65BE exhibited only anti-QS activity. The direct effect of antimicrobial substances on bacterial growth is a selective pressure leading to the development of antimicrobial resistance. The ideal QS inhibitors should exhibit a high degree of specificity for the QS regulator without toxic effect on becterial cells which prevent resistance.

4.2.4 Identification of active extracts

The active extract, AMA11CE was partially purified by a column chromatography. The three active fractions (5, 6 and 7) consisted of pentadecanoic acid and hexadecanoic acid, whereas fractions 6 and 7 contained additional

compounds including 3-nitro1,2-benzenedicarboxylic acid and quinoxaline-2-carboxamide. Pentadecanoic acid is a saturated fatty acid that is rare in nature. It is found in the milk fat of cows at the level of 1.2%. Hexadecanoic acid is a saturated fatty acid that commonly found in animals, plants and microorganisms including *Streptomyces* spp. (Kumar *et al*., 2014). Hexadecanoic acid exhibited larvicidal (Rahuman *et al*., 2000) and anti-inflammatory (Aparna *et al*., 2012) activities. However, pure hexadecanoic acid and pentadecanoic acid had no antibacterial activity (Table 14). 3-Nitro-1,2-benzenedicarboxylic acid, the major compound in the most active fractions 6 and 7, has also been reported in the partially purified fraction of *Nocardia levis* MK-VL_113 which showed antibacterial and antifungal activity (Kavitha *et al*., 2010). Accordingly, 3-nitro-1,2-benzenedicarboxylic acid might play an important antibacterial activity of these two fractions. Furthemore, quinoxaline-2-carboxamide was also found in fractions 6 and 7. Quinoxaline moiety is a part of many antibiotics such as echinomycin, levomycin and actinomycin having antibacterial activity against Gram-negative bacteria and anti-proliferative activities (Patidar *et al*, 2011). Levomycin and actinoleukin were the first antibiotics in the quinoxaline family produced by *Streptomyces* (Katagiri *et al.*, 2011). In this study, fraction 7 showed stronger antibacterial activity against both MRSA and *C. violaceum* than fraction 6 that exhibited moderate activity against only MRSA with 128 times higher MIC. Significant antibacterial activity was related with the ratio of these major compounds. Fraction 7 contained 3-nitro-1,2-benzenedicarboxylic acid and quinoxaline-2-carboxamide 2 times and 6.5 times higher than fraction 6. Therefore, the antibacterial activity of fraction 7 may be due to the synergistic activity among these two compounds.

GC-MS analysis of AMA50CH was also carried out. Seven fatty acids were found as major components in this extract including n-hexadecanoic acid, tetradecanoic acid, pentadecanoic acid, heptadecenoic acid, palmitoleic acid, hexadecanoic acid 2-hydroxyl-1-(hydroxymethyl) ethyl ester and hexadecanoic acid methyl ester. Several reports have been published on the antifungal activity of these fatty acids. Altieri *et al*. (2007) reported antifungal activity of pure n-hexadecanoic acid and tetradecanoic acid against *Aspergillus* and *Penicillium* species. In addition, tetradecanoic acid and palmitoleic acid have been reported to have antifungal property

against *C. albicans* (Kabara *et al*., 1972). Heptadecenoic acid produced by *Pseudozyma flocculosa* exhibited the inhibitory activity on growth and germination of *Botrytis cinerea* and *Cladosporium cucumerinum* (Avis and Belanger, 2001). The target of fatty acids is the lipid bilayer in fungal membrane. The antifungal fatty acids penetrate themselves into the membrane, disturb membrane functions causing an increased membrane fluidity and leakage of intracellular components and cell death. Moreover, another target is protein *N*-myristoyltransferase (NMT), an essential enzyme for growth and survival of several fungi and yeasts. Tetradecanoic (myristic) acid can bind to this enzyme and disturb protein function (Pohl *et al.*, 2011). Furthermore, Jain *et al*. (2012) reported that n-hexadecanoic acid has nematicidal activity. This compound may be responsible for the cytotoxicity of AMA50CH toward *C. elegans*.

4.3 Identification of potential actinomycetes

The 16S rRNA sequence analysis is a standard method for bacterial identification and taxonomy (Janda and Abbott, 2007). However, this method has a weakness in the taxonomy of some actinomycete genera such as *Mycobacterium* and *Streptomyces*. Some strains of these genera have multi-copy genes of 16S rRNA within one organism that affects different sequences (Kim *et al*., 2003). Several cumulative results suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65-83%). A minimum of 500-525 bp (ideal 1300-1500 bp) is important for phylogenetic study. There is no universal definition for species identification via 16S rRNA gene sequencing. However, the proposed criteria from Drancourt *et al*. (2000) are recommended for bacterial identification. These criteria for species identification based on a minimum of >99% similarity (ideal >99.5% similarity) of sequence that matched to type strain or reference strain. For matches with distance cores <0.5% to

the nest closest species, other properties such as phenotype should be considered in final species identification (Janda and Abbott, 2007).

From a total of 525 marine-derived actinomycetes obtained in this study, 63 isolates were chosen for molecular identification based on morphology and antimicrobial activity. Among them, 19 strains possessed strong antimicrobial activities. The majority of the strongly active strains in our study were in the genera *Actinomadura* (1 strain), Uncertain species (2 strains) and *Streptomyces* (16 strains).

4.3.1 Genus *Actinomadura*

The 16S rRNA gene sequence similarity calculation indicated that strain ASE 22 closest matched with *Actinomadura geliboluensis* A8036^T (100% similarity with no nucleotide difference). ASE22 grew well on ISP no. 2 medium. It produced white substrate and aerial mycelium that slightly distinguished from the type strain *A. geliboluensis* A8036^T (Sazak *et al*., 2012). Short chain spores often formed as spiral. A phylogenetic tree analysis showed strain ASE22 formed phyletic line with *A. geliboluensis* A8036^T (99% boostrap support). On the basis of morphological features and molecular data, ASE22 was identified as *A. geliboluensis*. Although members of the Genus *Streptomyces* are the common source of the antibiotic producer, but rare actinomycetes are being increasingly recognized as a good source of novel antibiotics (Shin *et al*, 2016). Many *Actinomadura* species produced chemical and biologically active compounds with antitumor, antifungal, anti-HIV and antibacterial activities (Igarashi *et al.*, 2012). In this study, all three extracts of ASE22 showed strong activity against *S. aureus* (MIC 1-4 µg/ml) and strong to moderate activity against MRSA (4-16 µg/ml) but could not inhibit the Gram-negative bacteria. Chandrananimycins A, B and C were isolated from the culture broth of *Actinomadura* sp. M048 derived from marine sediment in China. Among them, chandrananimycin C exhibited the strongest activity against *Bacillus subtilis* and *S. aureus* but none of the compounds was active against *E. coli* (Maskey *et al*. 2003). IB-00208 compound was isolated from marine *Actinomadura* sp. from the northern coast of Spain. It showed cytotoxic activity against tumor cells and also had a bactericidal activity against

Gram-positive bacteria (Malet-Cascon *et al.*, 2003). Our extracts showed bacterostatic activity against *S. aureus* and MRSA as their MBCs were higer than 4 times MICs.

4.3.2 Genus *Streptomyces*

Strain AMR92 showed moderate to strong activity against Gram-positive bacteria exhibiting bacteriostatic activity. Aerial mycelium of white-gray consisted of RF spore chains. Substrate mycelium was brown. The nearly complete 16S rRNA gene sequence and phyogenetic tree analysis revealed that AMR92 is closely related to *S. griseorubens* NBRC 12780^{T} (99.65% similarity, 5/1445 nt differences) with 73% boostrap support. Therefore, it was identified as *S. griseorubens*. In 2009, *S. griseorubens* WBF9 was isolated from marine sediment sample. Active fractions from its ethyl acetate extract showed antitumor activity. These fractions contained alkaloid, terpene, peptide and others (Ye *et al.*, 2009). *S. griseorubens* E44G isolated from terrestrial soil samples showed antifungal activity against various phytopathogenic fungi. In addition, strain E44G exhibited low antibacterial activity against Gram-negative bacteria such as *P. aeruginosa* and *E. coli* but no activity against Gram-positive bacteria (Al-Askar *et al.*, 2014).

AMR77 produced red substrate mycelium and white-gray aerial mycelium that formed spores with RA or S chains. Its morphological characteristics were distinguished from the type strain *S. spongiicola* HNM0071^{T}. However, sponge-associated *S. spongiicola* HNM0071^{T} was most closely related to AMR77 (99.49 % similarity, 7/1369 nt differences). Phylogenetic analysis revealed that AMR77 formed monophelytic line with *S. spongiicola* HNM0071^{T} (79% boopstrap support). Based on morphology and molecular approach, AMR77 was identified as *Streptomyces* sp. Strain AMR77 possessed strong activity against Gram-positive bacteria but weak activity against Gram-negative bacteria (MIC 200 µg/ml). Secondary metabolites from this strain exhibited bacteriostatic activity.

Phylogenetic analysis showed that strains AMR71, AMA50, AMA52 and AMA74 were clustered. All strains, except AMA74, exhibited strong antifungal

activity. AMA74 inhibited Gram-positive bacteria. Their morphological characteristics were consistent with members in the genus *Streptomyces*. They formed brown aerial mycelium which differentiated into S chains. Their substrate mycelia were yellow. Comparisons of 16S rRNA gene sequences with corresponding sequences available on the BioEZcloud server revealed that AMR71, AMA50, and AMA52 had the highest similarity with *S. phaeoluteichromatogenes* NRRL 5799^T (99.44, 98.70 and 99.51% similarity, respectively). Only AMA74 was closely related with *S. misionensis* DSM 40306^T (99.33% similarity). Their nucleotides were different from the closely related type strain more than 7 nucleotides. Therefore, all strains in this cluster were identified as *Streptomyces* spp. Ethyl acetate exctract from cells of AMR71 (AMR71CE) produced strong antifungal activity against *C. albicans* (MIC/MBC 4/4 µg/ml), *C. neoformans* (MIC/MBC 1/1 µg/ml) and *T. marneffei* (MIC/MBC 4/4 µg/ml), and AMR71CH exhibited strong activity to *T. marneffei* (MIC/MBC 4/4 µg/ml) and moderated activity to *C. neoformans* (MIC/MBC 32/64 µg/ml). SEM study of fungal cells treated with AMR71CE revealed pores, deep wrinkled and collapsed surfaces of *C. albicans* and *C. neoformans* as well as pores, wrinkled and flattened mycelia of the treated *T. marneffei*. Shin *et al*. (2003) found fugichromin from *S. padanus* PMS-72 that caused hyphal collapse in *Rhizoctonia solani*. This mechanism was similar to the function of polyenes group. Amphotericin B and nystatin are polyene macrolide antibiotics from actinomycetes which interact and damage cell membrane by forming permeable membrane channels on fungal hyphae (Fenical and Jensen, 2006). In this study, only AMA74 produced strong antibacterial activity against *S. aureus* and MRSA with MIC ranging from 4-16 µg/ml.

Strain AMA44 formed yellow substrate mycelium, white aerial mycelium producing RA and S spore chains. The highest 16S rRNA gene sequence similarity value was found with *S. levis* NBRC 15423^T (99.09%, 13/1425 nt differences). In phylogenetic tree, AMA44 consistently formed a distinct phyletic line with type strain *S. aurantiogriseus* NBRC12842^T (98.89% similarity, 16/1440 nt differences) by 59% bootstrap support. Based on the differences of morphology and position on phylogenetic analysis, AMA44 could only be identified as *Streptomyces* sp.

Secondary metabolites from *Streptomyces* sp. AMA44 exhibited strong antibacterial activity against Gram-positive bacteria such as *S. aureus* and MRSA. It showed bacteriostatic activity toward *S. aureus* but possessed bactericidal activity against MRSA.

AMR9 produced yellow substrate mycelium and green aerial mycelium that differentiated to S spore chains. It was related with *S. coeruleofuscus* NBRC 12757^T with 99.45% similarity (8/1443 nt differences). In phylogenetic tree, AMR9 formed a distinct cluster with type strains *S. naganishii* NBRC 12892^T (98.96% similarity) and *S. spiralis* NBRC 14215^T (98.95% similarity) by bootstrap support lower than 50%. Based on the differences of morphology and position on phylogenetic analysis, AMR9 could only be identified as *Streptomyces* sp. AMR9 extract exhibited strong activity against *S. aureus* and MRSA and weak activity against all strains of *A. baumannii*.

Strain AMA21 developed gray aerial mycelium and brown substrate mycelium on ISP2 medium. This strains was related to *S. violaceorubidus* LMG 20319^T with 98.61% similarity (20/1440 nt differences). A neighbor-joining tree based on 16S rRNA gene sequences showed the position of AMA21 that formed an independent clade with the type strains of *S. gramineus* JR-43^T, *S. griseoluteus* NBRC 13375^T, *S. recifensis* NBRC 12813^T and *S. seoulensis* NRRL B-24310^T. Its extract exhibited strong activity against *S. aureus* and MRSA, moderate activity against *A baumannii* NPRC001 and weak activity against *A. baumannii* NPRC002-005, 007.

Strain ALA175 revealed S spore chains on white-gray aerial mycelium and produced yellow substrate mycelium. 16S rRNA gene sequence showed that ALA175 was closely related to the type strain *S. thermoviolaceus* subsp. *thermoviolaceus* DSM 40443^T (98.96% similarity and 15/1440 nt differences). Phylogenetic tree revealed that its closest type strain was *S. caeni* HA 15955^T (98.82% similarity). Therefore, ALA175 was named as *Streptomyces* sp. Its extract exhibited strong activity against *S. aureus* and MRSA.

Strain AMR82 formed yellow substrate mycelium and white aerial mycelium which carried long RF chains of spores. It shared high percentage 16S rRNA gene sequence similarity values with *S. pluripotens* MUSC 135^T (99.86% similarity, 2/1440 nt differences). Phylogenetic analysis revealed that AMA82 formed a distinct clade with the type strain *S. pluripotens* MUSC 135^T at high bootstrap value of 99%. Therefore, AMR 82 could be identified as *S. pluripotens*. The type strain MUSC 135^T was isolated from mangrove soil in Malaysia. In addition, MUSC 135^T exhibited a broad-spectrum bacteriocin against MRSA ATCC BAA-44, *Salmonella* Typhi ATCC 19430^T and *Aeromonas hydrophila* ATCC 7966^T (Lee *et al*., 2014). In 2015, Ser *et al*. recently reported antioxidant activity of fermentation products from this strain. In their further study, they reported the draft genome sequence of MUSC 135^T and five clusters of biosynthetic genes. These genes were associated with secondary metabolite production including ectoine, albaflavenone, desferrioxamine, avtimycin and informatipeptin. In this study, BE extract from AMR82 exhibited a broad spectrum activity against all the bacteria tested with strong bactericidal activity against MRSA (MIC/MBC 4/8 µg/ml), moderate activity against *S. aureus* and *C. albicans* and weak activity against Gram-negative bacteria and *C. neoformans*.

Strain AMR67 was identified as *Streptomyces* sp. It produced brown substrate mycelium and gray aerial mycelium which diffentiated to RF spore chains. Phylogenetic analysis exhibited that AMR67 was closely related to *S. yogyakartensis* NBRC 100779^T, *S. javensis* NBRC 100777T and *S. violaceusniger* NBRC 13459^T at 93% bootstrap value. Strain AMR67 exhibited strong antibacterial activity and moderate to weak antifungal activity. SEM analysis of MRSA cells after treatment with AMR67CH revealed the damaged cells similar to the vancomycin-treated cells.

Strain AMR107 was found to belong to a distinct clade with *S. malaysiensis* NBRC 16446^T with 79% bootstrap value support (100% similarity). The brown substrate mycelium and the gray aerial mycelium was similar to the closest type strain. Therefore, AMR107 could be named as *S. malaysiensis*. All three extracts of AMR107 showed strong activity against *S. aureus* and MRSA with MIC values of 0.25 to 4 µg/ml, and ethyl acetate extract from culture broth (BE) presented moderate

activity against *C. albicans* and weak activity to *T. marneffei*. Activities of *S. malaysiensis* were determined by several studies. For instances, *S. malaysiensis* KC-153 isolated from Thailand showed a broad spectrum antimicrobial activity against *B. subtilis, Kocuria rhizophila, E coli, Xanthomonas campestris, C. albicans* and *Mucor racemosus* (Sripreechasak *et al*., 2013). Cheng *et al.* (2010) found azalomycin F complex that produced by *S. malaysiensis* MJM1968. It showed a broad spectrum antifungal activity against soil fungal pathogens. In addition, marine-derived *S. malaysiensis* O4-6 possessed algicidal activity to the harmful algal bloom (Zheng *et al.*, 2013).

The strains AMA11 and AMA12 were closely related with *Kitasatospora psammotica* NBRC13971^{T} (99.49 % similarity, 7/1386 nt differences and 99.30 % similarity, 10/1437, respectively) with high bootstrap support (90% bootstrap and 99.40% identity) in all three methods. The color of substrate mycelia of AMA11 and AMA12 was yellow-brown. The color of substrate mycelia of AMA11 and AMA12 was yellow-brown. The aerial mycelia at maturity formed many spores in spiral (S) or open loop (RA) chains. Colony color was green that can be distinguished from the type strain. Previous studies revealed the differences among the 16S rRNA-based phylogenetic trees leading to more questions on the validity of genus *Kitasatospora*. *K. psammotica* NBRC 13971^{T} was formerly named as *S. psammotica*. To confirm this validity, the multi-locus sequences analysis (MLSA) has been used to study the relationship among them and the result clearly observed the outside radiation of the genus *Kitasatospora* from the genus *Streptomyces*. This study has been held in the ARS Microbial Genomic Sequence database to find the differences of genes in the genera *Kitasatospora* and *Streptomyces*. Therefore, the type strains of many validly named *Streptomyces* species clearly belong within the genus *Kitasatospora* (Labeda *et al,* 2017). However, Figure 26b showed the other species of the genus *Kitasatospora* were monophelytic and were formed a tight cluster from *Streptomyces*. Therefore, two strains, AMA11 and AMA12 could not be identified as genus *Kitasatospora* or *Streptomyces*. They were marked as uncertain strains. AMA11 and AMA12 produced secondary metabolites exhibiting a broad spectrum bateriostatic activity aginst Gram-positive and Gram-negative bacteria.

Strain AMR69 produced RF spore chains on aerial mycelium like a closely related type strain *S. antibioticus* NBRC 12838^T. Its substrate mycelium was yellow color. AMR69 showed the highest similarity (99.75%, 3/1194 nt differences) with *S. antibioticus* NBRC 12838^T. In a rooted phylogenetic tree based on 16S rRNA gene sequence, AMR69 represented a distict phyletic line with type strain *S. antibioticus* NBRC 12838^T (97% boostrap support). Based on morphology and molecular approaches, AMR69 was identified as *Streptomyces* sp. AMR69 possessed the strong activity against Gram-positive bacteria and moderate to weak activity agaist Gram-negative and fungi. The BE and CH extracts exhibited strong activity against *S.aureus* and MRSA (MIC ≤0.03 to 0.13 µg/ml). Possible mechanisms of action of AMR69BE and AMR69CH against MRSA observed by SEM revealed the damaged cells similar to vancomycin treated cells. Oppegard *et al*. (2009) isolated simocyclinone D8 (SD8) from *S. antibioticus* Tü6040 and reported its antibacterial activity against *S. aureus*, *B. brevis* and *B. subtilis*. *S. antibioticus* M7 produced antibacterial metabolites such as actinomycins V, X_2 and D. All of these three actinomycins were effective against MRSA and VRE with MIC values of 1.95-2.25 µg/ml and 3.5-4.0 µg/ml, respectively. In addition, these compounds exhibited activity against *K. pneumonia*e sub sp. *pneumonia* and *B. subtilis* (MIC 1.95 and 31.25 µg/ml, respectively) (Sharma and Manhas, 2019). Antifungal compounds, antimycins A_{19} and A_{20} were isolated from *S. antibioticus* H74-18 which showed potential activity against *C. albicans* with MIC of 5-10 µg/ml (Xu *et al*., 2011).

Strains ALA166 and ALA167 formed gray substrate mycelium and white aerial mycelium that diffentiated to RA spore chains. Their morphological features were different from the type strain *S. bungoensis* DSM 4178^T. Results from BLAST algorithm indicated that ALA166 and ALA167 were closely related with *S. bungoensis* DSM 4178^T with 99.58 and 99.76% similarity (6/1442 and 3/1237 nt differences). A phylogenetic tree reconstructed with various *Streptomyces* type strains based on 16S rRNA gene sequencing showed that ALA166 and ALA167 formed a distinct line closest to *Streptomyces shaanxiensis* CCNWHQ 0031^T at 58% bootstrap support. Therefore, these two strains were identified as *Streptomyces* spp. Strains ALA166 and ALA167 produced secondary metabolites exhibiting strong activity

against *S. aureus* and MRSA, moderate activity against *C. neoformans* and week activity against *A. baumanii* strains and *C. albicans.* The effect of ethyl acetate extract from cells of ALA166 (ALA166CE) was determined by SEM. MRSA cells after treatment with ALA166CE have been found shrunken or broken cells as the vancomycin treated samples.

4.4 *Gordonia sediminis* sp. nov. AMA120^T, a novel species of marine-derived actinomycete

Marine environments are a rich source of diverse microorganisms including actinomycetes (Subramani and Sipkema, 2019). The majority of actinomycetes in this study was identified to genus level and may be considered as new species. Strains AMA120^T was a fast growing actinomycete that identified as *Gordonia* sp. Genus *Gordonia* was proposed by Tsukamura (1971) and to date, 39 species have been identified and published their names (http://www.bacterio.net/gordonia.html). They are originally isolated from sputa of patients with pulmonary disease (Tsukamura, 1971). In addition, members of the genus *Gordonia* are also predominant in mangrove habitat (Sowani *et al*., 2017). Strain AMA120^T was isolated from mangrove sediment. The chemotaxonomic and morphological study classified strain AMA120^T to the genus *Gordonia.* Phylogenetic trees baed on 16S rDNA sequence alignment with species of *Gordonia* revealed the distinct phyletic line of AMA120^T. The *gyrB* gene was chosen to confirm that strain AMA120^T is a novel species. The *gyrB* gene is an alternative target for the identification of *Gordonia* species because this gene is present only a single copy in bacterial species and cannot spread among different bacterial species (Shen *et al*., 2006). In addition, DNA-DNA relatedness values between AMA120^T and its closest neighbours were lower than 70% which is the cut-off point recommended by Wayne *et al* (1987). Likewise, the ANI values were below 94% that corresponded to the standard of the species demarcation (Konstantinidis and Tiedije, 2005). The differential genotypic properties of strain AMA120^T were

sufficient to classify it as novel species in the genus *Gordonia*. Similarly, the abilities to produce acids from mannitol, fructose and sucrose of strain AMA120^{T} differentiated it from the closest type strains. It is therefore proposed that AMA120^{T} should be recognized as the type strain of a novel species with the name *Gordonia sediminis* sp. nov.

CHAPTER 5

CONCLUSIONS

1. In this study, a total of 525 marine-derived actinomycetes were isolated from sediments and various marine organisms collected from the southern provinces of Thailand. They were preliminary classified into eight families based on morphological characteristics. Members of family *Streptomycetaceae* were the dominant population in this study.

2. Primary screening by a cross streak and hyphal inhibition tests against human pathogens revealed that 57.7% of representatives from all families except *Jianglellaceae* and *Nocardiaceae* showed inhibitory activity against at least one test strain. They were highly active against filamentous fungi, followed by Gram-positive bacteria, yeasts and Gram-negative bacteria.

3. The 104 fast-growing active actinomycetes were selected for fermentation and extraction. Among 287 extracts obtained, 160 showed a varying spectrum of activity and the most active type of extracts was the CE. For Gram-positive bacteria, *S. aureus* was found to be most susceptible to the CE and CH, whereas MRSA was most susceptible to the CE. The CH could most inhibit Gram-negative bacterium (*A. baumannii),* yeasts and filamentous fungi. In addition, only two BEs and one CE inhibited *E. coli*.

4. All active extracts were further determined for their MICs, MBCs/MFCs. The MIC values of extracts ranged from ≤0.03 to 200 µg/ml and the MBCs and MFCs from 0.25 to >200 µg/ml and 1 to >200 µg/ml, respectively. Thirty-nine extracts from 19 isolates showed strong inhibitory activity (MICs ≤8 µg/ml), 33 against bacteria and six against fungi.

5. AMA11CE exhibited the broadest activity against both Gram-positive bacteria, *S. aureus* and MRSA (MIC 0.5 µg/ml) and Gram-negative bacteria, *A. baumannii* (MICs 8-64 µg/ml) and *E. coli* (MIC 64/128 µg/ml). Furthermore, the evaluation antibacterial potential of AMA11CE revealed that it could inhibit biofilm formation by *S. epidermidis* and quorum sensing in *C. violaceum*.

6. For antifungal activity, AMR71CE had the broadest antifungal activity against *C. albicans, C. neoformans* and *T. marneffei* with MICs of 4, 1 and 4 µg/ml, respectively while AMA50CH showed the best MIC against *T. marneffei* (0.5 µg/ml). Thus, AMA50CH was further studied *in vivo* using *T. marneffei* infected *C. elegans*. This extract could protect the survival of *T. marneffei* infected *C. elegans* around 60-70%.

7. The composition of active extracts, AMA11CE and AMA50CH were determined by GC-MS analysis. Active fraction of AMA11CE contained two compounds (3-nitro-1,2-benzenedicarboxylic acid and quinoxaline-2-carboxamide) that may be associated with antibacterial activity. AMA50CH contained seven compounds as major components that have been reported on the antifungal activity. Scanning electron microscopic studies revealed that these active extracts may act on cell wall and cell membrane of pathogens.

8. Sixty-three marine-derived actinomycetes were identified by molecular technique based on the 16S rDNA sequence analysis. They belonged to six orders and eight genera namely *Streptomyces* (35 isolates), *Gordonia* (4 isolates), *Nocardia* (3 isolates), *Mycobacterium* (1 isolate), *Pseudonocardia* (4 isolates), *Actinomadura* (4 isolates), *Jiangella* (1 isolate), *Micromonospora* (9 isolates) and 2 isolates of uncertain species.

9. Strain AMA120^{T} was classified as a novel species in the genus *Gordonia* using a polyphasic study such as phenotypic, chemotaxonomic and genotypic (16S rRNA sequencing) characterisitics as compared to the closely related type strains. Therefore, the strain AMA120^{T} was proposed as the type strain of a novel species with the name *Gordonia sediminis* sp. nov.

10. This study can be concluded that marine environments in the South of Thailand would be a good source of novel and antimicrobial producing marine-derived actinomycetes.

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http://www.bacterio.net/

http://www.bacterio.net/gordonia.html

APPENDIX 1

1. M9 buffer	**1 litre**
KH_2PO_4	3.0 g
Na_2HPO_4	6.0 g
NaCl	0.5 g
NH_4Cl	1.0 g
pH	7.0
Autoclaved at 121^{o}C/15 min, 15 pounds/inch^2	

2. Chitin agar	**1 litre**
Colloidal chitin	20.0 ml
K_2HPO_4	0.7 g
KH_2PO_4	0.3 g
$MgSO_4 \cdot 5H_2O$	0.5 g
$FeSO_4 \cdot 7H_20$	0.01 g
$ZnSO_4$	0.001 g
$MnCl_2$	0.001 g
Agar	20.0 g
pH	7.2
Autoclaved at 121^{o}C/15 min, 15 pounds/inch^2	

3. Humic acid vitamin agar (HV)	**1 litre**
Humic acid	1.0 g
KCl	1.7 g
Na_2HPO_4	0.5 g
$MgSO_4$	0.5 g
$CaCO_3$	0.02 g
$FeSO_4$	0.01 g
Vitamin B stock solution	1.0 ml

Agar	20.0 g
pH	7.2

Autoclaved at 121^{o}C/15 min, 15 pounds/inch^{2} (Vitamin B stock solution is a filter sterilized that contained of 50 mg of vitamin B_1, vitamin B_2, niacin, vitamin B_6, D-calcium pantothenate, inositol, and para amino acid, 25 mg of biotin, and 100 mL distilled water. Vitamin stock solution added to autoclaved media after cooled down to 55°C)

4. International *Streptomyces* Project (ISP) 2 **1 litre**

Malt extract	10.0 g
Yeast extract	4.0 g
Glucose	4.0 g
Agar	15.0 g
pH	7.2

Autoclaved at 121^{o}C/15 min, 15 pounds/inch^{2}

5. Modified soil extract agar (A9) **1 litre**

Soil extract	100.0 ml
$CaSO_4 \cdot 2H_2O$	0.5 g
$MgSO4 \cdot 7H_2O$	0.05 g
K_2SO_4	0.03 g
KH_2PO_4	0.02 g
$NaHCO_3$	0.1 g
$CaCl_2 \cdot 2H_2O$	0.02 g
Trace salt solution	0.3 ml
Yeast extract	0.1 g
Casamino acids	0.1 g
Glucose	0.2 g
Agar	20.0 g
pH	7.0

Autoclaved at 121^{o}C/15 min, 15 pounds/inch^{2}

6. Nematode growth medium (NGM) **1 litre**

NaCl	3.0 g
Peptone	2.5 g
Agar	17.0 g
1 M $MgSO_4$	1.0 ml
1 M $CaCl_2$	1.0 ml
Cholesterol (5 mg/ml in ethanol)	1.0 ml
pH	7.0

Autoclaved at 121^{o}C/15 min, 15 pounds/inch^2 (Cholesterol added to autoclaved media after cooled down to 55°C)

7. Potato dextrose agar (PDA) **1 litre**

Potato	200.0 g
Glucose	20.0 g
Agar	15.0 g
pH	7.0

Autoclaved at 121^{o}C/15 min, 15 pounds/inch^2

8. Starch nitrate agar (SN) **1 litre**

Soluble starch	20.0 g
K_2HPO_4	1.0 g
KNO_3	2.0 g
$MgSO_4$	0.5 g
$CaCO_3$	3.0 g
NaCl	0.5 g
$FeSO_4$	0.1 g
$MnCl_2$	0.1 g
$ZnSO_4$	0.1 g
pH	7.2

Autoclaved at 121^{o}C/15 min, 15 pounds/inch^2

9. Starch-yeast extract-peptone-seawater agar (SYP-SW) 1 litre

Starch	10.0 g

Yeast extract	4.0 g
Peptone	2.0 g
NaCl	33.3 g
pH	7.0

Autoclaved at 121^{o}C/15 min, 15 pounds/inch^{2}

APPENDIX 2

Table 20 Type, characteristic and identification of marine samples and marine-derived actinomycete obtained from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
1	Mangrove forest/Songkhla	Sediment	5-10 cm	Dark grey sediment	AMA9, AMA10, AMA11, AMA12, AMA13, AMA14, AMA15, AMA16, AMA17, AMA18, AMA51, AMA52, AMA53, AMA54, AMA73, AMA74, AMA88, AMA89, AMA90, AMA101, AMA102, AMA103, AMA104, AMA105, AMA121, AMA130, AMA131, AMA132, AMA149, AMA150, AMA151, AMA152
2	Mangrove forest/Songkhla	Sediment	5-10 cm	Dark grey sediment	AMA1, AMA2, AMA3, AMA72, AMA75, AMA91, AMA92, AMA93, AMA94, AMA95, AMA106, AMA107, AMA108, AMA109, AMA110, AMA111,

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
2	Mangrove forest/Songkhla	Sediment	5-10 cm	Dark grey sediment	AMA112, AMA113, AMA119, AMA120, AMA122, AMA123, AMA133, AMA134, AMA135, AMA136, AMA137, AMA138, AMA139, AMA140, AMA141, AMA142, AMA154, AMA155, AMA156
3	Mangrove forest/Songkhla	Sediment	5-10 cm	Dark grey sediment	AMA4, AMA5, AMA6, AMA7,AMA8, AMA62, AMA63, AMA64, AMA124, AMA143
4	Mangrove forest/Songkhla	Sediment	5-10 cm	Dark grey sediment	AMA19, AMA20, AMA21, AMA22, AMA23, AMA24, AMA25, AMA26, AMA27, AMA28, AMA29, AMA30, AMA31, AMA32, AMA33, AMA34, AMA35, AMA36, AMA37, AMA38, AMA39, AMA40, AMA41, AMA42,

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
4	Mangrove forest/Songkhla	Sediment	5-10 cm	Dark grey sediment	AMA43, AMA44, AMA45, AMA46, AMA47, AMA58, AMA59, AMA60, AMA65, AMA66, AMA67, AMA68, AMA69, AMA70, AMA78, AMA79, AMA80, AMA81, AMA82, AMA83, AMA84, AMA85, AMA86, AMA87, AMA96, AMA97, AMA98, AMA99, AMA100, AMA114, AMA115, AMA125, AMA126, AMA144, AMA145, AMA157
5	Mangrove forest/Songkhla	Sediment	5-10 cm	Dark grey sediment	AMA48, AMA49, AMA50, AMA55, AMA56, AMA76, AMA77, AMA116, AMA117, AMA118, AMA127, AMA128, AMA129, AMA146, AMA147, AMA148, AMA158, AMA159, AMA160

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
6	Mangrove forest/Songkhla	Water	Surface water	-	-
7	Mangrove forest/Songkhla	Water	Surface water	-	-
8	Mangrove forest/Songkhla	Water	Surface water	-	-
9	Mangrove forest/Songkhla	Water	Surface water	-	-
10	Mangrove forest/Songkhla	Water	Surface water	-	-
11	KohBulon/Satun	Sediment	6.5 m	Sand	ASE13, ASE15, ASE16
12	KohBulon/Satun	Sediment	5.0 m	Sand	ASE2, ASE3, ASE4, ASE5, ASE7, ASE9, ASE10, ASE11, ASE12, ASE14, ASE17, ASE20, ASE21
13	KohBulon/Satun	Sediment	1.8 m	Sand	ASE1, ASE22,

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
14	KohBulon/Satun	Sponge	3.8 m	*Xestospongia* sp. 	-
15	KohBulon/Satun	Sponge	5.5 m	*Dysidea* sp. 	AMR1, AMR2, AMR3

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
16	KohBulon/Satun	Sponge	2.0 m	*Hyrtios* sp. 	-
17	KohBulon/Satun	Nudibranch	Coral reef		-
18	Sikao Bay/Trang	Sediment		Clay	
19	Sikao Bay/Trang	Sediment		Brown-reddish clay	ASE18, ASE19, ASE23, ASE25, ASE26, ASE27, ASE29, ASE30

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

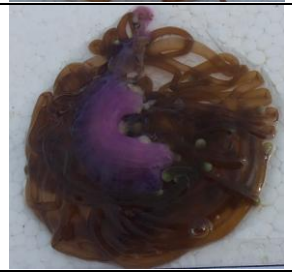
No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
20	Sikao Bay/Trang	Sea anemone			-
21	Sikao Bay/Trang	Sea anemone			-
22	Sikao Bay/Trang	Sea anemone			-

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
23	Sikao Bay/Trang	Algae		*Padina* sp. 	AMR4, AMR5, AMR6, AMR7, AMR8, AMR9, AMR10, AMR11, AMR12, AMR13, AMR14, AMR15, AMR16, AMR17, AMR18, AMR19, AMR20, AMR21, AMR23, AMR24, AMR25, AMR26, AMR27, AMR28, AMR29, AMR30, AMR31, AMR32, AMR33, AMR34, AMR35, AMR36, AMR37, AMR38, AMR39, AMR40, AMR41, AMR42, AMR43, AMR44, AMR45, AMR47, AMR48, AMR49, AMR51, AMR52, AMR53, AMR54, AMR56, AMR57, AMR59, AMR60, AMR62
24	Sikao Bay/Trang	Sediment		Clay and sand	-

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
25	Songkhla Lake/Songkhla	Sediment	0 - 5 cm	Black clay	ALA13, ALA26, ALA27, ALA28, ALA29, ALA30, ALA32, ALA34, ALA35, ALA36, ALA62, ALA94, ALA128, ALA165, ALA166, ALA167, ALA168, ALA169, ALA170, ALA171
26	Songkhla Lake/Songkhla	Sediment	6 - 10 cm	Black clay	ALA1, ALA2, ALA3, ALA4, ALA14, ALA15, ALA23, ALA24, ALA25, ALA63, ALA116, ALA120, ALA148, ALA172, ALA173, ALA174, ALA175
27	Songkhla Lake/Songkhla	Sediment	11 - 15 cm	Black clay	ALA5, ALA6, ALA7, ALA16, ALA17, ALA18, ALA21, ALA31, ALA33, ALA121, ALA176, ALA177, ALA181, ALA182

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
28	Songkhla Lake/Songkhla	Sediment	16 - 20 cm	Black clay	ALA8, ALA9, ALA10, ALA11, ALA12, ALA19, ALA20, ALA22, ALA178, ALA179, ALA180, ALA183
29	Songkhla Lake/Songkhla	Sediment	21 - 25 cm	Black clay	ALA37, ALA38, ALA39, ALA40, ALA41, ALA42, ALA43, ALA44, ALA45, ALA46, ALA47, ALA48, ALA49, ALA71, ALA72, ALA73, ALA76, ALA82, ALA83, ALA85, ALA97, ALA117, ALA122, ALA123, ALA129, ALA130, ALA131, ALA132, ALA133, ALA134, ALA135, ALA136, ALA137, ALA138, ALA139, ALA140, ALA141, ALA142, ALA143, ALA144, ALA145, ALA146

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
30	Songkhla Lake/Songkhla	Sediment	Depth of 26 - 30 cm	Black clay	ALA50, ALA51, ALA52, ALA53, ALA54, ALA55, ALA56, ALA57, ALA58, ALA59, ALA60, ALA61, ALA64, ALA65, ALA66, ALA67, ALA68, ALA69, ALA70, ALA74, ALA75, ALA77, ALA78, ALA79, ALA80, ALA81, ALA84, ALA86, ALA87, ALA88, ALA89, ALA90, ALA91, ALA92, ALA93, ALA95, ALA96, ALA98, ALA99, ALA100, ALA101, ALA102, ALA103, ALA104, ALA105, ALA106, ALA107, ALA108, ALA109, Ala110, ALA111, ALA112, ALA113, ALA114, ALA115, ALA118,

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
30	Songkhla Lake/Songkhla	Sediment	26 - 30 cm	Black clay	ALA119, ALA124, ALA125, ALA126, ALA127, ALA147, ALA149, ALA150, ALA151, ALA152, ALA153, ALA154, ALA155, ALA156, ALA157, ALA158, ALA159, ALA160, ALA161, ALA162, ALA163, ALA164
31	Phuket Coastal Fisheries Research and development Center/Phuket	Sea squirt		*Ascidia nigra* 	AMR130, AMR131, AMR135, AMR136, AMR137, AMR138, AMR139, AMR143, AMR144, AMR159, AMR160, AMR164

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
32	Phuket Coastal Fisheries Research and development Center/Phuket	Sea squirt	-	*Aplidium multiplicata* 	AMR113, AMR126
33	Phuket Coastal Fisheries Research and development Center/Phuket	Sea squirt	-	*Molgula* sp. 	AMR90, AMR91, AMR92, AMR107, AMR140, AMR162

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
34	Phuket Coastal Fisheries Research and development Center/Phuket	Sea squirt	-	*Didemnum* sp. 	AMR111, AMR112, AMR127, AMR128, AMR132, AMR133, AMR141
35	Phuket Coastal Fisheries Research and development Center/Phuket	Sea squirt	-	*Molgula* sp. 	AMR106, AMR108, AMR119, AMR120, AMR121, AMR122, AMR125, AMR134, AMR150

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
36	Phuket Coastal Fisheries Research and development Center/Phuket	Sea fan	-	*Melithaea* sp. 	AMR63, AMR64
37	Phuket Coastal Fisheries Research and development Center/Phuket	Algae	-	*Gracilaria* sp. 	AMR65, AMR69, AMR75, AMR76, AMR78

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
38	Phuket Coastal Fisheries Research and development Center/Phuket	Algae	-	*Acanthophora spicifera* 	AMR66, AMR67, AMR72, AMR73, AMR74, AMR80, AMR81, AMR83, AMR84, AMR85, AMR86, AMR87, MR129, AMR145, AMR146, AMR147, AMR148
39	Phuket Coastl Fishries Research and development Center/Phuket	Algae	-	*Padina* sp. 	AMR70, AMR71, AMR77, AMR88, AMR89

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
40	Khao Phlai Dam/Nakhonsrithammarat	-	On rock	Unidentified 	-
41	Khao Phlai Dam/Nakhonsrithammarat	Algae	On rock	*Sargassum* sp. 	-
42	Khao Phlai Dam/Nakhonsrithammarat	Algae	On rock	*Padina* sp. 	AMR68

Table 20 (Cont.) Type, characteristic and identification of marine samples including marine-derived actinomycete from each sample

No.	Site/Province	Type	Depth	Characteristic or identification of sample	Actinomycete code
43	Costal/Songkhla	Sponge		*Microciona* sp. 	AMR82, AMR93, AMR94, AMR95, AMR96, AMR97, AMR98, AMR99, AMR100, AMR101, AMR102, AMR103, AMR104, AMR105, AMR109, AMR110, AMR114, AMR115, AMR116, AMR117, AMR118, AMR123, AMR142, AMR151, AMR152, AMR153, AMR154, AMR155, AMR156, AMR157, AMR165, AMR166, AMR169
44	Costal/Songkhla	Sponge		*Callyspongia* sp. 	AMR158, AMR163, AMR167, AMR168
45	Pakmeng/Trang	Sponge		*Callyspongia* sp.	AMR161

Table 21 Actinomycete extracts presenting antimicrobial activity with the exception of 39 strong active extracts

Code	Extract	MIC/MBC or MFC (µg/ml)											
		SA	**MRSA**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
AMA4	BE	ND	ND	ND	ND	200/>200	ND	ND	ND	ND	ND	ND	ND
	CE	ND	200/>200	ND	ND	200/>200	ND	ND	ND	ND	ND	ND	ND
AMA6	BE	ND	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA10	CE	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	128/>200
AMA13	CE	ND	200/>200	ND	ND	ND	ND	ND	200/>200	ND	ND	ND	ND
AMA21	BE	64 / >200	128 />200	ND	ND	ND	200/>200	ND	ND	ND	ND	ND	ND
	CH	64/>200	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA22	BE	ND	200/>200	ND	ND	ND	200/>200	ND	200/>200	ND	ND	ND	ND
	CE	200/>200	16/>200	ND	ND	ND	200/>200	ND	200/>200	ND	ND	ND	ND
AMA26	BE	200/>200	200/>200	200/>200	ND	ND	200/>200	ND	ND	ND	ND	ND	ND
	CE	64/>200	64/>200	200/>200	ND	ND	200/>200	ND	ND	ND	ND	ND	ND
AMA30	BE	ND	ND	ND	ND	ND	200/>200	ND	ND	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA31	CH	128/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA32	CE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	64/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA44	CH	64/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA48	BE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA49	BE	200/>200	200/>200	ND	ND	ND	200/>200	ND	ND	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA51	CE	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	64/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA54	CE	200/>200	ND	ND	ND	ND	200/>200	ND	ND	ND	ND	ND	ND

Table 21 (Cont.) Actinomycete extracts presenting antimicrobial activity with the exception of 39 strong active extracts

Code	Extract	MIC/MBC or MFC (µg/ml)											
		SA	**MRSA**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
AMA59	BE	ND	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA64	BE	ND	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA66	BE	ND	200/>200	ND	ND	ND	200/>200	ND	ND	ND	ND	ND	ND
	CE	32/>128	32/>128	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA73	BE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA88	BE	32/>200	32/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA98	CE	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	32/>200	32/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA108	CH	64/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA110	CH	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA111	CE	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	64/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA122	CH	128/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA126	BE	16/>200	16/>200	ND	ND	ND	ND	ND	ND	200/>200	64/128	200/>200	200/>200
	CE	16/>200	16/>200	ND	ND	ND	ND	ND	ND	32/64	8/32	64/128	32/32
	CH	16/>200	16/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMA128	BE	ND	ND	ND	ND	ND	ND	ND	ND	200/>200	128/128	ND	200/>200
AMA142	CE	128/>200	200/200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ASE1	CE	ND	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ASE2	BE	ND	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 21 (Cont.) Actinomycete extracts presenting antimicrobial activity with the exception of 39 strong active extracts

Code	Extract	MIC/MBC or MFC (µg/ml)											
		SA	**MRSA**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
ASE2	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	200/>200	200/>200	200/>200	200/>200
ASE4	BE	32/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA1	CH	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA40	CH	128/>200	ND	ND	ND	ND	ND	ND	ND	16/>200	ND	ND	ND
ALA65	CE	32/>200	32/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	128/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA77	CH	128/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA88	CH	128/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA120	CE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	200/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA168	CH	16/>200	16/32	ND	ND	ND	ND	ND	ND	ND	ND	ND	200/>200
ALA174	CH	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA175	BE	32/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	16/>200	64/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ALA177	CE	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	200/>200
AMR1	BE	ND	ND	ND	ND	ND	200/>200	ND	ND	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	200/>200	200/>200	200/>200	200/>200
AMR2	BE	ND	ND	ND	ND	ND	200/>200	ND	200/>200	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	200/>200	200/>200	200/>200	200/>200
AMR4	CE	ND	ND	ND	ND	ND	ND	ND	ND	ND	32/64	ND	ND
AMR6	BE	ND	ND	ND	ND	ND	ND	ND	ND	128/128	128/>200	ND	64/64
	CE	ND	ND	ND	ND	ND	ND	ND	ND	128/128	128/>200	ND	64/64
AMR8	BE	128/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	128/>200	ND	ND
AMR9	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 21 (Cont.) Actinomycete extracts presenting antimicrobial activity with the exception of 39 strong active extracts

Code	Extract	MIC/MBC or MFC (µg/ml)											
		SA	**MRSA**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
AMR9	CH	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR10	BE	ND	64/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	32/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	200/>200	ND	ND
AMR12	CH	ND	ND	ND	ND	ND	ND	ND	ND	ND	128/>200	ND	ND
AMR19	BE	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	64/200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	200/200	200/200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR28	CH	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR30	BE	ND	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	64/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR44	BE	ND	ND	ND	ND	ND	ND	ND	ND	ND	200/>200	ND	ND
	CH	128/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR63	CH	128/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR64	CH	ND	64/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR71	BE	ND	ND	ND	ND	ND	ND	ND	ND	128/>200	64/200	200/200	128/128
AMR73	BE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	ND	ND	ND	ND	ND	ND	ND	16/>200	128/>200	ND	64/>200
	CH	ND	ND	ND	ND	ND	ND	ND	ND	200/>200	ND	ND	200/>200
AMR76	BE	32//>200	16/64	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	64/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR77	CH	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR86	CE	16/>200	16/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 21 (Cont.) Actinomycete extracts presenting antimicrobial activity with the exception of 39 strong active extracts

Code	Extract	MIC/MBC or MFC (µg/ml)											
		SA	**MRSA**	**AB001**	**AB002**	**AB003**	**AB004**	**AB005**	**AB007**	**CA**	**CN**	**MG**	**TM**
AMR90	CH	64/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR92	CE	16/>200	16/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	64/>200	32/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR93	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	64/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR100	CE	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR129	BE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR130	CH	128/>200	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR135	CH	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR151	BE	ND	ND	200/>200	200/>200	200/>200	200/>200	200/>200	200/>200	ND	ND	ND	ND
	CE	128/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	32/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AMR160	BE	128/>200	16/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CE	200/>200	64/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH	200/>200	200/>200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
vancomycin		1/2	1/2										
colistin				16/64	16/64	16/64	16/64	32/64	16/64				
amphotericinB										0.5/0.5	0.5/0.5		1/2
miconazole												2.2	

BE = ethyl acetate extract from culture broth CE = ethyl acetate extract from cells CH = hexane extract from cells SA = *Staphylococcus aureus* ATCC25923

MRSA = methicillin-resistant *S. aureus* SK1 AB = *Acinetobacter baumannii* NPRC001-005,007 CA = *Candida albicans* ATCC90028 CN = *Cryptococcus neoformans* ATCC90112

MG = *Microsporum gypseum* MU-SH4 TM = *Talaromyces marneffei* PSU-SKH1 AMA = actinomycetes from mangrove sediment

ALA = actinomycetes from Songkhla lake ASE = actinomycetes from coastal sediment AMR = actinomycetes from marine organisms

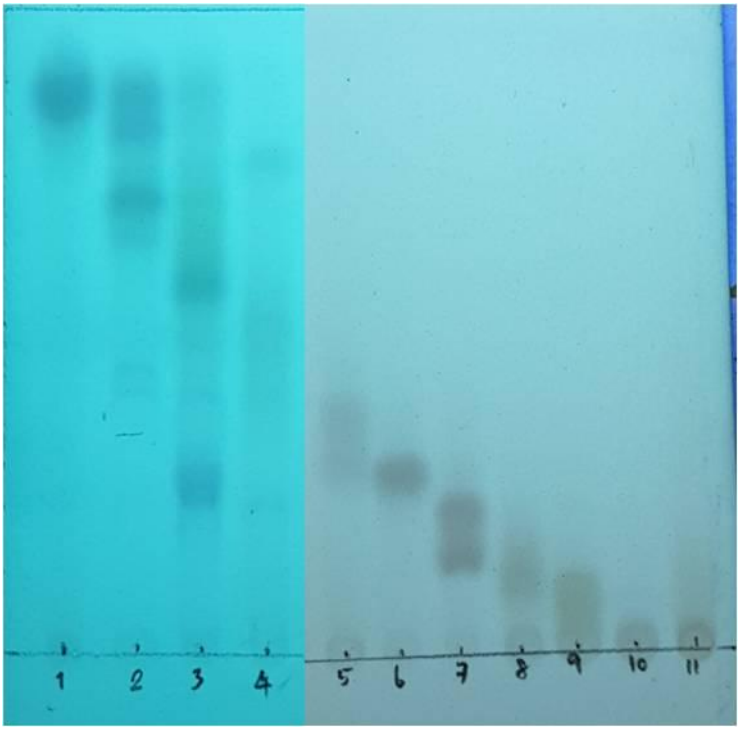


Figure 31 TLC of partial purification of ethyl acetate extract from cells of AMA11 (AMA11CE). Fraction 5,6 and 7 showed antibacterial activity and then they were analyzed by GC-MS.

Table 22 Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
Clade 1 (58% bootstrap support)				
AMR77	red	white-gray	RA,S	*Streptomyces* sp.
ASE1	yellow	white-gray	RA,S	*Streptomyces* sp.
ASE2	yellow	white-gray	S	*Streptomyces* sp.
S.spongiicola HNM0071^{T}	yellow	white-gray	S	
Clade 2 (100% bootstrap support)				
AMR92	brown	white-gray	RA	*S.griseorubens*
S.griseorubens NBRC 12780^{T}	yellow	gray	RA,S	
Clade 3 (99% bootstrap support)				
AMA22	red	gray	RA	*S.coelicoflavus*
S.coelicoflavus NBRC 15399^{T}	yellow	gray	S	
Clade 4 (100% bootstrap support)				
AMR1	yellow-brown	gray	RA	*S.olivaceus*
S.olivaceus NRRL B-3009^{T}	yellow	gray	RA, S	

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
Clade 5 (68% bootstrap support)				
S.misionensis DSM 40306^{T}		gray	S	
S.phaeoluteichromatogenes NRRL 5799^{T}		gray	RF	
AMR71	yellow	brown	RA, S	*Streptomyces* sp.
AMA50	yellow	brown	RA, S	*Streptomyces* sp.
AMA13	yellow	brown	RA, S	*Streptomyces* sp.
AMA30	yellow	brown	RA, S	*Streptomyces* sp.
AMA49	yellow	brown	RA, S	*Streptomyces* sp.
AMA52	yellow	brown	RA, S	*Streptomyces* sp.
AMA74	yellow	brown	RA, S	*Streptomyces* sp.
Clade 6 (74% bootstrap support)				
AMA44	yellow	white	RA, S	*Streptomyces* sp.
S.levis NBRC 15423^{T}		red	RA, S	

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
Clade 7 (96% bootstrap support)				
AMR82	yellow	white	RF	*S. pluripotens*
S. pluripotens MUSC 135^{T}	yellow	gray	RF	
Clade 8 (68% bootstrap support)				
ALA175	yellow	white-gray	S	*Streptomyces* sp.
S. thermoviolaceus subsp. *thermoviolaceus* DSM 40443^{T}		gray	S	
Clade 9 (51% bootstrap support)				
S. glomeratus LMG19903^{T}		gray	S	
AMA64	gray	gray	S	*Streptomyces* sp.
Clade 10 (85% bootstrap support)				
ASE4	yellow	green	RA	*Streptomyces* sp.
S. prasinopilosus NRRL B-2711^{T}		green	RA, S	
Clade 11 (90% bootstrap support)				
AMA11	yellow-brown	green	RA	Uncertain
AMA12	yellow-brown	green	RA	Uncertain
K. psammotica NBRC 13971^{T}		gray	RA	

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
Clade 12 (93% bootstrap support)				
AMR8	yellow	green	S	*Streptomyces* sp.
S. pseudovenezuelae DSM 40212^{T}		gray, red	RF	
Clade 13 (84% bootstrap support)				
AMA54	yellow	white	S	*Streptomyces* sp.
S. hyaluromycini NBRC 110483^{T}	red	gray	S	
Clade 14 (89% bootstrap support)				
AMA59	yellow	white	S	*Streptomyces* sp.
AMA66	yellow	white	S	*Streptomyces* sp.
S. coacervatus AS-0823^{T}		white	S	
Clade 15 (99% bootstrap support)				
AMA26	brown	white	RA	*Streptomyces* sp.
S. laurentii ATCC 31255^{T}		grayish yellow pink	RA	
Clade 16 (60% bootstrap support)				
AMR69	yellow	gray	RF	*Streptomyces* sp.
S. antibioticus NBRC 12838^{T}		gray	RF	

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
S. bungoensis DSM 41781^T	reddish brown	gray	S	
ALA166	gray	white	RA	*Streptomyces* sp.
ALA167	gray	white	RA	*Streptomyces* sp.
Clade 17 (100% bootstrap support)				
AMR107	brown	gray	S	*S. malaysiensis*
S. malaysiensis NBRC 16446^T	brown	gray	S	
Clade 18 (82% bootstrap support)				
AMA48	brown	gray	RF	*Streptomyces* sp.
S. catenulae NRRL B-2342^T		gray	RA,S	
AMR67	brown	gray	RF	*Streptomyces* sp.
S. yogyakartensis NBRC 100779^T		gray	S	
Clade 19 (100% bootstrap support)				
ASE5	yellow	gray	RA, S	*Streptomyces* sp.
S. specialis GW41-1564^T		pink-white	S	
Clade 20 (100% bootstrap support)				
G. rhizosphera NBRC 16068^T	pink to orange	not produced	rod-shaped	

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
AMR98	orange	not produced	rod-shaped	*G. rhizosphera*
AMA120	orange	not produced	rod-shaped and coccoid	*Gordonia* sp.
AMR3	red	not produced	rod	*Gordonia* sp.
G. sputi NBRC 100414^T				
AMA47	orange	not produced	rod	*Gordonia* sp.
G. alkanivorans NBRC 16433^T	orange	not produced	rod-shaped and coccoid	
Clade 21 (93% bootstrap support)				
AMR47	brown	white	fragment	*Nocardia* sp.
N. amamiensis TT 00-78^T		white	fragment	
AMA60	orange	white	fragment	*Nocardia* sp.
AMA36	orange	white	fragment	*N. nova*
N. nova NBRC 15556^T				
Clade 22 (100% bootstrap support)				
M. litorale CGMCC 4.5724^T	yellow	not produced	short rod, not formed endospore	
AMR130	yellow	not produced	short rod, not formed endospore	*Mycobacterium* sp.
M. florentinum DSM 44852^T	creamy	not produced	short rod, not formed endospore	

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
Clade 23 (100% bootstrap support)				
AMA3	brown	white	budding and fragment	*Pseudonocardia* sp
P. oroxyli D10^{T}	brown	pinkish brown	fragment	
ALA168	yellow	white	budding	*P. carboxydivorans*
P. carboxydivorans Y8^{T}	brown	white	budding and fragment	
ASE19	yellow	white	budding and fragment	*Pseudonocardia* sp.
P. nantongensis KLBMP 1282^{T}	yellow	white	rod-shaped and fragment	
ALA88	brown	white	rod-shaped and fragment	*P. antitumoralis*
P. antitumoralis SCSIO 01299^{T}	brown	white	rod-shaped	
Clade 24 (100% bootstrap support)				
A. maheshkhaliensis 13-12-50^{T}	yellow	white	hook to spiral	
AMA125	yellow	white	hook	*A. maheshkhaliensis*
AMA53	yellow	white	hook	*A. maheshkhaliensis*
ASE12	white	white	short chain and spiral	*A. geliboluensis*
ASE22	white	white	short chain and spiral	*A. geliboluensis*
A. geliboluensis A8036^{T}	orange-yellow	white	spiral	

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
Clade 25 (100% bootstrap support)				
J. muralis DSM 45357^T	white to gray	white	mycelium-like filament	
ASE10	yellow	white	mycelium-like filament	*J. alba*
J. alba DSM 45237^T	orange-yellow	white	mycelium fragment into short or elongated rods	
Clade 26 (94% bootstrap support)				
ALA1	orange and black	not produced	single	*Micromonospora* sp.
M. chalcea DSM 43026^T	red-orange	not produced	single	
AMR21	red-orange	not produced	single	*Micromonospora* sp.
AMA8	orange and black	not produced	single	*Micromonospora* sp.
M. maritima D10-9-5^T	dark grayish brown	not produced	single	
Clade 27 (98% bootstrap support)				
AMA141	red	not produced	single	*Micromonospora* sp.
M. zhanjiangensis 2902at01^T	red orange yellow	not produced	single	
Clade 28 (91% bootstrap support)				
ALA147	orange and brown	not produced	single	*Micromonospora* sp.

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
M. inositola DSM 43819^T	orange	not produced	single	
Clade 29 (75% bootstrap support)				
AMA2	orange	not produced	single	*M. auratinigra*
M. auratinigra DSM 44815^T	orange and black	not produced	single	
M. citrea DSM 43903^T				
Clade 30 (52% bootstrap support)				
ALA14	orange and deep brown	not produced	single	*M. endophytica*
M. endophytica DCWR9-8-2^T				
AMR101	orange and dark brown	not produced	single	*Micromonospora* sp.
AMR165	orange and dark brown	not produced	single	*Micromonospora* sp.
Single clade				
AMA21	brown	gray	RA	*Streptomyces* sp.
AMR2	brown	gray	RA	*Streptomyces* sp.

Table 22 (Cont.) Identification of 63 marine-derived actinomycetes and clades supported by bootstrap values greater than 50%

Taxa	Reverse color	Spore mass color	Spore feature	Identification
AMR6	brown	gray	S	*Streptomyces* sp.
AMR9	yellow	green	S	*Streptomyces* sp.
ALA181	brown	gray	S	*Streptomyces* sp.

Morphological characteristics of type strains obtained from previous study that published and presented on List of Prokaryotic Names with Standing in Nomenclature (LPSN)

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List of Publication and Proceeding

Sangkanu, S., Rukachaisirikul, V., Suriyachadkun, C. and Phongpaichit, S. 2017. Evaluation of antibacterial potential of mangrove sediment-derived actinomycetes. Microbial Pathogenesis. 112: 303-312.

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